Europ. J. Cancer Vol. 1, pp. 1–14. Pergamon Press 1965. Printed in Great Britain

Les Réactions Immunologiques (Spontanées ou à Visées Thérapeutiques) et l'Evolution des Cancers (Revue générale)

G. MATHÉ

Institut de Cancérologie et d'Immunogénétique, Hôpital Paul-Brousse, et Service d'Hématologie de l'Institut Gustave Roussy, Villejuif, Seine, France

IL EST en biologie, comme en clinique, certains sujets qui ne s'imposent que difficilement. C'est parce que les méthodes à eux disponibles manquent de sécurité, que les résultats des travaux qui leur sont consacrés ont un défaut de netteté et de concordance, que les hypothèses proposées sont multiples, voire contradictoires et trop peu étayées; tel fut le cas, jusqu'à ces dernières années, des relations immunologiques entre l'hôte et sa tumeur. Mais voici que sont "tombées" quelques données frappantes, que s'accumulent des observations convergentes, et que des hypothèses relativement soutenues par une structure de faits peuvent être proposées, certes encore fragiles, mais qu'il faut "remuer" pour disperser les nouvelles idées, et qui permettront peut-être de récolter, dans quelques années, des résultats applicables à la thérapeutique clinique. Nous n'envisagerons dans cette courte revue que les réactions immunitaires spécifiques.

I. Diverses études laissent à penser que les cellules tumorales peuvent manquer d'antigènes possédés par leurs "homologues" normales. Elles ont porté aussi bien sur des lignées tumorales de cancers transmis par greffes[1, 2], que sur des tumeurs humaines spontanées, leucémies [3, 4], cancers thyroïdiens [5] . . . , en passant par des cancers expérimentaux induits par des carcinogènes chimiques, singulièrement les hépatomes provoqués par les colorants azoïques ou le diméthylaminoazobenzène [6, 7]. Ces travaux ne sont pas exempts de critiques: n'est-il pas toujours difficile, sinon impossible, de trouver les cellules normales homologues de cellules tumorales données, les cancers étant souvent constitués de tissus indifférenciés, liés à la prolifération de cellules non différenciées, le tissu étudié comme homologue étant, lui, différencié? Il est, par ailleurs, impossible, en présence d'une délétion antigénique, de déterminer si elle est liée à des processus (mutation, sélection . . .) survenus au cours de l'évolution de la maladie tumorale, ou si elle relève directement de la carcinogénèse. On sait que cette dernière hypothèse a été soulevée par divers auteurs qui ont proposé d'expliquer la carcinogénèse chimique par une liaison du carcinogène et d'une protéine cellulaire, liaison qui entraîne la perte de celle-ci, perte qui peut-être responsable de la transformation maligne [8, 9].

II. Que les cellules cancéreuses possèdent des antigènes en plus de leurs homologues normales semble plus prometteur de spéculation, d'où la "ruée" récente des chercheurs depuis que Foley [10] annonça que des tumeurs induites chez la Souris par le méthylcholanthrène présentaient un caractère antigénique chez des animaux de la même lignée. Certes, une immunisation avait été produite contre des tumeurs greffées dans leur propre lignée d'origine, chez la Souris [11] et chez le Rat [12]; mais ce résultat avait été attribué à des mutations de cellules tumorales apparues au cours des passages successifs ou à des changements immunogénétiques survenus dans les lignées d'animaux [13, 14].

Prehn et Main [15] apportèrent des observations semblables à celles de Foley [10], montrant que l'exérèse des tumeurs induites par cette substance chimique laisse les animaux résistants à une nouvelle induction ou greffe de cette néoplasie. Sur trois tumeurs induites de la même façon, ces auteurs ne trouvèrent pas de résistance croisée. Cette notion fut confirmée par Old et coll. [16] qui, étudiant six tumeurs induites par le méthylcholanthrène ou le dibenzanthracène chez des souris de la même lignée, ne décelèrent aucune immunité croisée; par Klein et coll. [17] qui montrèrent que les animaux dont on a enlevé un cancer induit par le méthylcholanthrène sont spécifiquement résistants à la lignée tumorale; par Goberson et Feldman [18], etc . . . L'antigénicité spécifique persiste en général au cours de nombreux passages par greffe. Ainsi donc est acceptée la réalité d'une antigénicité du cancer, la spécificité de l'antigène, dans le cas des cancers induits par les carcinogènes chimiques, variant de tumeur à tumeur, même dans la même lignée.

Il convenait de rechercher si cette notion s'appliquait aux cancers induits par les virus. Habel [19], Sjögren et coll. [20] ont observé que des souris inoculées avec le virus polyome, et ne présentant qu'une infection inapparente, étaient résistantes à la greffe d'une tumeur du polyome isogénique, bien que celle-ci ne contienne pas de virus. L'interprétation proposée fut que les cellules cancéreuses possèdent un nouvel antigène; seuls les animaux inoculés dans la période périnatale tolèrent cet antigène et font des tumeurs; les animaux inoculés à l'âge adulte s'immunisent contre lui et rejettent les cellules cancéreuses. Evans et coll. [21] ont montré aussi que des tumeurs dérivées du papillome de Shope et ne contenant pas de virus, portent un antigène spécifique. Khera et coll. [22], Defendi [23], Habel et Eddy [24], Koch et Sabin [25] ont enregistré d'identiques résultats avec le virus SV40 chez le Hamster, montrant que cette résistance n'est pas due à des anticorps antivirus et, de plus, que l'immunisation induite par chaque virus est spécifique de celui-ci. Cette notion de spécificité liée au virus a été largement confirmé [26–30]. Il a tout au plus été observé que deux virus polyomes voisins induisent la même antigénicité. Il a été précisé que celle-ci n'est pas liée à des antigènes du virus: l'apparition des deux immunités, quand on conditionne les receveurs, est différente dans le temps [28].

La démonstration d'antigénicités spécifiques a également été apportée pour la leucémie induite par le virus de Rauscher [31], pour la tumeur plasmocytaire virale 70429 C_3H [32]; ces antigénicités ne confèrent pas de réactivité croisée avec la leucémie induite par le virus de Moloney. La leucémie induite par ce dernier virus n'induit pas de résistance croisée avec la leucémie de Gross [26].

Cet antigène spécifique demeure au cours de très nombreux passages en greffe [27–30]. Il est sensible à une irradiation des cellules de 800 r [30].

Il convient donc de souligner encore cette différence entre la carcinogénèse induite par le méthylcholanthrène, qui s'accompagne de l'apparition d'un nouvel antigène, spécifique de la tumeur, et la carcinogénèse virale, qui s'accompagne de l'apparition d'un nouvel antigène spécifique du virus.

Il n'est cependant pas impossible qu'existent des antigénicités croisées entre diverses variétés de tumeurs; ainsi Mirand et coll. [33] ont pu inhiber plus ou moins la greffe isogénique de trois tumeurs, l'une chimio-induite, l'autre spontanée, et la troisième virale, par l'inoculation de virus du polyome. Sjögren [27–30] a, par ailleurs, observé qu'une tumeur induite par le méthylcholanthrène et une tumeur spontanée étaient sensibles à la résistance induite par l'antigène spécifique des tumeurs induites par le virus polyome. Il a aussi pu rendre sensible une tumeur induite par le méthylcholanthrène, qui ne l'était pas, après l'avoir infectée par le virus du polyome *in vitro* [27–30].

Ainsi, la méthode immunogénétique fournit-elle une série de données concordantes et relativement solides. Elle n'est pas la seule méthode utilisée dans ce champ de recherche. Le test de cytotoxicié a permis d'accumuler de nombreux résultats, qui ont conduit à attribuer aux cellules leucémiques de la Souris un antigène spécial, l'antigène X [34, 35]. Des anticorps actifs contre les cellules des leucémies induites par les virus de Gross, Friend, Moloney ont en effet été révélés par ces tests qui se dirigent contre des antigènes spécifiques [36–38]. Un autre système antigénique (TL), "faiblement" représenté dans diverses leucémies spontanées et radio-induites, a été aussi mis en évidence par ce test [39].

Les antigènes cellulaires des leucémies induites par les virus de Friend et de Moloney sont distincts de ceux des cellules de la leucémie induite par le virus de Gross [36, 38]. Celui de la leucémie induite par le virus de Rauscher serait identique à celui de la leucémie de Friend [36]; celui de la leucémie de Moloney et celui de la leucémie de Rauscher sont communs [39]. Encore avec ce test, Old et coll.

[39] ont montré qu'une leucémie radio-induite présente une antigénicité croisée avec la leucémie spontanée A ou C58, mais pas avec les leucémies de Friend et de Gross.

Par contre, Klein et Klein [26] purent trouver chez les animaux rendus résistants par greffe des tumeurs, des anticorps par le test de cytotoxicité, ce test mettant cependant en évidence des réactions croisées non observées par la technique des greffes. Avec ce même test de cytotoxicité, Boyse [40] a montré que les cellules de cinq leucémies radio-induites C57Bl/6 étaient sensibles aux anticorps suscités par l'immunisation de souris C57Bl/6 avec des cellules d'une leucémie spontanée de la lignée A.

Quelles sont les relations entre les résultats de ces 2 tests, immunogénétique et de cytotoxicité? Koch et Sabin [25] n'ont pu mettre en évidence d'anticorps par le test de cytotoxicité chez les animaux devenus résistants à des antigènes spécifiques de cancers par greffes. Klein et Klein [26] trouvèrent une meilleure corrélation entre les résultats du test des greffes et la recherche d'antigène par les anticorps fluorescents.

Black et coll. [41] ont pu, par le test de fixation du complément, démontrer un antigène spécifique de la tumeur induite par le virus SV40 chez le Hamster, antigène différent de celui du virus.

Utilisant enfin, comme méthode, la diffusion en gélose, McKenna et coll. [42] avancent qu'ils ont mis en évidence dans des tissus cancéreux humains et dans les cellules HeLa un antigène soluble, particulier, commun, qu'ils n'ont pas retrouvé dans les tissus normaux. Un tel antigène avait été trouvé dans les cellules HeLa par le même test [43]. A encore été utilisé le test de l'hémagglutination passive [44]. Rappelons enfin que, par la méthode de l'anaphylaxie passive du Cobaye, Zilber [2, 45] a également enregistré des données qui le font parler depuis longtemps d'antigène spécifique du cancer.

Notons encore que, greffant à des humains leurs propres tumeurs, Southam et coll. [46] ont observé beaucoup moins de prises lorsqu'ils mélangeaient le tissu tumoral aux propres leucocytes des sujets que lorsqu'ils greffaient la tumeur seulement.

Ces antigènes spécifiques n'ont pas encore été soumis à des études génétiques ou biochimiques approfondies. Davies [47] a montré que la "spécificité X" est portée par les antigènes H–2. Dans les cas du lymphosarcome de Murphy-Sturm et du carcinome de Flexner, Hori et coll. [48] purent immuniser contre leurs tumeurs des animaux avec un lipolysaccharide extrait de celles-ci. Pilch et Metcham [49] ont pu immuniser, avec une fraction protéinique d'un fibrosarcome induit par l'éthiodol, des souris contre la greffe isogénique de cette tumeur.

III. S'il existe des antigènes spécifiques de divers cancers, pourquoi ne suscitent-ils pas une immunisation suffisante pour empêcher la formation de la tumeur?

1. Il est possible que trop peu de cellules néoplasiques soient détachées, et que trop peu d'antigène soit libéré. La greffe d'un nombre assez élevé de cellules est nécessaire pour protéger les animaux [17]

2. Une seconde hypothèse explicative est que l'organisme "se défend" généralement bien contre les cellules cancéreuses comme il le fait contre les microbes, et que cette "défense", comme dans le cas des microbes, échoue dans certaines circonstances, qui sont précisément des circonstances qui favorisent les cancers.

Divers carcinogènes ne sont en effet efficaces que s'ils sont appliqués en période néonatale, c'est-à-dire de faibles réactions immunitaires. Ainsi en est-il de certains virus, en particulier le virus de la leucémie AkR [50], le virus polyome [51]. Mais l'explication du phénomène peut résider dans les réactions immunitaires contre le virus.

Plus important donc est le fait que certaines carcinogènes chimiques ou physiques sont plus efficaces chez des sujets peu compétents immunologiquement: c'est ainsi que Pietra et coll. [52] ont observé qu'une dose donnée de diméthylbenzanthracène induit un pourcentage beaucoup plus élevé de lymphosarcomes si elle est administrée le jour de la naissance que si elle est administrée à l'âge adulte. Miller et coll. [53] ont pu augmenter l'effet d'un hydrocarbure en thymectomisant les souris pendant la période néonatale; Simpson et coll. [54] n'ont pas retrouvé ce résultat chez le Rat. Dans certaines circonstances, l'irradiation est également plus efficace lorsqu'elle est appliquée à l'animal nouveau-né [55].

Enfin, il est connu que, chez l'Homme, certaines néoplasies malignes sont plus fréquentes au cours de certains états réduisant les défenses immunitaires; ainsi la survenue relativement fréquente d'hématosarcomes chez des sujets atteints d'hypogammaglobulinémie, signalée par Page et coll. [56], et chez des patients atteints d'une affection que caractérise l'association d'ataxie, de télangiectasies, d'anomalies thymiques (absence de corpuscules de Hassal et de thymocytes dans le cortex),

d'insuffisance immunitaire enfin [57]. Talal et Bunim [58] ont également signalé plusieurs cas de développement d'hématosarcomes chez des patients atteints de syndrome de Gougerot–Sjögren, beaucoup d'entre eux présentant une hypogammaglobulinémie. Rappelons encore la grande fréquence des cancers épithéliaux chez les sujets atteints de leucémie lymphocytaire chronique [59], affection réduisant les défenses immunitaires. Il convient enfin de signaler que 23% des hypogammaglobulinémies de l'adulte sont associées, dans la statistique de Bernier [60], à des néoplasies des cellules sanguines ou hématopoïétiques (lymphosarcome, 10%; leucémie lymphocytaire chronique, 7%; maladie de Hodgkin, 2%; myélome multiple, 2%; leucémie aiguë lymphoblastique, 1%; affection myéloproliférative, 1%), 5% à des néoplasies malignes extra-hématologiques; bien que l'on considère généralement la néoplasie comme primitive, l'hypothèse inverse mérite d'être soulevée.

3. Une troisième hypothèse ne peut manquer de venir à l'esprit: les carcinogènes n'agissent-ils pas totalement, ou partiellement, en inhibant les réactions immunitaires?

Il est certain que de nombreuses substances connues pour être de puissants carcinogènes sont également connues pour être capables de reduire la production des anticorps; ainsi en est-il: *a.* des radiations ionisantes[61–63]; *b.* des agents alcoylants, dont l'action anti-immunitaire est connue pour beaucoup de composés, et dont le pouvoir carcinogène n'a été étudié et démontré que pour un nombre restreint d'entre eux [64]; *c.* des hydrocarbures polycycliques [65–69] (il a été signalé que le 3-méthylcholanthrène provoque d'importantes lésions du tissu lymphoïde, spécialement du thymus [20]; *d.* de l'uréthane [66, 71].

D'ou l'idée que les carcinogènes pourraient agir non seulement par l'induction d'une anomalie responsable de la malignité au niveau d'un clone cellulaire, mais encore en s'opposant à l'élimination par les réactions immunitaires de ce clone grâce à son antigénicité [72].

Le 3-méthylcholanthrène inhibe la formation des anticorps chez des souris DBA/2, susceptibles à la leucémogénèse par cet agent, mais non chez des souris non susceptibles, ce qui a été rapproché par Berenbaum [64] de l'observation de Davidsohn et coll. [65], selon laquelle l'irradiation déprimerait davantage la formation des anticorps chez de jeunes souris de la lignée C57Bl/6 (lignée sensible à la radio-leucémogénèse) que chez des souris d'autres lignées.

Il faut cependant noter que cette corrélation entre les actions carcinogène et immuno-dépressive est loin d'être parfaite: divers produits inhibent les fonctions immunitaires sans être carcinogènes: les stéroïdes cortico-surrénaliens, la colchicine [73–75]; d'autres, tels les antimétabolites, ne sont pas connus pour être carcinogènes. Il faut aussi remarquer que les doses d'irradiation ou de composés chimiques qui exercent ces deux types d'action diffèrent entre elles notablement. Même si l'on admet que les deux effets peuvent s'additionner, on ne comprend pas pourquoi les substances immuno-inhibitrices et non carcinogènes ne favorisent pas l'effet carcinogène des substances à action double [76].

On a même pu se demander si les virus carcinogènes eux-mêmes ne peuvent déprimer les défenses immunitaires: c'est ainsi que l'injection de virus de Gross à des nouveaux nés C3H, déprime leur réponse immunitaire contre le bactériophage T2 [77, 78].

III. Ces réactions immunitaires dont, somme toute, l'efficacité réelle dans la "défense" contre l'éclosion des cancers ne peut être estimée, jouent-elles un rôle important une fois la tumeur établie?

1. Il est d'abord possible qu'une sélection se fasse des cellules les moins antigéniques, et que ce soit à partir d'elles que se produise la tumeur qui persiste. Goberson et Feldman [18] ont observé qu'au cours de passages successifs en greffe, l'antigénicité de tumeurs induites par le benzopyrène diminue. Rappelons ici qu'Axelrad et Klein [79], comparant une tumeur primaire et ses métastases quant à leurs possibilités de passer chez des hybrides F1 obtenus par croisement avec une lignée résistante, ont observé que les cellules métastatiques présentaient un plus haut degré de spécificité de lignée que les cellules de la tumeur primaire.

2. Il apparaît d'un autre côté que les organismes porteurs de cancers présentent un affaiblissement de leurs "défenses immunitaires". Il a été observé que la production d'agglutinines contre les proteus [80] et l'anatoxine tétanique [81] est plus faible chez des patients porteurs de néoplasies diverses que chez des sujets normaux, cette insuffisance n'étant pas liée à l'altération de l'état général [81]. Southan et coll. [82], greffant des cellules HeLa ou d'autres cancers, ou encore des cellules normales cultivées *in vitro*, ont observé un nombre de prises très supérieures chez des cancéreux au nombre observé chez des individus normaux. Cette tolérance n'est pas davantage en corrélation avec la défaillance de l'état général.

3. Mais on ne peut envisager l'insuffisance immunitaire des cancéreux à l'égard des greffes de tumeurs, sans évoquer ce singulier phénomène de tolérance spécifique auquel on a donné le nom de favorisation ("enhancement"). Ce phénomène a fait l'objet, à lui seul, de tant d'études [83–85] que nous ne pouvons que renvoyer le lecteur à une revue générale récente [86] pour qu'il en trouve les bases expérimentales et les hypothèses proposées pour en expliquer le mécanisme d'action, auquel Möller [87] a consacré récémment d'importants travaux. Rappelons simplement ici que l'on désigne sous ce nom un phénomène qui facilite la prise de greffes allogéniques du fait de l'existence, chez le receveur, d'anticorps spécifiques, qui peuvent avoir été élaborés par l'hôte ou lui avoir été injectés.

Dans quelle mesure ce phénomène joue-t-il un rôle favorisant l'évolution dès tumeurs en clinique humaine? A cette question peut tenter de répondre une étude que nous avons conduite sur le seul cancer humain dont on peut être sûr qu'il porte des antigènes à l'égard de l'hôte, chez lequel on peut, de plus, rechercher facilement d'éventuels anticorps: le chorio-carcinome placentaire [88]. Etudiant, de 1957 à 1962, six patientes porteuses d'une telle tumeur, nous avons pu mettre en évidence dans leur sérum des anticorps efficaces sur les leucocytes et les plaquettes de leurs maris respectifs. Ayant ainsi démontré leurs réactions immunitaires contre leurs tumeurs respectives, nous avons étudié le sens de celles-ci en leur greffant des fragments de peau de leurs maris: ceux-ci furent tolérés plus longtemps que normalement. De semblables observations ont été faites sur deux malades par Robinson et coll. [89].

C'est peut-être un phénomène voisin qui explique les résultats de Kawamura [90] et de Shikata et coll. [91], qui, greffant à des cancéreux leur propre tumeur au moment de l'exérèse ou de l'essai d'exérèse, ont observé que les prises étaient d'autant plus fréquentes que le pronostic était plus souvent mauvais.

IV. Ainsi, les relations immunitaires spontanées entre la tumeur et son hôte, même démontrée l'existence d'antigènes spécifiques des cancers, sont-elles complexes, de même que le sont les relations entre un receveur et son greffon ou transplant allogéniques. Cette complexité, lourde pierre d'achoppement aux applications thérapeutiques, provient surtout du fait que les réactions de l'hôte peuvent se faire dans deux directions, favorable ou défavorable à la tumeur, défavorable donc ou favorable au cancéreux. C'est de la connaissance des facteurs qui gouvernent la raison de ces deux possibles directions, facteurs qui ne diffèrent probablement guère de ceux qui gouvernent le rejet ou la tolérance d'un greffon ou transplant incompatibles [92], que dépendront le succès des tentatives thérapeutiques. Celles-ci ont déjà été nombreuses. Nous n'envisagerons pas ici la stimulation des défenses non spécifiques.

L'immunothérapie peut être passive, active ou adoptive.

1. Il n'est pas déraisonnable d'espérer que l'immunothérapie passive puisse s'adresser d'abord aux éventuels virus carcinogènes, si de tels facteurs sont mis en évidence chez l'Homme. Encore sait-on que les anticorps ne sont pas efficaces sur les virus après leur pénétration dans les cellules.

L'activité sur les éléments néoplasiques d'anticorps injectés semble également très limitée. Gorer et Amos [93] ont montré que l'on peut prévenir la prise d'une greffe de tumeur par l'administration de sérum obtenu après immunisation allogénique, mais que celui-ci est inefficace lorsque la tumeur est établie. La raison pour laquelle le sérum immun n'est pas actif sur une tumeur avancée n'est pas claire: peut-être administre-t-on insuffisamment d'anticorps; peut-être ceux-ci sont-ils dans l'impossibilité d'atteindre toutes les cellules tumorales.

Une application de l'immunothérapie passive consiste au transport électif des composés chimiothérapiques aux cellules tumorales: nous avons montré l'intérêt d'anticorps hétérospécifiques de hamsters immunisés contre des cellules leucémiques de souris, comme transporteurs de substances chimiothérapiques dans le traitement de souris porteuses de la leucémie [94].

2. L'immunothérapie active, ou vaccination, a été montrée possible dans certaines conditions expérimentales: ne parlons pas de l'immunisation active d'un sujet "A" contre un cancer greffé "b" provenant d'un donneur "B", condition qui n'a qu'un seul correspondant en pathologie humaine, le chorio-épithéliome, et nous avons dit le sens possiblement défavorable pour le malade des réactions immunitaires.

Si les cellules "a" d'une tumeur spontanée ou induite chez un sujet "A" sont antigéniques, il sera peut-être possible d'auto-vacciner des individus cancéreux, par exemple pendant les phases de rémission. Si cet antigène est commun aux cellules "a" d'un cancer spontané ou induit, survenu chez un sujet "A", et aux cellules "b" d'un cancer survenu chez un sujet "B", il n'est pas déraisonnable d'espérer la possibilité de vacciner le sujet avant l'apparition de la maladie. Encore faudra-t-il trouver les

conditions de l'immunisation "défensive", et éviter celles de la "favorisation" (ou "enhancement"), dont nous avons montré la réalité chez l'Homme dans le cas du chorio-épithéliome.

A signaler l'observation de régression de tumeurs spontanées après greffe des propres cellules néoplasiques du patient, d'abord passées en greffes hétérospécifiques [95]. Weiss et coll. [96] ont enlevé des carcinomes mammaires chez des souris, et les ont regreffés chez leurs hôtes originels ou chez des animaux isogéniques: tandis que, chez ces derniers, les tumeurs prenaient, chez les premiers, les greffes étaient soient retardées, soit facilitées; et divers agents connus pour stimuler les réactions immunitaires se sont montrés capables d'une certaine action.

Il est possible que les guérisons de cancereux après exérèse chirurgicale relèvent de réactions immunitaires, l'exérèse n'ayant fait qu'enlever la masse principale, comme dans l'expérience de Prehn et Main [15], la récidive étant empêchée par ces réactions.

Haddow et Alexander [97] ont, par ailleurs, observé que l'effet d'une dose donnée d'irradiation sur un fibrosarcome primitif peut être augmenté par une autogreffe de la tumeur obtenue par biopsie, cette dernière étant faite dans le but d'amplifier les défenses immunitaires qui peuvent agir sur les cellules échappant à l'irradiation.

On peut encore se demander si la radiothérapie ne peut agir ainsi, et plus peut-être, si elle ne peut modifier la tumeur antigéniquement, ce qui concorderait avec les lésions chromosomiques qu'elle induit.

Il n'est pas exclu, enfin, qu'il en soit de même de la chimiothérapie: Sorn [98] a publié avoir immunisé des souris leucémiques AkR avec des cellules leucémiques isogéniques incubées en présence de chloréthylaminométhyluracile.

On a, bien sûr, pensé à l'emploi des adjuvants. Murphy [99] avait déjà montré que l'injection dans le système lymphatique d'acide oléique réduisait la fréquence des rechutes après ablation, chez des souris atteintes de cancer mammaire spontané. Graham et Graham [100], injectant à des sujets leurs propres tumeurs homogénéisées et mélangées à des adjuvants de Freund, disent avoir augmenté la radiosensibilité.

Mais il est à attendre que la vaccination la plus prometteuse consistera à immuniser avec les antigènes cancéreux spécifiques; déjà Goldner et coll. [101], vaccinant des hamsters avec l'antigène de tumeurs virales (polyome ou SV40) extrait sous forme de lipoprotéine, qu'ils appellent antigène de Bjorklund, après l'injection de virus ou avant, ont pu réduire notablement la fréquence des tumeurs; à noter qu'ils ont observé, dans certains cas, un phénomène de favorisation.

En ce qui concerne l'éventuelle immunisation active contre le (ou les) virus cancérigènes, si l'on sait que Charlotte Friend [102] a pu vacciner des souris par le virus formolé et rendre inefficace chez elles une nouvelle inoculation de virus actif, on comprend que cette vaccination ne puisse s'appliquer à l'Homme, tant que l'on n'a pas isolé de virus cancérigène. Le même résultat a été obtenu avec le virus de Rauscher par Fink et Rauscher [103].

3. D'où la supériorité de l'immunothérapie adoptive: on peut en effet utiliser non seulement son effet sur les cellules, mais aussi son action sur un éventuel virus, sans qu'il soit besoin d'avoir isolé ce dernier.

Soit un sujet "A", porteur de cellules "a" d'un cancer éventuellement dû à un virus "α". Une irradiation totale, d'environ 1000 rads, non seulement détruit une certaine proportion des cellules cancéreuses "a", mais permet une greffe de moelle osseuse "B". Les cellules myéloïdes de ce greffon repeuplent le tissu myéloïde et empêchent la mort par aplasie. Les cellules immunologiquement compétentes "B" du greffon repeuplent le tissu lymphoïde et s'immunisent contre les cellules cancéreuses "a" (l'addition de leur action à celle de l'irradiation permet d'obtenir l'éradication) et contre le virus "α". On peut enfin espérer que, si l'élimination de ce virus n'est pas complète, celui-ci, s'il est spécifique de l'individu (comme c'est le cas de certains virus leucémiques chez la Souris (virus de Charlotte Friend [102], virus de Gross [104]) ne sera pas capable d'induire un cancer "b" à partir des cellules "B".

La réalité de la greffe allogénique des cellules myéloïdes est démontrée dans plusieurs espèces animales: Souris, Rat, Lapin, Chien, Singe [voir 92], et chez l'Homme [105–110]; celle des cellules lymphoïdes et la production par elles de globulines du type du donneur, chez l'animal [111] et chez l'Homme [105, 106].

La faculté du greffon de réagir contre les cellules leucémiques a été démontrée, chez la Souris, sur des leucémies greffées [112–115], sur d'autres tumeurs greffées [116, 117] et sur des leucémies spontanées [118, 119].

La faculté d'un greffon hématopoïétique de réagir contre un virus leucémigène a été démontrée chez des souris porteuses de la leucémie virale de Ch. Friend, le greffon étant isogénique et provenant de souris vaccinées

[120], ou allogéniques (observations personnelles non publiées).

Malheureusement, les cellules immunologiquement compétentes du greffon "B" ne réagissent pas seulement contre les cellules ou antigènes leucémiques et les virus leucémigènes; elles réagissent aussi contre les antigènes normaux du receveur, induisant ainsi un syndrome dit "secondaire", d'expression plus ou moins rapide et sévère, observé dans les diverses espèces animales [voir 92] et chez l'Homme[110]. Des progrès récents ont été enregistrés dans son contrôle, curatif ou préventif, non seulement chez l'animal (traitement par antimitotiques chimiques qui peuvent d'ailleurs ajouter à l'effet antileucémique [113], conservation de la moelle osseuse à 37° [121, 122], utilisation des donneurs multiples [123], mais aussi chez l'Homme (stéroïdes corticosurrénaliens, acide amino-caproïque, maintien des sujets en conditions aseptiques, utilisation de donneurs multiples [105, 106, 110]

La possibilité de contrôler le syndrome secondaire a pu permettre d'observer, chez l'Homme, l'effet antileucémique de cette thérapeutique. Sur trois patients qui lui ont survécu: *a.* l'un [107] présentait une leucémie évoluant depuis 26 mois et sur laquelle la chimiothérapie avait obtenu trois rémissions, dont les durées respectives avaient été de 1 an, de 5 mois et de 3 semaines; après la greffe de moelle osseuse, une rémission de 1 an a été obtenue; *b.* le second [107] présentait une leucémie évoluant depuis 13 mois, sur laquelle on avait obtenu une première rémission de 8 mois et une seconde de 3 semaines, qui laissait la place à une rechute d'évolution rapide au moment où fut entrepris l'essai thérapeutique; une rémission de 9 mois a été obtenue; *c.* le troisième patient commençait, après 20 mois d'évolution, une troisième phase évolutive résistante aux stéroïdes, à la mercapto-6-purine et à la leurocristine; il est mort au vingtième mois d'une rémission complète, d'une encéphalite zostérienne, il ne présentait à l'autopsie, aucun indice visible de leucémie [105, 106]. Ce résultat laisse espérer que, dans un avenir assez proche, les essais de traitement de leucémiques par la greffe de moelle osseuse allogénique pourront se multiplier.

Des essais d'immunothérapie de diverses tumeurs solides ont enfin été conduits par l'administration de cellules spécifiques allogéniques après chimiothérapie, dont les résultats, bien que d'interprétation délicate, sont encourageants. [124]

Telles sont exposées, en se limitant surtout aux plus récentes, les principales données qui offrent enfin une base solide à l'immunologie des cancers, confrontées et discutées les quelques hypothèses, encore fragiles, jusqu'ici proposées, entrevus les espoirs qu'il ne faut encore accepter qu'avec de prudentes réserves, de l'immunothérapie.

RÉSUMÉ

L'auteur donne d'abord une brève revue des réactions immunitaires spontanées que les organismes semblent pouvoir mettre en jeu contre un éventuel cancer. Celles-ci sont, bien sûr, conditionnées par la présence, au niveau des cellules néoplasiques, d'antigènes particuliers: sont analysés les récents et nombreux travaux qui ont accumulé de multiples et convaincantes observations sur l'existence de tels antigènes dans les cancers induits par des carcinogènes chimiques ou par des virus.

Se pose alors la question suivante: pourquoi ces antigènes ne suscitent-ils pas une immunisation suffisante pour empêcher la formation des tumeurs? Une des hypothèses proposées pour répondre est que celle-ci pourrait résulter de l'application du carcinogène à des périodes d'insuffisance immunitaire (période néonatale par exemple), ou que le carcinogène pourrait lui-même induire une telle insuffisance (beaucoup de carcinogènes chimiques inhibent la production des anticorps; l'inoculation de certains virus pourrait faire de même); ajoutons que la fréquence des cancers est particulièrement élevée chez les humains atteints de certaines affections déprimant les défenses immunitaires.

Se pose ensuite une question découlant de la précédente: ces réactions immunitaires jouent-elles un rôle une fois la tumeur constituée? Une première réponse fait appel, là encore, à l'affaiblissement des défenses immunitaires chez les cancéreux, que met en évidence la baisse de production des anticorps ou la tolérance à certaines greffes incompatibles. Plus intéressante est la démonstration que l'auteur et ses collaborateurs ont apportée chez des femmes porteuses de chorio-carcinome placentaire, d'un état de favorisation ("enhancement") que caractérise ce paradoxe: présence d'anticorps tumoraux à l'égard du mari, tolérance de greffons de la peau de celui-ci.

Sont ensuite envisagées les possibilités actuelles de l'immunothérapie.

L'immunothérapie passive semble inefficace une fois la tumeur établie et fait courir le risque de favorisation.

L'immunothérapie active donne quelques résultats encourageants; s'adressant à certains virus leucémogènes, elle peut prévenir la cancérisation.

L'immunothérapie adoptive, que réalise la greffe de cellules immunologiquement compétentes, singulièrement celle de moelle osseuse, peut s'adresser aux cellules néoplasiques ou aux virus cancérigènes. Elle a donné, à diverses équipes, des résultats remarquables sur des leucémies et autres tumeurs de la Souris, et les premiers essais chez l'Homme conduits par l'auteur et son groupe sont encourageants.

SUMMARY

The author first gives a brief review of the spontaneous immunological reactions that the body seems to bring into play against the development of a cancer. These reactions are certainly conditioned by the presence of specific antigens on the cancer cells. Many recent observations provide convincing evidence for the existence of such antigens in cancers induced by chemical carcinogens or viruses. The following question arises: Why are these antigens unable to produce a state of immunity strong enough to inhibit the formation of the tumours? One possible answer is provided by the hypothesis that failure could be due to a decreased immunological activity coincident with the carcinogenic stimulus (for example during the neonatal period). Alternatively, the carcinogen itself, could be responsible for inducing a state of immunological unresponsiveness since many chemical carcinogens inhibit the production of antibodies; innoculation with certain viruses could have a similar blocking effect. In support of the immunological theory of cancer induction is the fact that the frequency of cancer appears increased in men, following certain diseases that are known to depress the immune defense reactions.

A further question arises from the preceding one: do these immunological reactions play any role once the tumour has been formed? A suggestion in this direction is represented by the poor immunological defense reactions observed in patients with cancer, as indicated by the reduction of antibody production or the tolerance to certain incompatible skin grafts. Related to this problem are the findings by the author and associates in women suffering from placental choriocarcinomata. These patients were observed to have an enhancement of tolerance and would accept skin grafts from their husbands, while at the same time antibodies were present which were active against the husband's cells; this situation presents a remarkable biological paradox.

There then follows a discussion of the actual possibilities of immunotherapy.

Passive immunotherapy seems to be ineffective once the tumour is established, indeed it may carry a risk of enhancing the growth of the tumour.

Active immunotherapy has given some encouraging results; these have been observed with certain viral leukaemias, in which immunization was able to prevent the development of neoplasia.

"Adoptive" immunotherapy is achieved by the grafting of immunologically competent cells, especially bone marrow; then these cells are able to deal with either the neoplastic cells or the carcinogenic virus. This approach has given some remarkable results in the treatment of murine leukaemia and other tumours in the mouse, the results being confirmed by several different groups of research workers. The preliminary trials of this type of therapy in man made by the author and his group have been encouraging.

ZUSAMMENFASSUNG

Verfasser gibt zunächst einen kurzen Uberblick über die spontanen Immunisationsreaktionen, welche von den Organismen anscheinend gegen eventuellen Krebs zum Einsatz gebracht werden können. Diese Immunisationsvorgänge sind sicher durch die Anwesenheit besonderer Antigene auf dem Niveau der neoplastischen Zellen bedingt: Es werden zahlreiche neuere Arbeiten analysiert, die viele überzeugende Beobachtungen bezüglich der Existenz derartiger Antigene bei künstlich mit karzinogenen Chemikalien oder Viren erzeugten Krebsen erbracht haben.

Es wird dann die Frage gestellt, warum diese Antigene keine zur Verhinderung der Tumorbildung ausreichende Immunisation bewirken. Eine der zur Erklärung vorgeschlagenen Hypothesen ist die, dass die Tumorbildung möglichcerweise eine Folge der Einwirkung eines Karzinogens zu Zeiten einer Immunisations-Insuffizienz (z.B. in der Neugeborenen Periode) wäre, wenn das Karzinogen selbst eine solche Insuffizienz erzeugen könnte (viele karzinogene Chemikalien hemmen die Antikörperproduktion; Einimpfung gewisser Viren könnte die gleiche Wirkung haben); ausserdem wäre zu erwäknen, dass Krebs besonders häufig vorkommt bei Patienten mit Krankheiten, durch welche die Immunisationsmechanismen unterdrückt werden.

Aus der ersten Frage ergibt sich die nächste, ob die Immunisationsreaktionen eine Rolle spielen, sobald sich der Tumor gebildet hat. Zunächst wird auch hier wieder auf die Schwächung der Immunisationsmechanismen bei Krebsleidern hingewiesen und das Sinken der Antikörperproduktion oder die Toleranz gegenüber gewissen, sonst unverträglichen Transplantaten wird hervorgehoben. Interessanter ist der von dem Autor und seinen Mitarbeitern an Chorionkarzinom-Patientinnen erbrachte Nachweis eines Begünstigungszustandes, der sich in folgendem Paradox ausdrücken lässt: Anwesenheit von Tumor-Antikörpern beim Ehemann, Toleranz von Hauttransplantaten, die diesem entnommen worden sind.

Sodann werden die gegenwärtigen Möglichkeiten der Immunisierung ins Auge gefasst.

Die passive Immunisierung scheint unwirksam, sobald sich der Tumor einmal festgesetzt hat und bringt das Risiko einer Begünstigung mit sich.

Die aktive Immunisierung liefert manche ermutigenden Resultate; richtet sie sich gegen gewisse leukämogene Viren, so lässt sich eine Verkrebsung verhüten.

Die adoptive Immunisierung, bei der immunologisch wirksame Zellen, vereinzelt aus dem Knochenmark, verpflanzt werden, kann sich gegen neoplastische Zellen older kanzerogene Viren richten. Sie hat bei verschiedenen Teams bemerkenswerte Ergebnisse bei Leukämie und anderen Tumoren der Maus gezeitigt und die ersten von dem Autor und seiner Gruppe am Menschen vorgenommenen Versuche sind ermutigend.

BIBLIOGRAPHIE

1. R. C. Nairn, H. G. Richmond, M. G. McEntegart et J. E. Fothergill, Immunological differences between normal and malignant cells. *Brit. Med. J.* **2,** 1335 (1962).
2. L. A. Zilber, Specific tumor antigens. *Advan. Cancer Res.* **5,** 291 (1958).
3. D. Nelken, Loss of leukocyte individual specific antigens in a case of acute leukemia. *Vo. Sang. (Basel)* **8,** 638 (1963).
4. M. Seligmann, Contribution à l'étude immunologique des leucémies. Thèse, Paris (1955).
5. R. B. Goudie et H. M. McCallum, Loss of tissue specific autoantigen in thyroid tumors. A demonstration by immuno-fluorescence. *Lancet* **7316,** 1035 (1963).
6. R. W. Baldwin, Immunological studies of rat liver cells antigens during azo-carcinogenesis. *Acta Un. Int. Cancr.* **19,** 545 (1963).
7. V. I. Kalnins et H. F. Stich, Loss of liver cell antigens in azo-dye induced hepatomas of rats. *Nature, Lond.* **200,** 189 (1963).
8. C. Heidelberger et M. G. Moldenhauer, Interaction of carcinogenic hydrocarbons with tissue constituents; quantitative study of binding to skin proteins of several C14-labeled hydrocarbons. *Cancer Res.* **16,** 442 (1956).
9. J. A. Miller et E. C. Miller, The carcinogenic aminoazo-dyes. *Adv. Cancer Res.* **1,** 339 (1953).
10. E. J. Foley, Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. *Cancer Res.* **13,** 835 (1953).
11. L. Gross, Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. *Cancer Res.* **3,** 326 (1943).
12. M. R. Lewis et M. B. Apterkman, Atrophy of tumors caused by strangulation and accompanied by development of tumor immunity in rats. *Cancer* **5,** 411 (1952).
13. T. S. Hauschka, Immunological aspects of cancer. *Cancer Res.* **12,** 615 (1952).

14. G. D. Snell, A. M. Cloudman et E. Failor, Inhibition and stimulation of tumor homotransplants by prior injection of lyophilized tumor tissue. *J.Nat. Cancer Inst.* **6,** 305 (1946).
15. R. T. Prehn et J. M. Main, Immunity to methylcholanthrene-induced sarcomas. *J. Nat. Cancer Inst.* **18,** 769 (1957).
16. L. J. Old, E. A. Boyse, D. A. Clarke et E. A. Carswell, Antigenic properties of chemically induced tumors. *Ann. N.Y. Acad. Sci.* **101,** 80 (1963).
17. G. Klein, H. O. Sjogren, E. Klein et K. E. Hellstrom, Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochtonous host. *Cancer Res.* **20,** 1561 (1960).
18. A. Goberson et M. Feldman, Antigenic specificity of benzopyrene induced sarcomas. *J. Nat. Cancer Inst.* **32,** 1229 (1964).
19. K. Habel, Resistance of polyoma virus immune animals to transplanted polyoma tumors. *Proc. Soc. Exp. Biol., N.Y.* **106,** 722 (1961).
20. H. O. Sjogren, I. Hellstrom et G. Klein, Resistance of polyoma-virus immunized mice to transplantation of established polyoma tumors. *Exp. Cell. Res.* **23,** 204 (1961).
21. C. A. Evans, L. R. Gorman, Y. Ito et R. S. Weiser, Antitumor immunity in the Shope papilloma-carcinoma complex of rabbits. I. Papilloma regression induced by homologous and autologous tissue vaccines. *J. Nat. Cancer. Inst.* **29,** 277 (1962).
22. K. S. Khera, A. Ashkenazi, F. Rapp et J. L. Melnick, Immunity in hamsters to cells transformed "*in vitro*" and "*in vivo*" by SV40. *J. Immunol.* **91,** 604 (1963).
23. V. Defendi, Effect of SV40 virus immunization on growth of transplantable SV40 and polyoma virus tumors in hamster. *Proc. Soc. Exp. Biol., N.Y.* **113,** 12 (1963).
24. K. Habel et B. E. Eddy, Specificity of resistance to tumor challenge of polyoma and SV40 virus-immune hamsters. *Proc. Soc. Exp. Biol. N.Y.*, **113,** 1 (1963)
25. M. A. Koch et A. B. Sabin, Specificity of virus-induced resistance to transplantation of polyoma and SV40 tumors in adult hamsters. *Proc. Soc. Exp. Biol., N.Y.* **113,** 4 (1963).
26. E. Klein et G. Klein, Antigenic properties of lymphomas induced by the Moloney agent. *J. Nat. Cancer. Inst.* **32,** 547 (1964).
27. H. O. Sjogren, Studies on specific transplantation resistance to polyoma-virus induced tumors. I. Transplantation resistance induced by polyoma-virus infection. *J. Nat. Cancer Inst.* **32,** 375 (1964).
28. H. O. Sjogren, Studies on specific transplantation resistance to polyoma-virus induced tumors. II. Mechanisms of resistance induced by polyoma-virus infection. *J. Nat. Cancer Inst.* **32,** 375 (1964).
29. H. O. Sjogren, Studies on specific transplantation resistance to polyoma-virus induced tumors. III. Transplantation resistance to genetically compatible polyoma tumors induced by polyoma tumor homografts. *J. Nat. Cancer Inst.* **32,** 645 (1964).
30. H. O. Sjogren, Studies on specific transplantation resistance to polyoma-virus induced tumors. IV. Stability of the polyoma cell antigen. *J. Nat. Cancer Inst.* **32,** 661 (1964)
31. A. R. Bianco, Immunity against iso-transplants of Rauscher virus-induced leukemia. *Proc. Amer. Ass. Cancer* **5,** 5 (1964).
32. E. J. Breyere et W. P. Jordan, Cross immunity elicited by tumors induced by a leukemia virus, *Proc. Exp. Amer. Ass. Cancer Res.* **5,** 8 (1964).
33. E. A. Mirand, J. T. Grace et J. G. Hoffman, Inhibition of mouse tumor transplants by polyoma virus inoculation. *Virology* **21,** 56 (1963).
34. P. A. Gorer, Antigenic structure of tumors. *Advan. Immunol.* **1,** 345 (1961).
35. P. A. Gorer, M. A. Tuffrey et J. R. Batchelor, Serological studies in the X antigens. *Ann. N.Y. Acaq. Sci.* **101,** 5 (1962).
36. L. J. Old, E. A. Boyse et F. Lilly, Formation of cytoxic antibody against leukemias, induced by Friend virus. *Cancer Res.* **23,** 1063 (1963)
37. B. Slettenmark-Wahren et E. Klein, Cytoxic and neutralization tests with serum and lymph node cells of isologous mice with induced resistance against Gross lymphomas. *Cancer Res.* **22,** 947 (1962).
38. B. Wahren, Cytotoxic assays and other immunologic studies of leukemia induced by Friend virus. *J. Nat. Cancer Inst.* **31,** 411 (1963).

39. L. J. Old, E. A. Boyse et E. Stockert, Antigenic properties of experimental leukemias. I. Serological studies "*in vitro*" with spontaneous and radiation induced leukemias. *J. Nat. Cancer Inst.* **31,** 977 (1963).
40. E. A. Boyse, Immune responses to experimental tumours. *Guy's Hosp. Rep.* **112,** 453 (1963).
41. P. H. Black, W. P. Rowe, H. C. Turner et R. J. Huebner, A specific complement-fixing antigen present in SV40 tumor and transformed cells. *Proc. Nat. Acad. Sci.* **50,** 1148 (1963).
42. J. M. McKenna, R. P. Sanderson et W. S. Blakemore, Studies of the antigens of human tumors. I. Demonstrations of a soluble antigen in HeLa cells and some human tumors. *Cancer Res.* **24,** 754 (1964).
43. J. Charney et L. L. Coriell, Demonstations and purification of soluble HeLa specific antigens. *Fed. Proc.* **22,** 271 (1963).
44. T. Itoh et C. M. Southam, Isoantibodies to human cancer cells in healthy recipients of cancer homotransplants. *J. Immunol.* **91,** 469 (1963).
45. L. A. Zilber, Studies on tumor antigens. *J. Nat. Cancer Inst.* **18,** 341 (1957).
46. C. M. Southam, A. Brunschwig, A. G. Levin et Q. Dizon, Inhibitory effect of autologous leukocytes on autotransplants of human cancer. *Proc. Amer. Ass. Cancer Res.* **5,** 60 (1964)
47. D. A. L. Davies, Occurrence of X antigenic specificity in histocompatibility antigens prepared from mouse leukemia cells. *Brit. J. Exp. Path.* **44,** 546 (1963).
48. M. Hori, R. Citron et B. Sokoloff, Immunization and auto-immunization of Murphy-sturm lymphosarcoma and Flexner rat carcinoma with tumor lipopolysaccharide. *Proc. Amer. Ass. Cancer Res.* **5,** 28 (1964).
49. Y. H. Pilch et A. S. Metchem, Induction of anti-tumor immunity by cell free extract of neoplastic tissue. *Proc. Amer. Ass. Res.* **5,** 51 (1964).
50. L. Gross, Transmission of Ak leukemic agent into new born mice of C57 brown/cd inbred line. *Proc. Soc. Exp. Biol., N.Y.* **86,** 734 (1954).
51. S. E. Stewart, B. E. Eddy et N. Borgese, Neoplasms in mice inoculated with a tumor agent carried in tissue culture. *J. Nat. Cancer. Inst* **20,** 1223 (1958).
52. G. Pietra, K. Spencer et P. Shubik, Response of newly born mice to a chemical carcinogen. *Nature, Lond.* **183,** 1689 (1959).
53. J. F. A. P. Miller, G. A. Grant et F. J. C. Roe, Effect of thymectomy on the induction of skin tumours by 3,4-benzopyrene. *Nature* **199,** 920 (1963).
54. W. L. Simpson, B. Bond, G. Leithauser, I. Yamaguchi et S. Horeglad, Effects of thymectomy on the response of sprague-Dawley rats to a single feeding of 7–12 dimethyl-benzanthracene. *Proc. Amer. Ass. Cancer Res.* **5,** 58 (1964).
55. A. C. Upton, F. F. Wolff, J. Furth et A. W. Kimball, A comparison of the induction of myeloid and lymphoid leukemias in X-radiated RF mice. *Cancer Res.* **18,** 842 (1958).
56. A. R. Page, A. E. Hansen et R. A. Good, Occurrence of leukemia and lymphoma in patients with agammaglobulinemia. *Blood* **21,** 197 (1963).
57. R. D. A. Peterson, M. Blaw et R. A. Good, Ataxia-telangiectasis: a possible clinical counterpart of the animals rendered immunologically incompetent by thymectomy. *J. Pediat.* **63,** 701 (1963).
58. N. Talal et J. J. Bunim, The development of malignant lymphoma in the course of Sjögren's syndrome. *Amer. J. Med.* **36,** 529 (1964).
59. J. Bousser et G. Mathe, Association de cancer épithélial et de lymphomatose *Sem. Hôp. Paris* **14,** 821 (1954).
60. G. M. Bernier, Adult hypogammaglobulinemia. Analysis of 100 cases. *Amer. J. Med.* **36,** 618 (1964).
61. F. J. Dixon, D. W. Talmage et P. H. Maurer, Radiosensitive and radioresistant phases in antibody response. *J. Immunol.* **68,** 693 (1952).
62. N. Gengozian et T. Makinodan, Relation of primary antigen injection to time of irradiation on antibody production in mice. *J. Immunol.* **80,** 189 (1958).
63. W. H. Taliaferro, L. G. Taliaferro et E. F. Janssen, Localization of X-ray injury to initial phases of antibody response. *J. Infec. Dis.* **91,** 105 (1952).
64. M. C. Berenbaum, Effects of carcinogens on immune process. *Brit. Med. Bull.* **20,** 159 (1964).
65. I. Davidsohn, K. Stern et L. Sabet, Immune response in mice and rats exposed to carcinogens. *Proc. Amer. Ass. Cancer Res.* **2,** 102 (1956).

66. R. A. Malmgren, B. E. Bennison et T. W. McKinley, Jr., Reduced antibody titers in mice treated with carcinogenic and cancer chemotherapeutic agents. *Proc. Soc. Exp. Biol., N.Y.* **79,** 484 (1952).
67. R. T. Prehn, Function of depressed immunologic reactivity during carcinogenesis. *J. Nat. Cancer Inst.* **31,** 791 (1963).
68. B. A. Rubin, Carcinogen-induced tolerance to homotransplantation of normal tissue. *Proc. Amer. Ass. Cancer Res.* **3,** 146 (1960).
69. B. A. Rubin et H. A. Kemp, Dependence of antitumor chemotherapeutic effect on host reactivity. *Proc. Amer. Ass. Cancer Res.* **2,** 340 (1958).
70. K. Yasuhira, Damage to the thymus and other lymphoid tissues from 3 methylcholanthrene and subsequent thymoma production in mice. *Cancer Res.* **24,** 558 (1964).
71. J. Borneff et K. Lennert, Experimentelle Untersuchungen über die Urethanwirkung auf die Antitoxinbildung. *Arch. Hyg. (Berl.)* **133,** 285 (1950).
72. F. M. Burnet, Immunological recognition of self. *Science* **133,** 207 (1961).
73. A. Fagraeus et H. Gormsen, Effect of colchicine on circulating antibodies, antibody producing tissues and blood cells in rat. *Acta Path. Microbiol. Scand.* **33,** 421 (1953).
74. C. Forman, J. Seifter et W. E. Ehrich, Effects of salicytates and other drugs on experimental serum disease. *J. Allergy* **20,** 273 (1949).
75. A. A. Hirata et M. Redlich, Effect of colchicine on antibody response in hamsters. *Proc. Soc. Exp. Biol., N.Y.* **109,** 628 (1962).
76. E. Hirschberg, Cancer chemotherapy screening data. XXI. *Cancer Res. Suppl.* **23,** 521 (1963).
77. R. A. Good, R. L. Vernier et R. D. A. Peterson, Decreased antibody-producing capacity in mice during the incubation period of passage A lymphatic leukemia. *J. Lab. Clin. Med.* **62,** 878, (1963).
78. R. D. A. Peterson, R. Hendnickson et R. A. Good, Reduced antibody forming capacity during the incubation period of passage A leukemia in C3H mice. *Proc. Soc. Exp. Biol., N.Y.* **114,** 517 (1963).
79. A. Axelrad et G. Klein, Differences in histocompatibility requirements between primary tumors and their metastases. *Transplant. Bull.* **3,** 100 (1956).
80. I. A. Parfentjew, E. E. Cliffton et F. Duran-Reynals, Alteration of immunological response in malignancy: decline of proteus agglutinin. *Science* **113,** 523 (1951).
81. B. Lytton, L. E. Hughes et A. J. Fulthorpe, Circulating antibody response in malignant disease. *Lancet* **2,** 69 (1964).
82. C. M. Southam, A. E. Moore et C. P. Rhoads, Homotransplantation of human cell lines. *Science* **125,** 158 (1957)
83. A. E. Casey et G. M. Scott, Experimental enhancement of malignancy in the Brown Pearce rabbit tumor. *Proc. Soc. Exp. Biol., N.Y.* **29,** 816 (1932).
84. N. Kaliss, Immunological enhancement of tumor homografts in mice. A review. *Cancer Res.* **18,** 992 (1958).
85. G. D. Snell, Enhancement and inhibition of the growth of tumor homolotransplants by pretreatment of the hosts with various preparations of normal and tumor tissue. *J. Nat. Cancer Inst.* **13,** 719 (1952).
86. G. A. Voisin, Greffes tumorales et facilitation immunologique ("immunological enhancement"). Revue critique. *Rev. Franç. Etud. Clin. Biol.* **8,** 927 (1963).
87. G. Moller, Studies on the mechanism of immunological enhancement of tumor homograft. *J. Nat. Cancer Inst.* **30,** 1153, 1177, 1205 (1963).
88. G. Mathe, J. Dausset, E. Hervet, J. L. Amiel, J. Colombani et G. Brule, Immunological studies in patients with placental chorio-carcinoma. *J. Nat. Cancer Inst.* **33,** 193 (1964).
89. E. Robinson, J. Shulman, N. Benhur, H. Zuckerman et Z. Neuman, Immunological studies and behaviour of husband and foreign homografts in patients with chorioepithelioma. *Lancet* **7276,** 300 (1963).
90. K. Kawamura, Approche immunologique de l'étude de la malignité du cancer (réaction antigène-anticorps après auto-greffe de cancer humain) (en japonais). *Clin. Surg., Tokyo* **18,** 336. (1963).
91. T. Shikata, Y. Uehara, M. Kodama, N. Fujimura, Y. Goto, T. Motosako, Y. Tanaka, A. Yamalata, T. Iwase, S. Sugeta, T. Oka, Y. Nakane et Y. Yasuno, Observations cliniques des autogreffes de cancers humains et effet des agents antitumoraux sur ces greffes (japonais). *Jap. J. Cancer Clin.* **9,** 429 (1963).

92. G. MATHE et J. -L. AMIEL, *La Greffe, Aspects Biologiques et Cliniques.* Masson et Cie, Paris (1963).
93. P. A. GORER et D. B. AMOS, Passive immunity in mice against C57 Bl leukosis E.L.4 by means of iso-immune serum. *Cancer Res.* **16,** 338 (1956).
94. G. MATHE, TRAN BA LOC et J. BERNARD, Effet sur la leucémie L 1210 d'une combinaison par diazotation d'A-méthoptérine et gamma-globulines de hamsters porteurs de cette leucémie par hétérogreffe. *C. R. Acad. Sci., Paris* **246,** 162 (1958).
95. C. W. RIGGS, Treatment of canine neoplasms with auntogeneous vaccinal preparations. *Nature, Lond.* **200,** 233 (1963).
96. D. W. WEISS, L. J. FAULKIN et K. B. DE OME, Acquisition of heightened resistance and susceptibility to spontaneous mouse mammary carcinomas in the original host. *Cancer Res.* **24,** 732 (1964).
97. A. HADDOW et P. ALEXANDER, An immunological method of increasing the sensitivity of primary sarcoma to local irradiation with X-rays. *Lancet* **7731,** 452 (1964).
98. F. SORN, The immunization of leukaemic Ak mice with isologous leukaemic cells incubated in 5-bis-(2-chloroethyl) aminomethyluracil. *Neoplasma* **10,** 217 (1963).
99. J. B. MURPHY, *Monograph of the Rockfeller Institut,* 21 (1924).
100. J. B. GRAHAM et R. M. GRAHAM, The effect of vaccine on cancer patients, *Surg. Gynec. Obstet.* **109,** 131 (1959).
101. H. GOLDNER, A. J. GIRARDI et M. R. HILLEMAN, Attempts to interrupt virus tumorigenesis by immunisation using homologous "Bjorklund-type" antigen. *Proc. Soc. Exp. Biol., N.Y.* **114,** 456 (1963).
102. C. FRIEND, Immunological relationships of a filterable agent causing leukemia in adult mice. I. The neutralization of infectivity by specific antiserum. *J. Exp. Med.* **109,** 217 (1959).
103. M. A. FINK et F. J. RAUSCHER, Immune reactions to a murine leukemia virus. I. Induction of immunity to infection with virus in the natural host. *J. Nat. Cancer Inst.* **32,** 1075 (1964).
104. L. GROSS, *Oncogenic viruses,* Pergamon, New York (1961).
105. G. MATHE, J. -L. AMIEL, L. SCHWARZENBERG, A. CATTAN et M. SCHNEIDER, Chimérisme hématopoïétique chez l'Homme après greffe de moelle osseuse allogénique. Contrôle du syndrome secondaire. Tolérance spécifique liée au chimérisme. *C. R. Acad. Sci., Paris.* **257,** 3527 (1963).
106. G. MATHE, J. -L. AMIEL, L. SCHWARZENBERG, A. CATTAN et M. SCHNEIDER, Hematopoietic chimerism following allogenic bone marrow transplantation in man. Control of secondary syndrome. Specific tolerance associated with the chimerism. *Brit. Med. J.* **2,** 1633 (1963).
107. G. MATHE, J. BERNARD, L. SCHWARZENBERG, M. J. LARRIEU, Cl. LALANNE, A. DUTREIX, P. DENOIX, V. SCHWARZMANN et B. CEOARA, Essai de traitement de sujets atteints de leucémie aiguë en rémission par irradiation suivie de transfusion de moelle osseuse homologue. *Rev. Franç. Etud. Clin. Biol.* **4,** 675 (1959).
108. G. MATHE, J. BERNARD, M. J. DE VRIES, Cl. LALANNE, A. DUTREIX, J. L. AMIEL et J. SURMONT, Nouveaux essais de greffe de moelle osseuse homologue après irradiation totale chez des enfants atteints de leucémie aiguë en rémission. Le problème du syndrome secondaire chez l'Homme. *Rev. Hématol.* **15,** 115, (1960)
109. G. MATHE, H. JAMMET, B. PENDIC, L. SCHWARZENBERG, J. F. DUPLAN, B. MAUPIN, R. LATARJET, M. J. LARRIEU, D. KALIC et Z. DJUKIC, Transfusions et greffes de moelle osseuse homologue chez des humains irradiés à haute dose accidentellement. *Rev. Franç Etud. Clin. Biol.* **4,** 226 (1959).
110. G. MATHE, M. J. DE VRIES, L. SCHWARZENBERG, J. -L. AMIEL, A. CATTAN, M. SCHNEIDER et M. TUBIANA, Etude du syndrome secondaire qui complique la greffe de moelle osseuse allogénique chezl' Homme. *Europ. Cancer J.* à paraître (1965).
111. P. GRABAR, J. COURCON, P. L. T. ILBERY, J. F. LOUTIT et J. P. MERRILL, Etude immuno-électrophorétique de souris irradiées par des doses létales de rayons X et protégées par des cellules de moelle osseuse de rats. *C. R. Acad. Sci., Paris* **245,** 950 (1957).
112. D. W. H. BARNES et J. F. LOUTIT, Treatment of murine leukaemia with X-rays and homologous bone marrow. *Brit. J. Haematol.* **3,** 241, (1957).

113. G. MATHE, J. -L. AMIEL et J. NIEMETZ, Greffe de moelle osseuse après irradiation totale chez des souris leucémiques, suivie de l'administration d'un antimitotique pour réduire la fréquence du syndrome secondaire et ajouter à l'effet anti-leucémique. *C. R. Acad. Sci., Paris* **254,** 3603 (1962).
114. G. MATHE et J. BERNARD, Essais de traitement de la leucémie greffée, L. 1210, par l'irradiation X suivie de transfusions de cellules hématopoïétiques normales (isologues ou homologues, myéloïdes ou lymphoïdes, adultes ou embryonnaires). *Rev. Franç. Etud. Clin. Biol.* **4,** 442 (1959).
115. M. J. DE VRIES et O. Vos, Treatment of mouse lymphosarcoma by total body irradiation and by injection of bone marrow and lymph node cell. *J. Nat. Cancer Inst.* **21,** 1117 (1958).
116. M. F. A. WOODRUFF, M. O. SYMES et N. F. ANDERSON, The effect of intra-peritoneal injection of thoracic duct lymphocytes from normal and immunized rats in mice inoculated with the Landschutz ascites tumor. *Brit. J. Cancer* **17,** 482 (1963).
117. M. F. A. WOODRUFF, M. O. SYMES et A. E. STUART, The effect of rat spleen cells on two transplanted mouse tumours. *Brit. J. Cancer* **17,** 320 (1963).
118. G. MATHE, J. -L. AMIEL et J. BERNARD, Traitement de souris AkR à l'âge de 6 mois par irradiation totale suivie de transfusions de cellules hématopoïétiques. Incidences respectives de la leucémie et du syndrome secondaire. *Bull. Cancer* **47,** 331 (1960)
119. G. MATHE et J. BERNARD, Essai de traitement par l'irradiation X suivie de greffe de cellules myéloides homologues de souris atteintes de leucémie spontanée très avancée. *Bull. Cancer* **45,** 289 (1958)
120. G. MATHE, J. -L. AMIEL et C. FRIEND, Essai de traitement de la leucémie de Charlotte Friend par la greffe de cellules hématopoïétiques de donneurs isogéniques vaccinés contre le virus. *Bull. Cancer* **49,** 416 (1962).
121. J. -L. AMIEL et G. MATHE, A comparison of the sensitivity to storage at 37°C in Tyrode Solution of immunologically competent cells and of myeloid cells. *Nature, Lond.* **200,** 1224 (1963).
122. G. MATHE, J. -L. AMIEL, L. SCHWARZENBERG et A. M. MERY, A method of reducing the incidence of the secondary syndrome in allogenic marrow transplantation. *Blood* **22,** 44 (1963).
123. G. MATHE, J. -L. AMIEL, M. MATSUKURA et A. M. MERY, Restauration hématopoïétique de souris irradiées par greffe de moelle osseuse allogénique de plusieurs donneurs de diverses lignées. *C. R. Acad. Sci., Paris* **225,** 3480 (1962).
124. M. F. A. WOODRUFF et B. NOLAN, Preliminary observations on treatment of advanced cancer by injection of allogenic spleen cells. *Lancet* **2,** 426 (1963).

Europ. J. Cancer Vol. 1, pp. 15–22. Pergamon Press 1965. Printed in Great Britain

Quantitative Aspects of the Storage of Bone Marrow Cells for Transplantation*

L. M. VAN PUTTEN

Radiobiological Institute TNO
Lange Kleiweg 151, *Rijswijk Z.H., Netherlands*

THE DATA on the storage of human bone marrow cells for transplantation have so far only provided suggestive evidence for the effectiveness of the storage methods used [1–6]. In experimental animal studies a wide choice of storage techniques has been demonstrated as qualitatively effective. (See the review by Phan The Tran and Bender [7]). Often a quantitative evaluation of a proposed storage technique has not been presented or a preference was based on the observation of better survival of irradiated animals after the injection of cells stored by one method than by another. Dependable evaluation is only possible from dose-effect curves and knowledge of the reproducibility of such curves [8–10]. Indirect methods for the evaluation of the repopulating capacity of haemopoietic cells have been widely used [9, 11–13]. These are useful as guides for choosing preservation methods but cannot be depended upon for direct application without confirmation by studies of the actual repopulating potential [8, 10, 12].

In an earlier report [14] on the quantitative analysis of storage effectiveness it was shown that the method giving best results for mouse bone marrow storage was completely ineffective in the Rhesus monkey. These negative results — which were obtained although every effort was made to simulate in the mice the conditions prevailing in primate studies where the aspiration of bone marrow from a living donor causes admixture of blood and heparin with the bone marrow cells — led to the conclusion that there are marked species differences in the effectiveness of bone marrow storage.

The present report describes further variations in mouse bone marrow and foetal liver cell storage techniques, in which use was made of polyvinylpyrrolidone, either alone or in a combination with glycerol or dimethylsulphoxide as additives during freezing. The methods showing best results were again tested in the monkey and the combination of glycerol and polyvinylpyrrolidone gave satisfactory results for monkey bone marrow storage. In contrast, some methods used for clinical bone marrow transplantation were found ineffective.

MATERIAL AND METHODS

The irradiation of mice and the methods for preparing mouse bone marrow cell suspensions as well as the system for testing the effectiveness of storage methods have been described previously [14]. Foetal liver cell suspensions were prepared from C57BL or (C57BL × CBA)F_1 hybrid foetuses between the fourteenth and seventeenth day of pregnancy. Twofold serial dilutions of fresh and stored cell suspensions were injected into groups of (C57BL × CBA)F_1 hybrid mice that had been irradiated with a supralethal dose of 910 r (875 rad). Survival at 30 days after irradiation was the endpoint. An equal volume of heparinized mouse blood was added to the mouse bone marrow cell suspensions to simulate the conditions encountered in the storage of autologous bone marrow in monkeys and man. The

*Part of this work was performed under contract with Euratom (European Atomic Energy Community) 51-53 rue Belliard, Brussels, Belgium.

addition of protective agents, glycerol, dimethylsulphoxide (DMSO) or polyvinylpyrrolidone (PVP) (Subtosan, Specia M.W. 37,000.) was performed by adding to the blood-bone marrow cell suspension an equal volume of a solution containing the protective agent in Tyrode's solution. Before these substances were added, in each case a portion of the suspension was set apart for testing the therapeutic capacity of the fresh cells. During freezing, cell concentrations for mouse bone marrow were 12 $\times$ 10^6/ml and for foetal liver 12–48 $\times$ 10^6/ml.

A standard method of freezing was used in all experiments: glass ampoules of a capacity of 2 or 5 ml were cooled in a slow freezing apparatus together with a similar ampoule in which a thermocouple was immersed in the suspending medium. Thus the temperature of the suspension was recorded at each freezing session. The temperature was lowered by 1°C/min from +20° to about −40°C. Thereafter the ampoules were rapidly cooled and stored in liquid air at −196°C. The storage times varied from 1 week to 3 months. Within the range tested in these experiments no influence of storage time upon recovery was noted. Immediately before use the ampoules were brought from liquid air into a waterbath kept at 38°C, where they were shaken to thaw the frozen solution as rapidly as possible. After thawing, the suspensions were handled in one of three different ways:

1. They were step-wise slowly diluted [15];
2. They were rapidly diluted 10 min after addition of hypertonic glucose [16];
3. They were used without dilution or, if necessary for adjustment of the cell dose, quickly diluted with Tyrode's solution without special precautions. The suspensions were then injected as soon as possible into irradiated hosts in volumes containing the desired number of bone marrow cells as calculated from the pre-freezing counts. The injections were made 1–6 hr after irradiation. No cell counts were made on the stored suspensions.

Primate studies were performed in young Rhesus monkeys (Macaca mulatta), weighing 2–4 kg. Autologous marrow was obtained before irradiation by puncture and aspiration of the femoral shaft through the knee-joint. The irradiation and post-irradiation treatment of the monkeys was similar to that described previously [17]. The duration of monkey marrow storage varied between 1 and 3 months. Supralethal radiation doses of 800–900 r equal to 770–865 rad, were given to preclude spontaneous haemopoietic recovery as an alternative to repopulation from the injected material. In earlier studies it was found that after irradiation with 600 r (575 rad) no indication of recovery of the peripheral blood cell counts occurred before the twentieth day in animals that had been kept alive with platelet transfusions. The highest radiation dose after which a single control animal survived with symptomatic treatment alone was 650 r (624 rad). No blood or platelet transfusions were given in the present study.

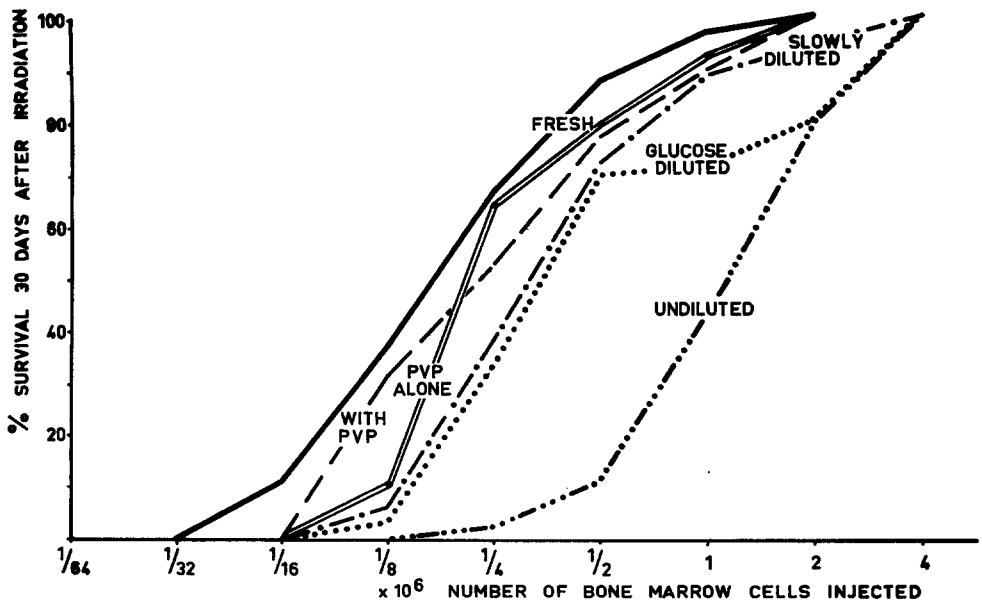


Fig. 1. Dose-effect-curves of fresh and stored C57BL *bone marrow cells in supralethally irradiated* (C57BL $\times$ CBA)F_1 *hybrids. Storage with glycerol.*

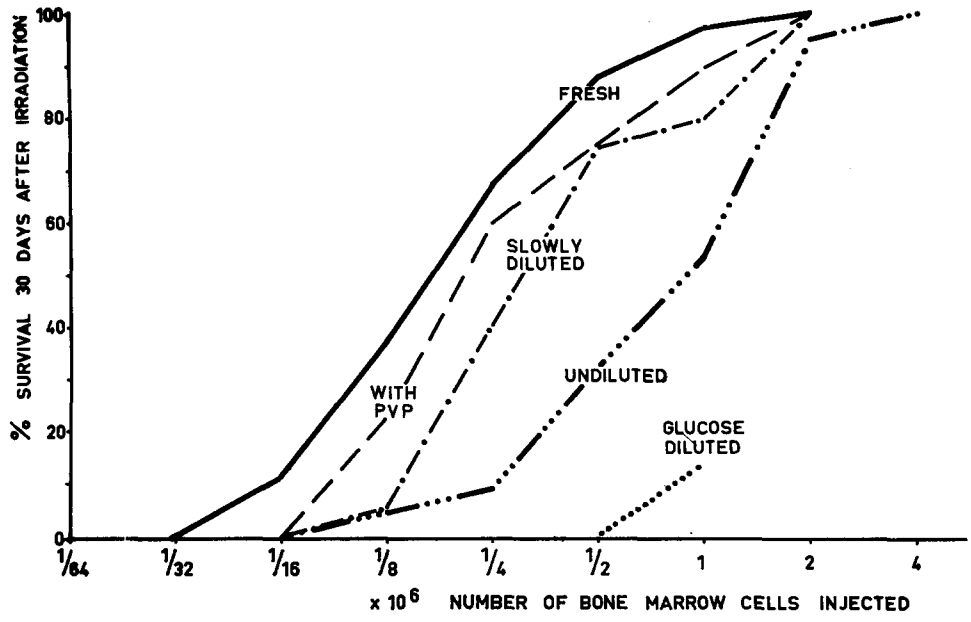


Fig. 2. Dose-effect-curves of fresh and stored C57BL *bone marrow cells in supralethally irradiated* (C57BL × CBA)F_1 *hybrids. Storage with* DMSO.

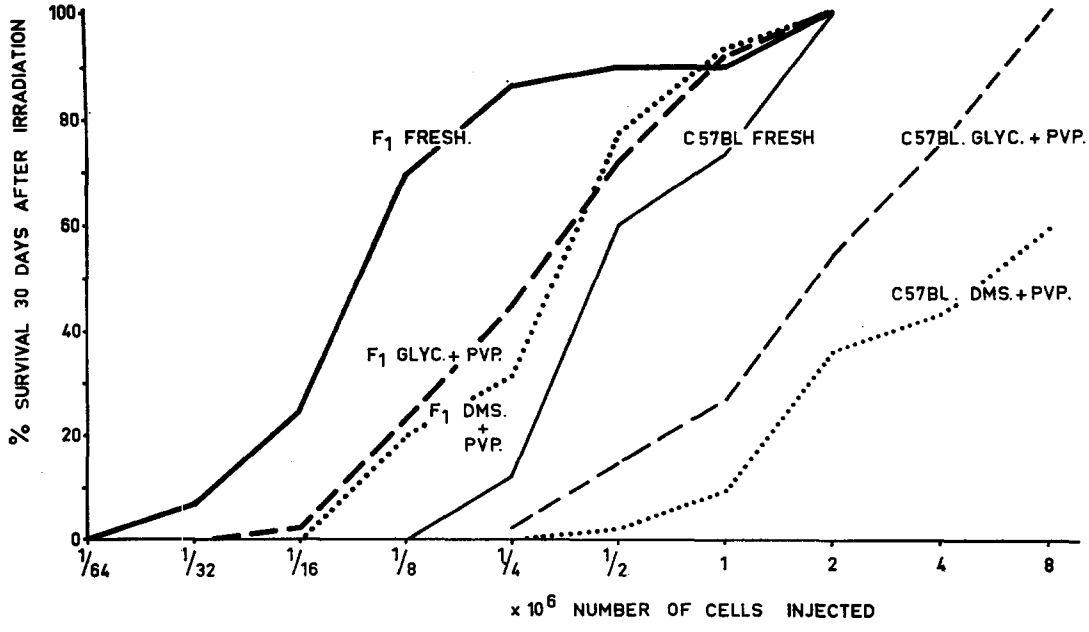


Fig. 3. Dose-effect-curves of fresh and stored C57BL *and isologous foetal liver cells in supralethally irradiated* (C57BL × CBA)F_1 *hybrids.*

RESULTS

Studies in mice

The survival of hybrid mice injected with the parental cell suspensions are plotted in Figs 1 and 2 for bone marrow and in Fig. 3 for foetal liver cells. The relative effectiveness of the various storage procedures and the statistical accuracy of the data are presented in Table 1. As was noted previously [14] there is a marked influence of the dilution technique used after thawing on the ultimate efficiency of the suspensions. A major improvement seems the combination of PVP with glycerol or DMSO. Not only does the addition of PVP make the precautions in the dilution technique unnecessary, it also improves average recovery over that obtained with optimal dilution methods without this substance. The observation of Persidsky and Richards [18] that PVP alone gave good protection, prompted us to use PVP alone. In mice it evidently gives similar protection as the PVP–glycerol mixture. Figure 4 shows that in isologous combinations 100 per cent effectiveness is obtained and obviously further improvements are unnecessary.

Table 1. Effectiveness cell of suspensions after different storage and dilution procedures.

Cell suspension	Additives and dilution procedure* after thawing	Number of mice used	ED_{50}† and 95% confidence limits (10^5 cells)	Effectiveness of stored suspensions (% of fresh material)
C57BL bone marrow	fresh	705	1·76 (1·58– 1·95)	—
	15% Glyc. S	285	3·51 (3·03– 4·07)	50
	15% Glyc. G	179	4·71 (3·65– 6·07)	37
	15% Glyc. U	168	11·01 (8·14–14·88)	16
	10% Glyc., 10% PVP U	324	2·46 (2·09– 2·90)	72
	15% DMSO S	275	3·52 (2·99– 4·15)	50
	15% DMSO G	101	>20	<10
	15% DMSO U	189	7·38 (6·16– 8·85)	24
	10% DMSO, 10% PVP U	130	2·50 (2·00– 3·05)	71
	10% PVP U	277	2·54 (2·16– 2·98)	69
Isologous (F_1) bone marrow	fresh	431	0·38 (0·32– 0·45)	—
	10% Glyc., 10% PVP U	341	0·44 (0·37– 0·52)	87
	10% PVP U	169	0·38 (0·31– 0·46)	101
C57BL foetal liver	fresh	201	5·16 (4·31– 6·17)	—
	10% Glyc., 10% PVP U	219	16·94 (12·7 -21·0)	30
	10% DMSO, 10% PVP U	208	>40	<13
Isologous (F_1) foetal liver	fresh	297	1·11 (0·93– 1·33)	—
	10% Glyc., 10% PVP U	268	2·79 (2·38– 3·27)	40
	10% DMSO, 10%PVP U	179	3·04 (2·49– 3·54)	37

*S=slow dilution according to Drasil [15].
G=glucose dilution according to Sloviter [16].
U=undiluted or rapid dilution with Tyrode's without special precautions.
†ED_{50}=cell number which produced 50% survival in lethally irradiated female (C57BL × CBA)F_1 hybrid mice.

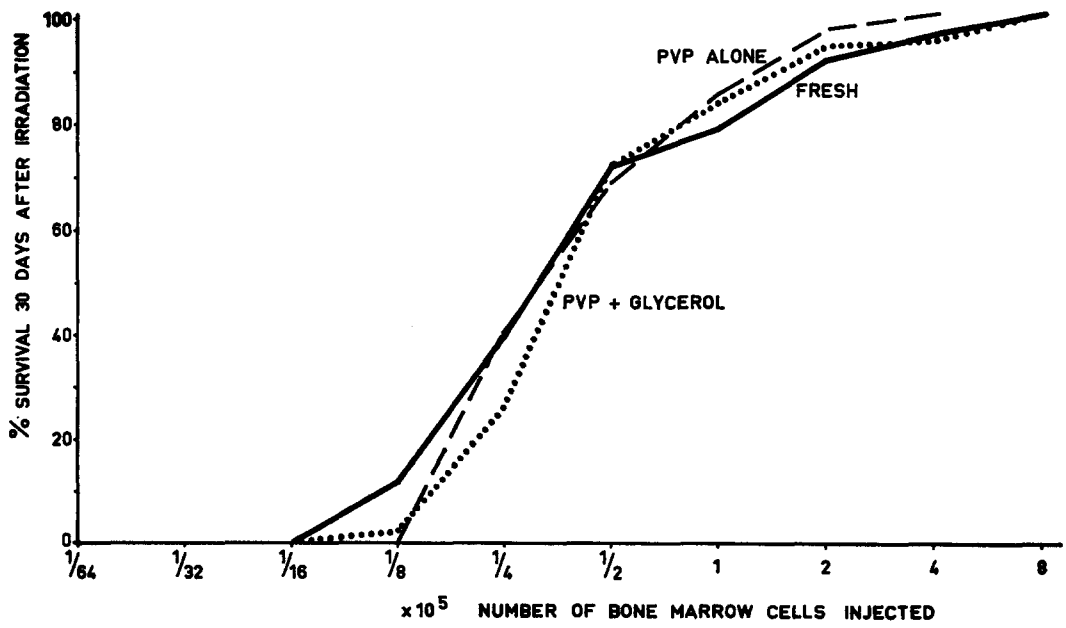


Fig. 4. Dose-effect-curves of fresh and stored isologous bone marrow cells in supralethally irradiated (C57BL × CBA)F_1 *hybrids.*

From Fig. 3 it is evident that storage efficiency for foetal haemopoietic cells is lower than for adult bone marrow cells. The curve for stored C57BL foetal liver in Fig. 3 has a distinctly lower slope than the others. This may have been caused by toxic effects of the large amounts of PVP and protective agents injected; at the highest cell dosages 30–40 mg pf PVP with the same amount of glycerol or DMSO were injected intravenously in mice weighing about 20 g.

Studies in monkeys

The 30-day survival of individual monkeys injected with fresh or stored autologous bone marrow suspensions after supralethal irradiation has been plotted in Fig. 5. The minimal effective number of fresh bone marrow cells is about $0{\cdot}4 \times 10^8$ per kg body weight. Monkey bone marrow cell suspensions stored with 15 per cent glycerol or 15 per cent DMSO that were kept for 5 min at room temperature after thawing, or diluted by the slow dilution or glucose dilution techniques showed appearance of a sticky viscous material in the suspension, presumably DNA, as has been described for thawed human marrow suspensions [13]. No attempts were made to inject these viscous suspensions in monkeys. If similar suspensions were not diluted but injected immediately after rapid thawing, the irradiated monkeys failed to show haemopoietic recovery and died with aplasia (Fig. 5). These methods which are widely used for storing human bone marrow, were thus completely ineffective in monkeys. The use of 10% PVP alone or in combination with DMSO resulted in the survival of some monkeys but frequent unexplained failures made these methods less dependable. A regular good protection was obtained with the use of a mixture of 10% PVP with 10% glycerol, although the storage efficiency of about 50 per cent is somewhat lower than for mouse bone marrow cells.

DISCUSSION

These studies confirm the usefulness of quantitative comparison of freeze storage techniques. Only in this way the best technique of storage, both for mice and monkeys, could be established. The test system that was used initially in mice: C57BL bone marrow injected into irradiated (C57BL × CBA)F_1 hybrids seems to differ from other parent→F_1 systems. More C57BL cells are needed for successful transplantation than in other P→F_1 combinations [19, 20] or even more than in homologous transplantations of C57BL cells [21]. The cause for this anomalous behaviour is not clear at present. Nevertheless, the results seem to indicate that the P→F_1 system reacts similarly to the isologous combination, although these show a slightly higher storage efficiency.

Since the report by Bender *et al.* [22] that 7% PVP gave no protection during freezing and the routine use of 3% PVP in procedures for testing other protecting agents [7], the good

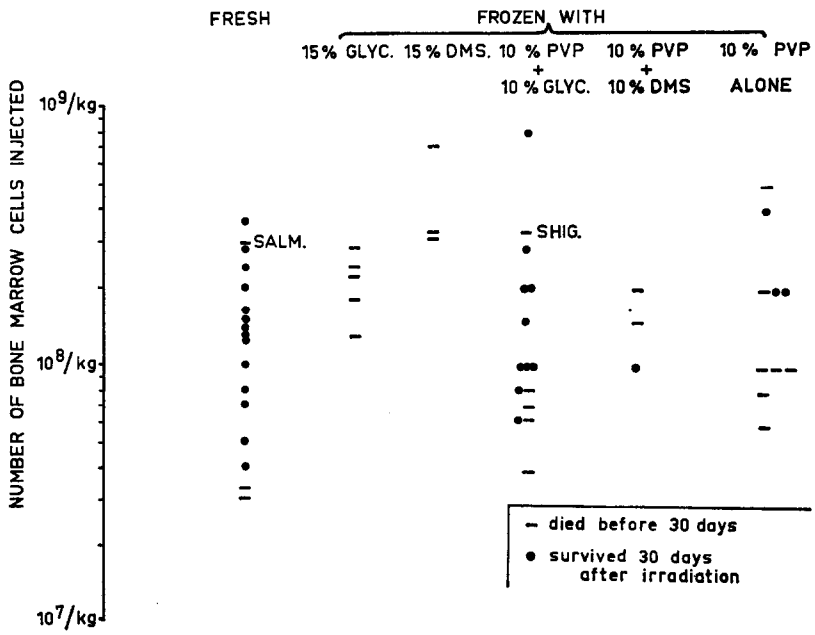


Fig. 5. Diagram showing the fate of supralethally irradiated monkeys injected with different numbers of autologous bone marrow cells. Fresh suspensions are compared with suspensions frozen with different additives.

protection with PVP alone as reported by Persidsky and Richards, was unexpected. Even more surprising are the present quantitative results which show PVP to be one of the best agents for protecting mouse bone marrow cells during freezing. Differences in the molecular weight of the PVP preparations used may perhaps explain these discrepancies.

Evidently, marked differences exist between the species regarding the ease with which their cells may be recovered after freezing. These differences are not only quantitative but also qualitative, notably the 10% PVP addition which ranks among the best methods in the mouse, seems to be far less effective in the monkey. Although many aspects of bone marrow transplantation in monkeys and man seem to be similar, this finding makes it impossible to recommend with certainty any technique as suitable for storage of human bone marrow.

A pertinent suggestion from these studies would be to test the glycerol–PVP mixture in the clinic since untoward effects are unlikely and infusion of limited quantities of PVP in man has been well tolerated.

One other important conclusion may be drawn from these data. Whatever number of cells is recommended for effective autologous or isologous transplantation in man, it seems necessary to double this number if stored cells are to be used, since a 50 per cent loss of effectiveness is to be expected.

RÉSUMÉ

Une étude quantitative de la conservation de la moelle osseuse de souris et de singe à basses températures a été fait afin de comparer l'efficacité de différentes méthodes. Le critère d'efficacité était la possibilité pas des suspensions de cellules médullaires de faire survivre des souris irradiées à doses léthales.

Les résultats suivants ont été obtenus:

1: *La meilleure protection des suspensions de cellules médullaires de souris a été observée par l'adjonction de* 10% *de polyvinylpyrrolidone (PVP) ou par les combinaisons de* 10% *de PVP avec* 10% *de glycérol et de* 10% *de PVP avec* 10% *de diméthylsulfoxide (DMSO). Pour les transplantations isologues de moelle osseuse, l'efficacité maxima, après congélation, était* 100%*; pour les cellules hépatiques foetales* 40%.

2: *Pour la conservation de la moelle osseuse autologue de singe, l'addition de glycérol ou de DMSO était sans effet. Une protection assez faible a été observée par l'addition de* 10% *de PVP et par la combinaison de* 10% *de PVP et* 10% *de DMSO. La combinaison de* 10% *de PVP et* 10% *de glycérol donnait, au contraire, une bonne protection jusqu' à* 50% *d'efficacité.*

Ainsi il y a des variations importantes, selon l'espèce, dans la technique optimale de conservation de moelle osseuse. Bien qu'il soit impossible de formuler des indications précises quant à la conservation de la moelle osseuse humaine, il semble que l'application de la combinaison PVP et glycérol mérite une étude clinique.

SUMMARY

Quantitative studies are presented of the effect of different storage techniques on mouse and monkey bone marrow cell suspensions. The ability to produce 30*-day survival in lethally irradiated hosts was used as an endpoint.*

The following results were obtained:

1: *Optimal protection of mouse bone marrow cell suspensions was found after addition of* 10% *PVP (polyvinylpyrrolidone) and with the combinations* 10% *PVP,* 10% *glycerol and* 10% *PVP,* 10% *DMSO (dimethylsulphoxide). For isologous mouse bone marrow optimal storage efficiency approximates* 100%*, for foetal mouse liver cells* 40%.

2: *For autologous monkey bone marrow storage methods based on the addition of glycerol or DMSO were found to be ineffective. Poor protection was afforded by addition of* 10% *PVP alone or by a* 10% *PVP,* 10% *DMSO mixture. Optimal preservation during storage (up to* 50 *per cent effectiveness) was found for monkey bone marrow with a* 10% *glycerol,* 10% *PVP mixture.*

It is concluded that although species differences in storage effectiveness preclude dependable recommendations for storage of human bone marrow, a careful trial of the glycerol–PVP combination in man seems indicated.

ZUSAMMENFASSUNG

Verschiedene Methoden zur Aufbewahrung von Knochenmarkzellen von Mäusen und Affen bei niedrigen Temperaturen wurden vergleichungsweise quantitativ geprüft. Das Kriterium des erfolgreichen Aufbewahrens war die Fähigkeit der Zellsuspensionen, letal bestrahlte Tiere ins Leben zu erhalten. Für Mäusezellen wurde eine optimale Konservierung nach Zusatz folgender schützenden Substanzen gefunden: 10% *Polyvinylpyrrolidon (PVP) oder eine Mischung von* 10% *PVP mit* 10% *Glyzerin oder* 10% *PVP mit* 10% *Dimethylsulfoxyd (DMSO). Die maximale Aufbewahrungseffizienz für isologe Knochenmarkzellen war* 100%, *für foetale Leberzellen nur* 40%.

Zusatz von ausschliesslich Glyzerin oder DMSO erwies sich für autologes Knochenmark bei Affen als unwirksam. Eine schwache Schutzwirkung wurde gefunden nach einfrieren der Zellen mit 10% *PVP oder mit* 10% *PVP und* 10% *DMSO. Optimale Effizienz bei der Aufbewahrung (bis* 50% *Wirksamkeit) wurde beobachtet bei Suspensionen denen eine Mischung von* 10% *PVP mit* 10% *Glyzerin zugefügt war.*

Diese Befunde weisen auf Unterschiede zwischen den optimalen Aufbewahrungsmethoden für verschiedene Tierarten. Eine Empfehlung einer optimalen Aufbewahrungsmethode menschlicher Knochenmarkzellen ist demnach nicht möglich. Trotzdem ist eine behutsame klinische Prüfung der Brauchbarkeit von der PVP — Glyzerin — Misschung als Konservierungsmethode für menschliche Zellen durchzuführen.

REFERENCES

1. P. Clifford, R. A. Clift and J. K. Duff, Nitrogen–mustard therapy combined with autologous marrow infusion. *Lancet* **1,** 687 (1961).
2. N. B. Kurnick, Autologous and isologous bone marrow storage and infusion in the treatment of myelo suppression. *Transfusion* **2,** 178 (1962).
3. J. J. McGovern, Jr., P. S. Russell, L. Atkins and E. W. Webster, Treatment of terminal leukemic relapse by total-body X-irradiation and intravenous infusion of stored autologous bone marrow obtained during remission. *New Engl. J. Med.* **260,** 675 (1959).
4. D. E. Pegg, J. G. Humble and K. A. Newton, The clinical application of bone marrow grafting. *Brit. J. Cancer* **16,** 417 (1962).
5. E. D. Thomas, H. L. Lochte, Jr., J. H. Cannon, O. D. Sahler and J. W. Ferrebee, Supralethal whole body irradiation and isologous marrow transplantation in man. *J. Clin. Invest.* **38,** 1709 (1959).
6. E. D. Thomas and J. W. Ferrebee, Prolonged storage of marrow and its use in the treatment of radiation injury. *Transfusion* **2,** 115 (1962).
7. Phan The Tran and M. A. Bender, La conservation de la cellule vivante. Applications à la moelle osseuse. *Rev. Franç. Étud. Clin. Biol.* **5,** 1020 (1960).
8. A. J. S. Davies, J. H. L. Playfair and A. M. Cross, Estimation *in vivo* of the viability of frozen and stored bone marrow. *Transplanation* **2,** 261 (1964).
9. A. Lengerova and J. Abraham, Preservation of haematopoietic tissues for the therapy of radiation disease and an indirect test of their viability. *Folia Biol. (Praha)* **6,** 16 (1960).
10. J. P. Lewis and F. E. Trobaugh, Jr., The assay of the transplantation potential of fresh and stored bone marrow by two *in vivo* systems. *Ann. N.Y. Acad. Sci.* **114,** 677 (1964).
11. L. F. Bélanger, R. K. Smiley and J. Martin-Villar, A method for the estimation of *in vitro* survival of human marrow cells. Utilizing radio-autographic detection of H^3-thymidine uptake. *Canad. Med. Ass. J.* **85,** 868 (1961).
12. J. P. Lewis, M. P. Farnes, M. Albala and F. E. Trobaugh, Jr., Transplantation potential of fresh and stored hematopoietic tissue as estimated by standard *in vitro* techniques. *Ann. N.Y. Acad. Sci.* **114,** 701 (1964).
13. H. L. Lochte, J. W. Ferrebee and E. D. Thomas, *In vitro* studies on the preser-

vation of marrow cells in glycerol at low temperatures. *J. Lab. Clin. Med.* **53,** 117 (1959).
14. L. M. VAN PUTTEN, The effectiveness of different freeze storage techniques for mouse bone-marrow-cell suspensions. *Ann. N.Y. Acad. Sci.* **114,** 695 (1964).
15. V. DRASIL, Effect of radiation on bone marrow cells at various temperatures *in vitro*. *Folia Biol. (Praha)* **6,** 359 (1960).
16. H. A. SLOVITER and R. M. TIETZE, A method for preparing thawed erythrocyte–glycerol mixtures for transfusion. *Amer. J. Med. Sci.* **231,** 437 (1956).
17. B. G. CROUCH, L. M. VAN PUTTEN, D. W. VAN BEKKUM and M. J. DE VRIES, Treatment of total-body X-irradiated monkeys with autologous and homologous bone marrow. *J. Nat. Cancer Inst.* **27,** 53 (1961).
18. M. D. PERSIDSKY and V. RICHARDS, Radiation protection of mice with bone marrow and spleen preserved at low temperature using polyvinylpyrrolidone. *Blood* **23,** 337 (1964).
19. R. A. POPP, Regression of grafted bone marrow in homologous irradiated mouse chimeras. *J. Nat. Cancer Inst.* **26,** 629 (1961).
20. R. A. POPP, G. E. COSGROVE and D. M. POPP, Relative ability of parental marrows to repopulate lethally irradiated F_1 hybrids. *Ann. N.Y. Acad. Sci.* **114,** 538 (1964).
21. E. A. MCCULLOCH and J. E. TILL, Repression of colony-forming ability of C57BL hematopoietic cells transplanted into non-isologous hosts. *J. Comp. Physiol.* **61,** 301 (1962).
22. M. A. BENDER, PHAN THE TRAN and L. H. SMITH, Preservation of viable bone marrow cells by freezing. *J. Appl. Physiol.* **15,** 520 (1960).

Europ. J. Cancer Vol. 1, pp. 23–32. Pergamon Press 1965. Printed in Great Britain

Studies on the Anti-Tumour Activity of Aliphatic Aldehydes — I

The Mechanism of Inhibition of Amino Acid Incorporation into Protein of Yoshida Ascites Hepatoma Cells

G. G. GUIDOTTI, L. LORETI and E. CIARANFI

Institute of General Pathology and C.N.R. Centre for Research in Cell Pathology, University of Milano, Italy

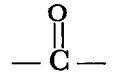
INTRODUCTION

EARLIER work in this Laboratory has established that DL-glyceraldehyde, whose L-isomer is known to inhibit glycolysis [1, 2], depresses amino acid incorporation into protein of normal and neoplastic tissues [3–5].

This effect has been confirmed by Drews *et al.* [6], who reported the dependence of the impairment of protein synthesis upon the inhibition of glycolysis. At variance, our results suggested that glyceraldehyde inhibits protein synthesis by mechanisms different from those involved in the inhibition of glucose metabolism [5].

The present work was undertaken to clarify the specific mechanism by which glyceraldehyde and other aldehydes interfere with protein metabolism.

Evidence will be presented that an inhibition of protein synthesis *in vitro* can be obtained with a number of aliphatic aldehydes* and that this inhibition depends on the presence of a reactive



group in the molecule. The condensation of this group with cysteine to form thiazolidine carboxylic acids and the consequent cell depletion of this SH-amino acid are likely to account, at least in part, for the inhibitory effects displayed by aliphatic aldehydes on protein synthesis in Yoshida ascites hepatomas.

*Formaldehyde, which is known to participate in an exceedingly large number of reactions with proteins, nucleic acids, etc. was not considered in the present study.

METHODS

Preparation of tissues

Yoshida ascites hepatoma cells (AH-130), harvested from tumour-adapted Wistar albino rats, were washed and suspended in phosphate or bicarbonate Ringer as described previously [5].

Incubation procedure

Incubations were performed in conventional Warburg apparatuses or in Dubnoff metabolic shakers, at 38°C in an atmosphere of air (aerobic conditions) or of 95% N_2: 5% CO_2 (anaerobic conditions). The basic incubation media were Krebs-Ringer phosphate [7] for aerobic and Krebs-Ringer bicarbonate [7] for anaerobic experiments. In routine experiments, the main compartment of the Warburg flasks contained 2·2 ml of medium, 0·2 ml of inhibitor (or substrate) solution and 0·3 ml of cell suspension (8–12 mg of dry tissue). The side arms of the flasks contained DL-leucine-1-C^{14} (0·25–0·5 μmoles; specific activity 3·8 mc/mmole) dissolved in 0·1 ml of water and, occasionally, 0·2

ml of substrate (or inhibitor) solution. When present, glucose was 15 mM (final). The content of the side arms was tipped into the main compartment after suitable equilibration periods. When oxygen consumption was measured, 0·2 ml of 20% KOH was present on a roll of filter paper in the central well. Inhibitors or substrates tested (aliphatic aldehydes, aldoses, amino acids, thiazolidine carboxylic acids) were added at different concentrations between 1 and 10 mM (final). The osmolarity of the incubation medium was always adjusted to 0·30–0·31.

Oxygen consumption and anaerobic glycolysis were measured by standard manometric Warburg procedures [7]. At the end of the incubation period, trichloroacetic acid was added (1%, final concentration) to stop the reaction and to precipitate proteins. The sedimented proteins were then purified [8], plated and counted beneath a mica-window Geiger-Mueller counter. The counting error was less then 2 per cent. Departures from this procedure will be described in the accompanying figures and tables.

Condensation of aldehydes with cysteine

The reaction of aldehydes with cysteine was followed in four ways:

a. Potentiometric measurements of pH changes occurring in neutral solutions of cysteine (50 mM) when neutral solutions of aldehydes (50 mM) were added under N_2 atmosphere (to prevent cysteine oxidation) and temperature strictly controlled (30°C);
b. Manometric measurements of CO_2 liberation from neutral solutions of cysteine (5 mM, final) in bicarbonate buffer to which neutral solutions of aldehyde (5 mM, final) were added (or vice-versa) by tipping the content of the side arm (0·2 ml) into the main compartment (2·8 ml) of Warburg flasks, previously equilibrated at 37°C with 95% N_2: 5% CO_2;
c. Measurement of the disappearance of free SH-groups in a mixture containing phosphate buffer (pH 7·2) and stechiometric amounts of aldehydes and cysteine (or other SH group-bearing molecules), incubated at 37°C in atmosphere of N_2. SH-groups were assayed by a nitroprusside method [9];
d. Chromatographic detection of the products resulting from the above condensation reaction. Descending chromatograms were run on Whatman No. 1 paper in a *n*-butanol, acetic acid, water system (4: 1: 5). Cysteine and its condensation products with aldehydes were located with ninhydrin; aldehydes were detected with aniline phthalate; labelled compounds were traced by radioautography of chromatograms.

Chemicals

Aldehydes were obtained as follows: acetaldehyde from Merck, Darmstadt, Germany; glyoxylic acid and DL-glyceraldehyde-3-phosphate (diethylacetal) from Sigma Chemical Co. St. Louis, U.S.A.; propionaldehyde, *n*-butyraldehyde, *iso*-butyraldehyde and crotonaldehyde from B.D.H., Poole, England; DL- and D-glyceraldehyde from Mann Research Lab., New York, U.S.A.; β-hydroxybutyraldehyde from Hopkin & Williams, Chadwell Heath, England; glycolaldehyde, glyoxal, lactaldehyde, L-glyceraldehyde, DL-*erythro, threo*-α, β-dihydroxybutyraldehyde, L-*erythro*-α,β-dihydroxybutyraldehyde, D-*threo*-α, β-dihydroxybutyraldehyde, 2-dihydroxyethyl-thiazolidine-4-carboxylic acid and 2-(1′,2′-dihydroxypropyl)-thiazolidine-4-carboxylic acid from Laboratori Farmitalia, Milano, Italy.

L-, D- and DL-Cysteine (hydrochlorides) and reduced glutathione were purchased from Mann Research Lab., New York, U.S.A. Other amino acids and aldoses were obtained from B.D.H., Poole, England or from Sigma Chemical Co. St. Louis, U.S.A.

DL-Leucine-1-C^{14} was purchased from C.E.A., Gif-sur-Yvette, France. DL-*erythro,threo*-α, β-dihydroxybutyraldehyde-U-C^{14} has been synthesized from $BaC^{14}O_3$ (EURATOM Contract No. 046-64-1 RISI). 2-(1′2′-dihydroxypropyl)-thiazolidine-4-carboxylic acid labelled with C^{14} was synthesized from DL-*erythro, threo*-α, β-dihydroxybutyraldehyde-U-C^{14} and unlabelled L-cysteine.

RESULTS

Metabolic effects of aliphatic aldehydes

The results presented in Table 1 show the effects of a number of 2-, 3- and 4-carbon aliphatic aldehydes on oxygen consumption, glycolysis and amino acid incorporation into protein of Yoshida ascites hepatoma cells.

All the saturated aldehydes tested displayed a marked inhibitory effect on aerobic incorporation of labelled leucine into protein at a concentration which had little effect on oxygen consumption. An exception was glyoxylic acid which is known to depress tissue respiration acting at the aconitase level [10]. As previously

Table 1. Effects of aliphatic aldehydes on oxygen consumption, glycolysis and incorporation of leucine-1-C^{14} *into protein of Yoshida ascites hepatoma cells.*

Cells (8–12 mg, *dry weight) were incubated at 38°C for 60* min *in Krebs Ringer phosphate (aerobic) or Krebs Ringer bicarbonate plus glucose 15* mM *(anaerobic conditions) containing 0·5* μmoles *of* DL-*leucine-1-*C^{14} *(s.a. 3·8* mc/m mole) *Medium volume, 3* ml. *Gas phases: air (aerobic) and 95%* N_2*: 5%* CO_2 *(anaerobic conditions). The results are mean values of three experiments.*

Compound	Conc. (mM)	Oxygen consumption*	Aerobic incorporation into protein*	Anaerobic glycolysis*	Anaerobic incorporation into protein*
Acetaldehyde	5	98	49	78	60
Glycolaldehyde	5	86	20	79	37
Glyoxylic acid	5	46	10	85	60
Glyoxal	5	87	10	86	20
Propionaldehyde	5	98	35	49	21
Lactaldehyde	5	103	39	60	37
D-Glyceraldehyde	5	96	68	100	90
L-Glyceraldehyde	5	90	35	8	2
DL-Glyceraldehyde	5	86	34	20	6
n-Butyraldehyde	5	102	23	66	50
iso-Butyraldehyde	5	98	29	78	82
β-Hydroxybutyraldehyde	5	95	32	80	62
DL-*Erythro,threo*-α,β-dihydroxybutyraldehyde	5	95	36	90	50
L-*Erythro*-α,β-dihydroxybutyraldehyde	5	93	25	90	37
D-*Threo*-α,β-dihydroxybutyraldehyde	5	90	43	74	53
Crotonaldehyde	2	27	2	45	8
D-Erythrose	10	102	111	—	—
DL-Glyceraldehyde-3-P	10	106	116	—	—

*Compared to 100 in control experiments.

described [5], L- and DL-glyceraldehyde, which in the presence of triose phosphates depress hexokinase activity [2], were found to inhibit almost completely the utilization of glucose under anaerobic conditions. A substantial decrease of anaerobic glycolysis was also observed with acetaldehyde, glycolaldehyde, propionaldehyde, lactaldehyde, *n*-butyraldehyde, *iso*-butyraldehyde, β-hydroxybutyraldehyde and D-*threo*-α,β-dihydroxybutyraldehyde. Glyoxylate, glyoxal, DL-*erythro*, *threo*-α,β-dihydroxybutyraldehyde and L-*erythro*-α,β-dihydroxybutyraldehyde were less effective and D-glyceraldehyde did not affect anaerobic glycolysis at comparable concentrations. A tentative explanation of non-specific effects of aldehydes on anaerobic glycolysis of surviving tissues has been advanced [5]. It postulated their condensation with dihydroxyacetone phosphate with consequent decrease in the amount of triose phosphates flowing through the Embden-Meyerhof pathway. Conversely, this condensation would decrease also the intracellular concentration of aldehyde available to inhibit amino acid incorporation [5]. Accordingly, under anaerobic conditions when an active glycolysis occurs, the inhibition of the incorporation reaction was lower than that observed aerobically in the absence of glucose. Exceptions with aldehydes exhibiting a strong inhibitory effect on glycolysis, are easily understandable since, under these circumstances, the depression of amino acid incorporation is the result of an energetic failure.

In keeping with the assumption that sugars with four or more carbons form cyclic haemiacetals thus lacking the reactive aldehyde group, D-erythrose and other aldoses (not shown) did not affect the incorporation reaction even at concentrations as high as 10 mM. The same was true for DL-glyceraldehyde-3-phosphate which fails to enter the cells. Crotonaldehyde and other unsaturated aldehydes (not shown) proved to be extremely toxic even at low concentrations.

Protective effects of cysteine

Figure 1 shows that, when a neutral solution of L-glyceraldehyde is added to a neutral solution of L-cysteine, a rapid shift of pH toward acidity occurs which can be followed by potentiometric or manometric measurements. All the aldehydes tested in these ways: acetaldehyde, glycolaldehyde, glyoxylic acid, glyoxal, propionaldehyde, D-, L- and DL-glyceraldehyde and α,β-dihydroxybutyraldehyde reacted with L-cysteine at comparable rates. D-Cysteine and DL-cysteine, but not glutathione could substitute for L-cysteine. No comparable reactions occurred when dihydroxyacetone, erythrose or

other aldoses were added to cysteine and when aldehydes were added to amino acids different from cysteine. That the SH-group of cysteine is involved in the above reactions, is shown in Table 2: upon addition of aldehyde solutions to equimolecular solutions of cysteine, free SH-groups disappeared almost completely after a short period of incubation. Chromatographic analyses of the mixture and radioautography of chromatograms (Fig. 2) showed that cysteine and aldehyde had disappeared almost completely and that new condensation products were formed, which had the chromatographic *Rf* of the corresponding thiazolidine carboxylic acid derivative. These compounds were remarkably stable toward acidity or to heat.

Under suitable experimental conditions, L-cysteine added *in vitro* to ascites hepatoma cells prior to the addition of L-glyceraldehyde, prevented completely its inhibitory effect on anaerobic glycolysis (Fig. 3). In the same conditions, a number of amino acids different from cysteine (glycine, alanine, valine, leucine, isoleucine, serine, threonine, phenylalanine,

Table 2. Effect of the addition of aldehydes to cysteine on the concentration of free SH*-groups at equilibrium.*
(Reaction mixture: K *phosphate buffer,* pH *7·2; cysteine, glutathione and aldehydes, 10-*^{3}M. *Incubation at 38°*C *in atmosphere of* N_2*; shaking, 50* c/min)

Compound	Addition	SH-Groups conc. (%)
L-Cysteine	Nil	100
L-Cysteine	Acetaldehyde	4
L-Cysteine	Glycolaldehyde	3
L-Cysteine	Glyoxylic acid	4
L-Cysteine	Propionaldehyde	4
L-Cysteine	Lactaldehyde	2
L-Cysteine	DL-Glyceraldehyde	6
L-Cysteine	D-Glyceraldehyde	6
L-Cysteine	L-Glyceraldehyde	5
L-Cysteine	DL-Glyceraldehyde-3-P	1
L-Cysteine	*n*-Butyraldehyde	7
L-Cysteine	*iso*-Butyraldehyde	6
L-Cysteine	β-Hydroxybutyraldehyde	25*
L-Cysteine	α,β-Dihydroxybutyraldehyde-C^{14}	6
Glutathione (SH)	Nil	100*
Glutathione (SH)	Glyoxylic acid	100*
Glutathione (SH)	DL-Glyceraldehyde	100*
Glutathione (SH)	α,β-Dihydroxybutyraldehyde	100*

*After 1 hr of incubation.

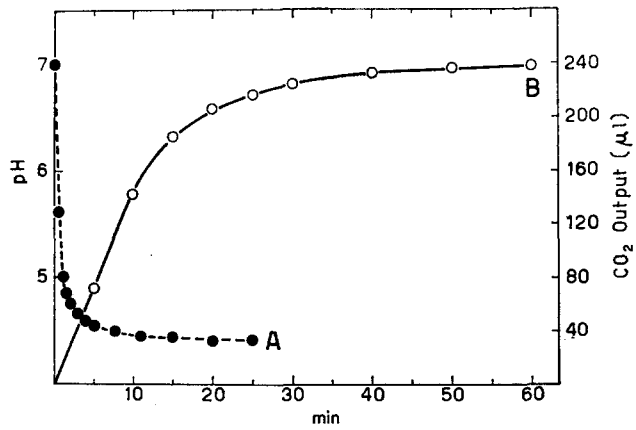


Fig. 1. Effects of the reaction of L*-cysteine with* L*-glyceraldehyde. Curve* A *represents* pH *changes occurring in a neutral water solution of cysteine* (50 mM) *when an equimolecular neutral solution of aldehyde is added at* 30°C *under* N_2 *atmosphere. Curve* B *represents* CO_2 *liberation from a neutral solution of cysteine* (5 mM, *final) in bicarbonate buffer to which a neutral solution of* L*-glyceraldehyde* (5 mM, *final) was added by tipping the content of the side arm* (0·2 ml) *into the main compartment* (2·8 ml) *of a Warburg flask; temperature:* 37°C; *atmosphere:* 95% N_2: 5% CO_2; *shaking:* 75 c/min.

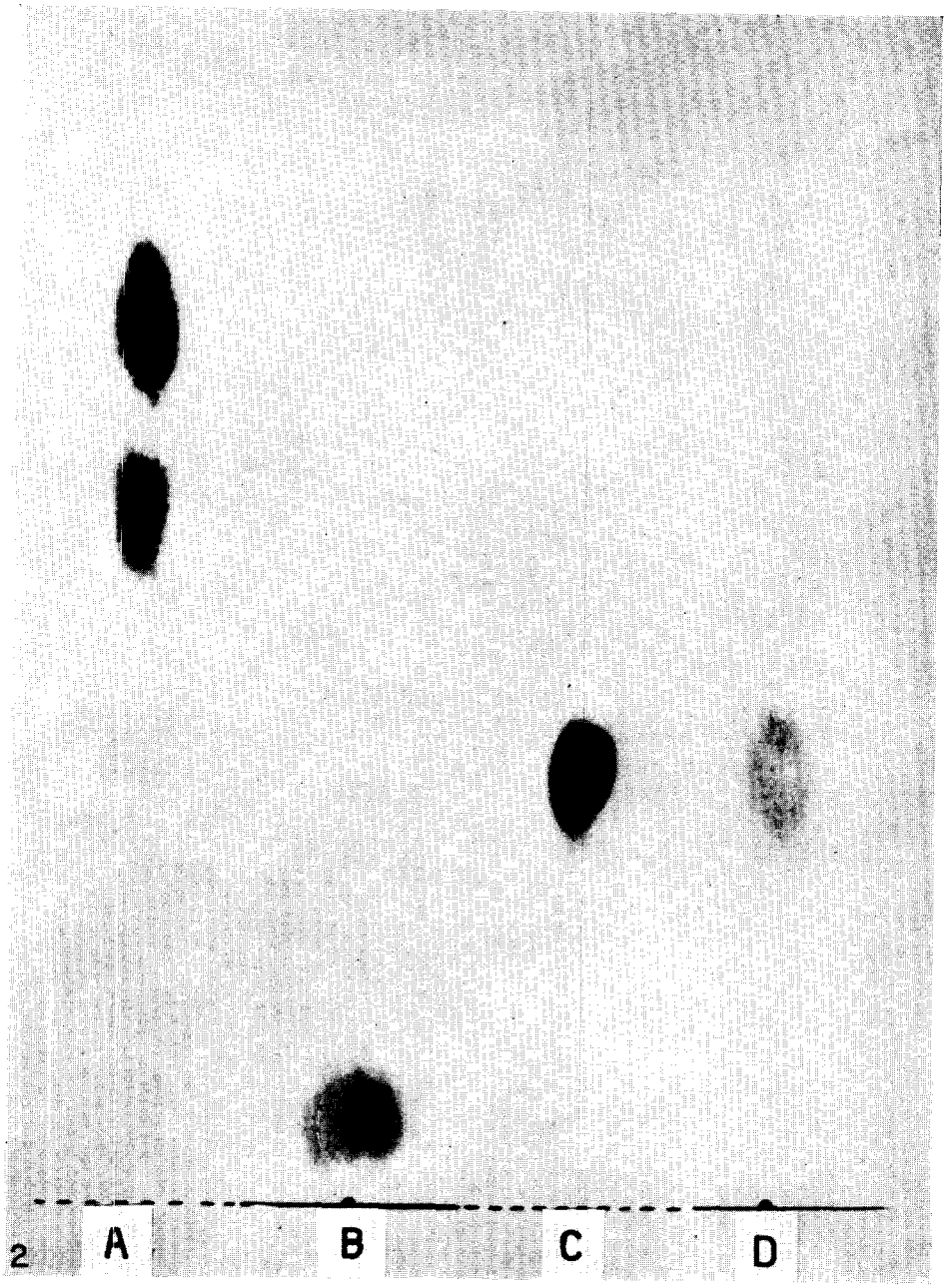


Fig. 2. Chromatographic and radioautographic detection of a thiazolidine carboxylic acid upon addition of cysteine to aldehyde. Incubation mixture contained α,β*-dihydroxybutyraldehyde-*U-C_{14} (A), L-*cysteine* (B) *and phosphate buffer,* pH 7·2. *After* 30 min *of incubation at* 37°C *in* N_2 *atmosphere, the only radioactive spot detected on chromatograms* (C) *coincided with that of unlabelled* 2-(1′, 2′*-dihydroxypropyl*)*-thiazolidine-*4*-carboxylic acid* (D). A *and* C *are autoradiograms;* B *and* D, *ninhydrin-sprayed chromatograms. Notice at* (A) *two spots corresponding to the* erythro- (*slow*) *and* threo- (*fast*) *isomers of* α,β*-dihydroxybutyraldehyde.*

(to face p. 26)

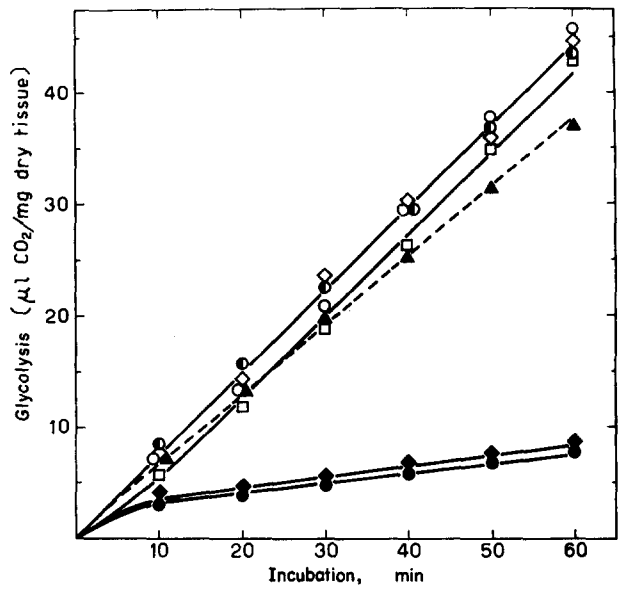


Fig. 3. Protective effect of cysteine on the inhibition of anaerobic glycolysis of Yoshida ascites hepatoma cells by L-*glyceraldehyde. Cells were incubated in Ringer bicarbonate at* 38°C *in the absence or in the presence of* 5 mM *amino acids; atmosphere:* 95% N_2: 5% CO_2. *Additions as follows:* L-*glyceraldehyde* (5 mM, *final*) *or 2-dihydroxyethyl-thiazolidine-4-carboxylic acid* (10 mM, *final*) *after* 20 min; DL-*leucine-*1-C^{14} (0·25 µmoles; *s.a.* 3·8 mc/mmole) *and glucose* (15 mM, *final*) *after* 40 min; *incubation lasted* 60 min *after the last addition. Legends:* I. *Cells pre-incubated in the absence of amino acids: glycolysis in the absence* (○) *and in the presence of* L-*glyceraldehyde* (●) *or 2-dihydroxyethyl-thiazolidine-4-carboxylic acid* (▲). II. *Cells pre-loaded with* L-*cysteine: glycolysis in the absence* (□) *and in the presence* (◑) *of* L-*glyceraldehyde.* III. *Cells pre-loaded with alanine: glycolysis in the absence* (◇) *and in the presence* (◆) *of* L-*glyceraldehyde. Points are means of triplicate determinations.*

tryptophan, methionine, proline, hydroxyproline, aspartic acid, asparagine, glutamic acid, glutamine, histidine, lysine and arginine) failed to hinder aldehyde effects. 2-Dihydroxyethyl-thiazolidine-4-carboxylic acid, the condensation product of L-cysteine and L-glyceraldehyde, did not appreciably affect, *per se*, anaerobic glycolysis.

A similar, although less effective protection was exerted by cysteine on the inhibition by aldehydes of the incorporation of labelled leucine into protein of ascites hepatoma cells (Table 3). Under these circumstances, 2-dihydroxyethyl-thiazolidine-4-carboxylic acid behaved as a weak inhibitor.

The rate of anaerobic incorporation of labelled amino acids into protein of ascites hepatoma cells in the absence of inhibitors proceeded as a linear function of time for at least 90 min (Fig. 4). In these conditions, the addition of L-*erythro*-α,β-dihydroxybutyraldehyde, which at a concentration of 5 mM has only a minor effect on anaerobic glycolysis (Table 1), markedly depressed incorporation. The addition of L-cysteine (10 mM, final) reversed, at least in part, this previously established inhibition.

C^{14}-labelled 2-(1',2'-dihydroxypropyl) - thiazolidine-4-carboxylic acid, whose saturated ring resembles that of proline, was not incorporated into protein and the C^{12}-compound did not interfere with the incorporation of C^{14}-labelled leucine into protein of Yoshida ascites hepatoma cells (Table 4).

DISCUSSION

Our data on the inhibition of amino acid incorporation into protein of Yoshida ascites hepatoma cells by a number of aliphatic aldehydes (Table 1) and results from other Laboratories which suggest comparable effects with α-ketoaldehydes [11], epoxyaldehydes [12] and unsaturated aldehydes [13, 14], indicate the existence of a common mechanism by which these compounds affect protein metabolism. The fact that similar activities are exhibited by *n*-aldehydes, α-, β-, α,β-substituted aldehydes and aliphatic glyoxals suggest that the —C(=O)—H group plays a major role in the mechanism of inhibition, whereas structural differences at sites other than the aldehyde carbon are relatively unimportant, unless they modify the

Table 3. Protective effect of cysteine on the inhibition of amino acid incorporation into protein of Yoshida ascites hepatoma cells by L-*glyceraldehyde.*

Cells (8–12 mg, *dry weight) were incubated in Ringer bicarbonate at 38°C in the absence or in the presence of 5* mM *amino acids; atmosphere: 95%* N_2: *5%* CO_2. *Additions as follows:* L-*glyceraldehyde (5* mM, *final) or 2-dihydroxyethyl-thiazolidine-4-carboxylic acid (10* mM, *final) after 20* min; DL-*leucine-1-*C^{14} *(0·25* μmoles; *s.a. 3·8* mc/mmole) *and glucose (15* mM, *final) after 40* min *of incubation. Suitable volumes of medium were added in control flasks. Incubation was stopped 60* min *after the last addition. The results are means of three determinations. Per cent inhibition in parentheses.*

Preloading	Additions	Incorporation into protein (counts/min/mg protein)	
Nil	Nil	980	
L-Cysteine	Nil	927	(5%)
Nil	L-Glyceraldehyde	33	(97%)
L-Cysteine	L-Glyceraldehyde	747	(24%)
Nil	2-Dihydroxyethyl-thiazolidine-4-carboxylic acid	792	(19%)
Glycine	Nil	982	(0)
Glycine	L-Glyceraldehyde	55	(94%)

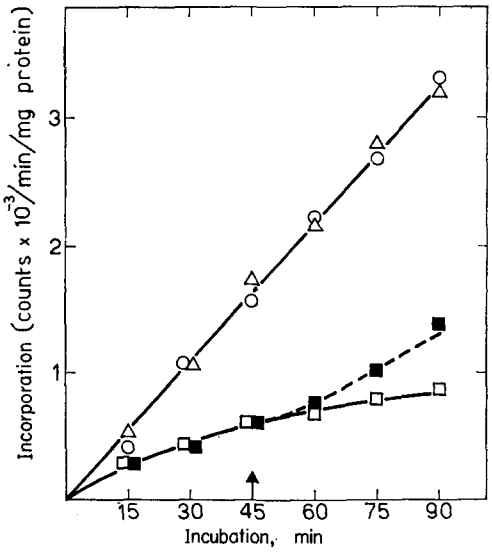


Fig. 4. Effect of cysteine on the inhibition of C^{14}*-leucine incorporation into protein of Yoshida ascites hepatoma cells by* L-erythro-α,β-*dihydroxybutyraldehyde. Cells were incubated in Ringer bicarbonate buffer at* 38°C; *atmosphere:* 95% N_2: 5% CO_2; *glucose concentration:* 15 mM. L-*Cysteine* (10 mM, *final) was added after* 45 min *of incubation* (⟶). *Curves represent leucine incorporation into protein in the absence of aldehyde without* (○) *or with* (△) *cysteine added and in the presence of dihydroxybutyraldehyde* (5 mM) *without* (□) *or with* (■) *cysteine added. Points are means of triplicate determinations.*

rate at which the molecule can be metabolized or transformed. Accordingly, any hypothesis based on a primary effect of aldehydes on glycolysis [6] can be disregarded and the concept reported previously [5] of an independent mechanism of inhibition on glucose and protein metabolism by molecules such as L-glyceraldehyde is strengthened.

Several possibilities can be considered to explain the inhibitory effect of aldehydes on protein synthesis and all arise from the unusual reactivity of the aldehyde group. Aliphatic aldehydes have been reported to react with proteins and with synthetic mixtures of amino acids with participation of primary amino groups [15]. However, the rate of these reactions is rather slow except for amino acids like serine, threonine and cysteine which possess an active group adjacent to the primary amino group, for arginine carrying a reactive amidine group and for histidine which, with aldehydes undergoes intramolecular cyclization. Under our experimental conditions, a reaction of aldehydes with proteins was doubtful and, among the above fast-reacting amino acids, only cysteine was able to antagonize the inhibitory effect of aldehydes on protein synthesis.

The reactivity of aldehydes toward sulphydryl groups and, in particular, their condensation with cysteine to form thiazolidine carboxylic acids are known since a long time [16, 17]. The formation of thiazolidine carboxylic acids upon addition of cysteine to tissue homogenates has been also reported [18].

Our results confirm these observations and suggest that, when exogenous aldehydes are added to surviving tissues, 2-R-thiazolidine-4-carboxylic acids (where R- is the remaining chain after the aldehyde carbon has been inserted between the S and N in the cysteine molecule) can arise at the expense of the intracellular amino acid. As a result, the intracellular concentration of cysteine will decrease

Table 4. Incorporation of DL-*leucine-1-*C^{14} *and* C^{14}*-labelled 2-(1',2'-dihydroxypropyl)-thiazolidine-4-carboxylic acid into protein of Yoshida ascites hepatoma cells.*

Cells (10–12 mg, *dry weight) were incubated at 38* °C *for 60* min *in Krebs Ringer bicarbonate in the absence and in the presence of glucose (15* mM). *Gas phase: 95%* N_2*: 5%* CO_2. C^{14}*-leucine (0·15* mM; *s.a. 3·8* mc/mmole), *unlabelled and* C^{14}*-labelled thiazolidine carboxylic acid (2* mM; *s.a. 0·26* mc/mmole) *were added after 10* min *of thermal equilibration. The results are mean values of three determinations.*

Labelled compound	Incorporation into protein (counts/min/mg protein)	
	No glucose	Glucose
C^{14}-Leucine	13	2194
C^{14}-Thiazolidine carboxylic acid	5	4
C^{14}-Leucine + C^{12}-thiazolidine carboxylic acid	15	1909

leading to an imbalance of the protein-synthesizing pool which, in turn, could impair protein synthesis. Furthermore, considerable experimental evidence has been accumulated in recent years, which outlines the importance of sulphydryl compounds as components of the enzyme systems capable of incorporating amino acids into protein [19, 20] and the hypothesis has been advanced that transient amino acid thiol esters may be intermediates in the biological condensation of amino acids to form proteins [21]. A decreased availability of sulphydryl compounds would then affect protein synthesis by lowering the efficency of the system concerned with the incorporation of amino acids. The antagonistic effect of exogenous cysteine (Table 3), the failure of other amino acids as protective compounds and the partial restoration of amino acid incorporation upon addition of cysteine after the inhibition by aldehyde had been established (Fig. 4) are consistent with both these hypotheses.

The possibility that the newly formed thiazolidine carboxylic acid, being a proline analogue, were incorporated into growing protein molecules thus disturbing the synthetic process, has been ruled out (Table 4). On the other hand, in the living animal, thiazolidine carboxylic acids can be re-converted into thio-amino acids [22], the first metabolic step being perhaps their oxidation by a proline oxidase system described in rat liver [23]. The possibility that neoplastic tissues lack the ability to metabolize these thiazolidine carboxylic acid derivatives would explain the anti-tumour activity and low toxicity of many aldehydes in the living animal [11, 24, 25].

It is recognized, of course, that mechanisms other than those described above could be operative in the inhibition of protein synthesis by aldehydes. An impairment of enzyme activities could result from binding of aldehydes to SH-groups of enzymes and inhibitory effects of aldehydes on the activity of SH-containing enzyme systems have been reported [14, 26]. However, in surviving tissues, many aldehydes which markedly inhibit protein synthesis, do not appeciably affect respiration or glycolysis, i.e. pathways in which several SH-containing enzymes are operative. In this connection, it should be emphasized that aldehydes form, with the majority of SH-bearing molecules, simple addition compounds which dissociate quite readily into their components, whereas the condensation products with cysteine are rather stable over a wide range of temperature and pH [16, 17].

In conclusion, in the Author's opinion, the reaction of condensation of aliphatic aldehydes with cysteine should be regarded as the primary step leading to a decrease of the intracellular concentration of this amino acid. The consequent imbalance of the protein-synthesizing amino acid pool and perhaps the lowered availability of sulphydryl compounds could explain the inhibitory effect of aliphatic aldehydes on the incorporation of amino acids into protein of Yoshida ascites hepatoma cells.

Acknowledgements — The authors are indebted to Prof. B. Camerino of the Laboratori Farmitalia, Milano, for the generous supply of aliphatic aldehydes and derivatives. Valuable technical assistance by Miss A. Arnaboldi is also acknowledged.

RÉSUMÉ

On a observé qu'un certain nombre d'aldéhydes aliphatiques à 2-, 3- *et* 4-*atomes carbone réduisent l'incorporation aérobique de la leucine marquée dans les proteines des cellules de l'hépatome ascite de Yoshida à une concentration qui ne réduit pas appréciablement la respiration. On a aussi observé une inhibition de l'incorporation anaérobique d'acides aminés. Dans de telles conditions l'effet inhibiteur n'était mineur que aérobiquement, sauf pour les aldéhydes qui déprimentfortement le métabolisme du glucose. Des mesures potentiométriques et manométriques démontrent que les aldéhydes aliphatiques se condensent avec la cystéine, et ce résultat a été confirmé en suivant la disparition des groupes SH libres de solutions de cystéine après l'addition d'aldéhydes. Le produit de condensation a un* R_f *chromatographique de l'acide thiazolidine-*4*-carboxylique correspondant.*

La L*-cystéine ajoutée* in vitro *avant l'addition d'aldéhydes prévient leur effet inhibitoire sur la glycolyse et sur l'incorporation des acides aminés dans les protéines des cellules des tumeurs ascite de Yoshida. Les autres acides aminés sont complètement inefficaces. La cystéine élimine, au moins en partie, l'inhibition préalablement établie.*

*Les acides thiazolidine-*4*-carboxyliques n'influencent pas la glycolyse et ont seulement un effet réduit sur la synthèse protéique. L'acide thiazolidine-*4*-carboxylique marqué avec* C^{14} *(obtenu avec l'aldéhyde* α,β*-dihydroxybutirique) n'est pas incorporé dans les protéines des cellules de la tumeur ascite de Yoshida.*

Ces résultats et la possibilité que les acides thiazolidine-carboxyliques puissent être formés aux dépens de la cystéine endogène suggèrent qu'une réduction de la concentration intracellulaire de cet acide aminé puisse être retenue comme la cause principale de l'inhibition de la synthèse protéique provoquée par les aldehydes aliphatiques.

SUMMARY

A number of 2-, 3- *and* 4*-carbon aliphatic aldehydes have been shown to depress the aerobic incorporation of labelled leucine into protein of Yoshida ascites hepatoma cells, at a concentration which does not appreciably affect respiration. Inhibition of anaerobic amino acid incorporation was also observed. Under these conditions, the inhibitory effect was lower than aerobically, except for aldehydes which strongly depressed glucose metabolism.*

Potentiometric and manometric measurements showed that aliphatic aldehydes condense with the amino acid cysteine and this result has been confirmed following the disappearance of free SH-groups from solutions of cysteine after the addition of aldehydes. The condensation product had the chromatographic R_f *of the corresponding thiazolidine-*4*-carboxylic acid.*

L*-Cysteine, added* in vitro *prior to the addition of aldehydes, prevented their inhibitory effects on glycolysis and amino acid incorporation into protein of Yoshida ascites tumour cells. Other amino acids were completely ineffective. Cysteine also relieved, at least in part, previously established inhibitions.*

*Thiazolidine-*4*-carboxylic acids did not affect glycolysis and had only a minor effect on protein synthesis. A* C^{14}*-labelled thiazolidine-*4*-carboxylic acid (from* C^{14}*-*α,β*-dihydroxybutyraldehyde) was not incorporated into protein of ascites hepatoma cells.*

These results and the possibility that thiazolidine carboxylic acids can be formed at the expense of endogenous cysteine suggest that a decrease in the intracellular concentration of this SH-amino acid may be regarded as the primary step in the inhibition of protein synthesis by aliphatic aldehydes.

ZUSAMMENFASSUNG

Für eine Anzahl von 2-, 3- *und* 4*-C aliphatischer Aldehyde konnte bewiesen werden, dass sie die aerobische Inkorporation von markiertem Leucin in das Protein der Yoshida Ascites Hepatomzellen verringern, und zwar bei einer Konzentration, welche die Atmung nicht besonders beeinflusst. Es wurde auch die Inhibition der anaerobischen Inkorporation der Aminosäuren beobachtet. Unter diesen Bedingungen, war die hemmende Wirkung niedriger als in Aerobiose, mit Ausnahme der Aldehyde, die den Glukosestoffwechsel stark reduzierten.*

Potentiometrische und manometrische Messungen zeigten, dass aliphatische Aldehyde mit der Aminosäure Cystein kondensieren und dieser Befund wurde bestätigt durch das Verschwinden der freien SH-Gruppen des Cysteins in der Lösung nach Zugabe von Aldehyden. Das Kondensierungsprodukt hatte den chromatographischen R_f-Wert der jeweiligen Thiazolidin-4-carboxyl-säure.

Die Zugabe von L-*Cystein* in vitro *vor der Zugabe der Aldehyde beugt die hemmende Wirkung auf die Glykolyse und auf die Aminosäureninkorporation in das Protein der Yoshida Ascites Hepatomzellen vor. Andere Aminosäuren waren vollkommen wirkungslos. Auch das Cystein eliminieate, wenigstens z.t., vorher stattgefundene Hemmungen.*

Thiazolidin-4-carboxyl-säuren waren wirkungslos auf die Glykolyse und zeigten eine schwache Wirkung auf die Proteinsynthese. Eine mit C^{14} markierte Thiazolidin-4-carboxyl-säure (aus C^{14}-α,β-dihydroxyl-butyraldehyd) wurde nicht in das Protein der Ascites Hepatomzellen eingebaut.

Diese Befunde und die Möglichkeit, dass Thiazolidin-carboxyl-säuren auf kosten des endogenen Cysteins gebildet werden können, weisen darauf hin, dass die Abnahme der intrazellulären Konzentration dieser Aminosäure als den ersten Schritt in der Inhibition der Proteinsynthese durch aliphatische Aldehyde angesehen werden kann.

REFERENCES

1. B. Mendel, F. Strelitz and D. Mundell, L-Glyceric aldehyde and tumor metabolism. *Science* **88,** 149 (1938).
2. H. A. Lardy, V. D. Wiebelhaus and K. M. Mann, The mechanism by which glyceraldehyde inhibits glycolysis. *J. Biol. Chem.* **187,** 325, (1950).
3. E. Ciaranfi and A. Fonnesu, Sui rapporti fra metabolismo enegetico e incorporazione di aminoacidi nelle proteine delle cellule di tumori ascite. *Atti Accad. Naz. Lincei, Rend., Classe Sci. Fis. Mat. Nat.* **32,** 835 (1962).
4. G. G. Guidotti, La gliceraldeide, un nuovo inibitore delle sintesi proteiche. *Tumori* **49,** 303 (1963).
5. G. G. Guidotti, A. Fonnesu and E. Ciaranfi, Inhibition of amino acid incorporation into protein of Yoshida ascites hepatoma cells by glyceraldehyde. *Cancer Res.* **24,** 900 (1964).
6. J. Drews, P. Wahl and E. Fölsch, Die Wirkung von Glycerinaldehyd auf Glycerinbau, Atmung und Gärung von Ascitestumorzellen. *Klin. Wschr.* **42,** 776 (1964).
7. W. W. Umbreit, R. H. Burris and J. F. Stauffer, *Manometric Techniques*, 3rd ed. Burgess, Minneapolis (1959).
8. M. Rabinovitz, M. E. Olson and D. M. Greenberg, Independent antagonism of amino acid incorporation into protein. *J. Biol. Chem.* **210,** 837 (1954).
9. R. R. Grunert and P. H. Phillips, A modification of the nitroprusside method of analysis for glutathione. *Arch. Biochem.* **30,** 217 (1951).
10. A. Ruffo, A. Adinolfi, G. Budillon and G. Capobianco, Control of the citric acid cycle by glyoxylate. 2. Mechanism of the inhibition of respiration in liver and kidney particles. *Biochem. J.* **85,** 593 (1962).
11. F. A. French and B. L. Freedlander, Carcinostatic action of polycarbonyl compounds and their derivatives. I. 3-Ethoxy-2-ketobutyraldehyde and related compounds. *Cancer Res.* **18,** 172 (1958).
12. C. H. Hine, R. J. Guzman, M. K. Dunlap, R. Lima and G. S. Loquvam, Studies on the toxicity of glycidaldehyde. *Arch. Environm. Hlth* **2,** 23 (1961).
13. R. A. Alarcon, Isolation of acrolein from incubated mixtures of spermine with calf serum and its effects on mammalian cells. *Arch. Biochem. Biophys.* **106,** 240 (1964).
14. E. Schauenstein, H. Esterbauer, G. Jaag and M. Taufer, Über die Wirkungen von Aldehyden auf gesunde und maligne Zellen. Hydroxy-octenal, ein neuer Fettaldehyd. *Mh. Chem.* **95,** 180, (1964).
15. G. E. Underwood, R. A. Siem, S. A. Gerpheide and J. H. Hunter, Binding of an antiviral agent (Kethoxal) by various metabolites. *Proc. Soc. Exp. Biol. (N.Y.)* **100,** 312 (1959).
16. M. P. Schubert, Compounds of thiol acids with aldehydes. *J. Biol. Chem.* **114,** 341 (1936).

17. S. RATNER and H. T. CLARKE, The action of formaldehyde upon cysteine. *J. Amer. Chem. Soc.* **59,** 200 (1937).
18. D. CAVALLINI, C. DE MARCO, B. MONDOVI and F. TRASARTI, Studies of the metabolism of thiazolidine carboxylic acid by rat liver homogenate. *Biochim. Biophys. Acta* **22,** 558 (1956).
19. W. C. HÜLSMANN and F. LIPMANN, Amino acid transfer from sRNA to microsome. I. Activation by sulfhydryl compounds. *Biochim. Biophys. Acta* **43,** 123 (1960).
20. J. M. FESSENDEN and K. MOLDAVE, Studies on aminoacyl transfer from soluble ribonucleic acid to ribosomes. *J. Biol. Chem.* **238,** 1479 (1963).
21. R. M. METRIONE and R. B. JOHNSTON, The enzymic condensation of a thiol ester-type carboxyl-activated acylamino acid with an amino acid amide to form a peptide. *Biochemistry* **3,** 482 (1964).
22. C. G. MACKENZIE and J. HARRIS, N-Formylcysteine synthesis in mitochondria from formaldehyde and L-cysteine via thiazolidine–carboxylic acid. *J. Biol. Chem.* **227,** 393 (1957).
23. A. B. JOHNSON and H. J. STRECKER, The interconversion of glutamic acid and proline. IV. The oxidation of proline by rat liver mitochondria. *J. Biol. Chem.* **237,** 1876 (1962).
24. O. WARBURG, K. GAWEHN, A. W. GEISSLER and S. LORENZ, Über Heilung von Mäuse-Ascites-Krebs durch D- und L-Glycerinaldehyd. *Z. klin. Chem.* **1,** 175 (1963).
25. E. CIARANFI, L. LORETI, A. BORGHETTI and G. G. GUIDOTTI, Studies on the anti-tumour activity of aliphatic aldehydes. II. Effect on survival of Yoshida ascites hepatoma-bearing rats. In preparation.
26. G. FERRI and S. RONCHI, Inibizione dell'acetato CoA ligasi da parte del gliossilato ed altre aldeidi. *Boll. Soc. Ital. Biol. Sper.* In press.

Europ. J. Cancer Vol. 1, pp. 33–40. Pergamon Press 1965. Printed in Great Britain

Action d'un dérivé de la Méthylhydrazine, le Ro4-6467, dans la Maladie de Hodgkin, dans d'autres Lymphomes Malins et dans des Leucémies

Y. KENIS, J. WERLI, J. HILDEBRAND et H. J. TAGNON

Service de Médecine Interne

Institut Jules Bordet — Centre des Tumeurs de l'Université Libre de Bruxelles

Groupe Européen de Chimiothérapie Anticancéreuse, Laboratoire Roche — Bruxelles, Belgique

Bollag et Grunberg ont démontré l'action inhibitrice des dérivés de la méthylhydrazine sur diverses tumeurs transplantables de la souris et du rat [1]. Le chlorhydrate de p-(N'-méthyl-hydrazinométhyl) - N - isopropyl - benzamide* (Fig. 1) est capable, par exemple, de provoquer une inhibition complète du carcinosarcome de Walker 256.

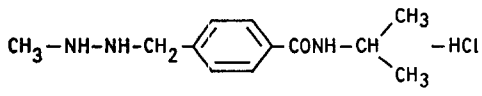


Fig. 1.

Les premiers essais cliniques avec ce produit ont démontré sa grande efficacité dans la maladie de Hodgkin et, à un moindre degré, dans les lymphomes malins [2–4]. Les résultats ont été nuls dans une petite série de malades avec tumeurs solides [2, 4].

Le présent travail est basé sur cinquante malades atteints de maladie de Hodgkin, d'autres formes de lymphomes malins et de leucémies, traités par le Ro 4-6467, à l'Institut Bordet, du 1er juillet 1963 au 31 mai 1964.

*Ce produit a été désigné pendant la période des essais cliniques par le numérocode Ro4-6467. Il a été préparé par Hoffmann-La Roche (Bâle) et nous a été aimablement fourni par la firme Produits Roche S.A. (Bruxelles).

MATERIAL ET METHODES

Maladie de Hodgkin

1. *Matérial clinique* — Trente-trois malades, dix-huit hommes et quinze femmes, âgés de 14 à 78 ans, étaient atteints d'une maladie de Hodgkin prouvée par une biopsie. Dans tous les cas, la maladie était étendue à plusieurs territoires ganglionnaires ou à plusieurs viscères. De nombreux malades avaient des phénomènes généraux, tels la fièvre et le prurit.

Tous les malades avaient été traités par la radiothérapie avant l'essai actuel. Treize malades avaient reçu, en outre, un ou plusieurs agents chimiothérapiques (agents alkylants, sulfate de vinblastine, 6-mercaptopurine, prednisolone). Le délai entre la fin du traitement précédent et le début de celui au dérivé de méthylhydrazine était d'au moins 1 mois, sauf dans trois cas traités par la prednisolone. Chez cas malades, le Ro4-6467 a été commencé immédiatement après l'arrêt de la prednisolone, qui était administrée à dose décroissante depuis plusieurs semaines et qui avait été donnée auparavant, sans effet, à forte dose, pendant une période d'au moins 2 mois.

2. *Mode d'administration du médicament* — Un seul malade a reçu le Ro4-6467 par voie parentérale. Il reçut des injections intraveineuses quotidiennes de 250 mg.

Les trente-deux autres cas reçurent le médicament par voie buccale.

La dose quotidienne maximum a été de 200 mg chez quatre malades, de 250 mg chez trois malades, de 300 mg chez vingt-quatre malades et de 400 mg chez un malade. La dose quotidienne a parfois varié, chez un même malade, en fonction des signes de toxicité. Le traitement était poursuivi, ou bien jusqu'à l'apparition de signes de toxicité (vomissements intolérables, leucopénie en dessous de 3.000 globules blancs/mm³, thrombocytopénie en dessous de 100.000 plaquettes/mm³), ou bien jusqu'à l'apparition d'une rémission, soit complète, soit incomplète mais ne s'améliorant plus. Le traitement a été interrompu, dans quatre cas, alors qu'il n'y avait ni rémission ni signe de toxicité. Dans ces quatre cas, la dose totale était de 5 g à 22,35 g. Sept malades ont reçu deux cures* de Ro4-6467. La dose totale pour une cure a été inférieure à 5 g dans cinq cas, de 5 à 8 g dans vingt-six cas, supérieure à 8 g dans neuf cas. La dose la plus forte était de 22,35 g. La durée du traitement, pour une cure, a été inférieure à 3 semaines dans cinq cas, de 3 à 5 semaines dans vingt-cinq cas, supérieure à 5 semaines dans dix cas. La plus longue cure a duré 106 jours.

Le Ro4-6467 a toujours été utilisé seul. Les malades n'ont reçu ni radiothérapie, ni hormonothérapie pendant toute la durée du traitement.

3. *Critères d'évaluation du traitement* — Tous les malades avaient des lésions mesurables: adénopathies palpables ou mises en évidence par lymphographie, nodules cutanés ou sous-cutanés, masses tumorales pulmonaires ou médiastinales visibles à la radiographie, épanchements pleuraux.

Les résultats ont été classés en effet subjectif et en effect objectif, probable ou certain.

Un *effet objectif certain* était défini par une diminution évidente des lésions mesurables, sans aucun signe d'évolution, pendant une durée d'au moins 1 mois. La disparition de la fièvre et le ralentissement de la vitesse de sédimentation, non accompagnés d'une diminution des masses tumorales, n'étaient pas considérés comme un effet objectif. Par contre, l'absence d'amélioration de ces deux manifestations empêchaient de classer un malade dans la catégorie des effets objectifs certains.

Un *effet objectif probable* était défini par: 1. une diminution des lésions mesurables, comme ci-dessus, mais pendant une période inférieure à 1 mois; 2. une stabilisation de toutes les lésions pendant au moins 6 mois, si ces lésions étaient évolutives avant le traitement; 3. une diminution de volume de certaines lésions, tandis que d'autres évoluent, ou que de nouvelles lésions apparaissent; 4. une diminution de lésions non mesurables (par exemple: lésions observées par cystoscopie, rectoscopie ou laryngoscopie; masses abdominales profondes).

Autres lymphomes malins et leucémies

1. *Matériel clinique* — Dix-sept malades, douze hommes et cinq femmes, âgés de 6 à 78 ans, constituaient le second groupe. Le diagnostic a toujours été posé par une biopsie ou par un examen de la moelle osseuse. Il s'agissait d'un lymphosarcome dans six cas, d'un réticulosarcome dans cinq cas, d'un myélome multiple dans deux cas, d'une leucémie lymphoïde chronique dans deux cas, et d'une leucémie myéloïde chronique dans deux cas.

Un cas de lymphosarcome, deux cas de réticulosarcome, et deux cas de leucémie myéloïde chronique, n'avaient reçu aucun traitement avant le Ro4-6467. Deux malades avaient été traités par la chimiothérapie seule et dix malades par la radiothérapie, seule ou associée à la chimiothérapie.

2. *Mode d'administration du médicament* — Tous les malades ont reçu le Ro4-6467 par voie buccale. La dose quotidienne maximale a été de 200 mg dans deux cas, 400 mg dans deux cas et 300 mg dans treize cas. Le traitement a consisté en dix-sept cures de 2.250 mg à 11.400 mg administrés en 10 à 45 jours. Le traitement a été interrompu par le décès du malade dans trois cas, par l'apparition de thrombocytopénie dans sept cas, d'une leucopénie dans trois cas, d'une érythrodermie dans un cas. L'apparition d'une rémission complète a amené l'arrêt du traitement dans les deux cas de leucémie myéloïde chronique. Un malade était encore en traitement au moment de la préparation du manuscrit.

3. *Critères d'évaluation du traitement* — Les critères d'évaluation étaient les mêmes que dans le groupe des cas souffrant de maladie de Hodgkin. Dans les cas de leucémie, les examens du sang et de la moelle osseuse étaient des éléments supplémentaires d'appréciation des résultats.

*Nous parlons de "deux cures" thérapeutiques lorsque les périodes de traitement sont séparées par un intervalle de plusieurs semaines. La durée de la cure comprend les courtes périodes d'interruption (maximum une semaine) nécessitées par les effets toxiques du médicament.

RESULTATS

Resultats thérapeutiques

1. *Maladie de Hodgkin* — Une *amélioration subjective* a été observée, au cours de la première cure, dans vingt-huit cas sur trente-trois et au cours de la deuxième cure dans trois cas sur sept. Le prurit, qui était une manifestation majeure de la maladie, a disparu ou a fortement diminué dans dix cas sur douze.

Un *effet objectif probable* a été observé dans deux cas au cours de la première cure et dans deux cas au cours du deuxième traitement. Trois fois, il s'agissait d'une diminution des adénopathies, de la splénomégalie et de la fièvre, pendant une période inférieure à 1 mois. Une fois, il s'agissait d'une diminution des adénopathies, pendant 1 mois, non accompagnée d'amélioration subjective.

Un *effet objectif certain* a été observé dans vingt-trois cas sur trente-trois au cours du premier traitement et dans un cas sur sept au cours de la deuxième cure. La rémission a duré de 2 à 6 mois dans huit cas. Quinze malades sont encore en rémission, de 1 mois à 7 mois après l'apparition des premiers signes objectifs d'amélioration. La durée de chaque rémission est indiquée dans la Fig. 2. Dans tous les cas ayant présenté un effet objectif certain, les premiers signes cliniques de l'effet objectif étaient présents deux semaines après le début du traitement.

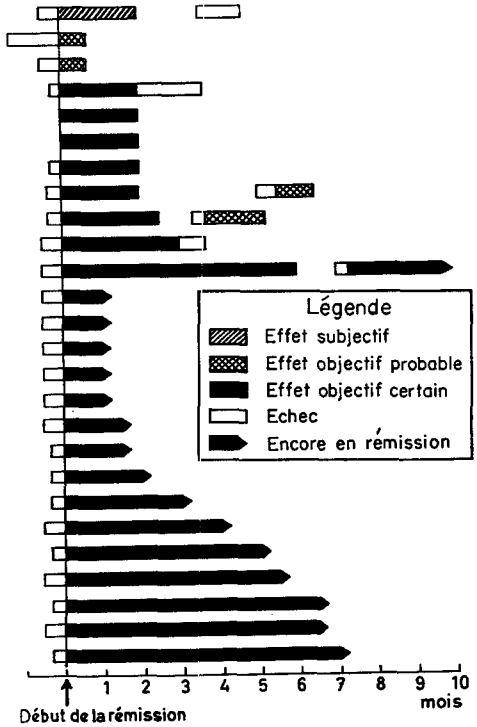


Fig. 2. Durée des rémissions dans la maladie de Hodgkin.

Les adénopathies ont diminué de volume dans vingt cas sur vingt-cinq. L'hépatosplénomégalie a diminué dans douze cas sur quatorze, les lésions pleuro-pulmonaires dans sept cas sur neuf (Figs. 3–4). Un ictère accompagné des signes cliniques et biochimiques de l'infiltration intra-hépatique a disparu dans les trois cas traités. Chez ces trois malades, le foie a diminué de volume et tous les tests hépatiques perturbés (bilirubine, phosphatase alcaline, transaminases oxalo-acétique et pyruvique, déshydrogénases lactique et isocitrique) sont revenus à des valeurs normales (Fig. 5).

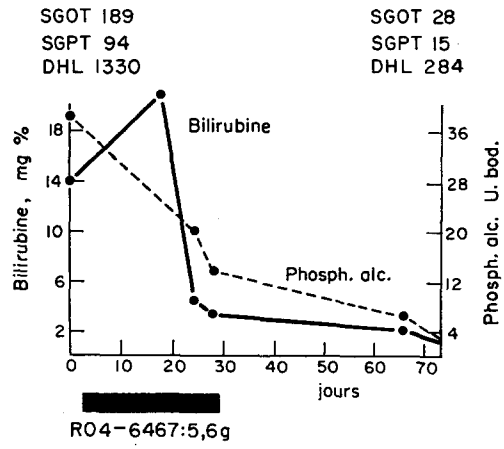


Fig. 5. Cas D . . . , M. (maladie de Hodgkin). Effet du Ro4-6467 *dans un cas d'ictère.*

Dans deux cas, une anémie importante a été corrigée pendant le traitement. La Fig. 6 montre l'effet de ce dérivé de la méthylhydrazine sur l'hémoglobine et sur les plaquettes, chez un malade qui n'avait pas répondu à de fortes doses de prednisolone. Ce patient, qui avait reçu 6 l. de sang en 70 jours avant le Ro4-6467, maintient un taux normal d'hémo-

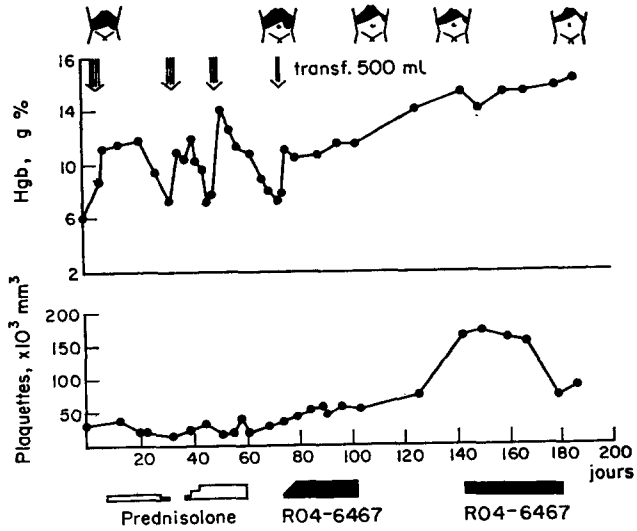


Fig. 6. Cas M . . . , M. (maladie de Hodgkin).

globine depuis 5 mois. Des cellules de Sternberg étaient présentes dans la moelle osseuse avant le traitement et avaient disparu à la suite de celui-ci.

2. *Autres lymphomes malins et leucémies* — Parmi les six cas de lymphosarcome, nous avons observé cinq échecs et un effet objectif certain. Ce malade est en rémission 10 mois après l'arrêt du traitement. Une volumineuse ascite et un épanchement pleural ont disparu. Les lymphographies ont montré une diminution des adénopathies (Figs. 7–8).

Quatre cas de réticulosarcome se sont montrés résistants au produit. Un effet objectif certain a été observé chez le cinquième patient, un enfant de 6 ans. La fièvre a cessé, les adénopathies et l'hépatomégalie ont disparu.

Les deux cas de myélome multiple ont été résistants au traitement. Un de ces malades est mort après 10 jours de traitement.

Les deux cas de leucémie lymphoïde chronique étaient à un stade terminal de la maladie et sont morts 19 jours et 34 jours après le début du traitement, sans que l'on puisse observer aucun effet favorable.

Un cas de leucémie myéloïde chronique a eu une rémission clinique et hématologique complète d'une durée de 2 mois. Les leucocytes sont restés en dessous de 20.000/mm³ pendant 4 mois. L'autre cas est en rémission complète depuis 2 mois.

Pour l'ensemble des malades, considérés comme un seul groupe, l'effet thérapeutique n'était pas directement lié à la valeur de la dose totale administrée en une cure. Un effet objectif était observé quatorze fois sur vingt-quatre, lorsque la dose totale était inférieure à 6 g (moyenne 4,62 g) et dix-huit fois sur trente-trois lorsqu'elle dépassait 6 g (moyenne 9,04 g).

Les résultats du traitement pour les différentes catégories de malades sont indiqués au Tableau 1.

Toxicité

Pour l'étude de la toxicité, nous considérons l'ensemble des malades en un seul groupe, puisque nous n'avons pas observé de différences selon le diagnostic de la maladie.

Effets sur l'hématopoïèse — Le Ro4-6467 a provoqué fréquemment une diminution des différents éléments figurés du sang. Les fréquences de l'anémie, de la leucopénie, et de la thrombocytopénie sont indiquées dans le Tableau 2. La chute des leucocytes était en général plus précoce que la chute des plaquettes, mais la valeur critique arbitraire de 3.000 leucocytes et de 100.000 plaquettes était atteinte en moyenne plus tardivement pour les premiers (37 jours) que pour ces dernières, (31 jours). De même, la valeur minimum pour les leucocytes était atteinte en moyenne en 52 jours (de 37 à 70 jours) et pour les plaquettes en

Tableau 1.

	Nombre de cas	Effet objectif		Echecs
		Certain	Probable	
Maladie de Hodgkin	33	23	2	8
Lymphosarcome	6	1	0	5
Réticulo-sarcome	5	1	0	4
Myélome multiple	2	0	0	2
Leucémie lymph. chron.	2	0	0	2
Leucémie myél. chron.	2	2	0	0
Totaux	50	27	2	21

Tabeau 2. Toxicité hématologique (total des cas: 50).

	Présente	Absente	Non évaluable
Anémie*	20	27	3
Leucopénie†	29	19	2
Thrombocytopénie‡	35	13	2

*Hémoglobine inférieure à 10 g pour cent.
†Leucocytes inférieurs à 3·000/mm³.
‡Plaquettes inférieures à 100·000/mm³

47 jours (de 32 à 71 jours). Ces moyennes ainsi que le retour aux valeurs normales sont représentées graphiquement dans la Fig. 9. Cette figure illustre le fait que la thrombocytopénie a été plus fréquemment la cause de l'arrêt du traitement que la leucopénie (respectivement vingt-trois fois et six fois). Elle montre aussi que les leucocytes n'atteignaient, en moyenne, leur valeur minimum que trois semaines après l'arrêt du traitement.

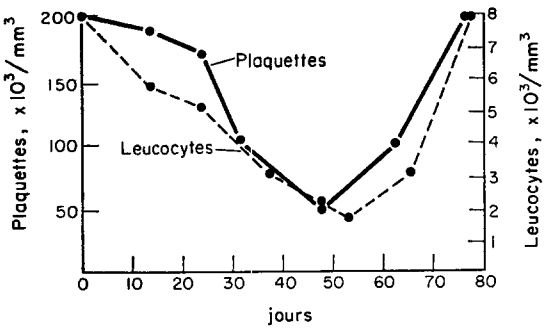


Fig. 9. Moyennes des numérations des leucocytes et des plaquettes.

La dépression médullaire était rarement très sévère. Les plaquettes sont tombées en dessous de 30.000/mm³ dans douze cas. Six malades de ce groupe ont présenté des manifestations hémorragiques sans gravité. Les leucocytes sont tombés en dessous de 1.500/mm³ dans six cas. Deux malades de ce groupe sont morts de septicémie au moment de la leucopénie. Ils avaient reçu 8.450 mg en 32 jours et 11.400 mg en 45 jours. En outre, un autre malade est mort de broncho-pneumonie, alors que les leucocytes étaient tombés à 1.800/mm³ après une dose de 2.450 mg donnée en 23 jours.

Il n'y avait pas de corrélation étroite entre la dose totale de Ro4-6467 et l'importance de la toxicité hématologique. Une toxicité hématologique sévère, définie par une leucopénie inférieure à 1.500 ou une thrombocytopénie inférieure à 30.000, a été observée dans cinq cas sur vingt-quatre, lorsque la dose totale donnée en une cure était inférieure à 6 g, et neuf fois sur vingt-trois lorsque la dose totale était supérieure à 6 g.

Nous n'avons pas observé de signes évidents d'hémolyse, mais nous n'avons pas pratiqué chez nos malades d'étude de la survie globulaire ni de mesure de l'excrétion totale d'urobilinogène. La bilirubine plasmatique non conjuguée et l'urobilinogène excrétée en deux heures n'ont été augmentées que dans un cas. Les réticulocytes ont varié de façon inconstante et étaient plus souvent diminués qu'augmentés.

Toxicité digestive — Les troubles digestifs étaient fréquents, au cours du traitement, chez la majorité des malades. Ils n'étaient complètement absents que dans dix-huit cas. Ils étaient modérés et souvent limités à la période initiale dans dix-sept cas. Ces malades avaient soit des nausées et des vomissements disparaissant malgré la poursuite du traitement, soit une anorexie durable. Chez quatorze malades, les vomissements et les nausées étaient importants, et persistaient pendant toute la durée du traitement. Le traitement a dû être interrompu à cause de ces troubles digestifs dans deux cas. Un malade a souffert d'une diarrhée sévère.

Autres signes de toxicité — Une alopécie modérée a été observée dans un seul cas.

Parmi les neuf femmes non ménopausées, une aménorrhée transitoire a été observée dans cinq cas (malades âgées de 31 à 45 ans). Les quatre patientes les plus jeunes (de 20 à 30 ans) n'ont pas présenté de modification des périodes menstruelles.

Huit malades ont présenté une intolérance à l'alcool; il y avait une vasodilatation intense au niveau du visage, parfois accompagnée de tendences lipothymiques. Ces symptômes apparaissaient quelques minutes après l'ingestion de bière ou de vin. Ils n'étaient pas présents lorsque ces malades ne recevaient pas de Ro4-6467.

Un malade a présenté une dermatite exfoliative avec érythrodermie, après 3 semaines de traitement. Une dose de 50 mg de Ro4-6467, administrée *per os* après un délai de 3 semaines sans traitement, a provoqué une nouvelle poussée d'érythrodermie.

Aucune altération des fonctions hépatiques et rénales n'a été observée.

Décès — Trente-sept malades étaient en vie, de 1,5 à 10 mois après le début du traitement, au moment de la préparation du manuscript.

Dix patients sont morts de l'évolution de la maladie, de 15 jours à 7 mois après le début du traitement.

Un cas de maladie de Hodgkin est décédé,un mois après le début du traitement, d'une broncho-pneumonie avec leucopénie. Un cas de lymphosarcome et un cas de réticulosarcome sont morts de septicémie avec leucopénie, un mois et demi après le début du traitment.

DISCUSSION

Nos résultats confirment ceux décrits dans la littérature [2–4]. Le Ro4-6467 est fréquemment efficace dans la maladie de Hodgkin et beaucoup plus rarement dans les autres lymphomes malins. Les deux cas de leucémie myéloïde chronique ont répondu au traitement par une rémission complète.

La durée des rémissions obtenues dans la maladie de Hodgkin est souvent assez courte. Dans neuf cas, elle n'a pas dépassé 3 mois. Notre expérience actuelle ne nous permet pas encore d'établir la longueur des rémissions les meilleures. Quinze malades sont encore en rémission de 1 à 7 mois après l'apparition des premiers signes d'amélioration.

Un deuxième traitement a été inefficace dans quatre cas, et peu efficace (effet objectif probable) dans deux cas. Une seule fois une rémission quasi complète a été obtenue au cours du deuxième traitement.

Il faut rapprocher ces observations des données expérimentales de Bollag [5] qui a observé l'apparition rapide d'une résistance chez les animaux de laboratoire porteurs de tumeurs. Par contre, notre matériel clinique permet d'établir qu'il n'existe pas, chez l'homme, de résistance croisée entre le Ro 4-6467 et la radiothérapie ou d'autres agents chimiothérapiques. Le Tableau 3 indique que les échecs étaient exceptionnels chez les malades qui étaient résistants aux précédentes thérapeutiques. Inversement, des malades résistants au Ro4-6467 ont répondu favorablement à un traitement ultérieur à la vinblastine (quatre cas), au cyclophosphamide (un cas), au chlorambucil (un cas), à l'ypérite azotée (un cas), à la radiothérapie (deux cas).

Certaines rémissions obtenues dans la maladie de Hodgkin ont été spectaculaires. Un patient avec un épanchement pleural très important et une volumineuse masse sous-cutanée a présenté une rémission apparamment complète, alors qu'il était résistant à la radiothérapie et à l'ypérite azotée intrapleurale (Fig. 4). Deux cas d'anémie, résistants à la prednisolone, ont parfaitement répondu au Ro4-6467. L'ictère, qui est en général considéré comme d'un mauvais pronostic dans la maladie de Hodgkin, a disparu chez les trois malades traités.

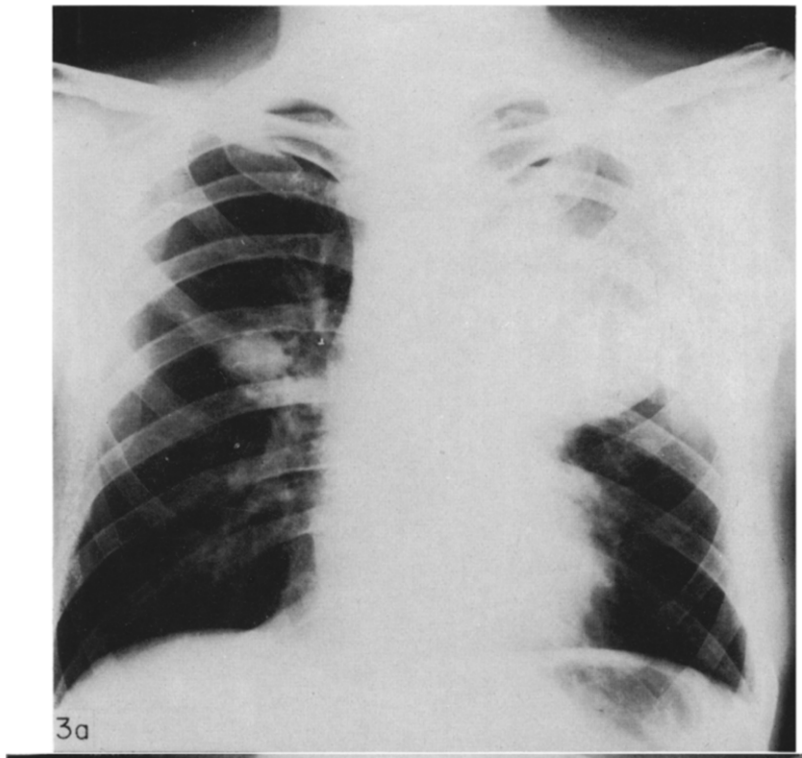
Les premiers signes d'amélioration sont toujours apparus rapidement, en 2 semaines maximum. La seule exception était un malade qui n'avait répondu qu'après 5 semaines de traitement: la rémission a duré moins d'un mois. On peut donc affirmer que la chance d'obtenir une rémission est très faible lorsqu'il n'y a pas eu de réponse après 3 ou 4 semaines de traitement, alors qu'on approche du seuil des effets secondaires.

Le principal inconvénient du traitement a été l'intolérance digestive. Les troubles digestifs étaient présents dans deux tiers des cas. Ils provoquaient une grave incommodité pour le patient dans un tiers des cas.

Comme pour tout nouvel agent chimiothérapique, nous nous sommes efforcés de donner la dose maximum tolérée. Nous avons donc observé un grand nombre de cas de thrombocytopénie et de leucopénie. La thrombocytopénie était plus fréquente que la leucopénie, contrairement à ce qu'on observe avec le cyclophosphamide et la vinblastine. L'effet toxique sur les globules blancs et sur les plaquettes est souvent tardif et s'accentue souvent pendent plusieurs semaines après l'arrêt du traitement. Il nous paraît prudent d'interrompre le traitement lorsque les plaquettes atteignent 100.000/mm³ et les leucocytes 3.000/mm³. Dans un cas, nous avons, cependant, continué l'administration du Ro4-6467 pendant 5 jours après une chute des globules blancs à 2.200/mm³. Ce malade est mort de septicémie avec une agranulocytose. Deux autres malades sont morts d'infection avec leucopénie sévère, alors que le traitement avait été arrêté lorsque les leucocytes atteignaient 3.000/mm³. La dépression médullaire a été réversible dans les autres cas, mais plusieurs malades ont eu un syndrome hémorragique. Nous estimons que la toxicité hématologique de ce dérivé de la méthylhydrazine est plus

Tabelau 3. Maladie de Hodgkin (total des cas: 33)

	Effet objectif		
	Certain	Probable	Echecs
Cas sensibles au traitement précédent	13	1	7
Cas résistants au traitement précédent	10	1	1



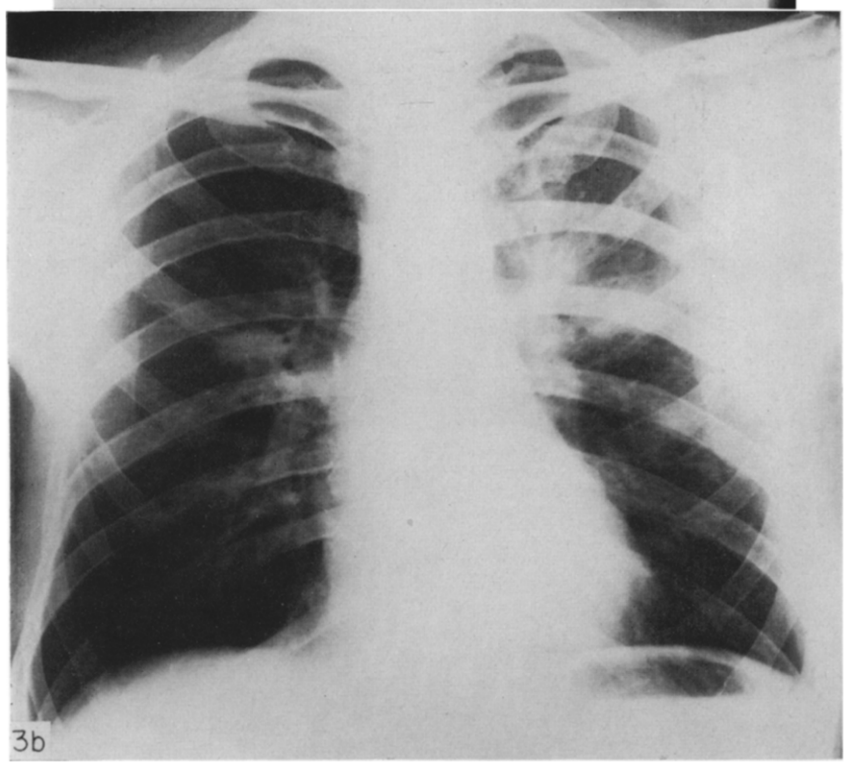


Fig. 3. Cas L . . . , P. (maladie de Hodgkin).
a. *avant le traitement ;* b. 46 *jours après le début du traitement.*

(to face p. 38)
Refer to p. 36

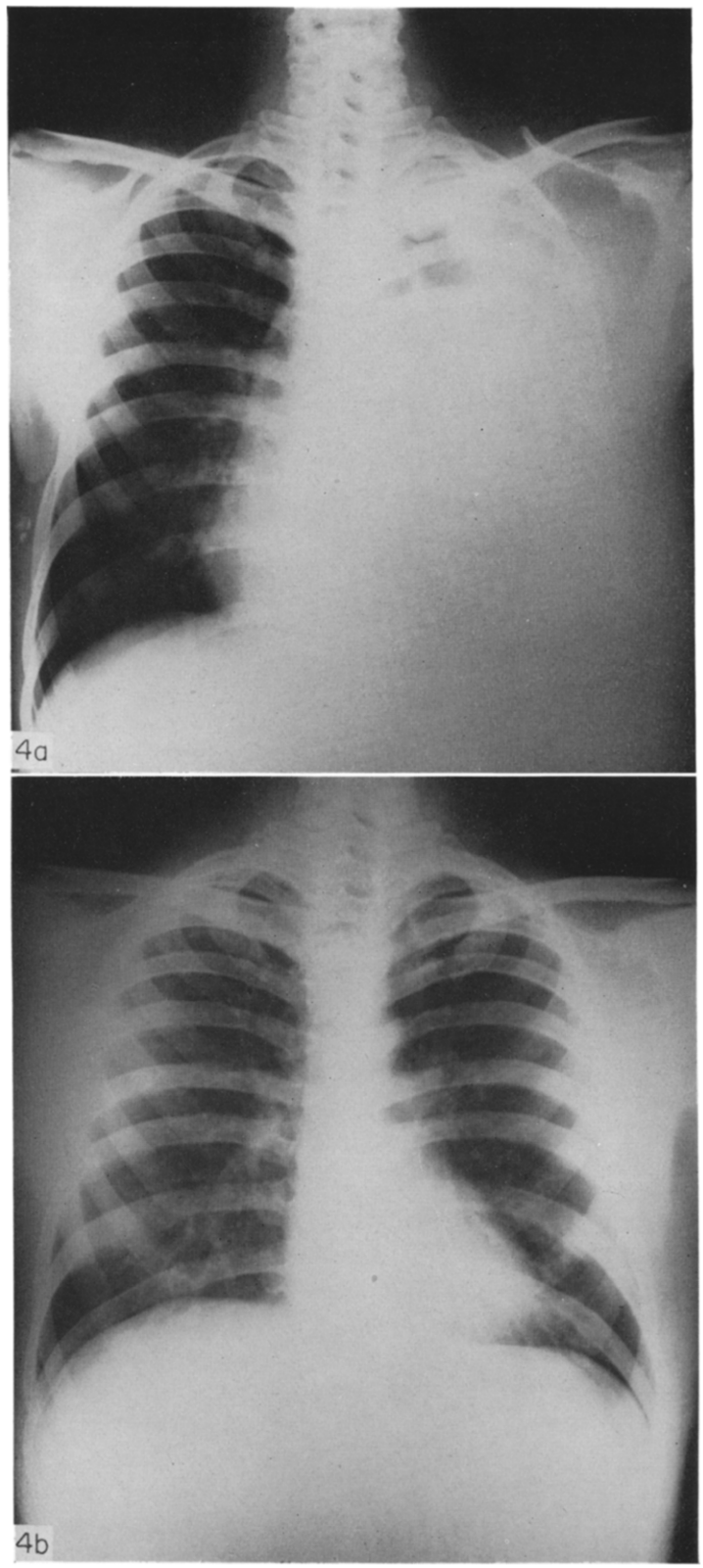


Fig. 4. Cas L . . . , J. (maladie de Hodgkin).
a. *avant le traitement;* b. 20 *jours après le début du traitement.*

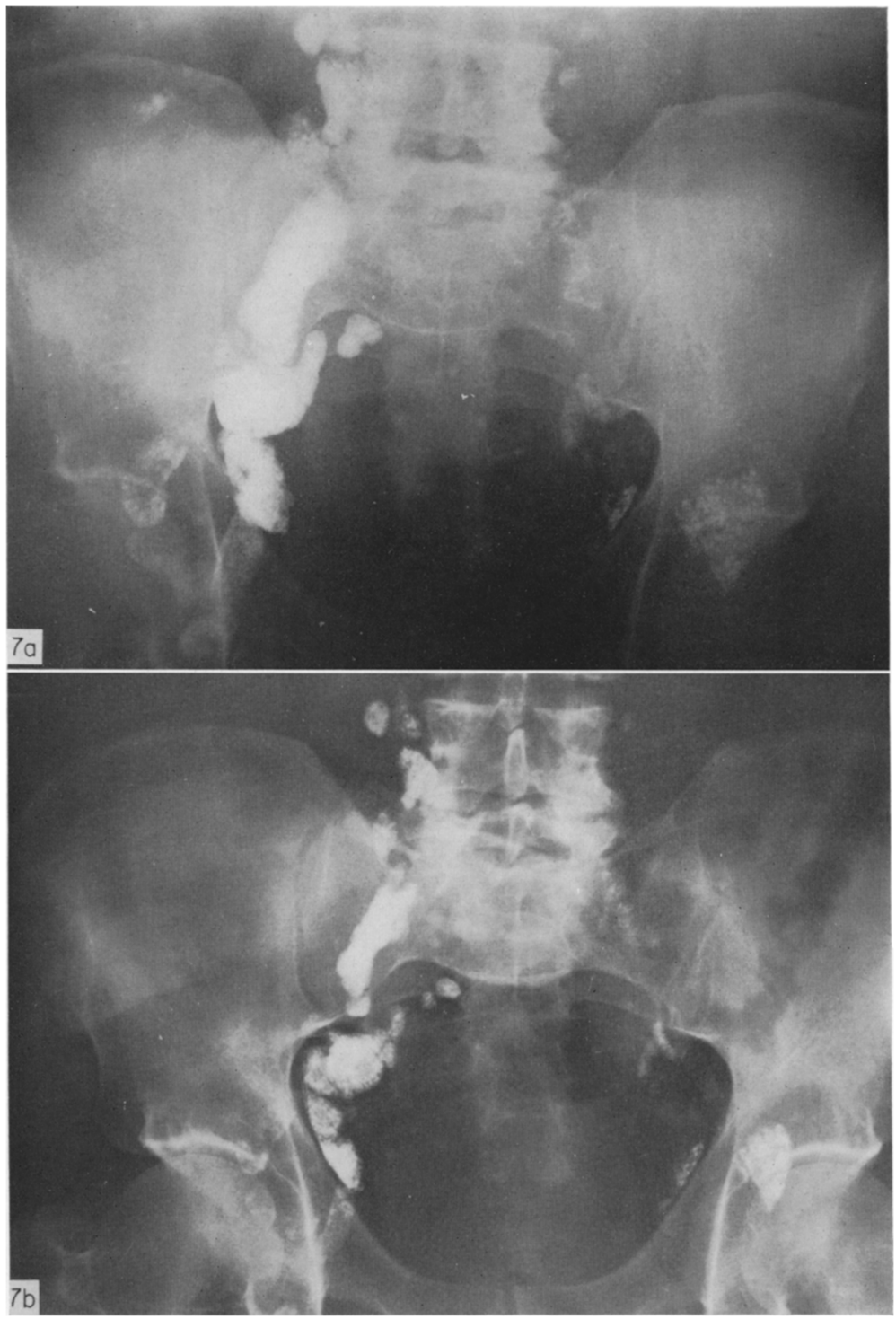


Fig. 7. Cas F . . . , G. (lymphosarcome).
a. *lymphographie avant le traitement;* b. *après une dose totale de* 4·450 mg *en* 29 *jours.*

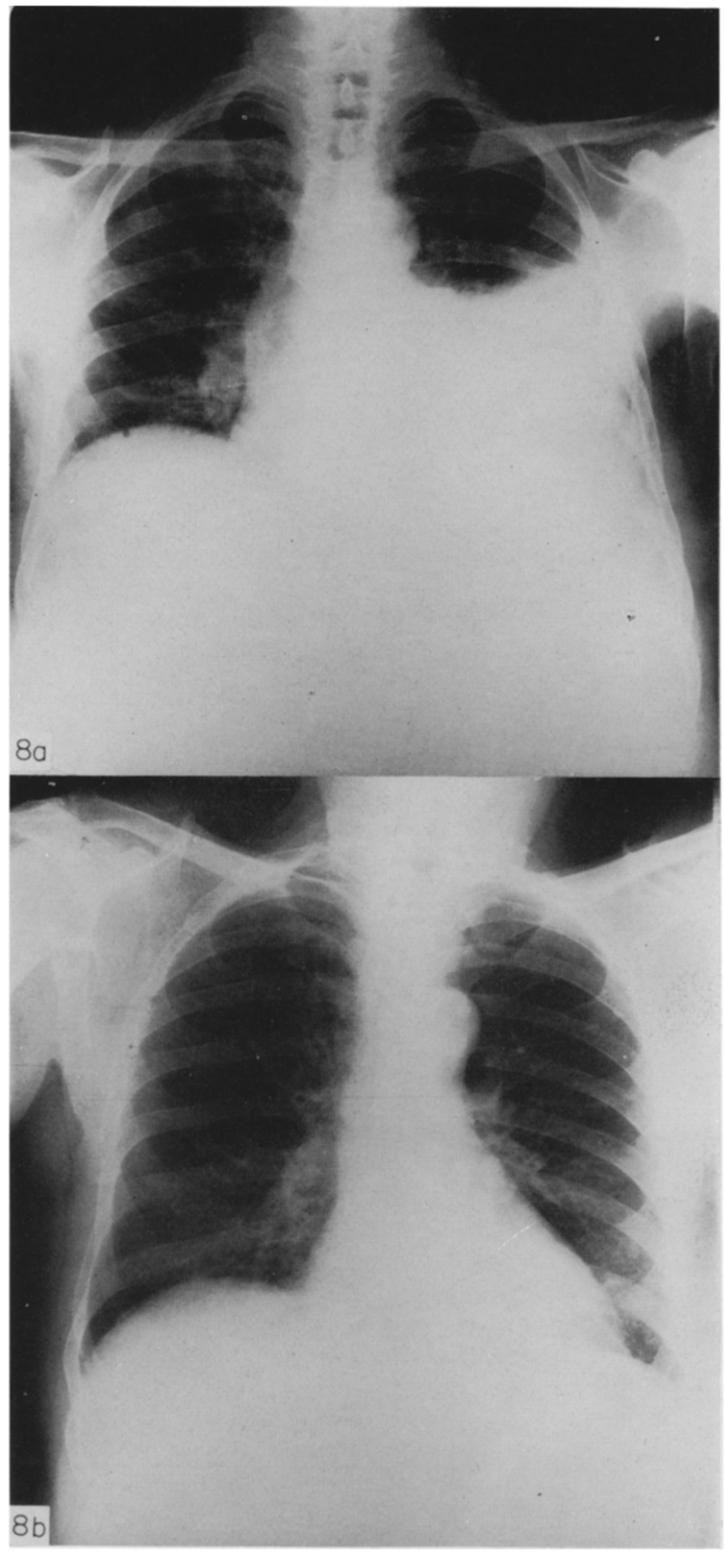


Fig. 8. Même cas.

importante que celle du cyclophosphamide [6] mais nettement plus faible que celle de la mitomycine C [7].

Nous n'avons pas observé de relation directe entre la dose totale administrée en une cure* et l'importance de la toxicité. Nous pensons que le facteur important est la dose quotidienne moyenne. Les malades qui ont reçu une dose quotidienne moyenne de 250 mg ou davantage ont présenté une toxicité hématologique sévère (voir plus haut) dans sept cas sur dix-sept (41 pour cent). Les malades qui ont reçu, en moyenne, moins de 250 mg par jour ont présenté une telle toxicité dans huit cas sur quarante (20 pour cent).

Les effets objectifs dans les deux groupes étaient respectivement de 41 pour cent (sept cas) et de 65 pour cent (vingt-six cas). Le rapport effet thérapeutique sur effet toxique était donc de 1·0 pour le premier groupe et de 3·2 pour le second. Ces chiffres indiquent que la dose la plus favorable est voisine de 250 mg par jour. En augmentant cette dose, on n'améliore pas les résultats thérapeutiques, mais on risque, par contre, de provoquer une dépression médullaire sévère. Il faut se rappeler que cette constatation s'applique à des cas traités par de petites doses quotidiennes. On ne peut pas en déduire la valeur du rapport effet thérapeutique sur effet toxique pour un mode d'administration différent, par exemple, par doses massives, espacées de plusieurs semaines.

Les autres manifestations toxiques étaient rares et semblables à ce qu'on observe avec d'autres agents: alopécie, aménorrhée, érythème cutané. Une complication spécifique est l'intolérance à l'alcool qui serait due à la libération d'acétaldéhyde [5].

CONCLUSIONS

Le chlorhydrate de p-(N'-méthyl-hydrazino-méthyl)-N-isopropyl-benzamide (Ro4-6476) est un agent chimiothérapique très efficace dans le traitement de la maladie de Hodgkin et dont l'efficacité dans d'autres formes de lymphomes malins et dans les leucémies reste à déterminer. Ce produit est relativement peu toxique. Il présente l'avantage considérable, du point de vue théorique, d'appartenir à une nouvelle classe d'agents chimiothérapiques anti-cancéreux.

Il est établi qu'il ne présente pas de résistance croisée avec les autres produits actifs dans la maladie de Hodgkin et semble donc destiné à prendre une place dans le traitement palliatif de cette maladie.

Remerciements — Nous tenons à remercier le Professeur S. Simon (Service de Radiothérapie) qui nous a confié de nombreux malades pour cette étude. Deux cas ont été traités, selon nos directives, à l'Hôpital Stuyvenberg (Anvers), par le Docteur M. E. Peetermans, que nous remercions aussi. Nous avons beaucoup apprécié l'aide compétente de Mademoiselle Van Leeuw, du Service des Archives Médicales de l'Institut Jules Bordet.

*Voir la note au bas de la page 4.

RÉSUMÉ

Le chlorhydrate de p-(N'-méthyl-hydrazinométhyl)-N-isopropyl-benzamide a été administré à trente-trois malades atteints de maladie de Hodgkin et à dix-sept malades atteints d'autres lymphomes malins et de leucémies.

Un effet objectif certain a été observé dans vingt-trois cas de maladie de Hodgkin, dans un cas de lymphosarcome, un cas de réticulosarcome, deux cas de leucémie myéloïde chronique. Des cas résistants à la radiothériape, aux agents alkylants, à la prednisolone, étaient sensibles à la méthylhydrazine.

Une diminution importante des leucocytes a été observée chez six malades et une thrombocytopénie sérieuse chez douze malades. Trois malades sont morts d'infection avec leucopénie.

Les autres signes de toxicité étalent des troubles digestifs (vomissements, anorexie, diarrhée), l'alopécie, l'aménorrhée, l'intolérance à l'alcool et une érythrodermie.

SUMMARY

p-(N'-methyl-hydrazinomethyl)-N-isopropyl-benzamide-hydrochloride has been administered to thirty-three patients suffering from Hodgkin's disease and seventeen patients suffering from other malignant lymphomas and leukaemias.

The preparation exerted a definite objective effect on twenty-three cases of Hodgkin's disease, one case of lymphosarcoma, one case of reticulum-cell sarcoma, and two cases of chronic myeloid leukaemia. Patients who did not respond to radiotherapy, alkylating agents or Prednisolone could be influenced by the methyl-hydrazine derivative.

A marked reduction in leukocytes was observed in six cases, and severe thrombocytopenia in twelve cases. Three patients died of infection accompanied with leukopenia.

Other toxic signs were digestive disturbances (vomiting, anorexia, diarrhoea), alopecia, amenorrhoea, alcohol intolerance, and erythrodermia.

ZUSAMMENFASSUNG

Das p-(N'methylhydrazinomethyl)-N-isopropyl-benzamid-hydrochlorid wurde drei und dreissig Patienten mit Morbus Hodgkin und siebzehn Patienten mit anderen malignen Lymphomen und Leukämien verabreicht. Ein sicherer positiver Effekt konnte in drei und-zwanzig Fällen von Morbus Hodgkin, in einen Fall von Lymphosarkom, in einen Fall von Retikulosarkom und in zwei Fällen von chronischer myeloischer Leukämie beobachtet werden. Fälle, die gegenüber Radiotherapie, alkylierenden Substanzen und Prednisolon resistent waren, sprachen auf das Methylhydrazinderivat an. Eine starke Verminderung der Leukozyten wurde bei sechs Patienten und eine Thrombozytopenie bei zwölf Patienten beobachtet. Drei Patienten starben an Infektionen.

Die anderen Toxizitätserscheinungen betrafen den Verdauungstrakt (Erbrechen, Anorexie, Diarrhoe), Alopezie, Amenorrhoe, Alkoholintoleranz und eine Erythrodermie.

BIBLIOGRAPHIE

1. W. Bollag and G. Grunberg, Tumour inhibitory effects of a new class of cytotoxic agents: methylhydrazine derivatives. *Experientia* **19,** 130 (1963).
2. A. d'Alessandri, H. J. Keel, W. Bollag und G. Martz, Erste Klinische Erfahrungen mit einem neuen Cytostatikum. *Schweiz. med. Wschr.* **93,** 1018 (1963).
3. G. Mathe, L. Berumen, O. Schweisguth, G. Brule, M. Schneider, A. Cattan, J. L. Amiel and L. Schwarzenberg, Methyl-hydrazine in treatment of Hodgkin's disease and various forms of haematosarcoma and leukemia. *Lancet* **II,** 1077 (1963).
4. G. Martz, A. d'Alessandri, H. J. Keel and W. Bollag, Preliminary clinical results with a new antitumor agent — Ro4-6467 (NSC-77213). *Cancer Chemother. Rep.* **33,** 5 (1963).
5. W. Bollag, Communication personnelle.
6. Y. Kenis, Action du cyclophosphamide (Endoxan) dans 41 cas d'affections néoplasiques. *Acta Clin. Belg.* **17,** 253 (1962).
7. Y. Kenis et P. Stryckmans, Action de la mitomycine C sur l'hématopoïèse dans le cancer en phase avancée. *Nouv. Rev. Franç. Hémat.* **4,** 15 (1964).

Europ. J. Cancer Vol. 1, pp. 41–50. Pergamon Press 1965. Printed in Great Britain

Responsiveness of Mouse Mammary Tumours to Pregnancy

R. VAN NIE and P. J. THUNG

Department of Experimental Pathology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

HORMONAL influences on the growth of experimental mammary tumours have been studied by Mühlbock [1], Noble [2], Furth and Kim [3] and others, using rats and mice. While in some strains of rats endocrine factors are known to influence the growth rate (Huggins, [4]), mammary tumours in mice, once established, are generally found to grow independently from the hormonal status of the host animal (Mühlbock, [1]).

Data have been published, however, indicating that in hybrids of some mouse strains, pregnancy may influence the growth rate of mammary tumours. Gardner [5] observed (C57 × CBA)F_1 hybrids with mammary tumours which grew during pregnancy but regressed following parturition. On this phenomenon more light was thrown by the experiments of Foulds [6], using forced breeding in F_1, F_3 and F_4 hybrids of C57BL and RIII mice. By careful observation of the mammary tumours, growth curves were obtained which revealed two main types of behaviour. "Unresponsive" tumours grew steadily throughout pregnancy and the postpartum period, while "responsive" tumours grew during pregnancy, reached maximum size shortly before parturition, and subsequently regressed after parturition. During the next pregnancy, the latter tumours recurred. Foulds' excellent analysis of the behaviour of these mammary tumours was the starting point for his concept of progression (Foulds [6, 7]).

According to Squartini [8], the hybrid state is not required for this responsiveness of tumour growth to pregnancy. This author found that 80·3 per cent of the mammary tumours observed in RIII breeders responded similarly by regressing after parturition. In BALB/cf mice, on the other hand, only 19 per cent of the mammary tumours showed this characteristic. In this latter strain, moreover, progression soon changed responsive tumours into unresponsive ones.

Recently, Heston *et al.* [9] described a new mouse strain with a high mammary tumour incidence, one characteristic of these tumours being the growth and regression of some of them, during and after pregnancy respectively.

In our laboratory, a similar relationship has now been observed in both breeders and forced-breeders of one strain and, more clearly, in hybrids derived from this strain, the present paper describing observations on these hybrids. The closely corresponding results from different laboratories, using different strains of mice, suggest a common mechanism which warrants further study.

MATERIAL AND METHODS

The mice described in this report, are F_1 hybrids of either RIII or C57BL mice and the GR/A strain described by Mühlbock [10]. In the present paper the abbreviation GR will be used for this strain. Since no differences were observed in our experiments, between (GR × RIII) and (RIII × GR) hybrids* or between (GR × C57BL) and (C57BL × GR) hybrids, reciprocal hybrids will be combined in the descriptions of our experimental groups.

Female hybrids were housed with males after weaning, and forced breeding was established

*In nomenclature of hybrids, the female parent is mentioned first.

by early removal of all litters. First litters were invariably found when the breeders were 2–2½ months of age.*

The mice were inspected twice weekly and the size and localization of mammary tumours were noted. Tumour size was assayed by matching with one of a series of beads, ranging in diameter from 5 to 28 mm. Intervals were ½ mm from 5 to 15 mm, and 1 mm from 15 to 28 mm dia.

For our experiments, mice were selected by measuring tumours during pregnancy, shortly before parturition. For some experiments (a), animals were taken when bearing at least one tumour with a diameter of more than 6 mm. In (GR × RIII) and reciprocal hybrids, mice fulfilling this criterium ranged in age from 82 to 262 days (mean age 126 days), and had experienced from 2 to 11 pregnancies.* In (GR × C57BL) and reciprocal hybrids, these data were, respectively, 105 to 301 days (mean age 175 days) and 2 to 11 previous pregnancies.*

For another experiment (b), a selection was made for larger tumours, only mice bearing at least one tumour with a diameter of more than 8 mm being used. Finally, for the experiments described under (c), (d) and (e), animals were selected in which, after the cessation of breeding, all tumours had regressed. These mice had previously been used in experiment (b) which served to analyze this regression.

In some experiments, hormones were administered.† Oestrone was given in drinking water in doses of 0·125, 0·5 or 2·0 mg/l. Progesterone was administered subcutaneously, either dissolved in olive oil or in pellets.

RESULTS

a. *Responsiveness of mammary tumours to pregnancy*

In a group of twenty-eight females (GR × RIII)F_1 and reciprocal hybrids, each bearing at least one mammary tumour with a diameter of more than 6 mm at the beginning of the experiment, the size and localization of the tumours was studied during a number of successive

*Data from the Biological Department of this Institute, Head Prof. O. Mühlbock. The tumour bearing mice used in our experiments, were obtained from this Department.

†Hormones were kindly supplied by N. V. Organon, Oss.

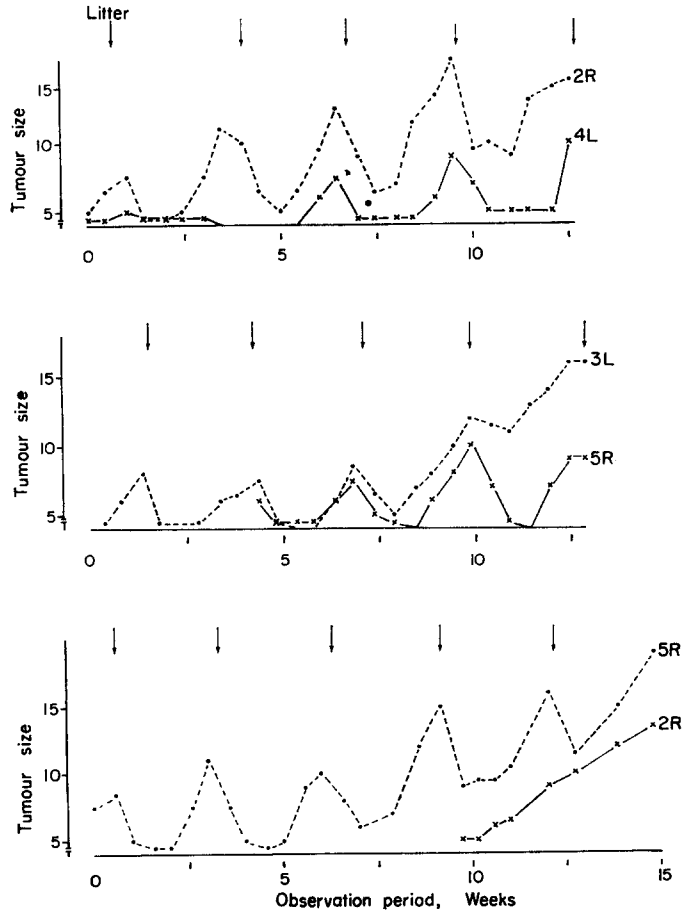


Fig. 1. Growth curves of mammary tumours in three forced-bred female F_1*-hybrids of* GR *and* RIII *mice, bearing two tumours each.*
2R *indicates a tumour localized in the second mammary gland on the right side,* 4 L *is the fourth gland on the left side etc.*

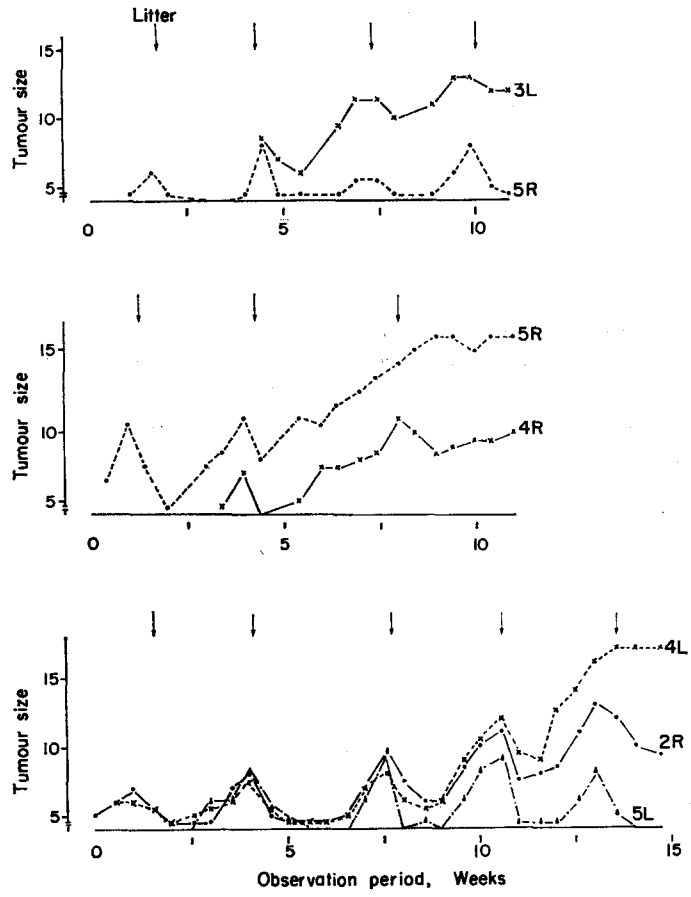


Fig. 2. Growth curves of mammary tumours in three forced-bred female F_1*-hybrids of* GR *and* C57BL *mice.* (cf. *Fig.* 1).

pregnancies. At least three successive pregnancies were observed in each mouse and at the end of the experiment a total number of sixty-four tumours had been followed in the course of 119 pregnancies.

Most tumours grew during the second half of pregnancy and underwent total or partial regression after parturition, thus showing some degree of responsiveness in relation to pregnancy. Growth curves of tumours observed in four mice from this experiment are shown in Fig. 1, illustrating the close similarity between our findings and those of Foulds [6]. Eight tumours, however, grew continuously from the time they were first noticed, without regression during postpartum periods.

All varieties of tumour behaviour mentioned by Foulds [6] were represented in our material. While some tumours regressed after parturition, to the extent of not being detectable by careful palpation, all tumours resumed growth during the next pregnancy. The maximal size of those tumours which showed partial regression only, increased during every following pregnancy. Multiple mammary tumours in one mouse may differ in responsiveness, unresponsive tumours often being found in animals bearing one or more responsive tumours. The phenomenon of "progression" (Foulds, [6]) was often observed too, postpartum regression frequently becoming less marked during successive pregnancies.

Apart from the mice mentioned above, twenty-nine females (GR × C57BL)F_1 and reciprocal hybrids were studied in the same way, a total number of sixty-one tumours being followed during 125 pregnancies. These tumours showed essentially the same characteristics as described above (Fig. 2).

Finally, it should be noted that unresponsive tumours, as well as those which regressed to a minor degree only, could often be distinguished macroscopically by their bluish colour which was visible through the skin of their hosts, especially in albino mice. This colour was due to the rich vascularity of these tumours which consisted of soft spongy tissue with many blood-filled cavities. Histologically, these tumours corresponded to the type B carcinomata of Dunn [11]. Markedly responsive tumours, on the other hand, usually had a more solid structure and showed the adenomatous and plaque-like characteristics described by Foulds [12].

Table 1. Relation between tumour size, and tumour behaviour after cessation of breeding in (GR × RIII) F_1 *and reciprocal hybrids.*

	Number (and percentage within each group) of tumours with a diameter of:			
	5–7½ mm	8–10 mm	10½ and more	all sizes
Regression	30 (100%)	58 (95%)	49 (74%)	137 (87%)
Partial or no regression	—	3 (5%)	17 (26%)	20(13%)
Total	30	61	66	157

Table 2. Relation between tumour size and tumour behaviour after cessation of breeding in (GR × C57BL) F_1 *and reciprocal hybrids.*

	Number (and percentage within each group) of tumours with a diameter of:			
	5–7½ mm	8–10 mm	10½ and more	all sizes
Regression	14 (82%)	17 (74%)	4 (12%)	35 (49%)
Partial or no regression	3 (18%)	6 (26%)	27 (88%)	36 (51%)
Total	17	23	31	71

b. *Tumour behaviour after cessation of breeding; short term effects*

In eighty-three forced breeders, F_1 hybrids of GR and RIII mice, each bearing at least one tumour with a diameter of over 8 mm, breeding was discontinued by removing the males. At that moment, these mice had in total 157 mammary tumours. According to their behaviour during the first few weeks after the last parturition, these tumours were subsequently classified as follows:

Regressing tumours, i.e. tumours which regressed to smaller than 5 mm dia. and which thereafter did not resume growth within 2 weeks.

Tumours showing no or partial regression, i.e. tumours which did not regress to smaller than 5 mm dia. or which resumed growth within 2 weeks after regression.

Of the 157 tumours, 137 were classified as regressing, the other twenty showing no or partial regression only. As is shown in Table 1, some relation was found between this aspect of tumour behaviour and the size of the tumours before cessation of breeding. No tumours smaller than 8 mm dia. belonged to the group of more or less unresponsive tumours. Most of these latter tumours were more than 10 mm dia. It should be noted, however, that among all tumours with a diameter of more than 10 mm, no difference in size was found between regressing and non- or partially regressing tumours, the mean diameters of these groups being 12 and 13 mm respectively.

A parallel experiment was done with thirty-eight F_1 hybrids of GR and C57BL mice, bearing a total number of seventy-one mammary tumours. The results are summarized in Table 2 and show that, among small, medium sized as well as large tumours, the relative number of more or less unresponsive tumours is higher in these mice than in the hybrids of GR and RIII.

c. *Tumour behaviour after cessation of breeding; long term effects*

From the previous experiment, fourteen hybrids of GR and RIII mice were taken in which all tumours had regressed and were not detectable by palpation. These mice were kept without males during the remainder of their lifespan and were checked twice weekly for mammary tumours, the maximal observation time being 300 days.

One animal survived for 291 days without showing tumour growth, while twelve mice developed mammary tumours and one mouse showed two tumours arising within 5 weeks of each other. Of the fourteen tumours observed,

six arose in hiterto unaffected glands, while eight tumours grew in the same mammary gland in which a regressed tumour was known to have occurred previously.

The results of this experiment are summarized in Table 3. These data indicate that

Table 3. Interval, in days, between involution of mammary tumours after cessation of breeding, and reappearance of the same tumour or appearance of new tumours, in non-breeding female mice (GR × RIII) F_1 *and reciprocal hybrids.*

	8 tumours, reappearing in a previously affected mammary gland, after:	6 tumours, appearing in a previously unaffected mammary glands, after:
	25	161
	60	196
	115	211
	140	220
	148	258
	163	297 days
	196	
	227 days	
Mean interval (± standard deviation)	134±62 days	224±67 days

tumours which recurred in previously affected glands, appeared earlier than the "new" tumours. It seems probable that the "recurring" tumours actually originated from remnants of regressed tumours. In mice sacrificed to study such remnants, these can usually be identified, upon dissection, as tough, pinhead-size white or yellowish nodules. Microscopically these consist of groups of small tubules with thick basal membranes and atropic epithelium, enveloped in reticular and collagenous fibres, lying between the tissues of the normal unstimulated mammary gland. The role of these remnants in the histogenesis of recurring tumours deserves more detailed study.

d. *Reactivation of regressed mammary tumours by resumed breeding*

For this experiment, five hybrids of GR and RIII mice were used, taken from experiment (b), which had previously borne nine tumours, all of which had regressed after cessation of breeding. The animals were housed with males again, the non-breeding interval varying from 40 to 133 days. During the first new pregnancy, all of the nine tumours reappeared in the same gland where they had previously been localized. The involution and reactivation of a typical tumour from this series, is illustrated in Fig. 3.

e. *Reactivation of regressed mammary tumours by hormonal treatment*

In the following experiments, it was tried to imitate the effect of pregnancy on regressed tumours, using exogenous hormones. The intention was first to reactivate tumour growth by hormonal treatment and subsequently to induce tumour regression by cessation of treatment. As in the experiments (c) and (d), hybrids of RIII and GR mice were used in which all mammary tumours had regressed after cessation of breeding.

Five females which had previously borne ten mammary tumours, were given oestrone in drinking water in doses of 0·5 or 2·0 mg/l. This treatment lasted 19 days, during which period no reactivation of regressed tumours was seen.

To four females, previously bearing nine mammary tumours, progesterone was given subcutaneously for 2 weeks (1 mg/day, or 2 mg every other day). During this period again no tumour growth occurred.

However, tumour growth did occur during combined treatment with oestrone and progesterone as specified in Table 4. Referring to this Table for details concerning this experiment, it must be emphasized that the results do not in all respects correspond to the effects of

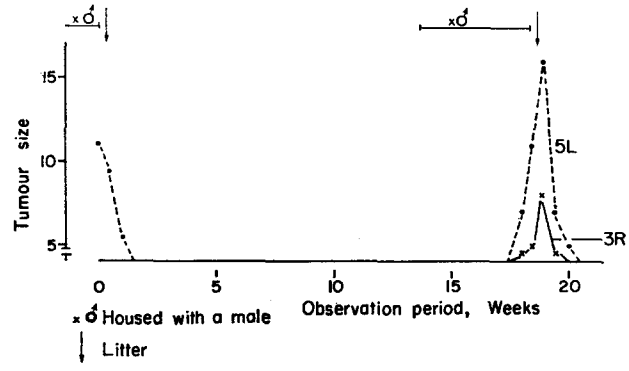


Fig. 3. Growth curves of mammary tumours in a female F_1 (RIII × GR) *mouse, bred discontinuously.*

Table 4. Reactivation, by exogenous hormones, of regressed mammary tumours in non-breeding (GR × RIII) F_1 *mice.*

During 18 days, all mice received one pellet (1½–2 mg) of progesterone, every other day. Additionally, oestrone was given in drinking water in the following concentration:	Number of mice	Number of previously regressed tumours	Number of tumours recurring during treatment, and( ) number of these with diameter over 7 mm:
2·0 mg	2 intact females	2	1 (0)
0·5 mg	4 intact females	7	5 (1)
0·125 mg	2 intact females	4	4 (2)
0·125 mg	2 castrate females	4	2 (2)
Total:	10 mice	17 regressed tumours	12 recurring tumours

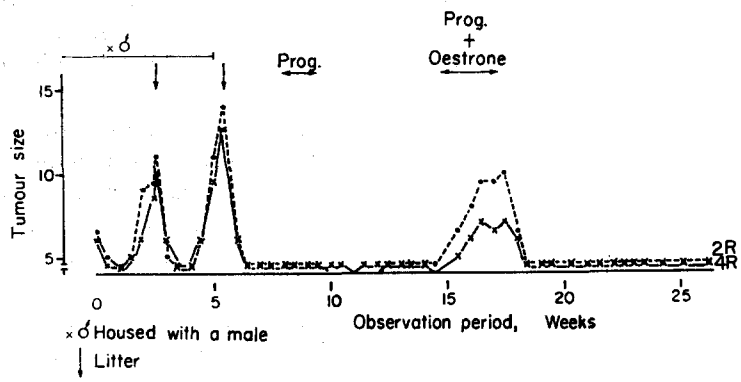


Fig. 4. Growth curves of mammary tumours in a female F_1 (GR × RIII) *mouse treated with progesterone and, later, with progesterone plus oestrone.*

pregnancy on these tumours. For one thing, not all regressed tumours reappeared during the hormonal treatment. Moreover, most of the recurring tumours did not reach the size which might have been expected during pregnancy. Only a few tumours grew to more than 7 mm dia. All tumours regressed after cessation of hormone treatment. Continued observation of the animals later disclosed spontaneous resumption of tumour growth in some of the mice, in accordance with the findings of experiment (c).

To illustrate the present experiment, Fig. 4 shows growth curves of mammary tumours in one of the animals from this series.

DISCUSSION

The tumours described in this report, differ markedly from the usual type of mammary tumour in high tumour strains of mice. The unresponsiveness of these latter tumours to hormonal conditions has been analysed by Mühlbock [1]. The present tumours, on the other hand, respond to pregnancy in a similar way to the tumours analysed by Foulds [6] and by Squartini [8].

Squartini and Severi [13] described different growth patterns in RIII mice which had and which had not been fosternursed by mice bearing a C3H-derived milk factor. Differences were also found between mammary tumours in BALB/c mice fosternursed by RIII and by C3H mothers (Squartini *et al.* [14]). It was concluded that these different growth characteristics reflected the fact that the RIII and the C3H strains carry different milk factors. With regard to the GR strain, this aspect is at present being studied in our laboratory.

Comparing the GR × RIII hybrids with the GR × C57BL hybrids, it was found that the latter combination results in a lower yield of responsive tumours. Moreover, around the end of pregnancy the mammary glands of (GR × C57BL)F_1 and reciprocal hybrids tend to become tensely swollen, which impedes the palpation of small tumours. For the analysis of hormone responsiveness of mammary tumours, we therefore prefer the GR × RIII hybrids.

In rats, effects of pregnancy on mammary tumour growth have been noted in passing by various authors, as reviewed by Dao and Sunderland [15]. The latter authors give an analysis of 3-methylcholanthrene induced tumours which grew rapidly in pregnant rats and decreased in size after parturition. Some of these tumours seemed to disappear entirely, while others later started to grow again, even though the animals were not breeding. It was found that the post-partum regression of these rat tumours was not influenced by lactation. This was similarly found in mice by Squartini [8].

Dao and Sunderland [15] also found that the growth of these carcinogen-induced rat tumours was somewhat stimulated during the first 5–9 days of pseudo-pregnancy. In mice, no stimulation of pregnancy-responsive tumours by pseuodopregnancy was detected by Foulds [6], which is in accordance with the fact that these tumours grow mainly during the second half of pregnancy.

Several workers studied the influence of changes in the hormonal balance of the hosts on the growth of primary mammary tumours induced by carcinogens in rats. Ovariectomy of the tumour-bearing rats resulted in varying degrees of tumour-regression (Huggins *et al.* [16]; Dao and Sunderland [15]; Kim and Furth [17]; Young *et al.* [18]; Daniel and Prichard [19]). A reactivation of the regressed tumours was seen after grafting a mammatropic pituitary tumour (Kim and Furth [17]) and after treatment with oestrogen and progesterone (Young *et al.* [18]). Sterental *et al.* [20] found that oestrogens stimulate mammary tumours which had regressed after combined ovariectomy and adrenalectomy.

After hypophysectomy most of the primary mammary tumours in carcinogen-treated rats decreased considerably (Huggins *et al.* [16]; Dao and Sunderland [15]; Kim and Furth [17]; Sterental *et al.* [20]; Daniel and Prichard [21].) The regression after hypophysectomy was more pronounced than that after ovariectomy. Kim and Furth [17] reactivated tumour growth in hypophysectomized rats with grafts of a mammotropic pituitary tumour. No stimulation of the regressed tumours in hypophysectomised animals could be observed by Sterental *et al.* [20] after treatment with oestrogen. The latter authors sustained the hypothesis of Furth and collaborators that stimulation by oestrogen of mammary tumours in rats is secondary to the activation, by the female hormone, of pituitary mammotropic cells [Kim *et al.* [22]).

Of special interest are the results of Huggins and Yang [23] obtained with primary mammary tumours induced in rats by dimethylbenzanthracene. In these tumours, combined treatment with oestradiol-17/β and progesterone, led to complete regression of established mammary tumours. It is a striking finding, that many of these tumours did not resume growth after cessation of this treatment.

In our experiments, partial reactivation of regressed tumours was obtained by combined treatment of intact female mice with oestrone and progesterone. Although these experiments are of a preliminary nature, artificial imitation of the effects of pregnancy on these tumours therefore seems feasible. Further experiments are called for, to ascertain which combination and doses of hormones may more fully approximate the effects of pregnancy. While such investigations may help to determine the exact endocrine factors involved in the pregnancy dependent growth of these tumours, the findings so far point to placental hormones as the main stimulators of this growth.

Finally, it should be noted that many of the observed tumours tended to lose their responsiveness to pregnancy. This phenomenon is identical to what has been described extensively by Foulds [6] as an aspect of tumour progression. The principles of progression as established by Foulds [6, 7], are of great importance for a better understanding of tumour behaviour in animals as well as in man. Apart from their endocrinological interest, the tumours described in the present report would seem to furnish promising material for the further study of this progression.

Acknowledgements — We thank Miss L. Wensink and Miss M. Molema for technical assistance; Prof. O. Mühlbock and Dr. L. Boot for advice during the preparation of the manuscript and Dr. L. Foulds for his enthusiastic stimulation throughout the work.

RÉSUMÉ

Une étude a été faite sur les caractères de la croissance des tumeurs mammaires des souris hybrides dérivées des souches GR et RIII, bienque GR et C57BL.

Ces tumeurs subissent un processus de croissance pendant la seconde moitié de la grossesse, et plusieurs d'entre elles régressent après l'accouchement.

Toutefois, cette régression se fait avec des variations quantitatives considérables; certaines tumeurs disparaissent entièrement, tandis que d'autres ne montrent qu'une faible diminution de taille après l'accouchement.

Lors de nouvelles grossesses, toutes les tumeurs croissent à nouveau et souvent atteignent une taille plus importante que celle acquise pendant la période de croissance préalable.

Après l'arrêt des périodes de gestation, la plupart des tumeurs montrent une régression apparamment complète surtout lorsqu'il s'agit de petites tumeurs (7,5 *mm dia.*).

L'étude portée sur une longue période après les gestations des souris hybrides GR × *RIII a mis en évidence que des tumeurs ayant régressé auparavant se développent à nouveau après plus ou moins* 134 *jours. Environ* 100 *jours plus tard, ces mêmes souris peuvent être atteintes par de nouvelles tumeurs se développant au niveau des glandes mammaires qui étaient indemmes lors des périodes de gestation.*

D'une façon semblable à l'effet de la grossesse, le traitement combiné de l'oestrone avec la progestérone a donné lieu à une nouvelle phase de croissance de la tumeur. L'arrêt du traitement entraîne la régression des tumeurs.

Tandis que quelques tumeurs se développent progressivement dès le moment ou elles sont détectées, la plupart d'entre celles qui réagissent pendant la période de grossesse, perdent plus ou moins rapidement la tendance à régresser après l'accouchement. Ce phénomène peut être considéré comme un aspect typique de la "progression" néoplasique.

SUMMARY

A study was made of the growth characteristics of mammary tumours arising in female hybrids of GR and RIII mice and of GR and C57BL mice. These tumours grow during the second half of pregnancy, and many of them regress after parturition. This regression shows considerable quantitative variation, some tumours disappearing entirely while others after parturition show a slight decrease in size only. During a following pregnancy, however, all tumours resume growth and often attain a larger size than was reached during the previous growth phase.

After cessation of breeding, most tumours tend to regress, complete regression being most frequent in small tumours (up to 7½ *mm dia.). Prolonged observation after cessation of breeding, showed recurrence of regressed tumours after a mean interval of* 134 *days in hybrids of the GR* × *RIII combination. Some* 100 *days later, these mice might also develop new tumours in glands which had not been affected during the breeding history.*

Recurrence of regressed tumours could be initiated by combined treatment with oestrone and progesterone which, at least partially, imitated the effect of pregnancy. Cessation of this treatment correspondingly led to regression of these tumours. While a few tumours grew continuously from the moment they were first detected, many pregnancy-responsive tumours more or less gradually lost the tendency to regress after parturition. This change of responsive into unresponsive tumours illustrates a typical aspect of tumour progression.

ZUSAMMENFASSUNG

Die Wachstumseigenart der Mammatumoren welche sich entwicklen in weiblichen Bastarden von GR und RIII, sowie von GR und C57L Mäusen, wurde analysiert. Diese Tumoren wachsen während der zweiten Hälfte der Schwangerschaft und bilden sich öfters zurück nach der Geburt der Jongen. Diese Rückbildung zeigt erhebliche quantitative Unterschiede: einige Tumoren verschwinden vollständig, während andere nach der Geburt nur einen geringfügigen Rückgang ihrer Grösse zeigen. Während einer nächsten Schwangerschaft aber zeigen alle Tumoren ein erneutes Wachstum und erreichen öfters eine Grösse, die die der vorhergehenden Wachstumsphase übersteigt.

Nach Beendigung der Züchtung neigten die meisten Tumoren zur Rückbildung, wobei völlige Rückbildung sich am häufigsten fand an kleinen Tumoren (Durchmesser bis zu 7½ *mm). Fortsetzung der Beobachtungen nach Beendigung der Züchtung zeigte auf, dass in den GR* × *RIII Bastarden die Tumoren nach einer durchschnittlichen Zeitspanne von* 134 *Tagen abermals wachsen. Ungefähr* 100 *Tage später kamen in diesen Mäusen auch neue*

Tumoren zur Entwicklung in Drüsen, welche während der Züchtung nicht betroffen gewesen waren.
Erneutes Wachstum der rückgebildeten Tumoren konnte hervorgerufen worden durch kombinierte Östron-Progesteron Behandlung, welche — wenigstens teilweise — den Einfluss der Schwangerschaft nachahmte. Beendigung dieser Behandlung führte in entsprechender Weise zur Rückbildung dieser Tumoren. Während einige wenige Tumoren nach dem ersten Nachweis ein ununterbrochenes Wachstum aufwiesen, zeigten viele Schwangerschaftabhängigen Tumoren einen allmählichen Verlust ihrer Neigung zu Rückbildung nach der Geburt der Jungen. Diese Abänderung von abhängigen in unabhängigen Tumoren erläutert ein charakteristisches Merkmal der Tumor Progression.

REFERENCES

1. O. Mühlbock, The influence of hormones on spontaneous experimental tumours. In: *Simposio sugli antimitotici San Remo*, p. 41–53 (1955).
2. R. L. Noble, Hormonal regulation of tumour growth. *Pharmacol. Rev.* **9,** 367 (1957).
3. J. Furth and U. Kim, Biological foundation of cancer control by hormones. In: *Biological Approaches to Cancer Chemotherapy*, pp. 259–276. Academic, London (1961).
4. C. Huggins, The hormone dependent cancers. *J. Amer. Med. Ass.* **186,** 481 (1963).
5. W. U. Gardner, The effect of estrogen on the incidence of mammary and pituitary tumors in hybrid mice. *Cancer Res.* **1,** 345 (1941).
6. L. Foulds, Mammary tumours in hybrid mice: Growth and progession of spontaneous tumours. *Brit. J. Cancer* **3,** 345 (1949).
7. L. Foulds, The experimental study of tumour progression: A review. *Cancer Res.* **14,** 327 (1954).
8. F. Squartini, Responsiveness and progression of mammary tumors in high-cancer-strain mice. *J. Nat. Cancer Inst.* **28,** 911 (1962).
9. W. E. Heston, G. Vlahakis and Y. Tsubura, Strain DD, a new high mammary tumor strain and comparison of DD with strain C3H. *J. Nat. Cancer Inst.* **32,** 237 (1964).
10. O. Mühlbock. In press.
11. T. B. Dunn, Morphology of mammary tumors in mice. In: *Physiopathology of Cancer*, 2nd Ed. pp. 38–84. Hoeber-Harper, New York, (1959).
12. L. Foulds, The histologic analysis of mammary tumors in mice. *J. Nat. Cancer Inst.* **17,** 701 (1956).
13. F. Squartini and L. Severi, Strain differences in the mammary tumour-inducing virus as detected by the characters and behaviour of neoplasms. In: *Tumour Viruses of Murine Origin*, pp. 82–106. Ciba Foundation, Symposium. Churchill, London (1962).
14. F. Squartini, G. Rossi and I. Paoletti, Characters of mammary tumours in BALB/c female mice fosternursed by C3H and RIII mothers. *Nature, Lond.* **197,** 505 (1963).
15. T. L. Dao and H. Sunderland, Mammary carcinogenesis by 3-methylcholanthrene I. Hormonal aspects in tumor induction and growth. *J. Nat. Cancer Inst.* **23,** 567 (1959).
16. C. Huggins, G. Briziarelli and H. Sutton, Rapid induction of mammary carcinoma in the rat and the influence of hormones on the tumors. *J. Exp. Med.* **109,** 25 (1959).
17. U. Kim and J. Furth, Relation of mammary tumors to mamatropes II. Hormone responsiveness of 3-methylcholanthrene-induced mammary carcinomas. *Proc. Soc. Exp. Biol. (N.Y.)* **103,** 643 (1960).
18. S. Young, D. M. Cowan and L. E. Sutherland, The histology of induced mammary tumours in rats. *J. Path. Bact.* **85,** 331 (1963).
19. P. M. Daniel and M. M. L. Prichard, The response of experimentally induced mammary tumours in rats to hypophysectomy and to pituitary stalk section. *Brit. J. Cancer* **17,** 446 (1963).
20. A. Sterental, J. M. Dominguez, C. Weissman and O. H. Pearson, Pituitary role in the estrogen dependency of experimental mammary cancer. *Cancer Res.* **23,** 481 (1963).

21. P. M. Daniel and M. M. L. Prichard, The response of experimentally induced mammary tumours in rats to ovariectomy. *Brit. J. Cancer* **17,** 687 (1963).
22. U. Kim, J. Furth and K. Yannopoulos, Observations on hormonal control of mammary cancer I. Estrogen and mammotropes. *J. Nat. Cancer Inst.* **31,** 233 (1963).
23. C. Huggins and W. C. Yang, Induction and extinction of mammary cancer. *Science,* **137,** 257 (1962).

Europ. J. Cancer Vol. 1, pp. 51–58. Pergamon Press 1965. Printed in Great Britain

Etude Clinique, Histologique et Electrophysiologique des Neuropathies Associées au Traitement par la Vincristine*

J. HILDEBRAND et C. COËRS

Service de Médecine et d'Investigation Clinique,
Institut Jules Bordet et
Département de Neurologie de la Clinique Médicale de l'Hôpital Brugmann,
Fondation Reine Elisabeth
Bruxelles, Belgique

La VINCRISTINE est un alcaloïde extrait de la pervenche (vinca rosea linn.), dont la structure chimique a été établie par NEUSS *et al.* [1]. Elle possède une action thérapeutique intéressante dans diverses formes de néoplasies, et notamment dans les leucémies aiguës. Malheureusement, elle est très toxique pour le système nerveux périphérique. Cette toxicité est telle qu'elle nécessite souvent l'arrêt du traitement.

Dans le dessein d'élucider la nature de cette neurotoxicité, nous avons fait une étude clinique, électrophysiologique et histologique des cas de neuropathies observés après emploi de ce médicament à l'Institut J. Bordet.

MATERIEL ET METHODES

Les six patients étudiés ont été traités à raison d'une injection intraveineuse de 3 à 5 mg de vincristine par semaine, pendant des périodes variables. On trouvera, dans le Tableau 1, l'âge des malades, l'affection dont ils étaient atteints ainsi que la conduite du traitement. L'examen neurologique, pratiqué chez ces malades, avant le traitement, était normal et leurs antécédents neurologiques sans particularité. Aucun des malades n'était diabétique ni n'avait d'antécédents éthyliques.

Les investigations ont porté sur les territoires neuro-musculaires des nerfs médian, cubital et sciatique poplité externe. L'état fonctionnel de ces territoires a été évalué par la mesure de la vitesse de conduction dans les fibres nerveuses motrices [2]. Les muscles biopsiés dans ces territoires ont été respectivement le petit et le grand palmaire, pour le nerf médian, et le jambier antérieur pour le sciatique poplité externe. Les valeurs normales de la vitesse de conduction des nerfs étudiés sont: 56,2 m/sec, $\pm$ 4,7 pour le médian et le cubital (segment coude-poignet), et 49,7 m/sec, $\pm$ 7,1 pour le sciatique poplité externe (segment tête du péroné-cou du pied) [2]. Les biopsies neuro-musculaires ont été prélevées dans les cas n° 1, 2, 5, 6 suivant la méthode décrite par l'un de nous [3].

Les préparations histologiques ont été soumises à un examen qualitatif et quantitatif portant sur les tissus musculaires (surface des fibres musculaires en coupe transversale et numération des noyaux musculaires périphériques et centraux), et sur l'innervation motrice terminale (aspect des axones moteurs

*Ce travail a été réalisé avec l'aide de la "Muscular Dystophy Foundation" et du F.N.R.M.

Tableau 1.

Cas	Age Sexe	Affection	Durée du traitement en semaines 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
1	M 66	Leucémie aiguë	3 3 3 3 3 3 – 3 mg
2	F 52	Epithélioma du col	3 3 3 mg
3	F 71	Leucémie aiguë	4 5 5 5 3 3 5 mg
4	M 57	Leucémie aiguë	3 3 3 3 5 5 5 5 mg
5	F 45	Leucémie aiguë	3 – 3 – – 4 3 – – 5 – – 5 2,5 5 5 mg
6	M 65	Leucémie lymph. chr. en poussée aiguë	3 3 3 3 3 3 mg

intramusculaires et planimétrie de l'aire de dispersion des appareils sous-neuraux). Les procédés utilisés pour ces mesures ont été exposés précédemment [4].

RESULTATS

Après une description du tableau clinique, nous exposerons les résultats des explorations électrophysiologiques et histologiques effectuées chez nos malades.

I. *Etude clinique*

Cinq patients ont développé une neuropathie sévère à prédominance motrice. La sixième malade (n° 2), décédée 24 jours après l'arrêt du traitement et qui avait reçu 9 mg de vincristine, n'a souffert que de paresthésies.

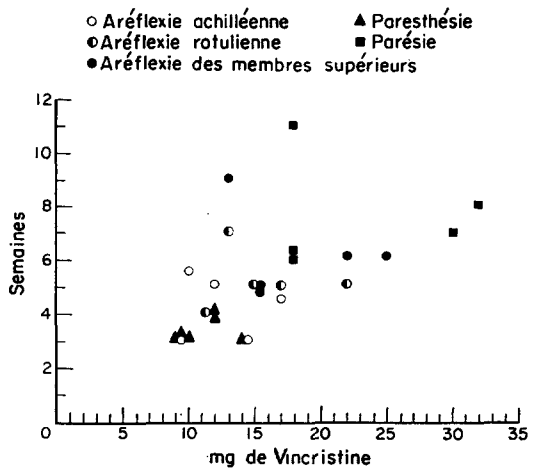


Fig. 1. Apparitions des symptômes neurologique en fonction de la dose de Vincristine reçue et de la durée du traitement.

a Fig. 1 indique le moment d'apparition des symptômes en fonction de temps écoulé depuis le début du traitement et de la dose de médicament reçue.

Les paresthésies sont apparues après l'administration de doses variant entre 9 et 14 mg et après 3 à 5 semaines de traitement. Les malades éprouvaient d'abord des sensations d'engourdissement dans les extrémités, puis des paresthésies douloureuses. Généralement, les membres inférieurs furent atteints quelques jours avant les membres supérieurs.

Les réflexes achilléens ont disparu après l'administration de 9 à 17 mg de vincristine et 3 à 6 semaines de traitement. L'aréflexie rotulienne est survenue après 4 à 7 semaines et pour une dose de 12 à 22 mg. Les réflexes des membres supérieurs furent abolis en dernier lieu, après 5 à 9 semaines de traitement et une dose de 15 à 25 mg de vincristine.

Le moment d'apparition du déficit moteur a été difficile à préciser avec certitude en raison de l'asthénie intense liée à l'affection dont étaient atteints nos malades. Ce déficit était manifeste après l'administration de 18 à 32 mg de vincristine et après 6 à 11 semaines de traitement.

Les parésies étaient symétriques et à prédominance distale. Elles s'accompagnaient toujours d'une importante atrophie musculaire.

Dans aucun cas, nous n'avons observé de déficit de la sensibilité objective profonde (perception des vibrations du diapason, sens de position des doigts et orteils) et superficielle (douloureuse et tactile).

L'arrêt du traitement n'a pas été suivi d'une régression rapide des symptômes neurologiques. Ceux-ci ont persisté chez une de nos malades (n° 5) 4 mois, et chez un autre (n° 4) 3 mois après l'arrêt du traitement. Deux patients (n° 1 et n° 3) sont décédés environ 1 mois après l'arrêt du traitement sans qu'on ait observé la moindre rémission des symptômes neurologiques. La malade n° 2 est décédée en cours de traitement et le patient n° 6 a quitté définitivement l'Institut après l'arrêt du traitement.

II. *Etude de la vitesse de conduction*

La vitesse de conduction dans les fibres nerveuses motrices a été mesurée dans deux conditions:

a. Au début du traitement, chez trois patients dont les symptômes neurologiques étaient, au plus, de légères paresthésies. Jusqu'à des doses totales de 9 mg de vincristine, on n'a constaté aucune tendance au ralentissement de la vitesse de conduction (Tableau 2);

b. Chez les patients ayant reçu un traitement prolongé (plus de 6 semaines), variant entre 21–35 mg (Tableau 3). Les quatre patients de ce groupe étaient tous atteints d'une polynévrite grave. Chez deux d'entre eux (n° 4 et 5), la vitesse de conduction était légèrement diminuée dans le nerf médian. Quatre autres mesures, effectuées deux fois dans le nerf médian, une fois dans le cubital et une fois dans le sciatique poplité externe, restaient dans les limites normales. Dans deux cas, la stimulation nerveuse du sciatique poplité externe n'évoquait pas de potentiel dans le muscle pédieux et nous avons considéré la vitesse de conduction comme nulle.

Tableau 2.

Cas	Nerf	Doses de vincristine 0 mg	3 mg	6 mg	9 mg
2	médian	56,7	—	—	65,7 m/sec
4	médian	—	—	57,3	52 m/sec
6	médian	60,1	58	54	57,1 m/sec

Tableau 3.

Cas	Nerf	Doses de vincristine	Vitesse de conduction
1	médian	21 mg	48 m/sec
	S.P.E.	idem	38,1 m/sec
3	médian	30 mg	54 m/sec
	S.P.E.	idem	0 m/sec
4	médian	27 mg	38,2 m/sec
5	médian	35,5 mg	44,2 m/sec
	cubital	idem	52 m/sec
	S.P.E.	idem	0 m/sec

S.E.P. = sciatique poplité externe.

III. *Etude histologique*

Une biopsie neuro-musculaire a pu être prélevée chez les patients n° 1, 2, 5, et 6. Les observations histologiques sont résumées dans le Tableau 4, qui indique également les valeurs normales chez dix patients exempts d'affection neuro-musculaire (six biopsies provenaient des muscles palmaires et quatre du jambier antérieur).

Cas n° 1 — La biopsie a été prélevée après 9 semaines de traitement. Le malade avait reçu une dose totale de 21 mg de vincristine. Au moment de la biopsie, le patient souffrait d'une importante atrophie musculaire et la vitesse de conduction dans le nerf médian était de 48 m/sec.

Les fibres musculaires sont de calibre très variable: certaines sont anormalement fines et d'autres considérablement hypertrophiées. La structure des fibres musculaires est bien conservée. Les noyaux périphériques sont en moyenne de 4, 2 noyaux par fibre et le pourcentage des fibres contenant des noyaux centraux est de 2% (Fig 2 et 3). On note d'importantes variations de taille des appareils sous-neuraux (Fig. 4 et 5).

Cas n° 2 — La biopsie a été prélevée après 24 jours de traitement, alors que la malade avait reçu 9 mg de vincristine et souffrait de légères paresthésies. Le muscle biopsié était le grand

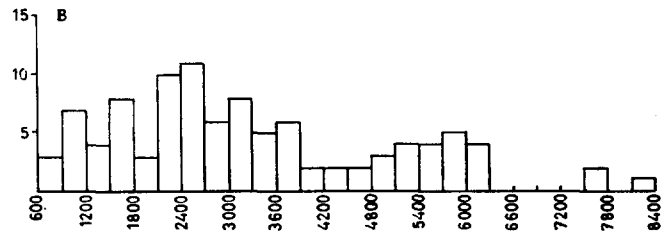


Fig. 3. Histogramme des surfaces des fibres musculaires en coupe transversale, cas n° 1.

Tableau 4.

	Muscle biopsié	Vit. de conduction lors de la biopsie	Tissu musculaire					Appareils sous-neuraux			
			Surf. moyen. μ^2	Valeurs extrêmes μ^2	% des valeurs anorm. −	% des valeurs anorm. +	Noyaux périph. par fibre	% des fibres ayant noyaux centr.	Surf. moyen. μ^2	Valeurs extrêmes μ^2	% des valeurs anorm. −	% des valeurs anorm. +
Valeurs normales			2285	900 à 4.500	0	0	3 à 5	max. 3	271	80 à 620	0	0
1	Palm.	48 m/sec (médian)	2047	600 à 8.100	3	25	4,2	2	236	40 à 680	4	1
2	Palm.	65,7 m/sec (médian)	2067	300 à 5.100	11	1,5	4,8	1	297	80 à 840	0	6
5	Jambier antér.	0 m/sec (S.P.E.)	2826	150 à 1500	28	21	4	4	—	—	—	—
6	Palm.	54 m/sec (médian)	2589	1200 à 4.800	0	2	3,1	1	250	80 à 440	0	0

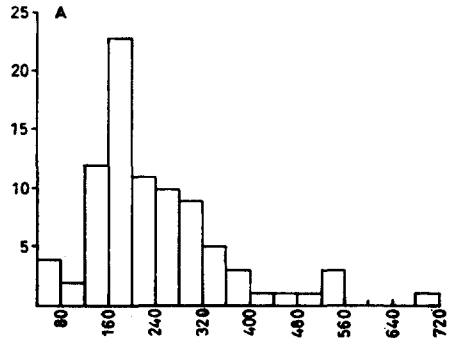


Fig. 5. Histogramme des surfaces des appareils sous-neuraux, cas n° 1.

palmaire. La vitesse de conduction dans le nerf médian était à ce moment de 65,7 m/sec.

Il existe une assez forte variation du calibre des fibres musculaires réalisant l'aspect histologique d'atrophie par fibres isolées (Fig. 6). La prédominance des fibres de petit calibre se traduit par un déplacement de l'histogramme vers des valeurs basses (Fig. 7).

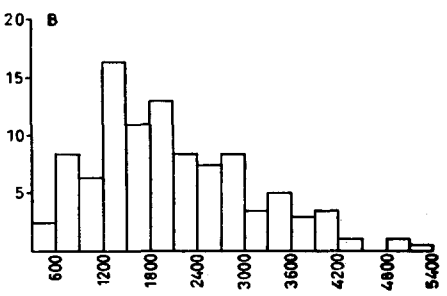


Fig. 7. Histogramme des surfaces des fibres musculaires en coupe transversale, cas n° 2.

Les noyaux périphériques sont de 4,8 noyaux par fibres et le pourcentage des fibres musculaires contenant des noyaux centraux de 1%. On note une dispersion anormalement grande des appareils sous-neuraux (Figs. 8–10). Les axones moteurs intramusculaires colorés par le bleu de méthylène ne sont pas anormalement ramifiés.

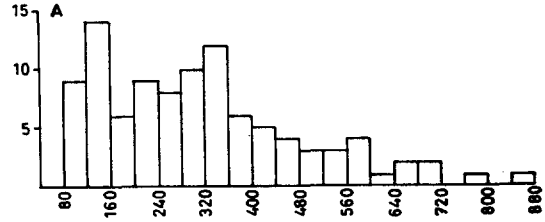


Fig. 10. Histogramme des surfaces des appareils sous-neuraux, cas n° 2.

Cas n° 5 — La biopsie a été prélevée dans le muscle jambier antérieur, 3 mois après l'arrêt du traitement. La malade avait reçu une dose totale de 35,5 mg de vincristine. Au moment où la biopsie fut prélevée, la malade était atteinte d'une neuropathie grave et la stimulation du nerf sciatique poplité externe n'évoquait aucune activité décelable dans le muscle pédieux.

Dans ce cas, seul le tissu musculaire a pu être examiné. Il montre une atrophie fasciculaire: petits groupes de fibres musculaires très atrophiées, voisinant avec des faisceaux de fibres fortement hypertrophiées et dont certaines atteignent, en coupe transversale,

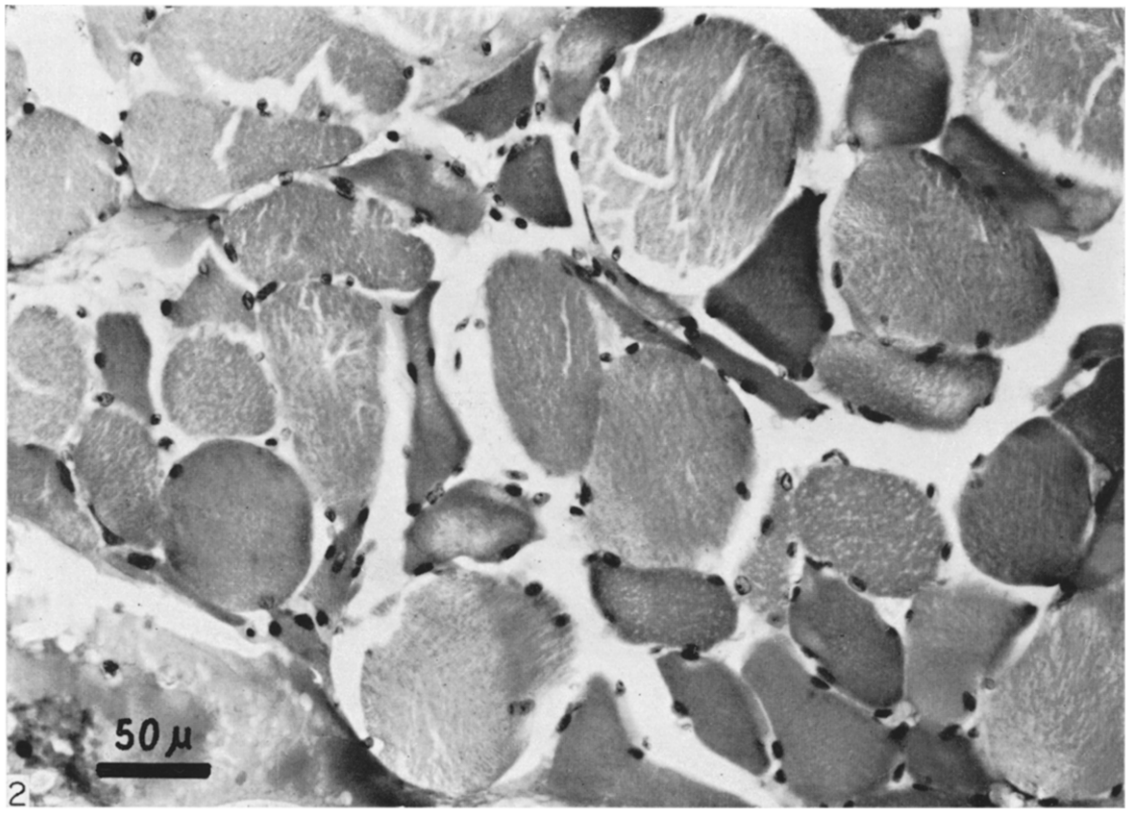


Fig. 2. Atrophie hétérogène musculaire, cas n° 1.



Fig. 4. Appareil sous-neural hypertrophié, cas n° 1.

(to face p. 54)

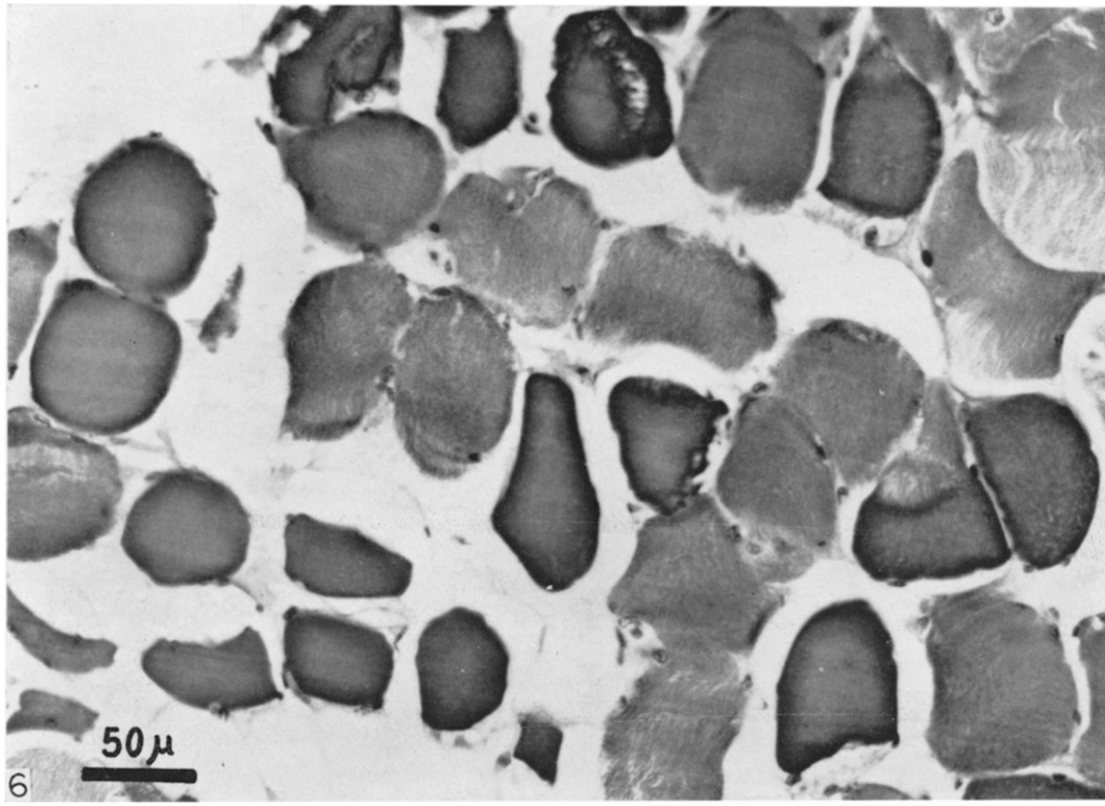


Fig. 6. Aspect d'atrophie musculaire hétérogène débutante, cas n° 2.

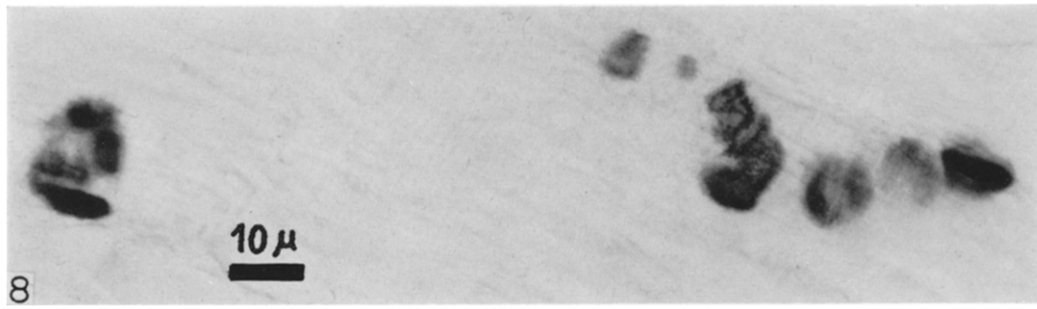


Fig. 8. Appareil sous-neural normal à gauche et hypertrophique à droite, cas n° 2.

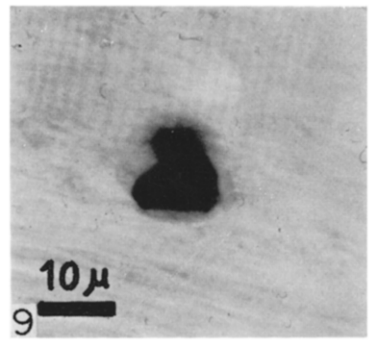


Fig. 9. Appareil sous-neural réduit à une unité, cas n° 2.

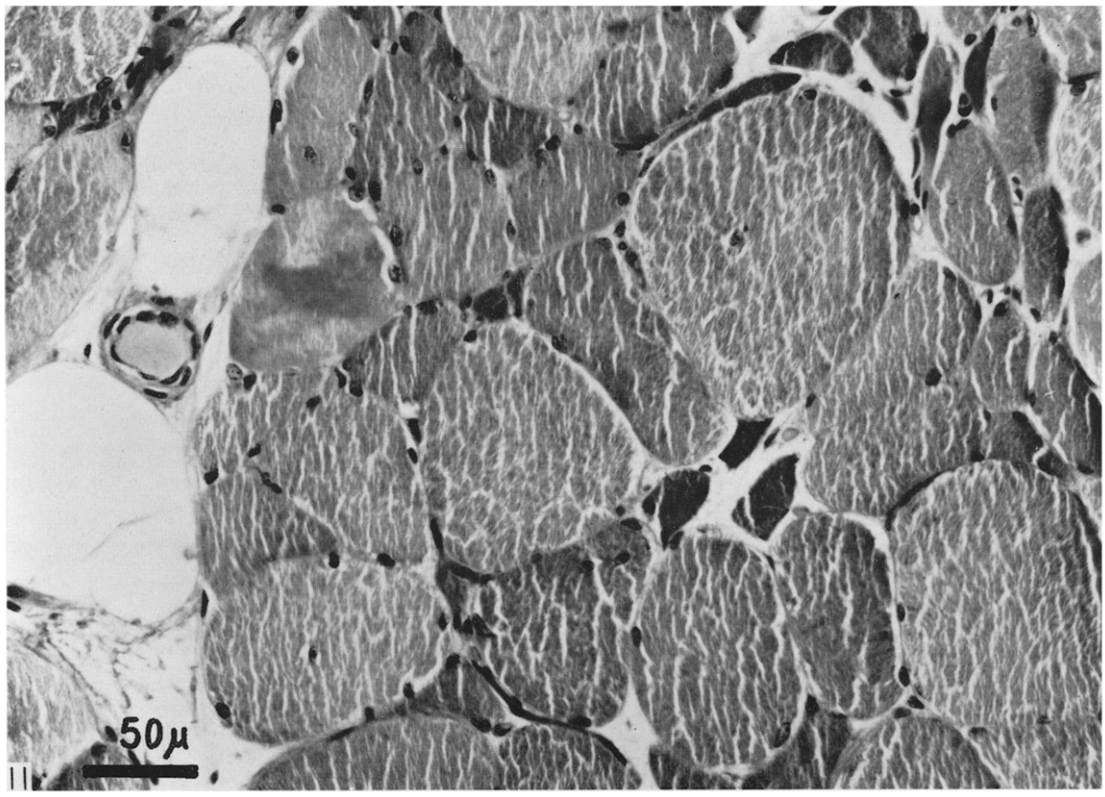


Fig. 11. Très importante atrophie hétérogène musculaire, cas n° 5.

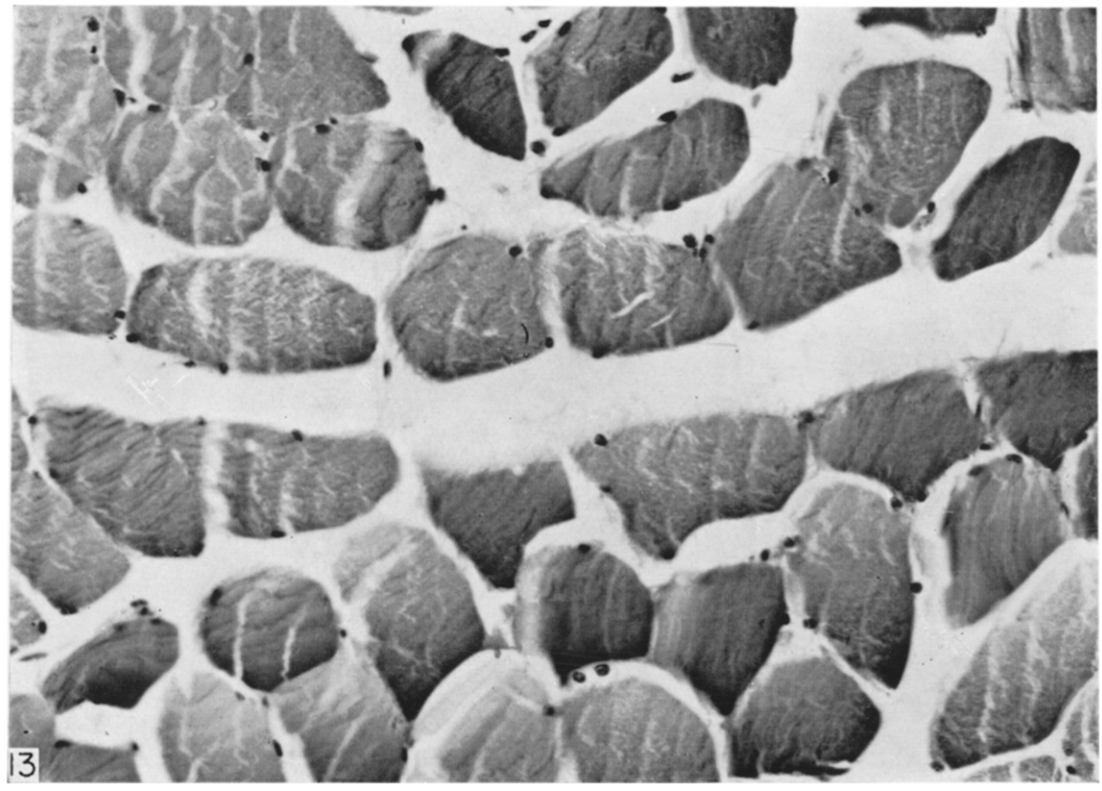


Fig. 13. Aspect normal des fibres musculaires en coupe transversale, cas n° 6.

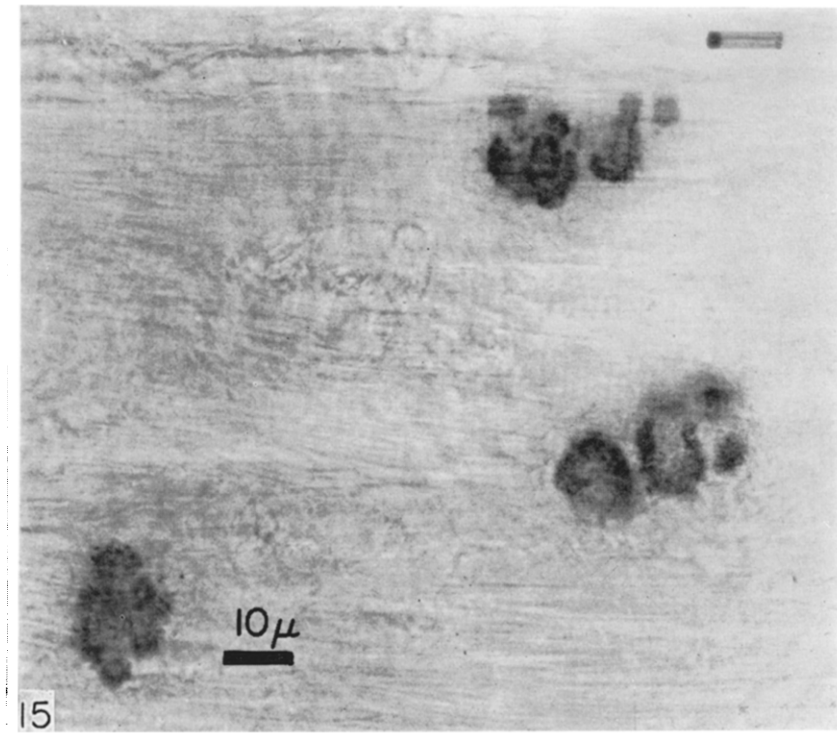


Fig. 15. Appareils sous-neuraux normaux, cas n° 6.

15.000 μ2 de surface (Figs. 11 et 12). Les noyaux périphériques sont de 4 noyaux par fibre et le pourcentage de fibres musculaires contenant des noyaux centraux de 4%.

Cas n° 6 — La biopsie a été prélevée après 15 jours de traitement, alors que le malade avait reçu 6 mg de vincristine. Il était indemne de

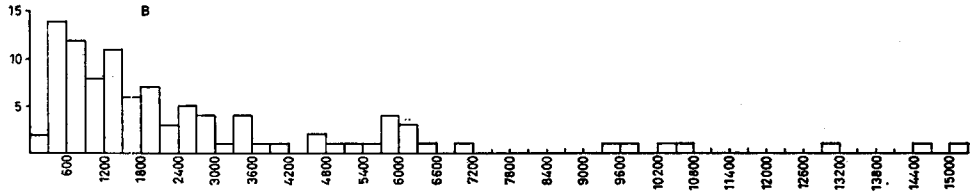


Fig. 12. Histogramme des surfaces des fibres musculaires en coupe transversale, cas n° 5.

tout symptôme de neuropathie. Le muscle biopsié était le grand palmaire et la vitesse de conduction dans le nerf médian était à ce moment de 54 m/sec. Les fibres musculaires ont une structure et un calibre normaux (Figs. 13 et 14). Les noyaux périphériques sont en

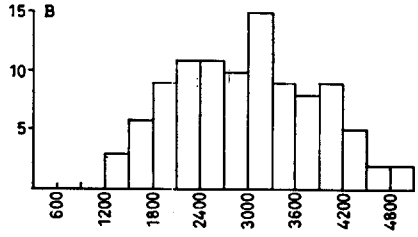


Fig. 14. Histogramme des surfaces des fibres musculaires en coupe transversale, cas n° 6.

moyenne de 3,1 par fibre et le pourcentage de fibres contenant des noyaux centraux est de 1%. On observe dans ce muscle quelques petits infiltrats leucémiques. Les surfaces des appareils sous-neuraux sont normales et l'aire de dispersion des valeurs individuelles n'est pas exagérée (Figs. 15 et 16). Les axones moteurs intra-musculaires colorés par le bleu de méthylène ne sont pas anormalement ramifiés.

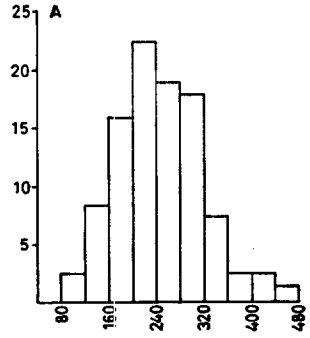


Fig. 16. Histogramme des surfaces des appareils sous-neuraux, cas n° 6.

Synthèse des données histologiques

Au début du traitement (cas n° 6), les fibres musculaires conservaient une taille et un aspect normaux. Quelques rares fibres avaient un calibre légèrement augmenté. A mesure que le traitement se prolonge, on assiste à une réduction du calibre de certaines fibres musculaires dont le nombre augmente progressivement (cas n° 1 et 2). Les fibres atrophiées, d'abord isolées, se groupent en petits faisceaux. En même temps, le processus d'hypertrophie d'autres éléments contractiles s'accentue.

Après l'arrêt du traitement (cas n° 5), l'atrophie fasciculaire reste très marquée et l'hypertrophie progresse. Certaines fibres atteignent une surface de 15,000 μ2 ce qui correspond à un diamètre théorique d'à peu près 200 μ2, soit plus de trois fois le diamètre moyen normal.

COMMENTAIRES

La polyneuropathie apparaît comme une complication très fréquente du traitement par la vincristine administrée aux doses usuelles. Selon Withelaw *et al.* [5], elle se produit dans 80% des cas; elle est susceptible de régressions partielles 3 mois après l'arrêt du traitement. Notre expérience confirme l'extrême toxicité de la vincristine à l'égard du système nerveux périphérique et souligne la gravité des symptômes dans les 3 mois qui suivent l'arrêt du traitement.

Pour apprécier la toxicité réelle du médicament, il faut évidemment tenir compte de la possibilité de neuropathies associées à la leucémie qui, chez cinq de nos patients, était la maladie causale. Cette affection est capable, par elle même, de produire des lésions neuromusculaires [6]. En faveur de la seule origine toxique des symptomes nerveux, nous pouvons invoquer les arguments suivants:

a. L'examen neurologique des malades était normal avant l'administration de la vincristine; les symptômes de neuropathie sont apparus pendant le traitement et ne se sont pas aggravés après l'arrêt de celui-ci.

b. L'allure parfaitement symétrique des symptômes n'est pas habituelle dans les polyneuropathies leucémiques [7].

Contrastant avec la gravité des signes cliniques, la vitesse de conduction nerveuse n'est diminuée que tardivement et d'une manière inconstante. D'Agostino et Jarcho [8] n'ont trouvé de réduction de la vitesse de conduction nerveuse que dans un cas sur trois. L'étude de nos malades a confirmé ces données en démontrant cependant que l'on pouvait observer tardivement une perte totale de toute conduction nerveuse dans le sciatique poplité externe.

La préservation de la vitesse de conduction nerveuse n'est pas propre à la polynévrite due à la vincristine. En effet, elle a été observée dans d'autres polynévrites d'étiologies variées. Dawnie [9], chez trois patients souffrant de porphyrinurie aiguë intermittente, dont deux étaient atteints de polynévrites sensitivo-motrices et un de polynévrite motrice, trouva des vitesses de conduction nerveuse normales dans les fibres motrices. Bannister et Sears [10], dans un cas de polynévrite aiguë idiopathique, constatèrent une vitesse de conduction à la limite inférieure de la normale: 49 m/sec dans le nerf médian (segment coude-poignet). Cette mesure fut effectuée au douzième jour de la maladie quand la force musculaire du malade était nulle [10].

Il apparaît donc que la neuropathie due à la vincristine se comporte comme ces polynévrites à évolution aiguë et se distingue nettement d'atteintes nerveuses chroniques comme celles qui accompagnent le diabète et l'intoxication éthylique. Dans ces dernières conditions, la vitesse de conduction nerveuse est souvent précocement diminuée, avant même que les signes cliniques ne fassent leur apparition [4].

Les premières recherches histologiques concernant les neuropathies dues à la vincristine, effectuées par d'Agostino et Jarcho [8], ont établi que les lésions se limitent aux nerfs périphériques et épargnent le système nerveux central.

A notre connaissance, aucune étude n'a été consacrée à l'histologie du tissu musculaire et de son innervation dans cette condition. L'analyse des biopsies neuro-musculaires, chez quatre de nos patients, a montré, en ce qui concerne le tissu musculaire, l'évolution habituelle des altérations consécutives aux lésions nerveuses périphériques qui se limitent à des modifications volumétriques aboutissant à une image d'atrophie fasciculaire avec hypertrophie compensatrice.

L'absence d'image de dégénérescence et d'infiltration du tissu musculaire permet d'exclure une participation myopathique ou myositique à la maladie.

On observe en général, dans les atteintes neurales partielles, une ramification collatérale exagérée des axones moteurs résiduels [4, 11], assurant la réinnervation d'un certain nombre de fibres musculaires, et dont le résultat semble être un agrandissement des unités motrices destiné à compenser la perte d'éléments contractiles. Ce processus à finalité compensatrice se complète probablement par l'hypertrophie des fibres musculaires réinnervées.

Pour autant que deux observations nous permettent de l'affirmer (cas n° 2 et 6), il semble que la régénération collatérale des axones intramusculaires ne se produise pas dans la neuropathie par la vincristine. Cette passivité de l'innervation motrice pourrait, dans une certaine mesure, rendre compte de la gravité et de l'irréversibilité des troubles moteurs. La persistance d'une importante proportion des fibres atrophiées, 3 mois après l'arrêt du traitement (cas n° 5), traduit cette carence de régénération nerveuse que l'hypertrophie de certaines fibres musculaires ne suffit pas à compenser.

Les variations exagérées de taille des surfaces synoptiques observées dans les cas n° 1 et 2 sont parallèles aux variations de calibre des fibres musculaires et paraissent se conformer à la règle générale selon laquelle les dimensions des plaques motrices sont proportionnelles au calibre des fibres musculaires [3]. Il ne semble pas que, comme dans les autres atteintes du système nerveux périphérique, la réduction de l'expansion des arborisations terminales puisse précéder les variations volumétriques des fibres musculaires. C'est tout au moins ce qui apparaît de l'examen des chiffres obtenus dans le cas n° 6.

Cette absence de variations structurelles précoces de l'innervation terminale, comme la pauvreté de prolifération collatérale axonique, pourraient être considérées comme l'expression d'une sidération fonctionnelle du système nerveux périphérique.

Cette faible réactivité des éléments nerveux contraste avec les importants remaniements axoniques que l'on observe dans les neuropathies chroniques frustes, de même que la réduction précoce de la vitesse de conduction nerveuse dans ces conditions, s'oppose à la relative préservation de cette transmission dans la neuropathie vincristinique. Le problème de la signification de cette corrélation reste posé.

RÉSUMÉ

1. *Le tableau clinique de la polyneuropathie causée par la vincristine est décrit chez six patients, en fonction de la dose de médicament administrée et de la durée du traitement.*

2. *La vitesse de conduction des fibres motrices ne diminue que tardivement et d'une façon inconstante.*

3. *Les altérations histologiques neuromusculaires se limitent à des modifications volumétriques consistant en un aspect d'atrophie fasciculaire classique, qui s'accompagne de variations exagérées de taille des plaques motrices. La régénération collatérale des axones moteurs n'a pas été observée dans des cas biopsiés au début du traitement.*

4. *Les données cliniques, physiologiques et morphologiques recueillies soulignent le caractère aigu et grave de l'atteinte nerveuse.*

SUMMARY

1. *Six cases of vincristine-induced polynevritis are reported. The clinical picture is described in relationship to the dosage and length of treatment.*

2. *The rate of nervous conduction in the motor fibres may diminish after a delay and not necessarily in all cases.*

3. *The histological alterations of the nerves and muscles are limited to a classical fascicular atrophy with an excessive variation in the size of the motor plates. There was no regeneration of collateral axones in the cases which were biopsied early in the course of treatment.*

4. *The clinical, physiological and morphological data underline the acute and serious aspects of the damage incurred by the peripheral nerves.*

ZUSAMMENFASSUNG

1. *Das klinische Bild der durch das Cytostaticum "Vincristin" hervorgerufenen Polyneuropathie wird bei 6 Kranken beschrieben, u.zw. in Abhängigkeit von der verabreichten Dosis und der Behandlungsdauer.*

2. *Die Leitungsgeschwindigkeit der motorischen Fasern sinkt erst spät, und auf einer unstäten Art ab.*

3. *Die histologischen neuro-muskulären Veränderungen beschränken sich auf volumetrische Abweichungen, kennzeichnet durch den klassischen Apect einer fasciculären Atrophie, welche wiederum von übermässigen Abwechslungen der motorischen Endplattengrösse begleitet wird. Man konnte keine collaterale Regeneration der motorischen Fasern bei den Kranken, wo am Anfang der Behandlung eine Biopsie durchgeführt wurde, beobachten.*

4. *Die gesammelten klinischen, physiologischen und morphologischen Angaben unterstreichten der akuten und ernsten Charakter der, an den Nerven beobachteten, Veränderungen.*

BIBLIOGRAPHIE

1. N. Neuss, M. German, H. E. Boaz and N. J. Cone, Vinca alcoloides IX Structure of vincristine and vincaleucoblastine. *J. Amer. Chem. Soc.* **84,** 1508 (1962).
2. P. K. Thomas, T. A. Sears and R. W. Gilliath, The range of conduction velocity in normal motor nerve fibres to the small muscles of the hand and foot. *J. Neurol. Neurosurg. Psychiat.* **22,** 175 (1959).
3. C. Coërs, Les variations structurelles normales et pathologiques de la jonction neuro-musculaire. *Acta Neurol. Belg.* **55,** 747 (1955).
4. C. Coërs and J. Hildebrand, Latent neuropathy in diabetes and alcoholism electromyographic and histological study. *Neurology* **15,** 19 (1964).
5. D. M. Whitelaw, D. H. Cowan, F. C. Cassidy and T. A. Patterson, Clinical experience with vincristine. *Canc. Chemother. Abstr.* July 13 (1963).
6. T. Alajouanine, R. Thurel, P. Castaigne et F. Lhermitte, Leucémie aiguë avec syndrome polynévritique et infiltration leucémique des nerfs. *Rev. neurol.* **81,** 294 (1949).

7. H. Williams, H. Diamond, C. Lloyd and H. Parsons. *Neurological complications of lymphomes and leukemias*, p. 86–98. Thomas, New York.
8. A. N. D'Agostino and L. W. Jarcho, A new neuropathy associated with vincristine therapy. *Clin. Res.* **12,** 106 (1964).
9. A. W. Dawnie, *Disorders of Voluntary Muscle*, pp. 529–531, edited by J. N. Wolton. Churchill, London (1964).
10. R. G. Bannister and T. A. Sears, The changes in nerve conduction in acute idiopathic polynevritis. *J. Nerol. Neurosurg. Psychiat.* **25,** 321 (1962).
11. C. Coërs et A. L. Woolf, The *Innervation of Muscle and Biopsy Study*. Blackwell, Oxford (1959).

Europ. J. Cancer Vol. 1, pp. 59–66. Pergamon Press 1965. Printed in Great Britain

Dysplasie Rénale Associée à un Cancer Hépatique Métastatique, avec Ostéomalacie et Phosphaturie

Revue de la Littérature

G. BUYSE, M. HENRY, F. LUSTMAN, et R. PARMENTIER

Service de Médecine interne de l'Institut Médico-Chirurgical de St. Gilles, et Laboratoire et Service d'Anatomie pathologique de l'Université Libre de Bruxelles

En 1946, Eisen [1] décrivait, dans un cas de cancer de la vésicule biliaire envahissant le foie, une transformation "adénomatoïde" des cellules de la capsule de Bowmann des glomérules rénaux. Depuis cette publication, quatre descriptions de modifications analogues de l'épithelium de la capsule de Bowmann et des tubes contournés proximaux ont été rapportées [2–5]. Dans tous les cas,il s'agissait de malades atteints de tumeurs primitives ou métastatiques du foie. Nous avons retrouvé dans la littérature une sixième observation analogue [6], qui avait été décrite comme métastase intraglomérulaire.

Nous avons eu la possibilité de suivre longuement l'histoire clinique d'une malade où, à l'autopsie, cette même lésion rénale a été retrouvée. Ici encore, il s'agissait d'une tumeur maligne envahissant le foie. De plus, dans notre cas, ainsi que dans celui rapporté récemment par Eulderink [5], on observait des troubles de l'élimination urinaire des phosphates qui pourraient être en rapport direct avec la dysplasie capsulaire et tubulaire.

La rareté du syndrome paraît établie par le fait qu'une étude du tube contourné proximal de mille reins prélevés à l'autopsie [7] n'a révélé aucune modification analogue. La signification fonctionnelle des lésions et leur association avec la néoplasie hépatique, soulèvent un problème de cancérologie qui nous paraît mériter de retenir l'attention.

OBSERVATION CLINIQUE

Cette malade, née en 1914, malgré de nombreux séjours dans les hôpitaux, posa des problèmes non résolus jusqu'à son décès en 1962.

L'affection débute en 1949, à 35 ans, par de l'anémie, de l'amaigrissement et des plaintes digestives (nausées, ballonnement post-prandial alternance de constipation et de diarrhée).

Les antécédents sont: une pleurésie à 18 ans, une appendicectomie à 32 ans, une amygdalectomie à 35 ans.

Au cours du 1er séjour à l'hôpital, en 1949, on constate une anémie hypochrome avec polychromatophilie, une leucocytose normale et un myélogramme normal. Il existe du sang occulte dans les selles. La rectoscopie et le tubage gastrique sont normaux.

Le deuxième séjour, en 1950, montre à nouveau une anémie hypochrome avec sang dans les selles et, cette fois, une achlorhydrie gastrique. Des radiographies digestives révèlent une dystonie de l'intestin grêle et de la stase dans le colon ascendant.

De 1950 à 1957, la malade subit des traitements multiples consistant en fer, vitamine B_{12}, extraits de foie et transfusions.

Elle accouche en 1952.

Le 1er séjour à l'Hôpital de St. Gilles se situe en 1957. Elle est admise pour anémie, plaintes

digestives, douleurs osseuses et ecchymoses spontanées.

L'examen montre une démarche de "canard", de la pâleur, des sclérotiques bleues et un foie paraissant légèrement hypertrophié. Au fond d'oeil: une petite tache hémorragique.

Les examens de laboratoire confirment l'anémie (3.430.000 GR/mm³) avec un fer sérique à 59 µg/100 ml. et la présence de sang dans les selles. On note une hypochlorhydrie gastrique; la gastroscopie et la rectoscopie sont normales, de même que les radiographies digestives.

Les épreuves de coagulation et de saignement sont normales. De nombreuses radiographies osseuses sont normales. Par contre, la phosphorémie est basse à 2,2 mg% avec une calcémie normale à 10,5 mg%. Les phosphatases alcalines sont augmentées à 15 U. Roberts.

Conclusion du séjour: Anémie ferriprive par pertes de sang digestives.
Fragilité vasculaire.
Ostéomalacie préradiologique.

Dans la suite, ont lieu de multiples séjours dans le même service, toujours pour anémie par pertes de sang occulte. On retrouve quasi constamment des douleurs osseuses généralisées (surtout au niveau des côtes, du bassin, des hanches, et des genoux) et des altérations du métabolisme phosphocalcique comparables à celles du 1er séjour. L'ensemble de ces analyses sera repris plus loin.

Les examens biologiques ne permettent pas de trancher formellement entre une néphropathie tubulaire entraînant de l'ostéomalacie, et une hyperparathyroïdie.

En 1959, apparition d'un melaena franc dont l'étiologie ne peut être précisée par des radiographies digestives.

Depuis 1960, une hépatomégalie de plus en plus importante se développe. Des taches angiomateuses tubéreuses ou stellaires apparaissent sur le thorax, l'abdomen et les extrémités des membres. A partir de la même époque, on note des fractures costales, et une transparence augmentée de la trame osseuse.

En fin 1961, apparaissent des altérations des tests hépatiques. Une laparoscopie confirme la grosse hépatomégalie et montre la présence de nodules évocateurs d'un processus néoplasique. Une laparotomie est pratiquée qui montre, en outre, une rate congestive et augmentée de volume, et la présence de sang dans le duodénum et le jéjunum. Les nodules hépatiques notés à la laparoscopie paraissent très vascularisés.

Dans le but de les visualiser, on pratique une artériographie hépatique par la méthode de Seldinger. Celle-ci met en évidence une volumineuse masse hypervascularisée dans le lobe droit (Fig. 1). Une biopsie osseuse effectuée dans un foyer costal ne montre aucune image de métastase osseuse. La moelle y est normale.

Le dernier séjour à l'hôpital de St. Gilles se place en février 1962; ce séjour est marqué par des melaena très importants et récidivants, nécessitant de fréquentes transfusions. L'hépatomégalie et la splénomégalie se sont encore accentuées.

En revoyant l'artériographie, on est frappé par une opacité se projetant aux environs de l'angle de Treitz et dont la localisation paraît correspondre à celle d'une lacune dans le transit grêle.

Dans l'éventualité d'une tumeur vasculaire du grêle responsable des hémorragies cataclysmiques de cette malade, on la transfère au Service de Chirurgie de l'hôpital St. Pierre (2/5/62).

Le 10/5/62, une splénoportographie montre une pression portale légèrement augmentée (18 cm H20).

Le 5/6/62, on pratique une splénectomie et une résection intestinale de 7 cm à partir de l'angle de Treitz pour tumeur hémorragique.

Le 10/6/62, la malade, âgée de 48 ans, décède à la suite d'un collapsus d'origine inexpliquée.

EXAMENS DE LABORATOIRE

Nous avons résumé l'ensemble des investigations biologiques subies par la malade, entre 1957 et 1962, de la façon suivante:

1. *Examens hématologiques:*
   Anémie ± bien corrigée en fonction des transfusions;
   Leucocytose normale;
   Plaquettes normales.
   Myélogrammes: normaux
2. *Valeurs des phosphorémies, calcémies et phosphatasémies:* Tableau 1.
3. *Réserves alcalines et chlorémies:* Tableau 2.
4. *Clearances du phosphore/24h:*
   31,2 ml/min (le 13/10/58)
   23,8 ml/min (le 29/6/60)

$$\frac{\text{Clearance du phosphore}}{\text{Clearance de créatinine}} \times 100 =$$

46,5% (le 13/10/58)
37,9% (le 29/6/60)

(Les valeurs normales[8] de la clearance du phosphore/24 hr sont: 10,8 ml/min, plus ou moins 2,7 ml.).

Tableau 1.

Dates	Calcémies (mg%)	Phosphorémies (mg%)	Phosphatasémies
14/6/57	10,5	—	—
4/7/57	11	2,2	15 U.R.
8/7/57	10,5	2,5	18 U.R.
13/8/57	11,5	2,2	18 U.R.
3/9/57	10,2	2,9	17 U.R.
25/11/57	10	2	21 U.R.
9/12/57	11	—	17 U.R.
29/8/58	11,7	1,5	32 U.R.
7/10/58	—	1,5	—
13/10/58	11,2	1,7	30 U.R.
11/3/60	9,3	0,9	13,2 U.B.
15/3/60	9,4	1	6,2 U.B.
30/3/60	—	1,3	7,7 U.B.
25/4/60	—	2,1	5 U.B.
24/6/60	—	3,4	—
25/7/60	9,7	3,6	16,4 U.B.
2/8/60	—	2,1	4,5 U.B.
4/5/61	9,8	2,9	8,7 U.B.
24/8/61	—	1,7	1,1 U.B.
11/9/61	—	1,9	3,2 U.B.
23/2/62	9,3	2,1	4 U.B.
valeurs moyennes	10,3 mg%	2,1 mg%	Moyenne des valeurs exprimées en Unités Roberts: 21 (N=3 à 5)
			Moyenne des valeurs exprimées en Unités Bodansky: 7 (N=1 à 4)

5. *Amino-acidurie:*
   598 mg N — aminé/24 hr (le 15/3/60);
   932 mg N — aminé/24 hr (le 4/5/60).
   (Valeurs normales: 100 à 150 mg N — aminé/24 hr).
   Technique: E. J. King and I. D. P. Wootton, [9]).
6. *Glucosurie: Toujours nulle.*
7. *Calciurie:* Valeur moyenne de quatre dosages, après un régime pauvre en calcium,
   82 mg/24 hr, 257 mg/24 hr, 210 mg/24hr, 202 mg/24 hr,;
   Moyenne=188 mg/24 hr.
8. *Sédiments urinaires:* Toujours normaux. Pas d'albuminurie.
9. *Urée sanguine:* Toujours normale.

Les diagnostics pre-mortem sont les suivants:

1. Tumeur vasculaire du grêle responsable des hémorragies répétées et de l'anémie chronique;
2. Métastases hépatiques de cette tumeur vasculaire;
3. Splénomégalie congestive;
4. Syndrome d'ostéomalacie par anomalie tubulaire.

PROTOCOLE D'AUTOPSIE

L'autopsie (62/270) est pratiquée 14 hr après la mort. Elle porte sur le cadavre d'une femme de petite taille ne présentant aucune lésion cutanée évidente. Il existe une cicatrice chirurgicale longue de 30 cm dans le flanc gauche.

L'examen de la *cavité thoracique* ne met en évidence aucune lésion notable. Le coeur est de volume normal (320 g) ses cavités et valvules sont normales. Il existe des adhérences au niveau de la plèvre droite mais les poumons (respectivement 470 et 535 g), à part une congestion, ne présentent aucun remaniement. A noter la consistance dure de la thyroïde qui est de poids normal (19 g).

La *cavité péritonéale* présente un aspect post-opératoire: il existe des adhérences entre les anses intestinales qui sont par endroits revêtues d'un exsudat fibrineux. Le péritoine est adhérent à la capsule de Glisson sur la face

antérieure du foie. La rate est absente de même que les portions terminales du duodénum et proximales du jéjunum.

Le foie est nettement augmenté de volume (3.200 g). La plus grande partie du lobe gauche est occupée par une tumeur arrondie d'environ 7 cm de diamètre, faisant saillie sur le rebord antérolatéral. A la coupe, on découvre une seconde tumeur de même volume dans le lobe droit. Ces masses se distinguent du parenchyme avoisinant par leur coloration rosée et leur consistance molle. A leur centre, on observe des foyers hémorragiques. Le restant du foie est d'aspect normal; il n'y a ni cirrhose évidente ni lésion vasculaire. La lumière de la veine porte est perméable bien que le moignon de la veine splénique soit obstrué par un caillot récent.

Les autres viscères abdominaux, notamment l'estomac, la vésicule biliaire, le colon, les surrénales (11 et 12 g) et les reins (160 et 165 g) sont d'aspect normal: on n'observe de lésion tumorale dans aucun autre organe. D'une manière générale, la sclérose vasculaire est peu prononcée. Les organes génitaux sont normaux.

Des prélèvements sont effectués au niveau de tous les viscères mais ce n'est qu'au niveau de la thyroïde, du foie et des reins que les *examens microscopiques* mettent des lésions en évidence:

—des réactions chroniques ont produit de profonds remaniements au niveau de la thyroïde: la glande est disséquée par des bandes fibreuses contenant des infiltrats à lymphocytes et des granulomes à cholestérides. La thyroïde présente un aspect pseudolobulaire avec vésicules de type peu actif contenant une colloïde dense englobant parfois des cristaux d'oxalate de calcium.

—les deux tumeurs hépatiques présentent un aspect identique, correspondant à celui observé au niveau de la biopsie prélevée au cours de l'intervention chirurgicale dans la paroi duodénale (protocole n° B62/551).

Ces tumeurs sont constituées de cellules allongées, d'allure fibrocytaire, grossièrement groupées en faisceaux (Figs 2 et 3). Entre ceux-ci, des fentes plus ou moins nombreuses et larges, selon les endroits, indiquent une différenciation angiomateuse. On y retrouve parfois des globules rouges. Une recherche soigneuse ne permet pas d'observer des mitoses. Cependant les irrégularités nucléaires et la présence de quelques monstruosités établissent le caractère malin de la prolifération et justifient le diagnostic d'angiosarcome. La nature neurofibromateuse de la tumeur duodénale, qui avait été évoquée à l'examen de la pièce opératoire, ne peut être entièrement écartée.

— les reins montrent, à côté des lésions de glomérulosclérose banale et peu importante, une dysplasie "adénomatoïde" de la capsule de Bowmann et du début des tubes proximaux (Fig. 4). Elle se caractérise par la présence de petites cellules disposées côte à cote. Ces cellules, dont on n'aperçoit souvent que les noyaux, sont de plus en plus nombreuses à mesure que l'on se rapproche de l'origine du tube proximal. Elles constituent une assise unistratifiée qui se prolonge fréquemment sur une certaine distance, dans le tube excréteur (Fig. 5). Cette disposition, la continuité avec l'épithélium tubulaire, l' absence de pluristratification et de mitoses différencient clairement cette dysplasie d'une localisation métastatique. Ces lésions sont observées au niveau d'environ 50 pour cent des glomérules.

En conclusion: status post-opératoire pour exérèse d'un sarcome intestinal. L'autopsie met en évidence deux volumineuses tumeurs hépatiques et permet d'observer une dysplasie glomérulaire et tubulaire "adénomatoïde" à petites cellules.

DISCUSSION

Nous avons groupé dans le Tableau 3 les principales caractéristiques des observations analogues relevées dans la littérature. Il convient de signaler que l'une d'entre elles, celle de Wuketich [6], n'est pas rapportée comme dysplasie, mais comme une forme particulière d'envahissement métastatique des cavités glomérulaires. Cependant, les illustrations reproduites correspondent d'une façon tellement précise aux lésions rencontrées dans les autres cas que nous n'hésitons pas à placer cette observation dans le cadre des dysplasies.

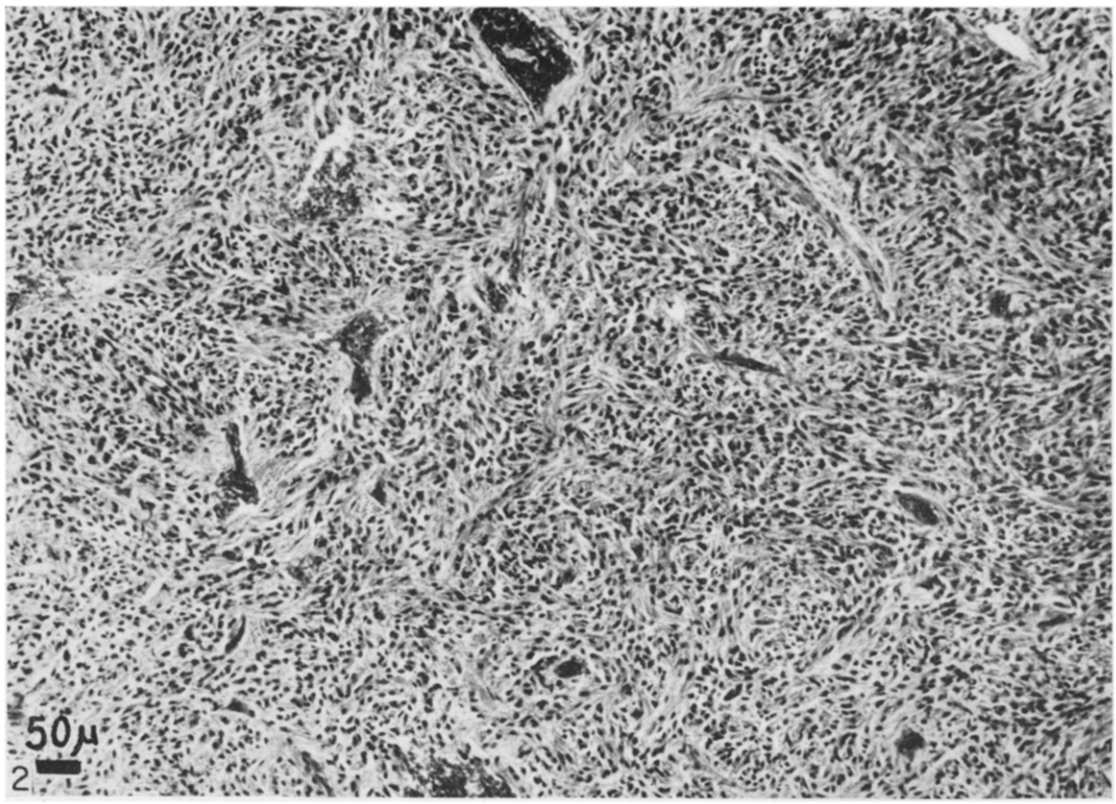
Tableau 2.

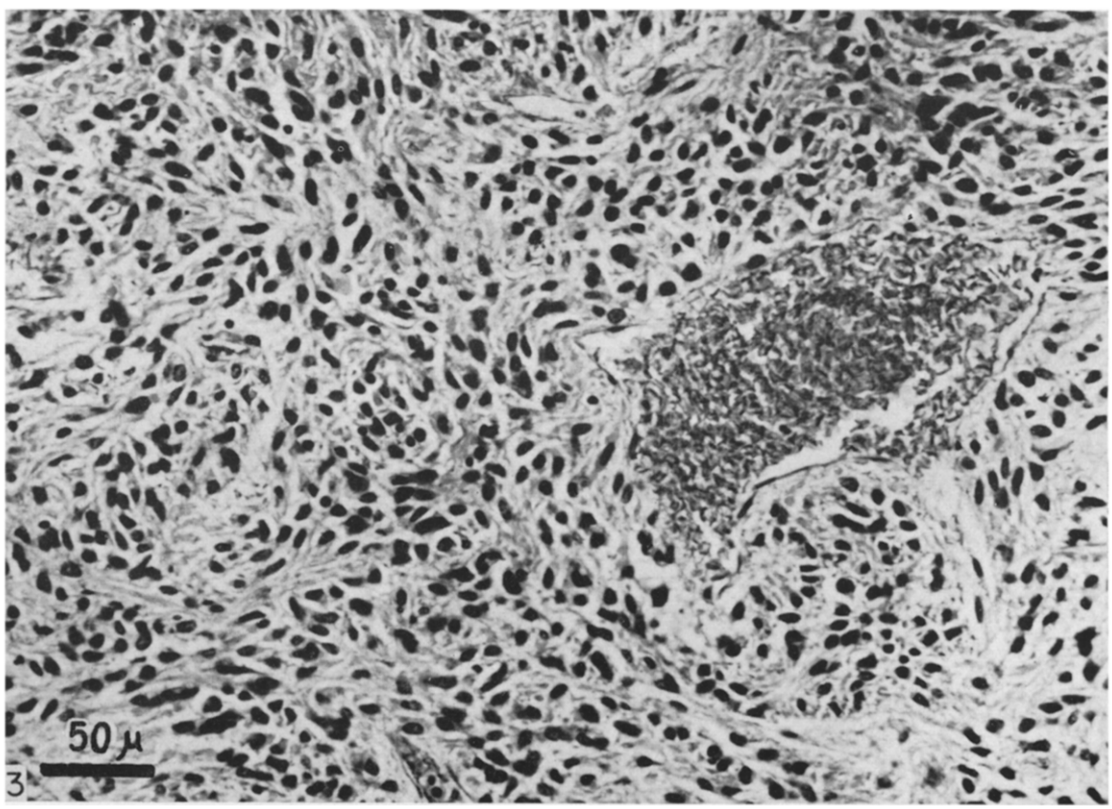
Dates	Réserves alcalines (mEq/l)	Chlorures (mEq/l)
30/7/57	23	100
29/8/58	22	120
6/10/58	20	106
15/10/58	20	90
17/10/58	23	110
9/12/58	18,2	—
11/3/60	17,3	—
15/3/60	24	83
30/3/60	18,2	—
25/4/60	21,6	119
25/7/60	28	—
2/8/60	19,5	—
4/5/61	30	107
24/8/61	23	112
Valeurs Moyennes	21,9 mEq/l	105 mEq/l



Fig. 1. Aortographie (injection par l'artère iliaque). Le réseau vasculaire de la plus volumineuse des métastases hépatiques, dans le lobe droit, est mis en évidence. Un réseau vasculaire assez dense, mais en partie caché par l'ombre aortique, s'observe dans la région duodénale.

(to face p. 62)
Refer to p. 60





Figs. 2 et 3. Aspects de la tumeur hépatique. En haut : faible grossissement. La richesse du réseau vasculaire est apparente. En bas, à un plus fort grossissement, la structure régulière des cellules et l'absence d'anomalies nucléaires sont bien visibles. Un vaisseau est limité par un endothelium directement appliqué contre le tissu néoplasique.

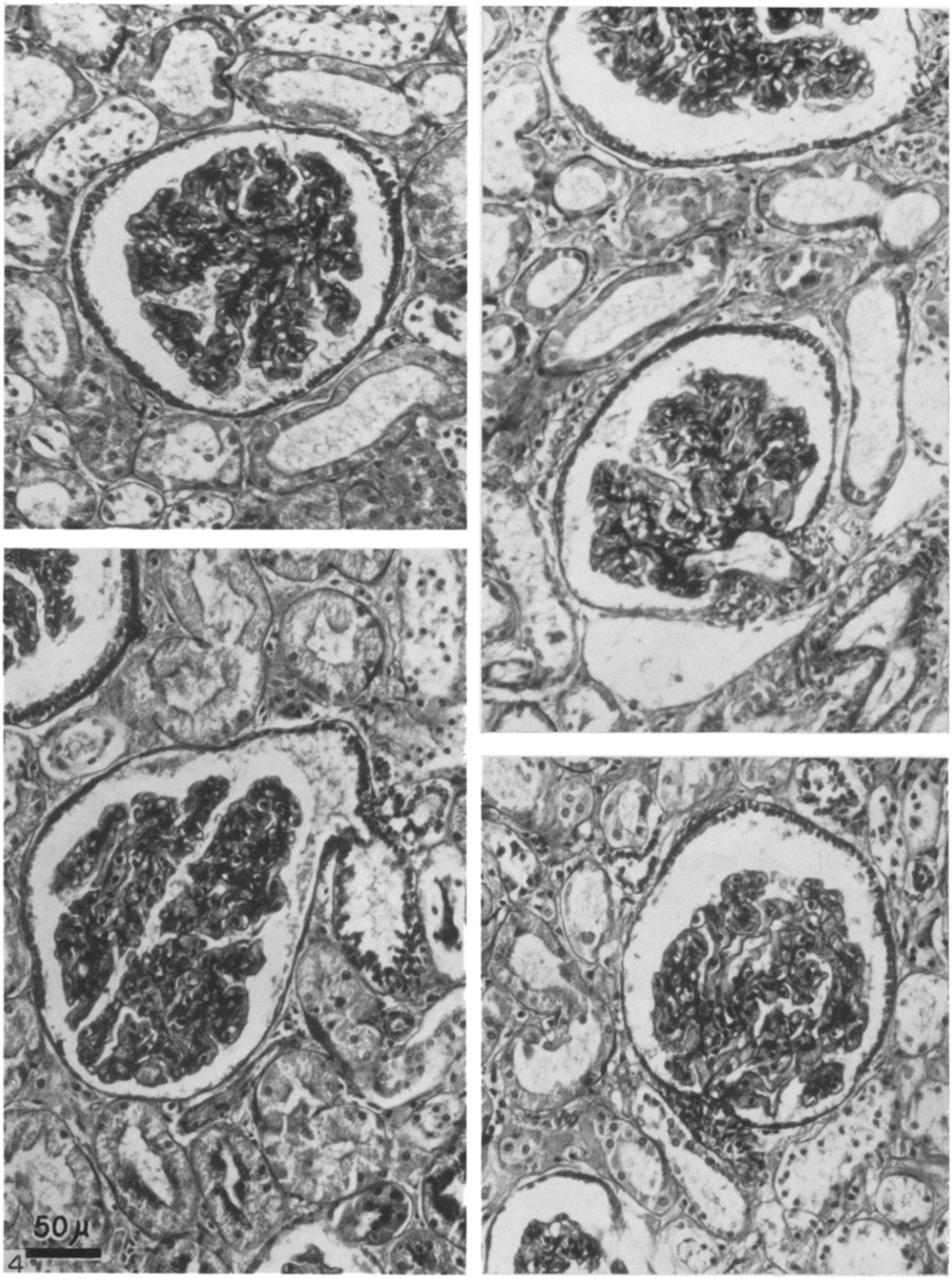
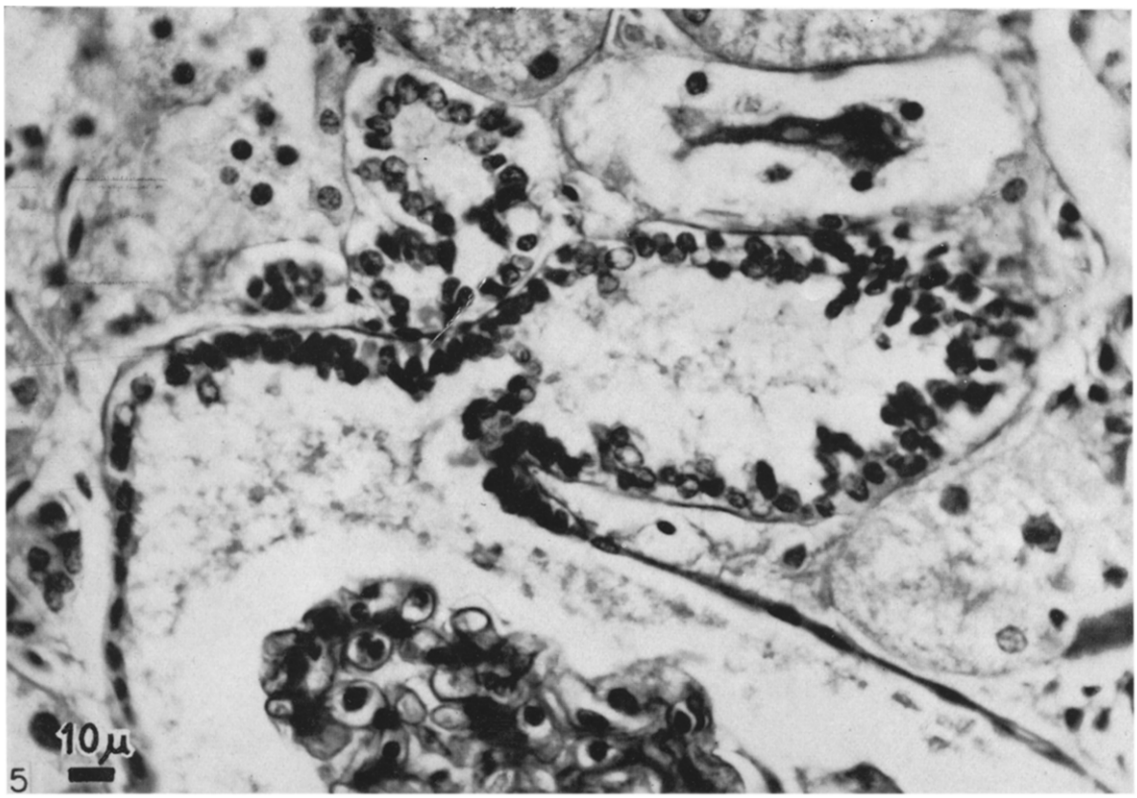


Fig. 4. Aspects des lésions glomérulaires. La capsule de Bowmann est limitée par de petites cellules, à cytoplasme difficilement visible, à noyaux ronds serrés les uns contre les autres. Ces cellules, qui occupent la plus grande partie de la capsule, se prolongent dans le tube rénal. Les glomérules sont par ailleurs de structure normale.



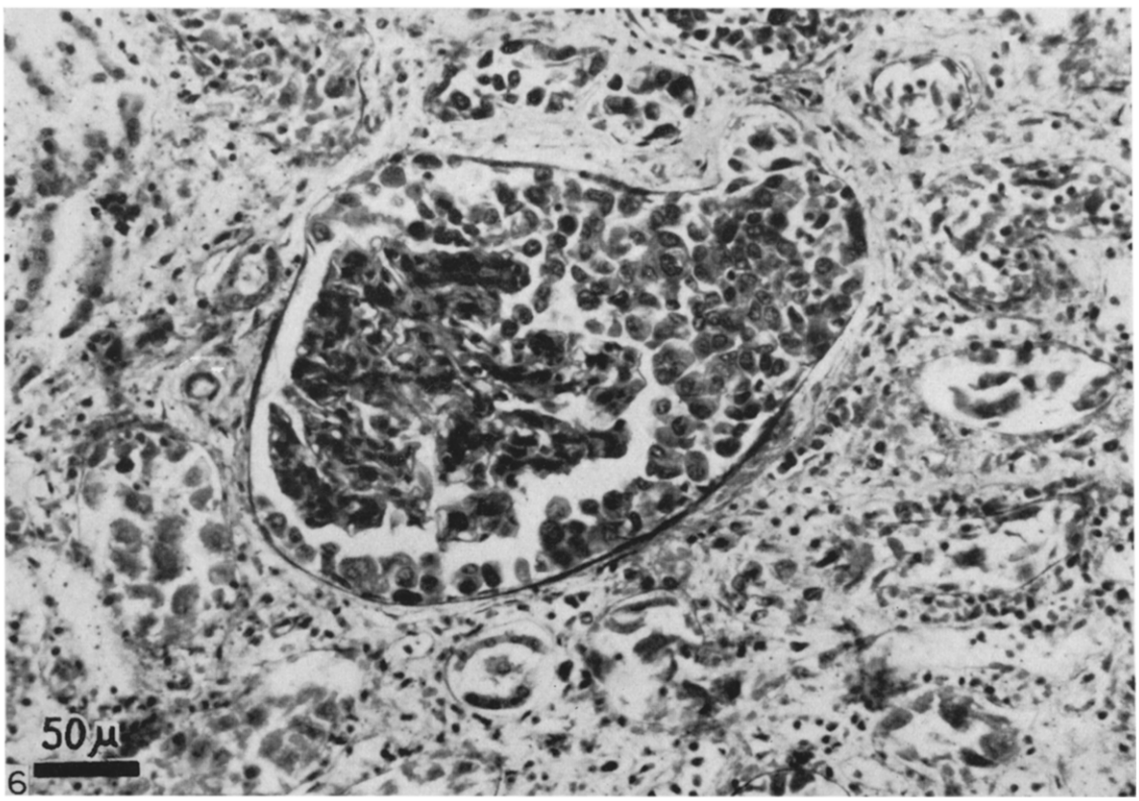


Fig. 5. Aspect à un plus fort grossissement de l'atteinte de la capsule de Bowmann et du segment proximal du tube contourné qui est coupé deux fois. Une partie de la capsule (à droite) présente un épithélium normal. L'épithélium du glomérule est tout à fait normal.

Fig. 6. Image d'infiltration glomérulaire par la métastase d'un épithélioma épidermoïde du col utérin, à cellules peu différenciées. Ce mode d'envahissement glomérulaire, qui est rare, est nettement distinct des lésions dysplasiques adénomatoïdes.

Tableau 3.

Cas	Age	Sexe	Tumeur primitive	Lésions hépatiques	Lésions rénales	Altérations hématologiques et autres complications.
1	54	F	Epithélioma glandulaire de la vésicule biliaire.	Métastases avec obstruction biliaire secondaire. Hypertension porte avec varices oesophagiennes.	Hypertrophie bilatérale (320 et 300 g) Pas de glycosurie.	Hématopoïèse extramédullaire étendue. Pas d'anémie importante.
2	16	F	Région surrénalienne neuroblastome ou carcinome atypique.	Métastases nécrotiques.	Dysplasie frappant 25 à 50% des glomérules.	Anémie.
3	6 m.	M	Hépatome	Tumeur primitive. Pas de cirrhose. Thrombose de la veine hépatique gauche.	Hypertrophie (63g au lieu de 32 g).	Splénomégalie congestive. Métastases médullaires et pulmonaires.
4	63	F	Epithélioma papillifère du foie.	Hépatomégalie (5245g)	Phosphaturie sans glycosurie depuis 20 ans. Poids total: 240g.	Anémie et thrombocytopénie depuis trois ans. Splénomégalie congestive (575g). Ostéomalacie.
5	80	F	Epithélioma de la vésicule biliaire.	Envahissement métastatique.	Lésions considérées comme métastatiques.	Erythropoïèse extramédulaire avec moelle active et anémie hypochrome.
6	40	F	Epithélioma de l'ovaire.	Envahissement métastatique.	Présence de métastases.	Pas d'anomalies mentionnées (anémie banale).
7	48	F	Sarcome du duodénum.	Deux volumineuses métastases.	Néphropathie tubulaire avec perte de phosphore et ostéomalacie.	Anémie secondaire depuis 13 ans. Hématopoïèse extramédullaire avec splénomégalie.

Références: Cas 1, Eisen, [1]. Cas 2, Chappell et Phillips, [2]. Cas 3, Nachman, [3]. Cas 4, Eulderink, [5]. Cas 5, Wuketich, [6]. Cas 6, Gordon, [4]. Cas 7, observation personnelle.

L'envahissement métastatique de la cavité glomérulaire peut exister mais son aspect est tout différent: la Fig. 6 qui montre un cas où des cellules épithéliales indifférenciées, provenant d'une tumeur du col utérin chez une femme de 66 ans, infiltraient certains glomérules, souligne ces différences.

Faut-il rappeler d'autre part que dans les séries de sept cas réunis dans le Tableau 3, la nature des tumeurs primitives et leurs caractères cellulaires différaient considérablement, tandis que les modifications glomérulaires étaient toujours du même type?* Lucke et Schlumberger [10], dans le fascicule consacré aux tumeurs rénales de l'Atlas des Forces Armées, écrivent laconiquement: "Eisen, and also Chappell and Phillips, have reported adenomatoid changes in the capsular epithelium of the glomerulus, but the presence of primary carcinoma elsewhere suggests the probability that these lesions are metastases". Il ne fait pas de doute que si les auteurs américains avaient rencontré un cas analogue dans leur matérial, ils n'auraient pas conclu de cette façon: on peut en déduire sans doute que la lésion dysplasique est fort rare.

Nous discuterons des diverses relations que l'on peut envisager entre les lésions rénales et les autres manifestations cliniques.

1. *Nature de la tumeur* — Une des preuves de la nature "adénomatoïde" et non métastatique des lésions glomérulaires est bien la variabilité extrême des types de tumeurs. En fait, le seul point qu'elles paraissent avoir en commun,

*Nous tenons à exprimer nos remerciements aux Drs. A. Gordon (Edinburgh) et S. Wuketich (Vienne) qui nous ont aimablement fourni quelques renseignements complémentaires sur le cas qu'ils ont décrit.

c'est l'atteinte du foie. Dans deux cas, il s'agit de tumeurs primitives du foie, très différentes l'une de l'autre, puisque l'une est un hépatome (cas 3) et l'autre un épithélioma papillifère provenant des voies biliaires (cas 4). En dehors de l'atteinte hépatique métastatique, les autres tumeurs sont très différentes. Le cas que nous rapportons est lui-même un exemple de tumeur exceptionnelle, puisqu'il paraît s'agir d'une transformation maligne d'une lésion duodénale présente depuis longtemps et se manifestant par des hémorragies (neurofibrome?). En dehors de deux nodules hépatiques bien limités, il n'y avait aucune autre métastase.

2. *Age* — On est frappé par la variabilité de l'âge, qui est sans doute en rapport avec celle des tumeurs. Il est remarquable que les glomérules d'un enfant de 6 mois (cas 3) puissent montrer des images superposables à ceux d'une femme de 80 ans (cas 5). L'aspect des petites cellules de la capsule de Bowmann n'est pas sans rappeler certaines images de reins immatures, et notamment des petites cellules épithéliales rondes qui recouvrent les glomérules du nourrisson. S'il est vrai que dans un cas au moins (celui d' Eulderink) des troubles de la fonction rénale étaient présents depuis très longtemps, il est difficile de croire que, chez les autres malades, une structure immature de la capsule glomérulaire ait persisté pendant toute leur vie.

3. *Sexe* — Sur sept cas, six sont des femmes et le septième, un nourrisson de 6 mois. Cette série est trop petite pour permettre une conclusion statistique, mais une corrélation entre le sexe féminin et les lésions ne doit pas être écartée.

4. *Troubles de l'hématopoïèse* — Plusieurs cas présentent des altérations assez sévères de l'hématopoïèse: étant donné l'intervention du rein dans la formation de l'érythropoïétine, on pouvait se demander si les lésions rénales n'étaient pas secondaires aux anomalies de la crase sanguine. Cependant, à l'examen des observations, on s'aperçoit que les troubles de la formation des hématies ont des causes très différentes. Dans notre cas, par exemple, la présence, depuis plusieurs années, d'une tumeur duodénale saignante rend compte de l'anémie, de l'hématopoïèse extramédullaire et de la splénomégalie. Cependant, il est intéressant de noter qu'une hématopoïèse extramédullaire (splénique) a été rencontrée dans deux autres cas. Dans celui décrit par Eisen, il n'y avait pas d'anémie importante ni d'hémorragies. Il faut noter toutefois la présence de varices oesophagiennes. Dans plusieurs cas, l'anémie est à mettre en rapport avec une splénomégalie congestive, elle-même secondaire à l'atteinte hépatique (cas 3 et 4). Dans les cas 2 et 6, l'anémie s'explique par l'existence d'une néoplasie généralisée.

Les troubles de l'hématopoïèse ne sont donc guère différents de ceux que l'on rencontre de façon banale chez des cancéreux porteurs de métastases hépatiques. Il ne semble pas que l'anémie ait précédé de longtemps la néoplasie, sauf dans notre observation, où la tumeur duodénale primitive a eu une longue évolution, marquée par des épisodes hémorragiques précédant probablement sa transformation maligne. Il ne semble donc pas que les lésions rénales soient à mettre en rapport avec les particularités de l'hématopoïèse relevées dans plusieurs des cas.

5. *Troubles de la fonction rénale* — Ceux-ci sont beaucoup plus importants, tout d'abord parce qu'ils pourraient être en rapport avec les lésions dysplasiques de la capsule de Bowmann et des tubes proximaux, ensuite parce que dans deux observations au moins, ils ont été la première manifestation pathologique et ont précédé de longtemps les signes produits par la prolifération néoplasique.

Mentionnons tout d'abord l'observation dans les cas 1 et 3 d'une hypertrophie pondérale des reins, dont l'explication ne nous est pas fournie par les données histologiques. Dans une observation (cas 5), des métastases rénales étaient présentes; nous avons dit pourquoi nous ne considérions pas comme métastatiques les lésions décrites par Wuketich.

Dans notre observation, le premier signe clinique a été l'anémie, attribuée à une perte de sang par le tube digestif. Cette anémie, avec hypochlorhydrie gastrique, s'est prolongée pendant plus de dix ans. En 1957, soit cinq ans avant le décès, une ostéomalacie est constatée, avec phosphorémie basse et calcémie normale, et élévation des phosphatases alcalines. L'ostéomalacie se manifeste par des douleurs osseuses qui seront retrouvées à tous les séjours. Des fractures costales ne sont apparues qu'après le développement d'une hépatomégalie métastatique. Les taux du calcium, du phosphore et des phosphatases sanguines sont repris au Tableau 1. La calcémie s'est maintenue à un taux satisfaisant, cependant que la phosphorémie demeurait toujours trop basse. L'étude de la clearance du phosphore a montré que celle-ci était augmentée, confirmant l'origine rénale de la perte de phosphore. Les valeurs de la calciurie sont légèrement trop élevées. De plus, les urines montrent une amino-acidurie très significative; cependant, on n'a jamais

rencontré de glycosurie, ce qui permet d'écarter le diagnostic de syndrome de Fanconi.

La malade observée par Eulderink (1964), âgée de 63 ans, souffrait depuis plus de 20 ans d'ostéomalacie consécutive à une phosphaturie. Cette anomalie ne put être améliorée par aucun traitement (vitamine D, éthinyloestradiol, diéthylstilboestrol, néostéron (stéroïde anabolisant). Il n'y avait pas de glycosurie. Les urines contenaient "une trace de protéine", mais l'élimination des acides aminés était normale. Le calcium urinaire était également normal. Le clearance du phosphate valait 17 ml/min, c'est-à-dire un taux trop élevé, mais moins que dans notre observation.

Les anomalies de l'élimination urinaire des phosphates sont très similaires dans ces deux cas, et on peut raisonnablement penser qu'elles découlent des structures anormales de la partie proximale des tubes contournés, région tubulaire active dans la résorption des phosphates. Ceci n'est pas l'opinion d'Eulderink qui pense que les lésions rénales sont secondaires à la tumeur maligne, et ne peuvent donc rendre compte d'une phosphaturie présente depuis si longtemps. L'observation d'un second cas présentant des anomalies quasi-identiques de la fonction rénale remet à notre avis cette opinion en question, et soulève le problème de savoir si les lésions rénales ne peuvent pas avoir *précédé* la néoplasie.

Cette hypothèse peut s'appuyer actuellement sur les images morphologiques qui, indubitablement, rappellent la structure embryonnaire du glomérule, et paraissent dysplasiques, ressemblant en ce qui concerne l'étroitesse du collet du tube contourné proximal, aux lésions du syndrome de Fanconi [11]. Rappelons que, dans cette affection, l'anomalie "en col de cygne" du tube contourné à son départ de la capsule de Bowmann est rendue responsable de la perte de phosphates par les urines. Nous n'avons toutefois pas connaissance d'observations de syndrome de Fanconi au cours desquels des tumeurs, hépatiques ou autres, aient fait leur apparition. Si l'on suppose que la lésion rénale a précédé la néoplasie — et l'observation d'Eulderink et la nôtre sont assez suggestives à ce point de vue — il faudrait admettre qu'il existe une dysplasie rénale particulière, dont nous avons rassemblé 7 cas, qui prédisposerait à la croissance néoplasique. A première vue, ce point de vue paraît acceptable: il suffit de penser aux lésions dysplasiques du mongolisme et leur relation avec l'augmentation de la fréquence des leucémies aiguës. Cependant, on est confronté alors avec le problème, qui nous apparaît insoluble, pour l'instant, de la variété des tumeurs et de l'atteinte du foie, atteinte très variable quantitativement, mais qui est le seul facteur constant dans toutes ces observations. L'on est ainsi ramené à supposer quelque relation de cause à effet entre les métastases ou les tumeurs primitives du foie et les lésions rénales, mais on ne peut alors que s'étonner de la rareté de telles lésions alors que les métastases hépatiques sont chose si fréquente.

Comme le montre cette brève discussion, l'association entre les dysplasies adénomatoïdes de la capsule de Bowmann et du tube contourné proximal, et l'apparition de tumeurs malignes au niveau du foie, soulève des problèmes qui demeurent sans solution. Nous avons pensé qu'il était intéressant d'attirer à nouveau l'attention sur cette entité morbide, car seules de nouvelles observations bien documentées pourront résoudre les questions posées.

RÉSUMÉ

Les auteurs rapportent un cas de dysplasie adénomatoïde de la capsule de Bowmann et du début des tubes contournés rénaux observé à l'autopsie d'une femme atteinte de sarcome duodénal avec métastases hépatiques. Cette malade présentait, depuis plus de cinq ans, de l'ostéomalacie consécutive à une phosphaturie excessive et une phosphorémie basse. Une aminoacidurie, sans glycosurie, était présente.

Sept observations semblables ont été rassemblées. Dans tous les cas, le foie était entrepris par une tumeur primitive ou secondaire. Six étaient du sexe féminin. Des troubles hématologiques, probablement secondaires, étaient présents dans tous les cas. Notre observation, ainsi que celle de Eulderink, suggère que les lésions rénales, à caractère dysplasique, pourraient avoir été présentes avant l'apparition de la tumeur maligne.

SUMMARY

A case of adenomatoid dysplasia of Bowmann's capsule and of the proximal part of the renal tubules was discovered at autopsy, in a patient suffering from a sarcoma of the duodenum with hepatic metastases. Osteomalacia had been noticed for five years. This

was associated with renal loss of phosphate, low blood phosphorus levels and amino-aciduria without glycosuria.

Seven similar observations have been tabulated. In all seven cases, hepatic neoplasia, either primitive or metastatic, was present. Six patients were female. In all cases, haematological disorders, probably secondary, were present.

Our case, as well as that recently reported by Eulderink, suggests the possibility that the renal dysplastic lesions may have preceded the malignant growth.

ZUSAMMENFASSUNG

Ein Fall von adenomatoider Dysplasie der Bowman'schen Kapsel und des proximalen Teiles der Nierentubuli wurde durch Autopsie bei einem Patienten mit Duodenalsarkom und Leber-Metastasen gefunden. Seit mehr als 5 Jahren war dabei eine Osteomalacie, verbunden mit erhöhter Phosphaturie und neidrigem Phosphorspiegel im Blut beobachtet worden, ausserdem eine Hyperaminoacidurie ohne Glykosurie.

Sieben vergleichbare Beobachtungen wurden zusammengestellt. In allen sieben Fällen befand sich ein Tumor in der Leber, entweder primär oder als Metastase. Sechs Patienten waren weiblich. In allen Fällen bestanden — vermutlich sekundäre — haematologische Veränderungen.

Sowohl unsere Beobachtung also auch die von Eulderink mitgeteilte deuten darauf hin, dass die dysplastiche Nierenschädigung schon von der Entstehung des malignen Tumors vorhanden gewesen sein könnte.

BIBLIOGRAPHIE

1. H. N. Eisen, Adenomatoid transformation of the glomerular capsular epithelium. *Amer. J. Path.* **22,** 597 (1946).
2. R. H. Chappell and J. R. Phillips, Adenomatoid changes of renal glomerular capsular epithelium associated with adrenal tumor. *Arch. Path.* **49,** 70 (1950).
3. R. L. Nachman, Metaplasia of parietal capsular epithelium of renal glomerulus. Report of an autopsied case of carcinoma of the liver. *Arch. Path.* **73,** 48 (1962).
4. A. Gordon, Renal glomerular adenomatosis. *J. Path. Bact.* **83,** 555 (1962).
5. F. Eulderink, Adenomatoid changes in Bowman's capsule in primary carcinoma of the liver. *J. Path. Bact.* **87,** 251 (1964).
6. S. Wuketich, Ungewönliche Erscheinungsformen der metastatischen Nieren-karzinose. *Verh. dtsch. path. Ges.* **46,** 365, (1962).
7. P. Dustin, L'athrocytose protidique dans le segment cervical du néphron humain. *Rev. belge Path.* **27,** 251 (1960)
8. L. H. Kyle, M. Schaaf and J. J. Canary, Phosphate clearance in the diagnosis of parathyroid dysfunction. *Amer. J. Med.* **24,** 240 (1958).
9. E. J. King and I. D. P. Wootton, *Micro-analysis in medical biochemistry*, p. 159. 3rd Ed. Churchill, London (1956).
10. B. Lucke and H. G. Schlumberger, Tumors of the kidney, renal pelvis and ureter. *A.F.I.P., Atlas of Tumor Pathology*, section VIII, fasc. 30. (1957).
11. R. D. Clay, E. M. Darmady and M. Hawkins, The nature of the renal lesion in the Fanconi syndrome. *J. Path. Bact.* **65,** 551 (1953).

Europ. J. Cancer Vol. 1, pp. 67–71. Pergamon Press 1965. Printed in Great Britain

A Cell Dispersion Technique for Use in Quantitative Transplantation Studies with Solid Tumours

H. S. REINHOLD

Radiobiological Institute TNO
Lange Kleiweg 151, Rijswijk Z. H., Netherlands

INTRODUCTION

FOR EXPERIMENTS concerning the survival of tumour cells after irradiation it was necessary to obtain large numbers of freely suspended cells from a solid tumour. Results obtained with purely mechanical disruption of solid tumours proved unsatisfactory as has also been the experience of other authors [1, 2].

An enzymatic procedure seemed therefore indicated. Trypsin has been widely used and some excellent procedures using that enzyme have been described [1, 2]. However, further improvement of these methods seemed desirable since our experiments required very large numbers of cells to be prepared within a short time.

MATERIAL AND METHODS

A combined mechanical and enzymatic method was employed. The tumour is mechanically chopped into particles of 1–2 mm^3, which are then "dispersed" [3] in a trypsin containing medium.

Mechanical equipment

The instrument consists mainly of parts available anywhere as standard equipment; the main part is a motor of continuously variable speed with extensions on both spindle ends (Fig. 1).

Attached to one side via a worm gear reduction is a crank which operates a razorblade holder; the rotation of the motor thus causes the razorblade to make a vertical chopping movement. This "chopper," using a stainless steel razorblade, is in fact a simplified version of the chopper of McIlwain and Buddle [4] and Gordy and Sieber [5]. The tumour tissue is placed on a silicone rubber inlay in a small petri dish, the latter being adjustable to the right position by a screw-lifting jack. At the opposite side, the motor spindle is connected with a flexible shaft, the end of which is fixed in a vertical position. In the chuck a stainless steel shaft is fitted with a round-edged razorblade, mounted perpendicularly. The latter can spin freely in a horizontal plane in the centre of a vessel of the type used in the Bühler homogenizer; a sphere with three whirling-ribs and a cylindrical neck. The distance of the razorblade to the ribs is about 3 mm and is similar for both vessel-sizes (150 and 400 ml). The razorblades in their holders as well as the silicone rubber and glass parts can be sterilized by dry heat.

Medium

The dispersion medium is a Hank's solution, modified after McLimans and contains the following ingredients in g/l: NaCl, 7·4, KCl, 0·2; $Na_2HPO.H_2O$, 0·75; KH_2PO_4, 0·05; $CaCl_2.6H_2O$, 0·05; $MgCl_2.6H_2O$, 0·18; glucose, 1·0. Trypsin 1/300 (NBC) 0·5; Phenol

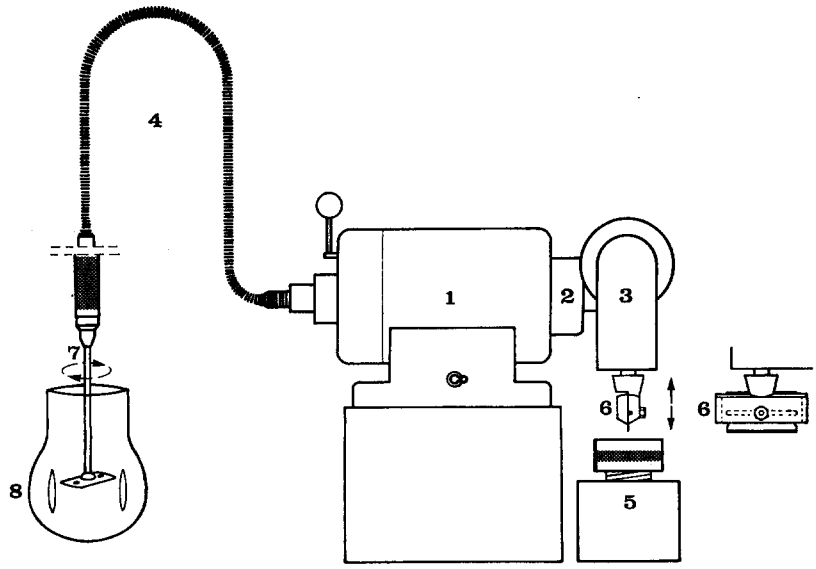


Fig. 1. Diagram of the instrument.

1, Motor; 2, reduction gear; 3, crank; 4, flexible shaft; 5, jackscrew; 6, razorblade holder (custom-made); 7, stainless steel shaft for razorblade (custom-made); 8, dispersion vessel.

red, 0·02. The pH should be about 7·35. The $MgCl_2$ is put in last, the $CaCl_2$ is dissolved separately.

Procedure

After weighing, the tumour is cut to pieces with scissors, and subsequently reduced to particles of 1–2 mm^3 by the chopper. This procedure takes only a few minutes, the tumour tissue being kept right under the chopping razorblade by turning the petri dish and gathering the pieces with a small spatule. The tumour brei is then transferred to the dispersion vessel, which should contain sufficient medium to fill it half-way up the neck. For most tumours not less than 30 ml medium per gramme tumour tissue should be used. This trypsinization phase is carried out at room temperature. Dispersion is started by spinning the razorblades at a speed of 1200–1600 rev/min for 10–20 min. Foaming must be avoided; if it occurs, it is usually due to a fibrous strand (occasionally present in spite of thorough chopping) which is caught by the razorblade, thus increasing the turbulence of the fluid. Such strands have to be removed. After the dispersion, the remaining particles are allowed to settle for 5 min. This prevents small particles from being caught in the sieve when the supernatant is subsequently decanted through a nylon filter. The single cell suspension so obtained is cooled rapidly to 0–4°C to stop the remaining trypsin activity. After centrifugation ($170 \times g$ for 10 min at 0°C) and resuspension in the desired amount of ice-cold Simms solution, the suspension is ready for use.

By adding fresh medium to the particles remaining in the dispersion-vessel after decanting the supernatant, a second "run" can be made and the new yield can be pooled with the first suspension. If a "clean" cell suspension is wanted, it is advisable to make a short first run of about 5 min and to discard the supernatant. Most erythrocytes will be removed by this procedure and the major part of necrotic material digested by the trypsin.

Yield

The average yield of the tumour most frequently used, a rhabdomyosarcoma (BA 1112) which originated in a Wistar rat, was 20–70 million viable cells per gramme tumour tissue. The number of viable cells was determined in a haemocytometer by adding a 0·02 per cent trypan blue solution. Only unstained cells were considered to be viable. The stained cell number was usually 10–20 per cent of the total number of cells in the suspension. The number of cells in a certain amount of tumour tissue was estimated by comparing the dry weight and the DNA content of that piece of tumour with the same parameters for the cells in suspension. From the dry weight of cells in suspension and from that of a solid tumour it was estimated that 1 g of tumour contains $5{\cdot}03 \times 10^8$ cells/g. Similar comparison of DNA content resulted in an estimate of $5{\cdot}7 \times 10^8$ cells/g. Thus these estimates suggest that this particular tumour contains about 5×10^8 cells/g, so that the yield of the cell dispersion technique was in the order of 10 per cent of the total number of cells.

Other types of tumours produce quite different yields. The following should illustrate the range of yields: from an irradiated human lymph node metastasis of a malignant melanoma only about 1 million viable cells per gramme were obtained; from a mouse osteosarcoma that had been transplanted many times, the yield was more than 10^9 viable cells per gramme.

Bioassay

For the *in vivo* determination of the viability of the processed cells of the rhabdomyosarcoma, graded numbers of cells were inoculated into isologous recipient animals. The results are presented in Table 1. The $E.D._{22}$ calculated

Table 1. Rat rhabdomyosarcoma BA 1112: pooled results of 11 experiments.
(Effectiveness of tumour cell suspensions in the production of a tumour at the injection site up to 100 days after injection of different numbers of cells).

Number of cells inoculated	Number of rats inoculated	Rats with tumour (%)
1000	79	92
400	20	80
100	100	72
40	40	67
10	110	25·5
4	40	12·5
1	90	1·1

according to the method of Litchfield and Wilcoxon [6] was forty cells; this means that after the inoculation of forty cells, as an average 50 per cent of the recipients will have a tumour at the site of inoculation within 100 days. The $E.D._{50}$ decreased to approximately ten cells after ten serial transplantations of the tumour.

Oxygen consumption

Since this parameter is of importance for radiobiological research, the oxygen consumption of tumour cells in suspension was determined for three different rat tumours; Two rhabdomyosarcomas (BA 1112 and LM 9963) and an adenocarcinoma (Nr. 4658). The QO_2 of the cells of these tumours was respectively 0·023 and 0·016 µl O_2/min/10^6 cells for the rhabdomyosarcomas and 0·029 µl O_2/min/10^6 cells for the adenocarcinoma. These values are within the range of data obtained by other investigators [2, 7] and may be useful for calculations of the distribution of partial oxygen pressure within solid tumours [7].

DISCUSSION

For an efficient action of trypsin on tumour tissue it is advisable to start the actual dispersion after the tissue has been reduced to a brei of small particles. A solid tumour, which always contains some tough connective tissue strands, is most conveniently dispersed with a mechanical device. Other advantages of a mechanical "chopper" are its speed and the reproducibility of the particle size. During the phase of enzymatic action and in particular after the intercellular cement has been digested by the trypsin, the cells have to be separated by some mechanical force, which is obtained by agitating the medium. Several systems have been described for this purpose. The simplest method, repeated pipetting or shaking of the bottle, is not suitable for large amounts of tissue. This also applies to the elegant method described by Boyse [1] in which the medium with suspended tumour particles is circulated through rubber tubing by means of a finger pump. A magnetic stirrer in a trypsinization bottle, as used in the method of Madden and Burk [2] works reasonably well, but has the disadvantage of a tendency to produce foam. A considerable part of the cellular yield can be lost in this foam. To avoid this complication, stirring must be done slowly which of course prolongs the dispersion time. To obtain intensive local agitation of the medium without excessive turbulence on the surface, a flat stirrer, rotating accurately in a horizontal plane is being used in the present method. A round-edged razorblade proved to be satisfactory for this purpose. Unwanted turbulence on the surface is reduced to a minimum by filling the vessel up to its narrow neck (small surface of the fluid and smooth lining of the walls).

The use of a phosphate buffer is preferred to a carbonate buffer; agitation of the medium easily causes CO_2 to escape, resulting in a decrease of the pH.

The method described in this paper seems satisfactory for many kinds of tumours. However, since tumours can differ in cellularity, amount of intercellular material etc., small variations in the dispersion technique may be necessary for different tumours.

The use of enzymes for the isolation of living cells from animal tissues has been extensively reviewed by Rinaldini [8]. For the moment trypsin seems to be the most useful enzyme [1, 2].

The use of other enzymes, such as collagenase [8] and pronase [9] or the use of chelating agents in combination with Ca- and Mg- free media have to be considered for those tumours, which do not respond properly to trypsinization. DNAse has been recommended [1, 2] to dissolve a DNA gel, originating from nuclei of

dead cells. Our experience however is that the use of DNAse can be avoided, provided that the dispersion technique as described here is carefully performed and the ratio of tumour volume to quantity of medium does not exceed 1/30. In the case of the mouse osteosarcoma a DNA gel developed in the final cell suspension in ice-cold Simms due to progressive desintegration of the cells. Addition of 10 per cent calf-serum proved to be effective to avoid this. Nevertheless, the use of DNAse might be necessary if such a cell suspension would have to be incubated subsequently.

With the described dispersion technique it is possible to obtain, in about 3 hr, as many as 10^9 cells from approximately 20 g of tumour tissue. It is possible to obtain two or three times as many cells within the same period, by alternating use of two dispersion vessels during the procedure. These large numbers of cells allow quantitative transplantation experiments using cells in suspension as well as for instance immunizations with large numbers of heavily irradiated cells.

For metabolic investigations cell suspensions have advantages as compared to solid tissue. With the described technique the equivalent of several grammes of tissue can be obtained as a concentrated suspension of viable cells. The method also makes it possible to obtain survival curves with cell suspensions obtained from solid tumours and will therefore be helpful in investigations of the response of tumours to radiotherapy.

Acknowledgements — I thank Mr. A. H. Andreas and Mr. J. E. Nelemans for their help in constructing the mechanical equipment, Dr. H. M. Klouwen and Dr. I. Betel for their assistance in making the medium and buffer and Dr. J. Kaalen of the Rotterdam Radiotherapeutic Institute for his advice concerning the calculations.

RÉSUMÉ

Une technique a été élaborée afin de réduire une tumeur solide en une suspension de cellules isolées.

La tumeur est divisée mécaniquement. Les cellules sont ensuite dispersées dans un milieu contenant de la trypsine paré une agitation appropriée.

La technique permit d'obtenir facilement des suspensions contenant plusieurs milliards de cellules. Quelques applications de cette technique ont été décrites.

SUMMARY

A method is described to obtain a single cell suspension from solid tumour tissue.

Combined mechanical and enzymatic procedures are employed; the tumour is mechanically chopped into small particles, which are then dispersed in a trypsin containing medium by means of a special stirrer.

With the processed cells from a rat rhabdomyosarcoma a mean of forty cells, inoculated into recipient animals, suffice to produce tumours in 50 *per cent of these animals at the inoculation site.*

With this technique a concentrated suspension containing several times 10^9 *viable cells can be obtained.*

ZUSAMMENFASSUNG

Ein Verfahren ist beschrieben zur Erhaltung einer Suspension isolierter lebender Zellen aus soliden Tumoren. Die Tumoren werden zuerst mechanisch zerkleinert und dann werden die Zellen isoliert in einem Trypsin enthaltenden Medium mittels eines geeigneten Rührapparats.

Mit diesem Verfahren konnten konzentrierte Suspensionen von einigen Milliarden Zellen bereitet werden.

Einige Anwendungen dieser Technik weder beschrieben.

REFERENCES

1. E. A. Boyse, A method for the production of viable cell suspension from solid tumours. *Transplant. Bull.* **7,** 100 (1960).
2. R. E. Madden and D. Burk, Production of viable single cell suspensions from solid tumours. *J. nat. Cancer Inst.* **27,** 841 (1961).

3. S. T. JACOB and P. M. BHARGAVA, A new method for the preparation of liver cell suspension. *Exp. Cell Res.* **27,** 453 (1962).
4. H. MCILWAIN and H. L. BUDDLE, Techniques in tissue metabolism. I. A mechanical chopper. *Biochem. J.* **53,** 412 (1953).
5. E. GORDY and G. SIEBER, An automatic tissue chopper with quick-change sterilizable cutting parts. *J. appl. Physiol.* **18,** 191 (1963).
6. J. T. LITCHFIELD and F. WILCOXON, A simplified method of evaluating dose-effect experiments. *J. Pharmacol. exp. Ther.* **96,** 99 (1949).
7. R. H. THOMLINSON and L. H. GRAY, The histological structure of some human lung cancers and the possible implication for radiotherapy. *Brit. J. Cancer* **9,** 539 (1955).
8. L. M. J. RINALDINI, The isolation of living cells from animal tissues. *Int. Rev. Cytol.* **VII,** 587 (1958).
9. R. B. GWATKIN and J. L. THOMSON, A new method for dispersing the cells of mammalian tissues. *Nature, Lond.* **201,** 1242 (1964).

Europ. J. Cancer Vol. 1, pp. 73–74. Pergamon Press 1965. Printed in Great Britain

Announcements

International Symposium on the Biological Characterisation of Human Tumours

Chairman: Professor FRANZ BERGEL, F.R.S.

The proceedings of this Symposium, to be held on May 27 *and* 28, 1965 *will be published in the European Journal of Cancer*

Eleanor Roosevelt International Cancer Fellowships of the American Cancer Society 1966

THE INTERNATIONAL UNION AGAINST CANCER, with the funds provided by the American Cancer Society will award fellowships for research on cancer.

The awards will be granted to experienced investigators who have demonstrated their ability for independent research and who wish to broaden their experience by a period of study at a single institution in another country.

Fellowships will be granted only to persons on the staff of universities, teaching hospitals, research laboratories or similar institutions.

Awards will be made to investigators who are devoting themselves either to the experimental or the clinical aspects of cancer research.

Fellowships will not be granted to persons who wish primarily to perfect their training in methods of cancer detection or in therapeutic techniques, or who wish to visit briefly several institutions abroad. The duration of the fellowships ordinarily will be one year but this period may be longer or shorter in special circumstances.

The stipend will be based on the current salary of the applicant and the salary of persons of comparable qualifications in the place where the applicant expects to study.

An allowance will be made for the cost of travel of the fellow and of those dependants who will accompany him from his place of residence to the institution where he will work, and return.

Applications must be received prior to 1 September 1965, for a fellowship to begin within the twelve months' period following 1 March 1966.

Application forms and additional information may be obtained from:

International Union Against Cancer
P.O. Box 400
1211 *Geneva* 2
Switzerland

L'UNION INTERNATIONALE CONTRE LE CANCER, grâce à une subvention de l'«American Cancer Society», alloue chaque année des bourses de recherche sur le cancer.

Ces bourses sont attribuées à des chercheurs ayant l'expérience et les capacités requises pour la recherche indépendante et qui désirent élargir le champ de leurs connaissances par une période d'études dans un institut d'un autre pays.

Elles ne sont accordées qu'à des personnes appartenant au personnel scientifique d'universités, d'hôpitaux universitaires, de laboratoires ou d'institutions similaires. Ces bourses sont destinées à des chercheurs se livrant à des travaux sur le cancer soit dans le domaine expérimental soit dans le domaine clinique.

Elles ne sauraient être accordées à des personnes désirant se perfectionner dans les méthodes de dépistage ou de traitement ou qui désirent simplement visiter plusieurs centres à l'occasion d'un bref voyage d'étude à l'étranger.

La durée de ces bourses sera généralement d'un an. Cependant, dans certaines circonstances particulières, leur durée pourra être plus longue ou plus courte.

Le montant de la bourse sera fixé compte tenu du salaire actuel du candidat et du salaire de chercheurs d'un niveau équivalent dans l'institut où le candidat désire travailler.

Une allocation spéciale est prévue pour couvrir les frais de voyage aller et retour du boursier et des personnes à sa charge qui l'accompagneront, de son domicile à l'institut qui l'accueillera.

Les demandes de bourses doivent être déposées avant le 1er septembre 1965 pour une bourse devant commencer dans les douze mois qui suivent le 1er mars 1966.

Les formules de demande de bourses ainsi que tous renseignements complémentaires sont fournis par:

Union Internationale Contre le Cancer
Boîte postale No 400
1211 *Genève* 2
Suisse

Revue Francaise d'Etudes Cliniques et Biologiques. Mai 1965 — Vol. X — No. 5

Tribune Editoriale

L'hypoplasie thymique du nourrisson. M. LAMY et CH. NEZELOF.

Revues Generales

La prolifération des lymphocytes humains *in vitro*. E. H. COOPER et J. L. AMIEL.

Les altérations du métabolisme énergétique dans les tissus cancéreux. R. NORDMANN.

Memoires

Modifications des lipides plasmatiques dans les troubles de l'absorption intestinale. J. REY, J. FRÉZAL, J. POLONOVSKI et M. LAMY.

Résistance naturelle des staphylocoques à la méthicilline et l'oxacilline. Y. A. CHABBERT, J. G. BAUDENS, J. F. ACAR et G. R. GERBAUD.

Myélomes, maladies de Waldenström zrt groupes de gamma-globulines Gm et Inv. C. ROPARTZ, L. RIVAT, P. Y. ROUSSEAU et J. M. FINE.

Tuberculose rénale et hypertension artérielle: à propos de 31 cas. P. TCHERDAKOFF et P. MILLIEZ.

Analyses

Notes Originales

Autoanticorps à spécificités multiples, anti-HI, anti-AI, anti-BI, dans certains éluats d'anémie hémolytique. CH. SALMON, J. C. HOMBERT, G. LIBERGE et F. DELARUE.

Dosage des protéines totales et de certaines globulines du L.C.R. I. Application de l'analyse automatique aux techniques de fractionnement chimique. P. CASTAIGNE, J. CAMBIER et E. SCHULLER.

II. Dosage des gamma-globulines du L.C.R. P. CASTAIGNE, J. CAMBIER et E. SCHULLER.

III. Les mucoprotéines de L.C.R. P. CASTAIGNE, J. CAMBIER et E. SCHULLER.

Réflexions sur une hyperlipidémie complexe révélée au cours d'un traitement par l'Isoniazide. H. BOUR, B. SWYNGHEDAUW et M. CLAUVEL.

Notes de Technique

Dosage du glutathion dans les érythrocytes humains. G. WELLERS.

Le reflexogramme achilleen. H. DESCHAMPS et H. BRICAIRE.

Informations

Europ. J. Cancer Vol. 1, pp. 75–113. Pergamon Press 1965. Printed in Great Britain

Les Divers Aspects du Syndrome Secondaire Compliquant les Transfusions Allogéniques de Moelle Osseuse ou de Leucocytes chez des Sujets Atteints d'Hémopathies Malignes*

G. MATHE, L. SCHWARZENBERG, M. J. de VRIES,† J. L. AMIEL, A. CATTAN, M. SCHNEIDER, J. L. BINET, M. TUBIANA, C. LALANNE, V. SCHWARZMANN et R. NORDMANN

Institut de Cancérologie et d'Immunogénétique,‡ et Service d'Hématologie de l'Institut Gustave Roussy§

NOTRE expérience des transfusions de moelle osseuse allogénique chez l'Homme, après irradiation totale à dose létale, qui porte sur dix-neuf essais (Mathé *et al.* [1–6]), a montré la réalité de la greffe de ce tissu dans notre espèce, comme dans les autres espèces animales étudiées au laboratoire, Souris (Barnes *et al.* [7, 8]; Van Bekkum et Vos [9]; Cole [10]), Rat (Balner [11]; Balner *et al.* [12]), Lapin (Porter [13–15]), Chien (Thomas *et al.* [16]), Singe (de Vries *et al.* [17]; Crouch *et al.* [18]) Mais elle a également montré que cette greffe se complique chez l'Homme, comme dans les autres espèces, d'un syndrome secondaire de gravité variable, mais fréquemment sévère, véritable pierre d'achoppement à l'utilisation courante de la greffe de moelle osseuse allogénique en thérapeutique humaine (Mathé et Amiel [19]). (Mathé [20–22]; Mathé *et al.* [1–6, 23]).

Notre expérience des transfusions de leucocytes concerne trente essais (Schwarzenberg *et al.* [24]): six se sont compliquées de manifestations semblables à celles qui caractérisent le syndrome secondaire à la greffe de moelle osseuse allogénique.

Nous nous proposons de rassembler dans le présent travail tous les cas de ce syndrome, que nous avons observés, d'en analyser les divers aspects, de les comparer entre eux et avec ceux décrits dans les autres espèces animales.

*Travail accompli grâce à l'aide de l' "European Office, Aerospace Research" contract no. 61(052)816.

†Institut de Radiobiologie TNO, Rijswijk, Hollande.

‡INSERM et Association Claude Bernard, Hôpital Paul Brousse, 14, Avenue Paul Vaillant Couturier, Villejuif (Seine). France.

§16 bis, Avenue Paul Vaillant Couturier, Villejuif (Seine) France.

I. METHODES

A. *Transfusion de moelle osseuse allogénique après irradiation totale chez les leucémiques*

Quatorze sujets atteints de leucémie aiguë lymphoblastique ont été l'objet de tels essais: dans trois cas, la greffe n'a pas pris et les patients sont morts d'aplasie myélo-lymphoïde due à l'irradiation; dans neuf cas, parallèlement à la prise de la greffe est apparu un syndrome secondaire; dans un cas la greffe n'a pas pris, mais la malade a pu être restaurée par la moelle osseuse de sa soeur jumelle monozygote; dans un autre cas, la prise de la greffe ne s'est pas accompagnée des signes majeurs du syndrome secondaire.

Les conditions de l'irradation et de la transfusion de la moelle osseuse ont été respectivement décrites dans d'autres publications (Mathé *et al.* [1-4]; Lalanne et Surmont [25]); les premières sont résumées au Tableau 1.

Le traitement symptomatique a été dirigé par les indications données au Tableau 2, adaptées au problèmes particuliers posés à chaque moment par les patients.

Tableau 1. *Les conditions de l'irradiation appliquée avant la transfusion de la moelle osseuse.*

1. Patients soumis à une irradiation par une source unique de Co–60

Patient	Date d'irradiation		Doses tissulaires en rads								Dose tissulaire moyenne (estimation)		Remarques
			Abdomen		Thorax		Tête		Pieds				
	1ère	2ème	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Ensemble du corps sauf abdomen	Abdomen	
D.V.	12.1.59	13.1.59	760	870	850	910	820	920	870	920	870	820	
V.L.	13.2.59	14.2.59	380	460	430	480	390	430	450	490	875	820	
			Doses mesurées partiellement										
E.D.	8.6.59	9.6.59	800	840	950	1050	850	950	950	1030	950	820	En outre, 220 r ont été distribués sur une adénopathie cervicale gauche, sur un champ de 4×7 cm
M.V.	7.7.59	8.7.59	890	905			865	905	855	940	880	885	

2. Patients soumis à une irradiation simultanée par deux sources de Co–60 opposées

Patient	Date et dose d'irradiation par séquence		Dose tissulaire instantanée (rad/min)	Temps total d'exposition	Dose tissulaire totale abdomen compris (rads)*	Etalement (h)	Remarques
	1ère	2ème					
P.M.	4.6.62 350 r	5.6.62 400 r	5,2	152′	750	25	A reçu le 29.5.62, 50 r *in toto*
N.P.	16.10.62 400r	17.10.62 400r	5,8	138′	800	25	
B.F.	4.2.63 400r	5.2.63 400r	5,2	154′	800	26	
B.B.	17.4.63 400r	18.4.63 400r	4,76	168′	800	24	
J.L.C.	4.12.63 400r	5.12.63 400r	4,56	175′	800	35	
D.F.	6.2.64 400r	7.2.64 400r	4.43	180′	800	25	
P.P.	6.2.64 400r	7.2.64 400r	4.48	178′	800	22	
J.P.C.	27.4.64 413r	28.4.64 380r	4,20	188′	793	24	

*Pour la plupart des malades, les écarts de doses d'une région à l'autre sont de l'ordre de ±2%. Ils restent dans tous les cas au-dessous de 5%. Seules les extrémités des membres inférieurs reçoivent des doses plus élevées (environ 10%), en raison de leur faible épaisseur.

Tableau 2. Les mesures du traitement symptomatique et leurs indications pendant l'aplasie induite par l'irradiation et le syndrome secondaire.

Indications	Mesures
Anémie (nombre d'hématies $<2\cdot10^6$)	Transfusions de sang
Thrombocytopénie (nombre de plaquettes $<10^5$)	a. Acide ε-amino-caproïque b. Transfusions de thrombocytes c. Δ-1-cortisone si les traitements ci-dessus sont inefficaces.
Leucopénie	
a. Température normale	a. Maintien du malade en appartement aseptique (irradiation U.V., air filtré et pressuré; aliments et objets stérilisés . . .). b. γ-globulines. c. Nystatine. d. Antibiotique antibactérien si un germe pathogène est décelé.
b. Avec fièvre	
1. Culture positive avec signes d'infection	Antibiotique (selon l'antibiogramme).
2. Culture négative avec ou sans signes d'infection	Antibiotiques à large spectre (sauf chloramphénicol). Nystatine γ-globulines Traitement symptomatique de la fièvre

B. *Transfusions de leucocytes*

1. *Transfusion à une radio-chimère hématopoïétique de leucocytes du donneur dont il est tolérant* — Un sujet atteint de leucémie aiguë lymphoblastique et qui reçut, après une irradiation totale, une transfusion de moelle osseuse de six donneurs, devint tolérant à celle de l'un d'entre eux. Six mois après l'irradiation, dans le but d'accentuer la réaction du greffon contre l'hôte (afin d'augmenter son éventuel effet anti-leucémique), le patient reçut en l'espace de 3 semaines, quatre injections de leucocytes prélevés dans le sang périphérique du donneur auquel il était tolérant (au total: $1{,}63.10^8$ leucocytes).

2. *Transfusion de leucocytes de patients atteints de leucémie myéloïde chronique à des sujets atteints de leucémie ou d'hématosarcome compliqués d'aplasie myélo-lymphoïde* — Trente malades atteints de leucémie ou d'hématosarcome compliqués d'asplasie myélo-lymphoïde ont été l'objet de transfusions de globules blancs prélevés dans le sang périphérique de patients porteurs de leucémie myéloïde chronique: dans six cas, ces transfusions ont été suivies de l'apparition de complications rappelant étroitement les manifestations du syndrome secondaire qui complique la greffe de moelle osseuse allogénique. Les conditions de ces transfusions sont résumées au Tableau 3.

II. OBSERVATIONS

A. *Le syndrome secondaire après greffe de moelle osseuse*

1. *Résumés d'observations antérieurement publiées* — Cinq observations ne seront que brièvement résumées au Tableau 4, puisqu'elles ont fait l'objet de publications antérieures (Mathé *et al.* [1–6]) auxquelles nous renvoyons pour les détails.

2. *Observations inédites* — Nous donnons ici le résumé de cinq observations concernant des essais de greffe de moelle osseuse allogénique au cours desquels sont apparus des signes quelconques de syndrome secondaire; deux observations qui concernent les échecs de la greffe, au cours desquels le patient n'a pas présenté la moindre manifestation de syndrome secondaire, seront aussi rapportées: a) l'une d'elles (N.P.), car la moelle osseuse administrée dans son cas a été conservée pendant 2h à 37°, un travail expérimental (Mathé *et al.* [26], Amiel et Mathé [27]) nous ayant révélé que ce traitement des cellules réduit la fréquence du syndrome secondaire; b) l'autre concerne un échec de la greffe et s'ajoute à titre de témoin à nos cas F.B. (Mathé *et al.* [2]) et P.F. (Mathé *et al.* [3]) antérieurement publiés.

a) P.M. 28 ans, étudiant en droit, était atteint de leucémie aiguë lymphoblastique depuis juillet 1961. La première phase évolutive avait été traitée par la Δ-1-cortisone (60 mg par jour) associée à la mercapto-6-purine (150 mg par jour). Une rémission apparemment complète avait été obtenue, pendant la durée de laquelle le patient avait reçu 50 mg de mercapto-6-purine par jour. En avril 1962, l'examen de la moelle osseuse révélait la présence de 39% de lymphoblastes et de 5% de monoblastes. Une cure de leurocristine fut inefficace. Le 15 mai, le nombre des lymphoblastes dans la moelle osseuse était de 90%.

Sur les instances du malade, un essai de greffe de moelle osseuse allogénique après irradiation totale était décidée. Après une première irradiation totale de 50 rads, le 30 mai 1962, l'irradiation totale de 750 rads fut effectuée en deux séances les 4 et 5 juin. Le jour suivant l'irradiation, le malade reçut une transfusion de moelle osseuse prélevée sur sa femme, dont le phénotype érythrocytaire et les antigènes leucocytaires s'étaient révélés très proches (Tableau 5): 425 cm^3, soit 8.10^9 cellules nuclées. Le 10 juin, 4 jours après la transfusion, la température s'éleva à 39°, en même temps qu'apparaissaient des hémorragies: gingivorragies, pétéchies. Une seconde transfusion de moelle osseuse du

Tableau 3. *Conditions des transfusions de leucocytes de donneurs porteurs de leucémie myéloide chronique à des patients atteints de leucémie ou d'hématosarcome, compliqués de syndrome secondaire.*

Receveurs	Affections	Caractères de l'aplasie	Causes de l'aplasie	Nombre de transfusions et nombre de leucocytes injectés	Recherches de la prise de la greffe et résultats
J.B.	Maladie de Hodgkin	Leucopénie Thrombocytopénie	Chimiothérapie: Méthylhydrazine	Neuf transfusions de leucocytes frais: du 16 au 27.3.64 ($3{,}7 \cdot 10^{12}$)	Disparition du syndrome infectieux
G.M.	Leucémie aiguë lymphoblastique	Leucopénie Thrombocytopénie	Chimiothérapie: Leurocristine	Sept transfusions de leucocytes frais: du 8 au 18.6.64 ($1{,}24 \cdot 10^{12}$)	Appendices nucléaires des granulocytes (persistance de polynucléaires type donneur au 19e jour après la dernière transfusion). Disparition du syndrome infectieux. Rémission complète de la leucémie.
C.A.	Leucémie aiguë lymphoblastique	Leucopénie Thrombocytopénie	Chimiothérapie: Cyclophosphamide	Six transfusions de leucocytes conservés: du 12 au 20.8.64 ($2{,}39 \cdot 10^{11}$)	Rémission complète de la leucémie
C.C.	Lymphoblastosarcome transformé en leucémie aiguë	Anémie Leucopénie Thrombocytopénie	Evolution terminale de l'affection causale	Trois transfusions de leucocytes conservés: les 8, 9, 10.9.64 ($1{,}05 \cdot 10^{11}$)	Décès quatre jours après la dernière transfusion de leucocytes
P.T.	Leucémie aiguë lymphoblastique	Anémie Leucopénie Thrombocytopénie	Phase évolutive de l'affection	Six transfusions de leucocytes conservés: du 27·9 au 2.10.64 ($1{,}30 \cdot 10^{11}$)	Rémission complète de la leucémie
M.J.	Maladie de Hodgkin	Anémie Leucopénie Thrombocytopénie	Chimiothérapie: méthylhydrazine, vinca leucoblastine	Cinq transfusions de leucocytes frais du 15 au 20.1.65 ($7{,}99 \cdot 10^{11}$)	Présence du chromosome Ph^1 dans toutes les mitoses des cellules de la moelle osseuse.

Tableau 4. Résumé des observations de syndromes secondaire compliquant la greffe de moëlle osseuse antérieurement publiées.

Nom	Preuves de la prise de la greffe de moelle	Début du syndrome secondaire	Manifestations du syndrome secondaire	
			Cliniques	Hématologiques
D.V.	Erythrocytes (M)	45e jour après la greffe	Fièvre; dermite staphylococcique; asthénie; anorexie, nausées, vomissements, diarrhée; amaigrissement, fonte musculaire; adénopathie transitoire.	Lymphocytopénie (Fig. 1)
V.L.	Erythrocytes ($\bar{c}$)	33e jour après la greffe	Fièvre; dermite streptococcique; toux; asthénie; anorexie; nausées, vomissements, diarrhée; amaigrissement; adénopathie transitoire.	Lymphocytopénie
E.D.	Appendices nucléaires des granulocytes liés au sexe	5e jour après la greffe	Erythrodermie desquamative et bulleuse; fièvre, coryza, toux (sans signes pulmonaires); stomatite à muguet; torpeur, tuphos; anorexie totale, nausées, vomissements, diarrhée profuse; fonte musculaire considérable.	Lymphocytopénie
M.V.	Erythrocytes (E et $\bar{c}$)	5e jour après la greffe	Erythrodermie desquamative; fièvre; stomatite érythématopultacée; tremblement; anorexie, nausées, vomissements, diarrhée profuse.	Lymphocytopénie
B.B.	Erythrocytes: $\bar{c}$, S, s et Le^a; immuno-globulines: groupe sérique Inv; Chromosomes: absence de toute radiolésion; Greffe de peau: tolérance spécifique liée au chimérisme.	7e jour après la greffe	Erythrodermie desquamative laissant des séquelles de pigmentation (Figs. 3 et 4); fièvre; algies musculaires; anorexie, vomissements importants, diarrhée; amaigrissement (15 Kg); micropoly-adénopathie transitoire (8 jours); hépatomégalie; surinfection miliaire tuberculeuse.	Lymphocytopénie; présence de cellules hyperbasophiles dans la moelle; éosinophilie (jusqu'à 55% dans le sang) (Fig. 5).

Tableau 4 *(Suite)*

Manifestations du syndrome secondaire		Thérapeutique du syndrome secondaire	Durée du syndrome secondaire	Evolution
Histologiques	Biochimiques			
Aplasie lymphoïde (ganglion prélevé par biopsie).	Signes d'insuffisance digestive, sidération des fonctions iléo-coecales et coliques; hypergammaglobulinémie (jusqu'à 42 g/l); augmentation des transaminases (SGOT, SGPT) et des aldolases.	Antibiothérapie; mesures diététiques; ferments digestifs; perfusions de solutés d'acides aminés.	80 jours	Guérison
Aplasie lymphoïde (ganglion prélevé par biopsie).	Signes d'insuffisance digestive; hypogammaglobulinémie; augmentation du taux des aldolases et des transaminases.	Antibiothérapie; mesures diététiques; ferments digestifs; perfusion de solutés d'acides aminés; injection de gammaglobulines.	73 jours	Guérison
Autopsie: aplasie lymphoïde totale; peau: hyperkératose générale folliculaire; foie: infiltration graisseuse périportale; rate: aplasie lymphoïde totale; intestin: disparition des cryptes iléales et coliques.	Hypogammaglobulinémie (jusqu'à 3 g/l); hypo-albuminémie (jusqu'à 30 g/l); augmentation des α-2-globulines (jusqu'à 15 g/l).	Δ-1-cortisone; antibiothérapie; hormonothérapie mâle; alimentation parentérale.	26 jours	Aggravation par transfusion de sang du donneur de moelle. Décès.
Autopsie: aplasie lymphoïde totale; peau: hyperkératose générale et folliculaire, parakératose de l'épiderme; foie: nécrose et dissociation du parenchyme, cirrhose périportale; rate: aplasie totale des follicules de Malpighi; intestin: disparition des cryptes.	Hypogammaglobulinémie (jusqu'à 4 g/l); hypo-albuminémie (jusqu'à 29 g/l); augmentation des α-2-globulines (13,5 g/l); augmentation des aldolases et des transaminases; hyperbilirubinémie (60 mg 1).	Δ-1-cortisone; antibiothérapie; alimentation parentérale.	9 jours	Aggravation par transfusion de sang du donneur de moelle. Décès.
Ganglion cervical prélevé par biopsie: aplasie lymphocytaire (Fig. 6), prolifération de cellules hyperbasophiles à allure histiocytaire (Fig. 7); biopsie de peau: légère acanthose, dyskératose (Fig. 8), infiltration éosinophilique vacuolisation des cellules basales, infiltration dermique par des cellules lymphoïdes (Fig. 9).	Hypogammaglobulinémie (jusqu'à 6 g/l), diminution des β-2A et β-2M-globulines; augmentation des β-1A et α-2 macroglobulines; augmentation des transaminases (SGOT surtout), de l'ornithine-carbamyl-transferase, de l'aldolase, de la lactico-déshydrogénase; signes d'insuffisance digestive.	Δ-1-cortisone; acide ε-aminocaproïque; antibiothérapie; maintien prolongé en conditions aseptiques (80 jours).	10 semaines	Réactivation par transfusion de leucocytes du donneur compatible. Guérison. puis décès par encephalite zozterienne.

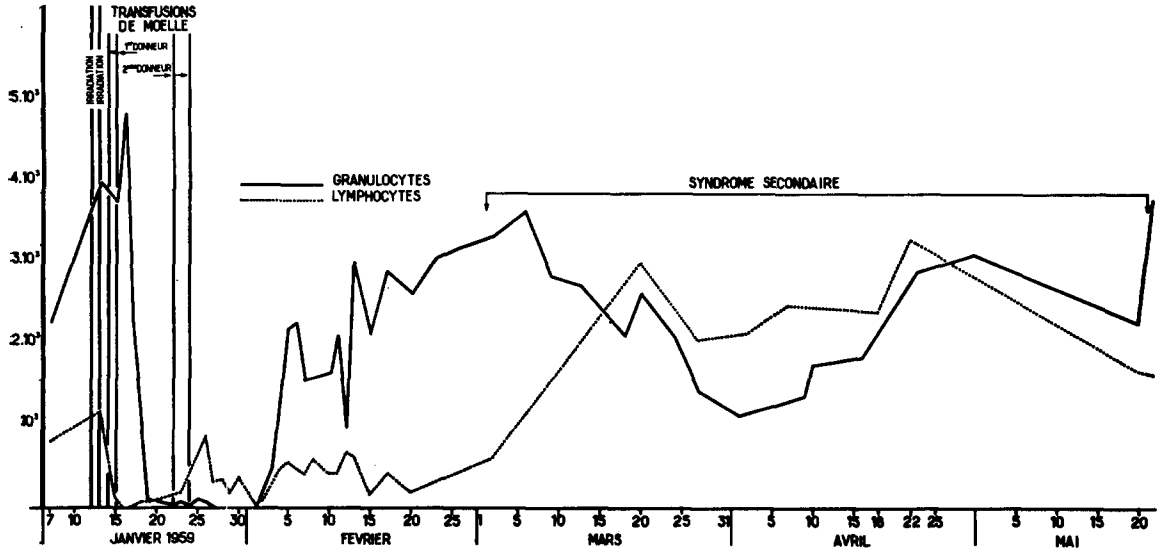


Fig. 1. *Le taux des lymphocytes comparé à celui des granulocytes dans le sang périphérique au cours des phases de radio-aplasie et du syndrome secondaire chez D.V.*

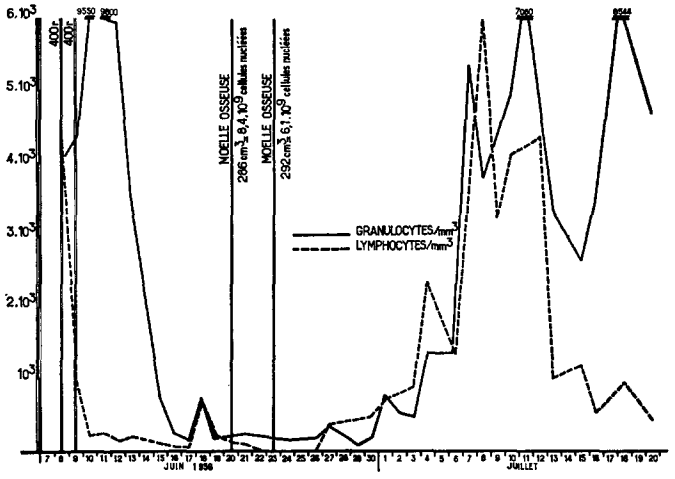


Fig. 2. *Le taux des lymphocytes comparé à celui des granulocytes dans le sang périphérique au cours des phases de radio-aplasie et du syndrome secondaire chez E.D.*

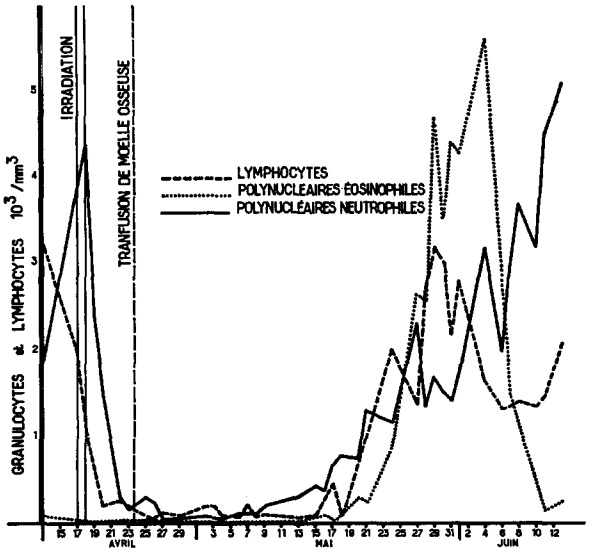


Fig. 5. *Le taux des éosinophiles comparé à celui des polynucléaires neutrophiles et des lymphocytes dans le sang périphérique au cours du syndrome secondaire chez B.B.*

Tableau 5.

1. *Phénotypes érythrocytaires de P.M. et de son donneur*

P.M.	A_2, M/S, P_1, CCDee, K−, Kp (a−), Le (a−b+), Fy (a−), Jk (a+)
Epouse	A_1, N/S, P_1, CCDee, K−, Kp (a−), Le (a−b−), Fy (a+), Jk (a−)

2. *Antigènes leucocytaires de P.M. et de son donneur*

	Identités		Différences	
P.M.	+	—	+	—
Epouse	+	—	—	+
	4	38	2	3
	42 fois identiques		5 fois différents	

même donneur fut effectuée une semaine plus tard, le 13 juin: 225 cm³ soit 4.10^9 cellules nucléées.

Cette deuxième injection fut suivie d'une éruption papuleuse qui devint vite prurigineuse, confluente, surtout marquée dans le dos, mais ne respectant aucun territoire cutané, et réalisant, le 16 juin, une véritable érythrodermie. En même temps, apparaissaient une anorexie rapidement totale, une diarrhée liquide importante, atteignant sept selles par jour, dans lesquelles les coprocultures répétées mettaient en évidence une flore banale et que n'influença aucune antibiothérapie. Ce syndrome secondaire, apparu le 11 juin, s'aggrava progessivement; des hémorragies gingivales, cutanées, digestives, avec melana et hématémèse, s'y associèrent. Enfin, la dégradation de l'état général fut rapide; le 16 juin, 10 jours après la première injection de moelle, alors que l'éruption cuivrée était généralisée, apparurent des troubles sphinctériens, une polypnée et un hoquet; le malade mourut le 19 juin au matin, malgré l'antibiothérapie massive, la correction des désordres hydroélectrolytiques, les transfusions de sang irradié, les corticoïdes à fortes doses.

L'autopsie révéla la cause immédiate du décès: la rupture d'une varice oesophagienne et de multiples plaques d'infarcissement au niveau des poumons. La moelle osseuse montrait un début de régénération, surtout nette pour l'érythropoïèse. L'aplasie lymphoïde était généralisée: on n'observait aucun ganglion dans les régions cervicale, médiastinale, abdominale, pelvienne. Dans deux petits ganglions para-aortiques, l'aplasie du tissu lympoïde était quasi-totale; on ne notait que des cellules réticulaires et histocytaires. Cette aplasie lymphoïde se retrouvait dans la rate, qui était le siège d'une sclérose et d'une congestion de la pulpe, dans l'iléon d'où tout follicule lymphoïde avait disparu, dans le rectum, d'où la sous-muqueuse n'était plus faite que de tissu conjonctif sans aucun élément figuré. Le foie (1500 g) était le siège d'une stéatose diffuse, mais surtout centro-lobulaire, avec quelques petits foyers de nécrose du parenchyme. La peau n'a pu être prélevée.

(b) N.P., 6 ans et demi, était atteinte de leucémie aiguë lymphoblastique depuis décembre 1961. Un traitement par la Δ-1-cortisone (40 puis 100 mg par jour) avait permis d'obtenir une rémission complète pendant laquelle l'enfant était demeurée sans traitement. Elle avait reçu au début de sa maladie cinq transfusions de sang, soit 625 cm³. En octobre 1962, s'amorça une nouvelle phase évolutive: l'hémogramme était normal (3.775.000 hématies par mm³, 5700 leucocytes par mm³ dont 3200 polynucléaires, 240.000 thrombocytes par mm³), mais la moelle osseuse contenait 14% de lymphoblastes, et l'on notait à l'examen clinique une adénopathie inguinale bilatérale.

L'irradiation totale eut lieu les 16 et 17 octobre 1962: 400 rads furent administrés en 100 min le premier jour, en 90 min le second. La première séance fut bien tolérée; la deuxième fut suivie de quelques vomissements. Les jours qui suivirent, l'état général fut satisfaisant, en dehors d'une tachycardie variant de 90 à 120. Le 23 octobre, 8 jours après l'irradiation, la moelle était aplasique et le sang périphérique contenait par mm³: $3 \cdot 10^6$ erythrocytes, 780 leucocytes, dont 400 polynucléaires, et 150.000 thrombocytes; les réticulocytes avaient disparu. On injecta à la malade, par voie veineuse, la moelle osseuse de cinq donneurs du sexe masculin, dont les phénotypes érythrocytaires sont indiqués au Tableau 6. Cette moelle osseuse fut préalablement conservée pendant 2 h à 37°. On transfusa 1360 cm³ soit $28 \cdot 10^9$ cellules nuclées, et l'on retira 1200 cm³ de sang par saignée.

Les jours suivants, l'enfant présenta une fièvre presque permante à 40°, une tachycardie à 120, un muguet buccal, des selles diarrhéiques assez abondantes et quelques signes hémorragiques (épistaxis, pétéchies sur le thorax, ecchymoses gingivales), contre lesquels on lutta par des transfusions de plaquettes quotidiennes. Le 29 octobre, 14 jours après l'irradiation, 7 jours après la transfusion de la moelle, il n'existait dans le sang aucun signe de restauration; 1.880.000 hématies par mm³; 90 leucocytes et 28.000 thrombocytes par mm³; on ne comptait aucun réticulocyte sur 10.000 éléments. Mais un myélogramme révéla que, si la moelle osseuse était pauvre, elle n'était pas aplasique: 19% d'éléments granulocytaires (myéloblastes, promyélocytes), 8% d'érythroblastes: quelques rares mégacaryoblastes. Les jours suivants, la température s'abaissait, oscillaint autour de 38°; la diarrhée s'atténuait, les signes hémorragiques disparaissaient, l'anémie se réparait sous l'effet de transfusions de sang; mais le muguet se généralisa aux muqueuses buccales et nasales: on le traita par l'amphotéricine en application lacales.

Le 5 novembre, au quatorzième jour après l'injection de moelle, l'état général s'altérait à nouveau: la température s'élevait à 40°, l'asthénie était intense et l'enfant s'alimentait difficilement. La pancytopénie périphérique persistait et la moelle était à nouveau aplasique. Devant l'aggravation de l'état clinique et hématologique, on fut conduit à transfuser la moelle de

Tableau 6.

Phénotypes érythrocytaires de P.P. et de ses donneurs.

N.P. ♀	O, MN/ss, P_2, ccDEe, K−, Le (a−b+), Fy (a+), Jk (a+)
A.G. ♂	O, N/ss, P_1, CcDEe, K−, Le (a−b−), Fy (a−), Jk (a+)
P.C. ♂	O, MN/S, P_2, CcDee, K−, Le (a+b−), Fy (a+), Jk (a+)
P.E. ♂	O, M/S, P_2, CcDEe, K−, Le (a−b+), Fy (a+), Jk (a−)
R.P. ♂ (père)	O, N/S, P_2, CcDEe, K−, Le (a−b+), Fy (a+), Jk (a+)
M.P. ♀ (soeur)	O, MN/ss, P_2, ccDEe, K−, Le (a−b+), Fy (a+), Jk (a+)

la soeur jumelle monozygote. L'étude des phénotypes érythrocytaires et salivaires et celle des groupes sériques confirmèrent qu'il s'agissait de vraies jumelles; l'étude des antigènes leucocytaires (Dr. J. Dausset) seule fut en discordance, révélant quarante-quatre identités et trois différences. Le 7 novembre, 16 jours après la première transfusion de moelle allogénique, on injecta à l'enfant 282 cm^3 de moelle, soit $10 \cdot 10^9$ cellules nucléées. Les jours qui suivirent furent difficiles. La température ne dépassa pas 39°, mais l'enfant dormait mal; le muguet buccal s'accompagnait d'une hypersialorrhée et d'une dysphagie intenses; la souffrance rendait l'alimentation impossible. On lui administra 25 mg de Δ-1-cortisone par jour. Le 12 novembre, 5 jours après l'injection de moelle isogénique, on put observer sur le myélogramme quelques cellules souches (promyélocytes et érythrocytes), traduisant le début de la restauration myéloïde. Le 17 novembre, la température était à 37°2. Le 20 novembre, commença une crise réticulocytaire: 35.000 par mm^3, 10.500 le 22 novembre, 340.000 le 24 novembre. Le 30 novembre, hémogramme et myélogramme étaient normaux. L'enfant sortait de la chambre stérile le 3 décembre, 48 jours après y être entrée, en rémission hématologique complète. Elle présenta pendant quelques jours une fièvre à 38° sans aucune explication clinique.

Des greffes de peau des différents donneurs furent effectuées: au dix-neuvième jour, toutes les greffes de peau des donneurs allogéniques étaient éliminées (celles de deux donneurs, dont le père de l'enfant, attendront le dix-septième jour pour être nécrosées): la greffe de peau de sa soeur jumelle était en parfait état et le demeura. Les leucocytes (polynucléaires et mononucléaires) étaient du sexe féminin.

L'état hématologique demeura normal jusqu'au 30 avril; à cette date, le myélogramme révéla la présence de vingt-six lymphoblastes pour 100 éléments. Le 28 mai, la moelle osseuse était envahie de lymphoblastes: on était au septième mois après l'irradiation. L'enfant mourait le 9 juillet 1963, 9 mois après l'irradiation.

(c) B.F., 5 ans était atteinte de leucémie aiguë lymphoblastique depuis juin 1962. Un premier traitement par la Δ-1-cortisone (50 mg par jour), poursuivi pendant 2 mois, n'entraîna pas de rémission. Durant cette période, la malade avait reçu trois transfusions de sang. Un traitement par la leurocristine (1 mg puis 2 mg par semaine) fut entrepris en août 1962: il comporta en tout dix injections; le 14 novembre, la malade était en rémission apparemment complète; un traitement d'entretien comporta l'administration de mercapto-6-purine (50 mg par jour).

L'enfant avait une soeur jumelle dizygote; leurs groupes érythrocytaires et leucocytaire sont indiqués au Tableau 7. Le 8 janvier 1963, B.F. fut hospitalisée pour un essai de greffe de moelle osseuse de cette soeur. Avant l'irradiation, l'hémogramme donna les résultats suivants; érythrocytes, 2.650.000 par mm^3, dont 10.000 réticulocytes; leucoytes; 3680 par mm^3, dont 52% de polynucléaires; thrombocytes, 350 000 par mm^3. La moelle osseuse était pauvre mais ne contenait pas de formes anormales. L'irradiation fut effectuée en deux séances, les 4 et 5 février 1963, à l'aide de deux sources de Cobalt-60. Le 12 février, l'hémogramme était le suivant: érythrocytes: 1.950.000 par mm^3; absence totale de réticulocytes: leucocytes: 170 par mm^3, dont 60 polynucléaires: lymphocytes: 110 par mm^3; thrombocytes, 150 000 par mm^3. La moelle osseuse était aplasique. Le 13 février, l'enfant reçut une transfusion de moelle

Tableau 7.

1. *Phénotypes érythrocytaires de B.F. et de son donneur*

B.F.	O, N/Ss, P_1, CcDee, K−, Kp^a−, Le (a+b−), Fy (a+), Jk (a+)
Soeur jumelle dizygote	O, MN/ss, P_2, CcDee, K−, Kp^a−, Le (a−b−), Fy (a−), Jk (a+)

2. *Antigènes leucocytaires de B.F. et de son donneur*

	Identités		Différences	
B.F.	+	−	+	−
Soeur jumelle dizygote	+	−	−	+
	3	36	2	5
	39 fois identiques		7 fois différents	

osseuse prélevée chez sa soeur jumelle (476 cm³ soit $1{,}1 \cdot 10^{10}$ cellules nuclées). L'injection fut couplée avec une exanguino-transfusion de 400 cm³. Elle fut bien tolérée. La cytopénie se compléta; le taux des thrombocytes était à de 48.000 par mm³ le 15 février; à partir de cette date, l'enfant reçut chaque jour une transfusion de plaquettes.

L'état demeura stationnaire jusqu'au 17 février, date à laquelle une aggravation brusque se produisit; la température monta à 39°, tandis qu'une éruption érythémateuse, violacée par plaques, et des troubles digestifs (diarrhée, anorexie absolue, vomissements) apparaissaient. L'examen ne décelait qu'une candidose buccale: les coprocultures en révélaient qu'une flore banale; les hémocultures étaient négatives; il n'y avait pas de germes dans les urines. Malgré un traitement symptomatique intensif (correction des désordres hydro-électrolytiques, antibiotiques), l'état général s'aggrava rapidement; le 18 février, l'enfant tomba dans un tuphos profond. Le 19, la température était à 41°; l'éruption s'intensifia et devint purpurique. L'aplasie périphérique était toujours totale: érythrocytes: 2.800.000 par mm³; leucocytes, 260 par mm³ dont 120 polynucléaires; thrombocytes, 8000. Cependant, une ponction osseuse, faite le même jour, soit 15 jours après l'irradiation et 6 jours après la transfusion de moelle, montrait un début de restauration myéloïde et de nombreuses cellules hyperbasophiles: cellules réticulaires, 4%; série granulocytaire, 20,5% (myéloblastes, 1,5; promyélocytes neutrophiles, 3; myélocytes neutrophiles,5,5; métamyélocytes neutrophiles, 2,5; polynucléaires éosinophiles, 1,5); série érythroblastique, 6% (érythroblastes basophiles, 0,5; érythroblastes polychromatiques, 2,5; érythroblastes acidophiles, 3); série non myéloïde, 73,5% dont: plasmocytes, 3,5; cellules hyperbasophiles 28,5%. Le 20 février, l'enfant mourait dans un brusque collapsus cardio-vasculaire. L'autopsie montra un début de régénération myéloïde, des poumons oedématiés, des hémorragies pétéchiales de la muqueuse intestinale. La peau présentait une éruption généralisée, avec hyperkératose folliculaire, dyskératose généralisée, par endroit, des cellules basales vacuolisées, une infiltration et de l'épiderme par des lymphocytes. Il existait dans le foie des foyers isolés de nécrose cellulaire (Fig. 10). Dans le colon et l'intestin grêle, on notait la désintégration des cryptes, celles-ci étant par plages, complètement disparu (Fig. 11) on note encore une infiltration diffuse de la muqueuse par des lymphocytes (Fig. 12).,

(d) P.P., 7 ans, était atteint de leucémie aiguë lymphoblastique depuis juin 1963. Un traitement associant la Δ-1-cortisone (40 mg par jour) et la mercapto-6-purine (50 mg) n'avait pas permis d'obtenir une rémission lorsque l'enfant, qui habitait la Réunion, arriva à Paris en Décembre 1963. Le sang péripherique contenait 280 lymphoblastes par mm³; l'examen clinique montrait des adénopathies inguinales et cervicales bilatérales, une hépatomégalie, une splénomégalie, et l'examen radiologique du médiastin montrait une volumineuse opacité polycyclique latéro-trachéale droite. Pendant un mois, l'enfant reçut 100 mg de Δ-1-cortisone par jour, jusqu'au 16 janvier 1964, et, devant l'absence d'efficacité notable de celle-ci, de la leurocristine pendant 15 jours (2 mg par semaine). Le 2 février, il n'existait aucune amélioration clinique; les adénopathies et la rate avaient nettement augmenté de volume. A noter que le malade n'avait jamais reçu de transfusion de sang.

L'irradiation fut précédée par l'administration, pendant 4 jours, de méthyl-nitro-imidazolyl-mercaptopurine (150 mg). Elle eut lieu les 6 et 7 février 1964; 800 rads furent administrés à l'aide de deux sources de Cobalt 60. Le 14 février, 8 jours après l'irradiation, la moelle était désertique, et le sang périphérique contenait 2.800.000 hématies, 285 leucocytes, dont 75 polynucléaires par mm³. On injecta 2000 cm³ de moelle osseuse contenant $50 \cdot 10^9$ cellules nucléées, provenant de six donneurs différents, sans aucun lien de parenté avec l'enfant, dont les phénotypes érythrocytaires sont indiqués au Tableau 8; 1700 cm³ de sang furent soustraits; on administra le même jour 150 mg de Δ-1-cortisone. Les deux jours suivants, l'enfant était fatigué, mais apyrétique. Le 17 février, la température remonta à 39°. Le 19 février, apparut une angine nécrotique. L'enfant était anorexique, fébrile (39°), mais l'état clinique demeurait satisfaisant; le foie n'était plus perceptible, mais la rate débordait les fausses côtes de 5 cm. Le 24 février, 15 jours après l'irradiation, 7 jours après l'injection de moelle, apparurent une polypnée à 38/min, une hyperthermie à 40°2, une toux avec crachats, signes d'une brocho-pneumonie. Une hémoculture montra la présence de staphylocoques sensibles à la kanamycine et à la novobiocine. La moelle était très pauvre ne contenant que huit granulocytes pour 100 éléments. La semaine qui suivit, du 24 février au 1er mars, le traitement associa oxygénothérapie, antibiothérapie intensive et corticothérapie. L'enfant présenta quelques pétéchies, malgré des transfusions quotidiennes de thrombocytes et l'administration d'acide ε-aminocaproïque. Le 1er mars, une crise d'oedème pulmonaire aigu (avec hypertension à 18–11 et râles dans les deux champs pulmonaires) put être jugulée. Le 2 mars apparut une hépatomégalie atteignant l'ombilic. Le sérum prit une teinte verdâtre, les urines ne montrant ni pigments, ni sels biliaires. Le 3 mars, 24 jours après l'irradiation, 17 jours après l'injection de moelle, l'enfant mourut après une crise convulsive, dans un tableau hémorragique (melaena, épistaxis, purpura), alors que le myélogramme, pauvre, montrait neuf granulocytes pour 100.

La vérification anatomique révéle des foyers confluents de broncho-pneumonie avec lésions oedémateuses, un oedème cérébral, des lésions de stéatose hépatique, et, dans le médiastin, une volumineuse masse ganglionnaire tumorale. L'autopsie confirma l'oedème pulmonaire, accompagnant des embolies de graisse. L'infiltration de la moelle osseuse, de la rate, des ganglions mésentériques et des espaces portes par des cellules immatures, avec de nombreuses mitoses, témoignait d'une reprise du processus leucémique. La moelle osseuse présentait un début de régénération avec quelques éléments myéloïdes. La peau se caractérisait par une atrophie de l'épiderme, une hyperkératose folliculaire, une légére dyskératose, une infiltration lympho-histiocytaire de l'épiderme; le colon, par une dégénérescence des cryptes intestinales, légère, mais diffuse; le foie, par une infiltration graisseuse et la dégénérescence des cellules hépatiques.

(e) D.F., 11 ans, était atteinte de leucémie aiguë lymphoblastique depuis octobre 1963. Un traitement par la Δ-1-cortisone (60 mg par jour) entraina une rémission complète en janvier 1964; au cours de cette période, des douleurs extrêmement violentes de la colonne dorso-lombaire avaient immobilisé l'enfant. La malade n'avait jamais reçu de transfusions de sang.

L'irradiation (800 rads) eut lieu les 6 et 7 février 1964, précédée par l'administration, pendant 4 jours, de méthyl-nitro-imidazolyl-mercaptopurine (150 mg par jour). Les jours qui suivirent, l'état du malade était

Tableau 8.

1. *Phénotypes érythrocytaires de P.P. et de ses donneurs*

P.P. ♂	A_1, N/ss, P_1, CcDee, K−, Le (a−b−), Fy (a+), Jk (a−), Lu (a+)
S.C. ♀	A_1, M/ss, P_1, ccDEe, K+, Le (a+b−), Fy (a−), Jk (a+), Lu (a−)
M.G. ♀	A_1, N/S, P_1, CcDee, K−, Le (a−b−), Fy (a+), Jk (a−), Lu (a−)
F.M. ♂	A_1, Mn/ss, P_1, CcDee, K−, Le (a+b−), Fy (a+), Jk (a+), Lu (a−)
S.G. ♀	A_1 N/S, P_1, CcDee, K−, Le (a+b−), Fy (a−) Jk (a−), Lu (a+)
M.R. ♂	A_1, MN/ss, P_1, CcDee, K−, Le (a−b−), Fy (a+), Jk (a−), Lu (a−)
F.M. ♀	A_1, N/S, P_1, ccDEe, K−, Le (a−b−), Fy (a−), Jk (a−), Lu (a−)

2. *Antigènes leucocytaires de P.P. et de ses donneurs*

(N'ont pu être établies que les identités et les différences entre trois donneurs et le receveur).

	Identités		Différences	
P.P.	+	−	+	−
M.G.	+	−	−	+
	7	6	21	0
	13 fois identiques		21 fois différents	
P.P.	+	−	+	−
S.C.	+	−	−	+
	1	6	27	0
	7 fois identiques		27 fois différents	
P.P.	+	−	+	−
F.M.	+	−	−	+
	13	5	15	1
	18 fois identiques		16 fois différents	

bon, mais une angine érythémato-pultacée entraîna la prescription d'antibiotiques le 10 février, alors que le sang montrait 420 leucocytes, dont 240 polynucléaires par mm^3, 3.250.000 érythrocytes et 250.000 thrombocytes par mm^3. Le 12 février, 5 jours après l'irradiation, on transfusa la moelle osseuse de six donneurs n'ayant aucun lien de parenté avec la malade, dont les phénotypes érthrocytaires et leucocytaires sont indiqués au Tableau 9: 2000 cm^9, soit $57 \cdot 10^9$ cellules nuclées; l'injection fut couplée avec une exsanguino-transfusion de 1700 cm^3 de sang; en fin d'opération, la température était à 37°8. On administra, au moment de l'injection de moelle, 200 mg de Δ-1-cortisone. Dès le lendemain, la fièvre oscilla entre 40° et 38°, entraînant la prescription d'antibiotiques.

Trois jours plus tard, l'etat général s'améliora un peu, mais quelques troubles digestifs apparurent, selles diarrhéiques, vomissements bilieux, tandis que la fièvre persistait; on trouva du citrobacter dans les urines et les fècès. Le 20 février, 8 jours après la transfusion de la moelle, l'enfant allait mieux: température à 37°; mais apparaissait dans la gorge un muguet. Les jours qui suirvirent, l'enfant vomit fréquemment; on pouvait noter une desquamation au niveau des bras, des jambes, de la partie basse de l'abdomen; elle demeurait fébrile. Une hémoculture montra la présence dans le sang de staphylocoques sensibles à la flabelline et à la novobiocine.

Le 24 février, 17 jours après l'irradiation, 12 jours après la transfusion de cellules médullaires, la moelle, 'pauvre' contenait 11% de granulocytes neutrophiles, et 12% d'érythroblastes, le 26 février, le sang conterait $2 \cdot 10^6$ érythrocytes par mm^3, 470 leucocytes dont 80 polynucléaires et 78 000 thrombocytes (on transfusait quotidiennement des suspensions de thrombocytes). Une crise de tachycardie avec hépatomégalie, suivie le 27 février d'un collapsus avec une hypotension à 7, entraîna la prescription de corticoïdes; la tension remonta à 11. Le myélogramme était un peu moins pauvre et montrait 32% de granulocytes. Le 1er mars, dans la nuit, l'enfant présentait une crise d'oedème aigu du poumon, alors que la température était à 37° depuis la veille, et mourait.

La vérification anatomique montra des signes caractéristiques d'oedème aigu du poumon; des signes de début de régénération myéloïde: quelques éléments myéloïdes et érythroblastiques, peu de mégacaryocytes. La peau présentait essentiellement une hyperkératose folliculaire de l'épiderme; le foie une stase et une stéatose des zones péricentrolobulaires, une nette hyperplasie des cellules de Kupffer. L'atrophie lymphoïde était totale, aussi bien

Tableau 9.

1. *Phénotypes érythrocytaires de D.F. et de ses donneurs*

D.F.	♀	B, MN/S− s− U−, P_1, ccDEe, K−, Le (a−b−), Fy (a−), Jk (a−)
G. de P.	♀	B, MN/S, P_1, CcDEe, K−, Le (a−b+), Fy (a+), Jk (a−)
J.L.	♂	B, MN/S, P_1, CcDee, K−, Le (a+b−), Fy (a+), Jk (a−)
G.P.	♀	B, MN/ss, P_1, CcDee, K−, Le (a−b+), Fy (a+), Jk (a−)
F.H.	♀	B, N/S, P_1, ccDEe, K−, Le (a+b−), Fy (a+), Jk (a−)
C.L.	♀	B, MN/ss, P_1, CcDee, K+, Le (a−b−), Fy (a−), Jk (a−)
L.Q.	♀	B, MN/S, P_1, CcDEe, K− Le (a−b+), Fy (a−), Jk (a+)

2. *Antigènes leucocytaires de D.F. et de ses donneurs*

	Identités		Différences			Identités		Différences	
D.F.	+	−	+	−	D.F.	+	−	+	−
G. de P.	+	−	−	+	F.H.	+	−	−	+
	25	2	17	0		17	1	25	1
	27 fois identiques		17 fois différents			18 fois identiques		26 fois différents	
D.F.	+	−	+	−	D.F.	+	−	+	−
J.L.	+	−	−	+	C.L.	+	−	−	+
	16	0	26	2		19	1	23	1
	16 fois identiques		28 fois différents			20 fois identiques		24 fois différents	
D.F.	+	−	+	−	D.F.	+	−	+	−
G.P.	+	−	−	+	L.Q.	+	−	−	+
	27	1	15	1		23	1	19	1
	28 fois identiques		16 fois différents			24 fois identiques		20 fois différents	

3. *Phénotypes sériques de D.F. et de ses donneurs*

D.F.	Gm (a+b+x−e− like−), Inv (1+a+)
G. de P.	Gm (a+b−x+e− like−), Inv (1−a−)
J.L.	Gm (a+b+x+e+ like−), Inv (1−a−)
G.P.	Gm (a+b+x−e+ like−), Inv (1−a−)
F.M.	Gm (a−b+x−e+ like−), Inv (1−a−)
C.L.	Gm (a−b+x−e+ like−), Inv (1−a−)
L.Q.	Gm (a+b−x+e− like−), Inv (1−a−)

au niveau des ganglions que de la rate: les amygdales se réduisaient à des zônes lymphocytaires très pauvres, avec une sclérose récente, des cellules réticulaires et des histiocytes; la muqueuse du grêle était complètement atrophiée, et l'aplasie lymphoïde y était complète; hémorragies de la muqueuse colique; paroi rectale sans aucun follicule lymphoïde; ganglions périrectaux aplasiés; pulpe blanche splénique pratiquement disparue (une rate accessoire présentait les mêmes caractères).

(f) J.P.C., 4 ans, était atteint de leucémie aiguë lymphoblastique depuis octobre 1961. Un traitement par la Δ-1-cortisone et l'améthoptérine avait permis d'obtenir une rémission complète pendant laquelle l'enfant avait été traité par la mercapto-6-purine. Le patient avait reçu trois transfusions de sang.

Le 8 avril 1964, l'enfant était en phase évolutive, et des traitements successifs par la Δ-1-cortisone, l'améthoptérine, la mercapto-6-purine et la leurocristine étaient sans effet; le sang périphérique contenait 22.000 lymphoblastes par mm^3. Une cure de leurosine (50 mg par semaine) entraîna une amélioration transitoire, mais le 22 avril, le patient avait encore 2400 lymphoblastes par mm^3 de sang.

Après une prémédication de 4 jours par la méthyl-nitro-imidazolyl-mercaptopurine (150 mg/jour), l'enfant fut irradié les 27 et 28 avril, 400 rads chaque fois. Il était fébrile (39°4, 40°5) les soirs qui suivirent les irradiations, et tachycardique. Le lendemain 29 avril, alors que sa moelle contenait 52% de lymphoblastes et pas un seul granulocyte, on transfusa la moelle osseuse de 5 donneurs de sa famille: son père, son frère, sa soeur et deux cousins, dont les phénotypes érythrocytaires sont indiqués au Tableau 10: 1463 cm^3, soit $42{,}67 \cdot 10^9$ cellules nucléées; on administra, avant l'injection, 200 mg de Δ-1-cortisone. Une antibiothérapie intensive systématique fut administrée en perfusion veineuse (flabelline+colimycine). Dans la soirée, on injecta 0,5 mg d'améthopterine par kilo de poids; une administration de 0,2 mg par kilo sera répétée les troisième, cinquième, septième et neuvième jours après la transfusion de moelle.

Tableau 10.

1. *Phénotypes érythrocytaires de J.P.C. et de ses donneurs*

J.P.C.	♂		O, MN/ss, P_1, ccDEe, K−, Le (a−b−), Fy (a−), Jk (a+)
J.C.	♂	pere	O, MN/ss, P_1, CcDee, K−, Le (a−b+), Fy (a−)
M.C.	♀	soeur	O, N/ss, P_1, CcDee, K−, Le (a−b+), Fy (a−)
J.M.C.	♂	frère	O. N/ss, P_1, CcDEe, K−, Le (a−b+), Fy (a−)
R.C.	♂	cousin	O, M/S, P_1, CcDee, K−, Le (a+b−), Fy (a−)
A.C.	♀	cousine	O, M/S, P_1, CcDee, K−, Le (a+b−), Fy (a+)

2. *Phénotypes sériques de J.P.C. et de ses donneurs*

J.P.C.	Gm (a−b+x−e+), Inv (1+a+)
J.C.	Gm (a+b+x−e+), Inv (1+a+)
M.C.	Gm (a−b+x−e+), Inv (1−a−)
J.M.C.	Gm (a−b+x−e+), Inv (1−a−)
R.C.	Gm (a+b+x−e+), Inv (1−a−)
A.C.	Gm (a−b+x−e+), Inv (1−a−)

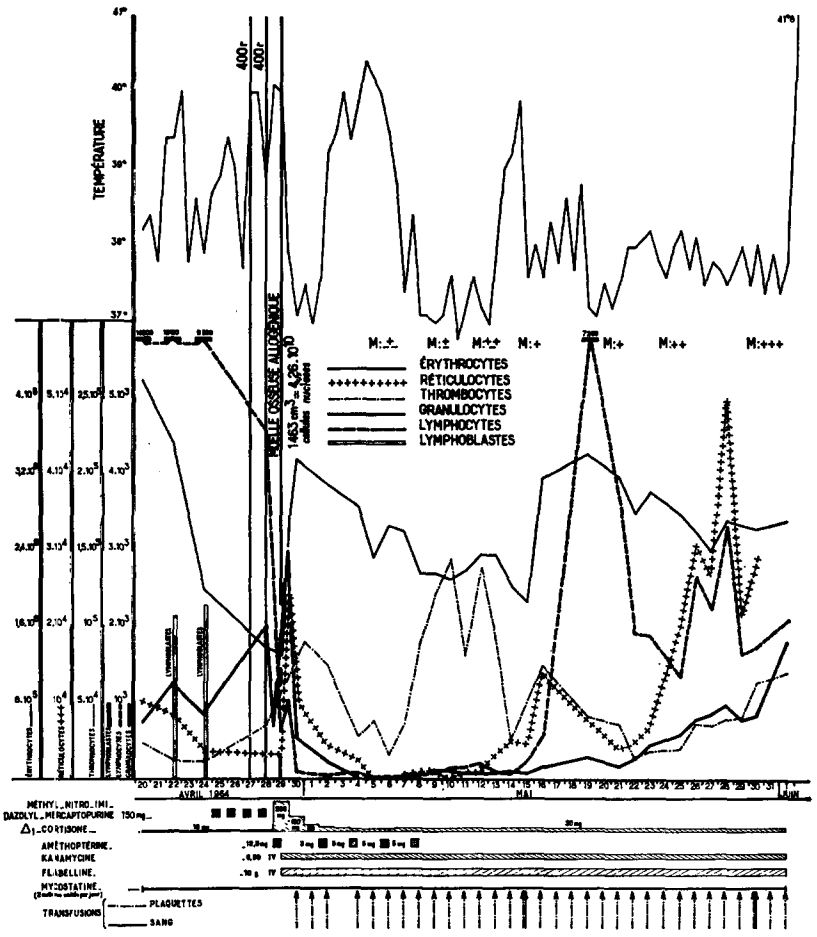


Fig. 13. *Evolution d'ensemble depuis l'irradiation jusqu'à la mort chez J.P.C. Signes hématologiques. Température. Traitements. Remarquer l'hyperlymphocytose transitoire.*

Les deux jours qui suivirent l'injection de moelle, le malade n'eut pas de fièvre; la bouche était propre. Le 5 mai, 6 jours après l'injection de moelle, l'hémogramme était le suivant: érythrocytes: 2.000.000 par mm³; leucocytes: 35 par mm³ dont 10 polynucléaires; aucun réticulocyte. Le myélogramme montrait 900 éléments nucléés par mm³, dont 20% de granulocytes. Ce même jour, la température remontait à 40°. L'administration de 30 mg par jour de Δ-1-cortisone fut poursuivie. Le 8 mai, la fièvre tombait à 37° et la moelle contenait 2100 eléments nucléés par mm³, dont 33% de granulocytes. L'enfant reçut ce jour la dernière injection d'améthoptérine, la dose totale s'élevant à 32,5 mg Les jours suivants, le patient retrouvait un bon état général, demeurait sans fièvre, s'alimentant; il était cependant tachycardique, anémique ($2 \cdot 10^6$ érythrocytes par mm³), leucopénique (225 leucocytes dont 75 polynucléaires par mm³), thrombocytopénique (80.000 plaquettes par mm³) (on lui injectait quotidiennement une suspension de thrombocytes). Le 14 mai, la température remontait à plus de 40°, l'anémie était à 1.800.000 érythrocytes par mm³; les polynucléaires apparaissaient dans le sang circulant le 16 mai: 150 par mm³.

La température redescendait le 17 mai, mais une éruption morbiliforme, évoquant un syndrome secondaire, apparaissait; des crises d'excitation et de délire conduisirent à interrompre l'administration d'acide ε-amino-caproïque. Le sérum était de teinte verdâtre. Le 19 mai, le myélogramme montrait 26% de granulocytes et l'enfant, en assez bon état clinique, s'alimentait; mais l'hémogramme révélait ce jour la présence de 7200 lymphocytes et 280 polynucléaires par mm³ (Fig. 13); le sérum avait retrouvé sa couleur normale. Le 23 mai, le taux de granulocytes continuait à s'élever dans la moelle: 41%; ils apparurent dans le sang circulant le 26 mai: 780 par mm³; le 27 mai: 845 par mm³. Ce même jour, apparurent des lésions cutanées sous forme de papules, se transformant le lendemain en vésicules typiques d'une éruption de varicelle; ces lésions n'étaient pas nécrotiques; on injecta le 30 mai et le premier juin, 6 cm³ de gammaglobulines; et le premier juin, bien que la température fût à 37°4, eut lieu une troisième éruption, varicelleuse: aucun signe méningé; le malade s'alimentait; l'hémogramme montrait 3900 leucocytes, dont 1800 granulocytes par mm³, 50.000 thrombocytes par mm³; le myélogramme était celui d'un individu normal.

Tableau 11.

1. *Phénotypes érythrocytaires de J.L.C. et de ses donneurs*

J.L.C.	♂	A_1, MN/Ss, P_1, CcDEe, K−, Le (a−b+), Fy (a+)
D.C.	♀ (mère)	A_1, MN/SS, P_1, ccDEE, K−, Le (a+b−), Fy (a−)
O.K.	♀	A_1, MN/SS, P_1, CcDEe, K−, Le (a−b+), Fy (a−)
L.M.	♀	A_1, MN/SS, P_1, CCDee, K+, Le (a+b−), Fy (a+)
M.T.L.	♀	A_1, MN/ss, P_1, CCDee, K−, Le (a+b−), Fy (a+)
A.T.	♀	A_1, MN/ss, P_1, CcDee, K−, Le (a−b+), Fy (a+)
G.D.	♀	A_1, MN/ss, P_1, CcDEe, K−, Le (a−b+); Fy (a−)

2. *Antigènes leucocytaires de J.L.C. et de ses donneurs*

	Identités		Différences			Identités		Différences	
J.L.C.	+	−	+	−	J.L.C.	+	−	+	−
D.C.	+	−	−	+	M.T.L.	+	−	−	+
	5	40	2	1		7	26	0	15
	45 fois identiques		3 fois différents			33 fois identiques		15 fois différents	
J.L.C.	+	−	+	−	J.L.C.	+	−	+	−
O.K.	+	−	−	+	A.T.	+	−	−	+
	3	38	4	2		6	24	1	17
	41 fois identiques		6 fois différents			30 fois identiques		18 fois différents	
J.L.C.	+	−	+	−	J.L.C.	+	−	+	−
L.M.	+	−	−	+	G.D.	+	−	−	+
	5	34	2	7		7	28	0	12
	39 fois identiques		9 fois différents			35 fois identiques		12 fois différents	

3. *Phénotypes sériques de J.L.C. et de ses donneurs*

J.L.C.	Gm (a−b+x−e+), Inv (1−a−)
D.C.	Gm (a−b+x−e+), Inv (1−a−)
O.K.	Gm (a−b+x−e+), Inv (1−a−)
L.M.	Gm (a−b+x−e+), Inv (1+a+)
M.T.L.	Gm (a−b+x−e+), Inv (1−a−)
A.T.	Gm (a−b+x−e+), Inv (1−a−)
G.D.	Gm (a−b+x−e+), Inv (1−a−)



Fig. 3. *Aspect macroscopique de la peau à la phase aiguë du syndrome secondaire chez B.B.*

Fig. 4. *Aspect macroscopique de la peau, avec l'importante desqaumation, au cours du syndrome secondaire, chez B.B.*

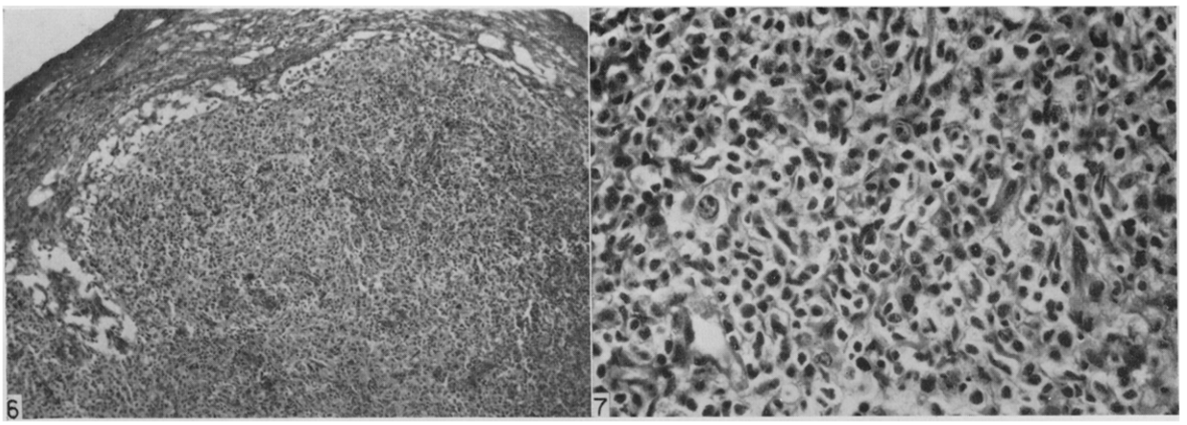


Fig. 6. *Ganglion lymphatique au cours du syndrome secondaire chez B.B. Atrophie du tissu lymphoïde. Remarquer l'absence de follicules et la rareté des lymphocytes.*
(*Hématoxyline—éosine—safran* × 120)

Fig. 7. *Ganglion lymphatique au cours du syndrome secondaire chez B.B. Importante diminution des cellules de type lymphoide. Infiltration par des cellules ressemblant à des histiocytes. Remarquer plusieurs mitoses.*
(*Hématoxyline—éosine—safran* × 120)

(to face p. 88)
Refer to p. 80

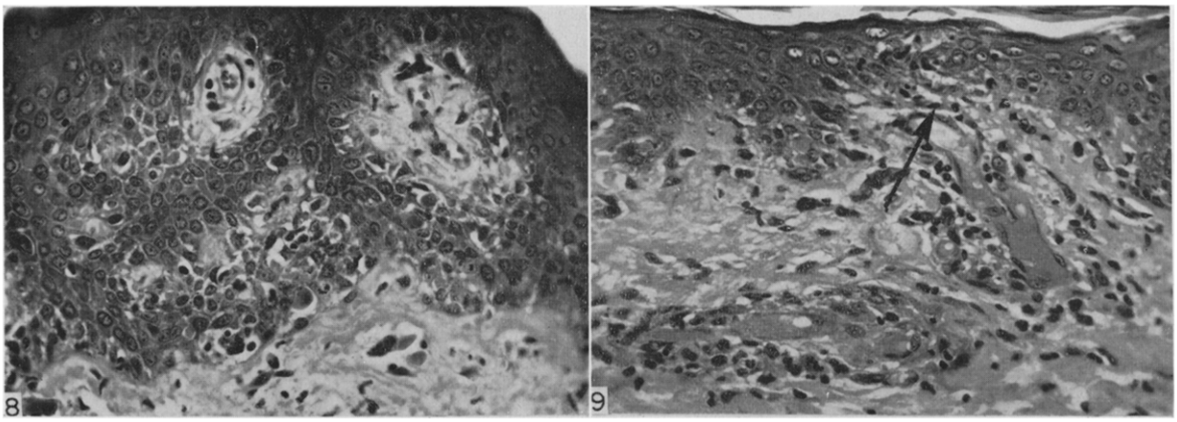


Fig. 8. *Biopsie de peau au cours du syndrome secondaire chez B.B. Infiltration de l'épiderme par des cellules de type lymphoïde; des cellules épidermiques au voisinage des cellules lymphoïdes sont le siège d'une vacuolisation et d'une dyskératose.*
(*Hématoxyline—éosine—safran* × 300)

Fig. 9. *Biopsie de peau au cours du syndrome secondaire chez B.B. Infiltration lymphoïde périvasculaire à la partie supérieure du derme. Remarquer la vacuolisation des cellules basales et la destruction des cellules épidermiques par l'infiltration lymphoïde (flèche).*
(*Hématoxyline—éosine—safran* × 300)

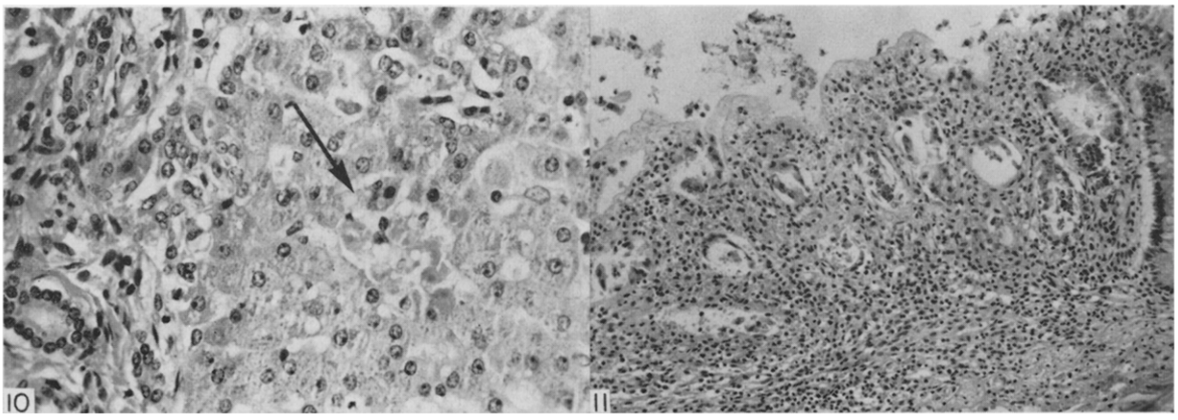


Fig. 10. *Examen histologique du foie chez B.F. Un amas de cellules hépatiques nécrotiques est indiqué par la flèche. Légère infiltration graisseuse.*
(*Hématoxyline—éosine—safran* × 300)

Fig. 11. *Examen histologique du colon chez B.F. De nombreuses cryptes sont altérées dans leur structure. Dénudation localisée de la muqueuse; on peut voir une crypte normale à droite. Infiltration massive de cellules lymphoïdes.*
(*Hématoxyline—éosine—safran* × 120)

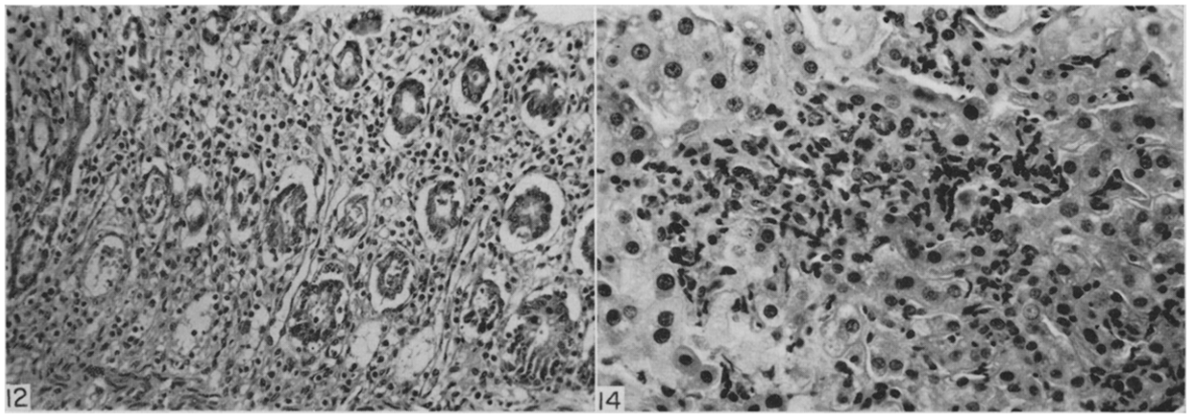


Fig. 12. *Examen histologique du colon chez B.F. Infiltration de la muqueuse par des cellules de type lymphoïde; nombreux caryorhexis et desquamation des cellules épithéliales des cryptes.* (*Hématoxyline—éosine—safran* × 190)

Fig. 14. *Biopsie de foie au cours de la réactivation du syndrome secondaire chez B.B. après injection de globules blancs. Infiltration par des cellules ressemblant à des histiocytes et des ceulluls de type lymphocytaire.* (*Hématoxyline—éosine—safran* × 300)

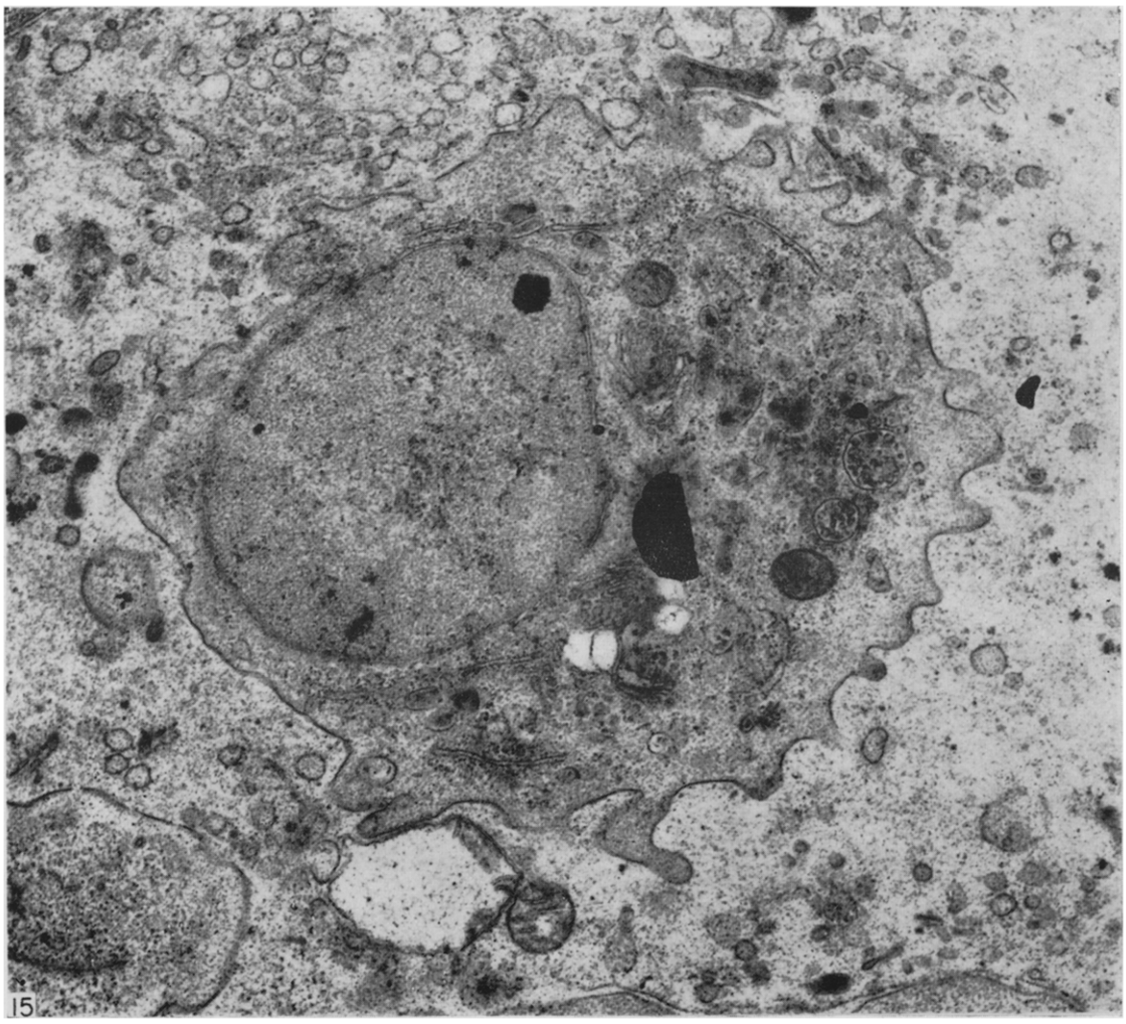


Fig. 15. *Biopsie de foie au cours de la réactivation du syndrome secondaire chez B.B. après injection de globules blancs. Cellule ressemblant à un histiomonocyte observé en microscopie électronique.*

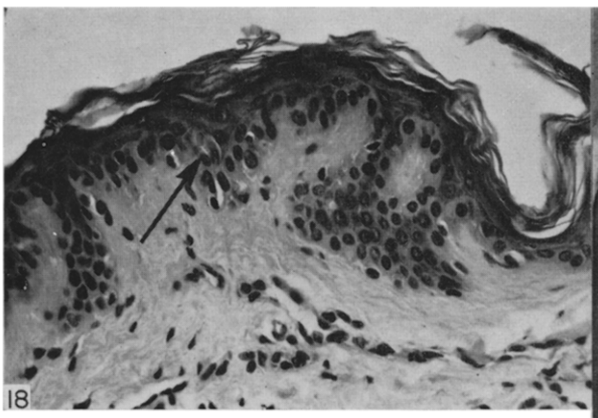
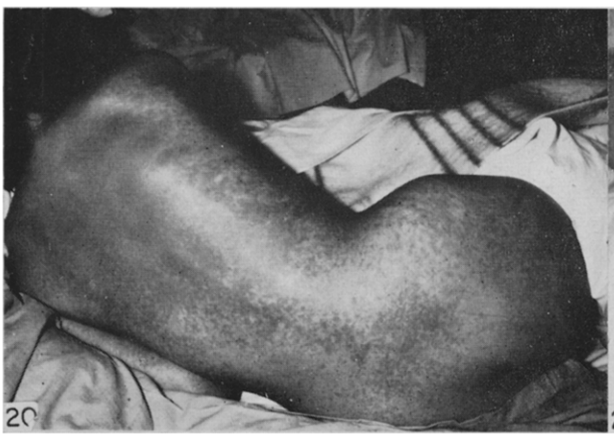




Fig. 18. *Biopsie de peau au cours de l'éruption cutanée présentée par C.A. après transfusions de globules blancs. Atrophie de l'épiderme et des follicules pileux; hyperkératose folliculaire. Remarquer la légère infiltration lymphoïde; plusieurs cellules basales vacuolisées et dyskératose (flèche).*
(*Hématoxyline—éosine—safran* × 300)

Fig. 19. *Aspect macroscopique de la peau chez C.C. au 4 ème jour après l'injection de globules blancs.*



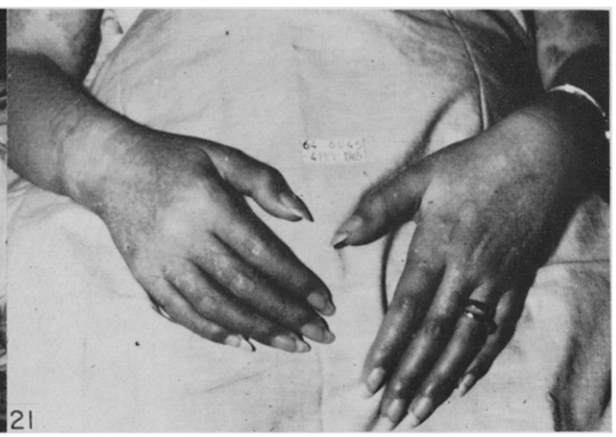


Fig. 20. *Aspect macroscopique de la peau de M.J. au* 11*ème jour après transfusion de globules blancs.*

Fig. 21. *Oedème infiltrant les deux mains de M.J., au* 11*ème jour après transfusion de globules blancs.*

Brutalement, le premier juin, à 19 heures, des crises convulsives témoignant d'une méningo-encéphalite varicelleuse, survinrent, alors que le malade n'avait pas cessé d'être sous traitement par la Δ-1-cortisone (30 mg/jour); elles entraînèrent la mort dans l'hyperthermie (41°8), malgré un traitement symptomatique intense.

L'autopsie montra une aplasie lymphoïde marquée; aucune formation ganglionnaire médiastinale; aucun ganglion dans les mésos coliques; existence dans l'intestin grêle de quelques très petits amas lymphoïdes de la sous-muqueuse; disparition de la pulpe blanche de la rate; un foie ferme, jaune comme du mastic à la coupe, avec une stéatose massive diffuse.

(g) J.L.C., âge de 16 ans, était atteint de leucémie aiguë lymphoblastique depuis mars 1963. L'irradiation eut lieu les 4 et 5 décembre 1963, précédée par l'administration pendant 4 jours de méthyl-nitro-imidazolyl-mercaptopurine (300 mg/jour). Le 10 décembre, 5 jours après l'irradiation, on transfusa la moelle osseuse de six donneurs dont cinq n'ayant aucun lien de parenté avec le sujet, dont les phénotypes érythrocytaires et leucocytaires sont indiqués au Tableau 11. On injecta ainsi 1900 cm^3 de moelle osseuse soit $56 \cdot 10^9$ cellules nucléées, en soustrayant en même temps 1500 cm^3 de sang. Le soir même, la température s'éleva à 40° et se maintint pendant une semaine à plus de 39°. Le malade, asthénique, s'alimenta peu. La semaine suivante, c'est-à-dire du 7e au 14e jour après l'injection de moelle, la fièvre fut moins élevée (38°, 38°5). Le 24 décembre, la température s'éleva à nouveau à 40°; une parotidite bilatérale importante apparut, qui s'améliora en 48 h sous perfusion de flabelline. Une polypnée (quarante respirations par minute) s'installa; l'examen radiographique des poumons montra des opacités micronodulaires liées à la septicémie; la peau présentait quelques pustules liées aussi à l'infection.

Le 2 janvier, le myélogramme, très pauvre, montrait 2 polynucléaires pour 100 éléments, et des plasmocytes; l'hémogramme, 60 leucocytes dont 20 polynucléaires, 22 000 thrombocytes et 3 300 000 hématies par mm^3. Devant l'absence d'efficacité des perfusions massives d'antibiotiques (pourtant actifs sur le germe *in vitro*), on décida de pratiquer des exsanguino-transfusions quotidiennes de 6000 cm^3 de sang frais. En fin d'exsanguino-transfusion, la température redescendait régulièrement autour de 37°; mais elle remontait à 40° la nuit suivante. Dès la deuxième exsanguino-transfusion, les hémocultures étaient devenues négatives. Le 5 janvier, survint un ictère; le malade présenta un iléus paralytique. Les exsanguino-transfusions quotidiennes étaient bien supportées par le malade qui s'alimentait malgré une polypnée croissante (50/mn). Le 8 janvier, 35 jours après l'irradiation et 30 jours après l'injection de moelle, 2 h après la sixième exsanguino-transfusion, le décès survint brusquement.

L'autopsie montra des signes de septicémie, d'oedème pulmonaire bilatéral, d'iléus paralytique; elle ne montra aucun signe de régénération myéloïde.

B. *Le syndrome secondaire après transfusion de leucocytes*

Dans six cas, des transfusions de leucocytes ont entraîné chez les receveurs des manifestations cliniques et des signes biologiques rappelant étroitement ceux observés au cours du syndrome secondaire survenant après greffe de moelle osseuse allogénique.

1. *Transfusion à une radio-chimère hématopoïétique de leucocytes du donneur dont il est tolérant.*— B.B., 26 ans, atteint de leucémie aiguë lymphoblastique depuis 1962, avait été traité par greffe de moelle osseuse allogénique après irradiation totale fin avril 1963 (Mathé *et al.* [4]). Huit jours après la transfusion de moelle osseuse, commença un syndrome secondaire aigu, associant des troubles cutanés (érythrodermie desquamative généralisée) (Fig. 3), digestifs (anorexie, vomissements, diarrhées), hépatiques [hépatomégalie, perturbation des taux des enzymes sériques (Tableau 12), et des taux des acides organiques (Tableau 13)], psychiques (asthénie extrême), sanguins (éosinophilie sanguine atteignant 49% le 25 mai 1963) (Fig. 5). Après un mois d'évolution, apparut une miliaire tuberculeuse. Le traitement associa la Δ-1-cortisone à une chimiothérapie anti-tuberculeuse (isoniazide+streptomycine). Le syndrome secondaire était très amélioré et les lésions radiologiques guéries en octobre 1963. Le patient montrait alors une tolérance complète pour un seul des six donneurs de moelle; il produisait 100% de ses érythrocytes et de ses immunoglobulines à partir de ce donneur.

Six mois après l'irradiation, dans le but d'accentuer la réaction du greffon contre l'hôte, on lui administra

Tableau 12. *Taux sériques de divers enzymes chez le patient B.B.*

Dates	L.D.H.	M.D.H.	G.O.T.	G.P.T.	Aldolase	O.C.T.
22.4.63	210	115	12	6		
13.5.63	360	90	55	34	0,25	0,33
20.5.63	400	76	10	46	0,24	*1,21*
29.5.63	*600*	113	*310*	*92*	*0,75*	*2,10*
13.6.63	*550*	115	*220*	*73*	0,35	*1,4*
14.6.63	340	152	*164*	38	*0,48*	*0,69*
18.6.63	295		38	46	0.07	
1.10.63	195	95	6	10	0,29	
15.10.63	145	185	12	20	0,30	
22.10.63	200	105	46	170	*0,96*	
18.11.63	200	195	*146*	*410*	*1,44*	*2,85*
10.12.63	245	85	*275*	*650*	*1,22*	*1,42*

Pour la LDH (Lacticodéshydrogénase), et la MDH (Malicodéshydrogénase), les résultats sont exprimés en unités spectrophotométriques dites de Wroblewski et sont rapportés au ml de sérum et à la minute. Pour les deux transaminases, GOT et GPT, les résultats sont exprimés en unités Frankel et rapportés là encore au ml de sérum/minute. Pour l'Aldolase, les résultats sont exprimés en unités Meyerhof. Pour l'OCT (Ornithine-Carbamyl-Transférase), les résultats sont exprimés en micromoles d'N-NH_3 formés par ml de sérum par 24 h à 40°.

Tableau 13. *Détermination des taux de divers acides organiques chez le patient B.B.*

Dates	Jours par rapport à L'irradiation	Jours par rapport à La greffe de moelle osseuse	Dans le sang: Acide pyruvique *	Acide lactique *	Acide citrique †	Acide α céto-glutamique †	Acide malique †	N.A.D. *	Dans l'urine: Acide citrique †	Acide α céto-glutamique ‡	Acide malique †
	Chiffres normaux		0,85±	10,0±	1,99±	0,171±	0,043±	2,13±	380±	22±	3,7±
			0,08	1,4	0,13	0,012	0,006	0,11	88	9	1,4
17·4	0		0,73	7,5	1,97	0,219	0,055	1,79	375	16	2,3
18·4	1		1,33	12,8	2,48	0,475	0,084	2,40	280	16	3,5
22·4	5		0,94	13,6	1,74	0,267	0,081	1,70	375		3,6
25·4	8	2	1,46	15,1	1,22	0,255	0,083	1,71	230		2,4
9·5	22	16	0,99	7,5	1,02	0,237		1,13			
13·5	26	20				0,124		1,23			
14·5	27	21							135	56	5,1
20·5	33	27	1,37	12,1	1,47	0,143	0,131	1,13			
27·5	40	34									
28·5	41	35	1,40	22,7	1,72	0,195	0,057	1,55	370	66	5,6
29·5	42	36								35	1,7
24·6	68	62									
5·7	79	73	0,75	6,01	1,57	0,404	0,101	2,41	100	38	1,1
15·7	89	83	2,20	26,2	2,14	0,650	0,123	2,70	205	55	5,8
26·7	100	94									
2·9	138	132									
3·9	139	133	1,02	9,9	2,42	0,303	0,058	2,61			
1·1	167	161	0,90	7,1	1,53	0,288	0,071	3,02	95	25	1,6
15·1	181	175									
22·1	188	182									
18·11	216	210									
27·11	225	219									
10·12	238	232									

*mg/100 ml de sang total.
†mg/100 ml de plasma.
‡µg/min.

Tableau 14. *Le syndrome hépatique réapparu chez BB après transfusions de leucocytes du donneur toléré.*

	$32 \cdot 10^6$ ↓	$22 \cdot 10^6$ ↓	$28 \cdot 10^6$ ↓	$49 \cdot 10^6$ ↓		
Dates	15.10.63		8.11.63	27.11.63	10.12.63	6.1.64	29.1.64
Bilirubinémie (mg/l)	4,8		11	42	80,5		6,3
Réaction au thymol (unités Vernes)	8		30	56	70	37	19
Réaction de Hanger	−		++	++	++		−
Cholesterolemie (g/l)	1,66		2,34	3,36	3,37	2,32	1,56
γ-globulines (g/l)	11			18	18	16,9	11
Temps de Quick %			100	75	93	85	
Prothrombine (facteur II) %			90	64	69	71	
Proaccélérine (facteur V) %			170	200	156	140	
Proconvertine (facteur VII) Stuart Prower (X) %			90	75	95	90	

quatre injections de leucocytes du donneur auquel il était tolérant. Il reçut ainsi, du 15 octobre au 8 novembre 1963, $1{,}63 \cdot 10^8$ leucocytes. Dès le lendemain de la première injection, le malade présent ait une certaine fatigue. Une semaine plus tard, la seconde injection de leucocytes fut suivie d'une asthénie extrême, analogue à celle survenue lors de son hospitalisation, "reconnue" par le malade, et le forçant à s'aliter. En même temps, réapparaissaient des symptômes déjà accusés durant le mois qui avait suivi l'injection de moelle osseuse: prurit, phénomènes vaso-moteurs marqués au niveau des pieds: érythème, sueurs, légère desquamation; des troubles de la fonction hépatique (Tableaux 12 et 14) étaient notés dont une hyperaccelérinémie, déjà rencontrée au cours d'autres syndromes secondaires (Tableau 15); une hépatomégalie était à nouveau perceptible. Le 18 novembre, 10 jours après la dernière injection de leucocytes, les taux dans le sérum de diverses enzymes atteignaient des niveaux significatifs. Le 18 décembre, une biopsie du foie montrait (Fig. 14) des cellules hépatiques modérément riches en glycogène, quelques capillaires distendus par des thrombi biliaires, un élargissement prononcé des cellules de Küpffer, une prolifération de cellules à allure histiocytaire au niveau de deux foyers intralobulaires (Fig. 15); on pouvait suspecter en marge d'un de ces foyers une unique cellule hépatique nécrotique; apparaissait autour des espaces portes une legère infiltration lymphocytaire et plasmocytaire; on trouvait dans les sinusoïdes un nombre plutôt élevé de lymphocytes. Un traitement par la Δ-1-cortisone (à la dose de 10 à 50 mg/jour) permit de contrôler ce syndrome).

En février 1964, le patient présenta un zona, qui s'accompagna d'une méningo-encéphalite zostérienne, hyperaebuminorrahie (16g/l), 300 lymphocytes par mm^3 dans le L.C.R. hémiplégie droite avec aphasie. La régression en fut spontanée mais lente; une patiente rééducation et des soins appropriés entrainèrent une très nette amélioration. Il persista cependant des troubles de la vigilance, s'accompagnant par moments de troubles du comportement, évoqaunt les troubles observés au cours de l'encephalite léthargique. Le 17 décembre 1964 le malade mourut en quelques heures dans un tableau de coma neurologique; le 15 décembre 1964, 20 mois après l'irradiation l'hémogramme et le myélogramme étaient normaux.

2. *Transfusion de leucocytes de donneurs porteurs de leucémie myéloïde chronique à des patients atteints de leucémie ou d'hématosarcome compliqué d'aplasie myélo-lymphoïde* — Ces transfusions furent effectuées dans un but symptomatique chez des malades fébriles, avec signes de septicémie, que ne parvenait pas à contrôler l'administration massive d'antibiotiques. Dans cinq cas, on a pu observer une éruption cutanée de type morbilleux, généralisée pour trois malades, localisée pour deux autres malades.

(a) J.B., âgée de 30 ans, était atteinte de maladie de Hodgkin depuis novembre 1960. Elle fut irradiée à de nombreuses reprises, de décembre 1960 à août 1963, et traitée par divers antimitotiques, de mars 1961 à mars 1964 (Dégranol, Vinca leucoblastine, Oenanthoyl-éthylène-imine-quinone, Chlorhydrate de 1-méthyl-2-p (isopropyl-carbamoyl) benzyl-hydrazine).

Le 14 mars, la malade dut être hospitalisée en raison d'une fièvre à 39° et d'une importante leucopénie (200 leucocytes par mm^3 de sang, dont 160 polynucléaires). Quatre jours plus tard, la fièvre ne diminuant pas malgré l'antibiothérapie instituée, et la leucopénie ne se réparant pas, il fut décidé d'injecter à la malade des leucocytes prélevés le jour même sur un sujet porteur de leucémie myéloïde chronique. La patiente reçut ainsi du 16 mars au 27 mars 1964 neuf transfusions de globules blancs qui lui apportaient en moyenne 4 à $5{,}10^{11}$ granulocytes à chaque fois. La fièvre demeura en plateau autour de 40° pendant 10 jours, commença à décroître vers le 27 mars, et, après plusieurs grandes oscillations, disparut le premier avril, au moment même où le sang périphérique commençait à compter de nouveau 1000 polynucléaires par mm^3.

Le 23 mars, au huitième jour après la première injection de leucocytes, la malade présenta une éruption généralisée, surtout marquée au niveau du dos. Cette éruption, faite de macules confluentes au niveau du tronc, espacées au niveau des membres, rouge-violacée, chaude, sèche, prurigineuse, s'accompagnait, chez cette malade fébrile (40°), d'anorexie, de nausées, de diarrhée et d'une très grande asthénie. La biopsie d'un élément cutané montra un épiderme d'une épaisseur sensiblement normale, une légère hyperkératose, quelques lymphocytes pénétrant dans l'épiderme, plusieurs cellules basales vascuolisées; une cellule dyskératosique était

Tableau 15. *Evolution des facteurs de l'hémostase chez les patients décédés de syndrome secondaire aigu compliquant des greffes de moelle osseuse allogénique.*

Tests	Patient M.V. Dates par rapport à l'irradiation								Patient E.D. Dates par rapport à l'irradiation						Normales
	1 mois avant	24 h avant	48 h après	8e jour après (trait. par la vit. k_1)	14e jour après	20e jour après	23e jour après	27e jour après	Avant	5e jour après	10e jour après	21e jour après	24e jour après	42e jour après	
Temps de saignement (min)	4	3,30			8,30				2,30		2,30	2,30			2–4
Nombre de plaquettes par mm³)	300.000	220.000	220.000	200.000	80.000	65.000	75.000	55.000	220.000	230.000	130.000	85.000	90.000	120.000	250.000
Consommation de prothrombine (sec)	60	23	60	60	19	20	16	10	35	60	49	23	38	13	60
Activité plaquettaire du sérum	25	7	16	11	1				7	16	7		5	3	15
Quick (%)	37	75	50	100	75	90	100	95	90	100	100	45	65	45	100
Accélérine (%)	65	90	75	85	130	120	95	150	120	120		120	170	100	100
Complexe VII (%)	75	100	50	70	80	70	*35*	80	100	65		35	60	60	100
Prothrombine (%)	85	100	85	100	100	100	80	100	95	100		68	80	92	100
Lyse (Euglobines)		Normale	Normale		Normale						Normale			Normale	Normale
Fibrinogène (g/l)	4,3	5,5		6,3	6,5		3,5		3		3,7	5		3,30	3–4
Temps de thrombine (sec)	15/15	21/15	20/22	18/19	18/18	21/21	20/20	26/25	15/15	18/19	19/19	19/19	22/21	28/19 absence d'anti-thrombine directe	

même trouvée; la couche sous-épidermique montrait quelques foyers d'homogénéisation. Cette éruption dura plusieurs jours, s'atténua à partir du 27 avril, au moment des grandes oscillations thermiques, et disparut le 2 avril, 10 jours plus tard, en même temps que la malade devenait apyrétique.

La malade put repartir chez elle le 10 avril, son sang périphérique contenant alors 9800 polynucléaires par mm³. Elle mourut le 30 mai 1964.

(b) G.M., âgé de 15 ans, était atteint de leucémie aiguë lymphoblastique depuis décembre 1963. Un traitement associant l'administration de fluoro-Δ-1-hydrocortisone et de mercapto-6-purine avait permis d'obtenir une rémission incomplète lorsque le malade fut examiné pour la première fois le 16 mars 1964. En prévision d'une éventuelle greffe de moelle, il n'avait pas été transfusé. La moelle osseuse contenait 18% de lymphoblastes. Le traitement fut poursuivi: on doubla la dose de mercapto-6-purine (100 mg/jour); on augmenta la corticothérapie progressivement de 5 mg par jour jusqu'à administrer 200 mg de Δ-1-cortisone le 2 avril.

Au milieu d'avril, devant l'aggravation de l'état clinique (fièvre importante atteignant 40°, asthénie considérable) et hématologique (3.400.000 hématies, 720 leucocytes, 92.000 thrombocytes par mm³ dans le sang périphérique; 54 lymphoblastes et seulement 1 polynucléaire pour cent éléments dans la moelle osseuse), l'administration de mercapto-6-purine fut cessée et l'on associa la Δ-1-cortisone et la leurosine (50 puis 100 mg par semaine), en même temps qu'on mettait en oeuvre une antibiothérapie massive (30 millions d'unités de pénicilline, 1 gramme de kanamycine). Le 19 mai, la température était retombée à 37°, l'état clinique était bon, l'hémogramme comptait 6000 leucocytes dont 4100 polynucléaires par mm³; on décida d'administrer la Δ-1-cortisone à doses décroissantes, tout en continuant la Leurosine. Mais 8 jours plus tard, le 26 mai, le malade était à nouveau fébrile, le sang périphérique contenait 6400 lymphoblastes et seulement 300 polynucléaires par mm³, et la moelle osseuse était envahie de lymphoblastes (87%). Une hémoculture fournit la preuve d'une septicémie en mettant en évidence des colibacilles. Une antibiothérapie intensive et polyvalente fut instituée, associant l'administration par voie veineuse, suivant l'antibiogramme, de colimycine, tétracycline, chloramphénicol et flabelline, et l'administration orale de trimethoprim. Par ailleurs, on injecta 2 mg de leurocristine. Pendant 10 jours, la température demeura très élevée, entre 40° et 41°3. Le 8 juin, on commença à transfuser quotidiennement des leucocytes prélevés le jour

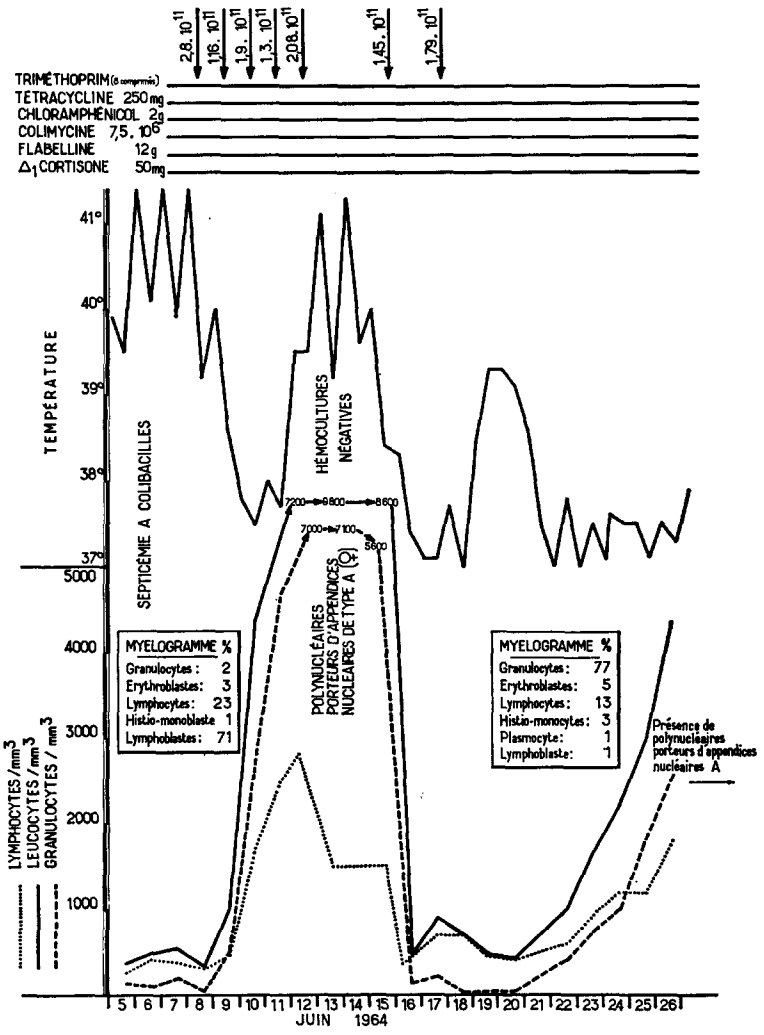


Fig. 16. *Evolution de la température et du taux des leucocytes dans le sang périphérique chez G.M. après transfusions de globules blancs.*

même sur un sujet de sexe féminin porteur de leucémie myéloïde chronique. En deux jours, la température descendit de 41°2 à 37°5, le chiffre des polynucléaires dans le sang périphérique passa de 40 à 2700 par mm³. Le 22 juin, 5 jours après le début des injections de leucocytes, le chiffre de ceux-ci atteignait 9800 par mm³ dans le sang périphérique, dont 7000 polynucléaires (Fig. 16); ce chiffre se maintint pendant 4 jours: 5600 polynucléaires par mm³ le 16 juin, pour retomber brutalement le 18 juin: 700 leucocytes par mm³ dont pas un seul polynucléaire. L'examen des granulocytes allait montrer qu'ils étaient bien du type donneur, leurs appendices nucléaires, avec une proportion notable d'éléments du type A ("drumsticks"), étant du type femelle.

En même temps que cette augmentation rapide du taux des granulocytes dans le sang circulant, trois éléments pouvaient être notés: la réapparition d'une fièvre élevée avec hémocultures répétées négatives et qui disparaîtra en même temps que les granulocytes étrangers; un érythème important de tout le corps avec une desquamation généralisée au niveau du dos, ou des lésions cutanées (vergetures) de la corticothérapie, et une importante escarre, rendaient difficile l'appréciation exacte de l'éruption spécifique; un myélogramme fait le 13 juin, au moment de la nouvelle phase thermique et de l'ascension granulocytaire, montrait 77 granulocytes et un lymphoblaste pour 100 éléments. Le malade était en rémission complète. On entreprit l'administration de Leurocristine (2,5 mg par semaine). Le chiffre des polynucléaires remonta à nouveau régulièrement: on notait par mm³: 740 le 23 juin, 800 le 25, 2560 le 26, 4800 le 29, 7600 le 30.

Le 4 juillet, l'examen des appendices nucléaires décelait encore la présence de granulocytes du type femelle (15 jours après la dernière injection de globules blancs). L'éruption s'effaça en même temps que les lésions cutanées s'amélioraient; il n'avait pas été possible de faire de biopsie.

La malade repartit chez lui le 25 juillet, en rémission hématologique complète depuis un mois et demi, avec l'hémogramme suivant: 3.400.000 hématies, 10.000 leucocytes dont 8200 polynucléaires, 260.000 thrombocytes par mm³, et le myélogramme suivant: 58% de granulocytes, 35% d'erythroblastes, 7% de lymphocytes. Il devait y mourir fin août, alors que l'état sanguin était excellent, à la suite d'un collapsus d'origine septicémique.

(c) C.A., âgée de 20 ans, était atteinte de leucémie aiguë lymphoblastique depuis novembre 1963. Un traitement associant l'administration de mercapto-6-purine et de Δ-1-cortisone permit d'obtenir une rémission incomplète et de courte durée (3 semaines) en mars 1964. Le 12 mai 1964, le sang périphérique contenait 60% de lymphoblastes par mm³ et le traitement associa dès lors à la Δ-1-cortisone, la Leurocristine (1,5 mg/semaine). Le 6 juin la malade était en rémission presque complète, la moelle ne comprenant plus que neuf lymphoblastes pour 100 éléments. Mais une nouvelle phase évolutive survenait presque aussitôt, malgré l'augmentation des doses de Δ-1-cortisone (200 mg/jour) et de Leurocristine (2,5 mg/semaine): 64% de lymphoblastes dans le moelle le 29 juin. Le 9 juillet était commencée l'administration quotidienne de 200 mg de méthyl-glyoxal-bis (guanyl-hydrazone) qu'on se trouvait dans l'obligation d'arrêter le 20 juillet, alors que le chiffre de lymphoblastes médullaires n'atteignait plus que 10%. On administra *per os* à la malade les 1er et 8 août 1 500 mg de cyclophosphamide. Le 12 août, lamalade était cytopénique (par mm³ de sang: 2.000.000 d'hématies, 40 leucocytes, tous lymphocytes, 44.000 thrombocytes), et fébrile (39°), sa moelle comptait trente-six lymphoblastes pour 100 éléments. Il fut décidé de lui injecter des globules blancs prélevés 3 mois plus tôt sur un sujet porteur de leucémie myéloïde chronique, et conservés à −70° dans une solution de diméthyl-sulfoxide à 12,5%. Il lui fut ainsi injecté, du 12 au 20 août 1964, $2{,}3 \cdot 10^{11}$ leucocytes. Le lendemain de la sixième injection, 8 jours après la première, l'amélioration clinique était remarquable, la température était à 37° (Fig. 17), la malade, euphorique, présentait une éruption cutanée localisée au thorax, à l'abdomen et à la face, faite de placards érythémateux très prurigineux; le prurit était même généralisé à tout le corps, plus discret au niveau des régions respectées par l'éruption. Cette éruption aura durée 3 jours, du 21 au 24 août; en même temps qu'elle s'effaçait, apparaissaient des troubles digestifs: la malade présenta une intolérance digestive absolue du 24 au 26 août, avec vomissements faciles et répétés pendant deux jours. Une biopsie cutanée effectuée au niveau d'un placard érythémateux montrait (Fig. 18) des modifications atrophiques légères mais nettes de l'épiderme et des follicules pileux; la courche basale était indistincte avec de nombreuses cullules vacuolisées; des cellules dyskératosiques, dégénérées, diffusaient à travers l'épiderme; de plus, on pouvait voir régulièrement des mitoses; il existait une légère infiltration lymphocytaire de la partie supérieure du derme, s'étendant à l'intérieur de l'epiderme.

Le myélogramme effectué le 22 août, le surlendemain de la dernière injection, permettait d'affirmer la rémission complète: pour la première fois depuis le début de sa maladie, 9 mois plus tôt, la malade n'avait plus un lymphoblaste dans sa moelle, qui comptait 81 granulocytes pour 100 éléments. Dans le sang périphérique, le chiffre des polynucléaires par mm³ augmentait régulièrement: 1180 le 21 août, 15.000 le 31 août, 27.000 le 5 septembre, 44.000 le 7 septembre. Mais cette rémission allait être de courte durée: le 16 septembre, le myélogramme comptait à nouveau 95% de lymphoblastes.

Un traitement par l'améthoptérine (7,5 g/jour) fut institué pendant plusieurs jours, puis l'administration de méthyl-glyoxal(bis-guanyl-hydrazone) put être reprise. Mais le 22 octobre, le chiffre de lymphoblastes atteignait dans le sang périphérique 3 920 par mm³.

(d) C.C., garçon âgé de 14 ans, était atteint de lymphoblastosarcome depuis mars 1964 avec localisation primitive au niveau de l'intestin grêle. Une dissémination aux chaînes ganglionnaires axillaires, inguinales, lombo-aortiques fut traitée par la Leurosine (100 mg/semaine) qui entraîna en 3 semaines une diminution très nette des adénopathies. Une adénopathie inguinale gauche fut en outre traitée par radiothérapie (3000 R/T en 15 jours). En juin 1964, aucune adénopathie n'était perceptible. Au milieu de juillet, l'apparition d'une masse hypogastrique et d'une lacune de l'aile iliaque droite firent décider l'irradiation de toute la cavité péritonéale (3000 R/T); des lésions orbitaires droites entrainèrent l'irradiation temporale (2000 R/T) du fond de l'orbite de la fente sphenoïdale; furent également irradiés: deux nodules sous-cutanés de l'hypochondre gauche et du flanc gauche (2500 R/T), une masse testiculaire (700 R/T), un champ lombaire postérieur gauche (1000 R/T). Une chimiothérapie fut ensuite entreprise; la leurocristine fut administrée du 23 juillet au 13 août; le glucoside de la podophyllotoxine (de 65 mg à 300 mg par jour), du 21 août au 31 août, en

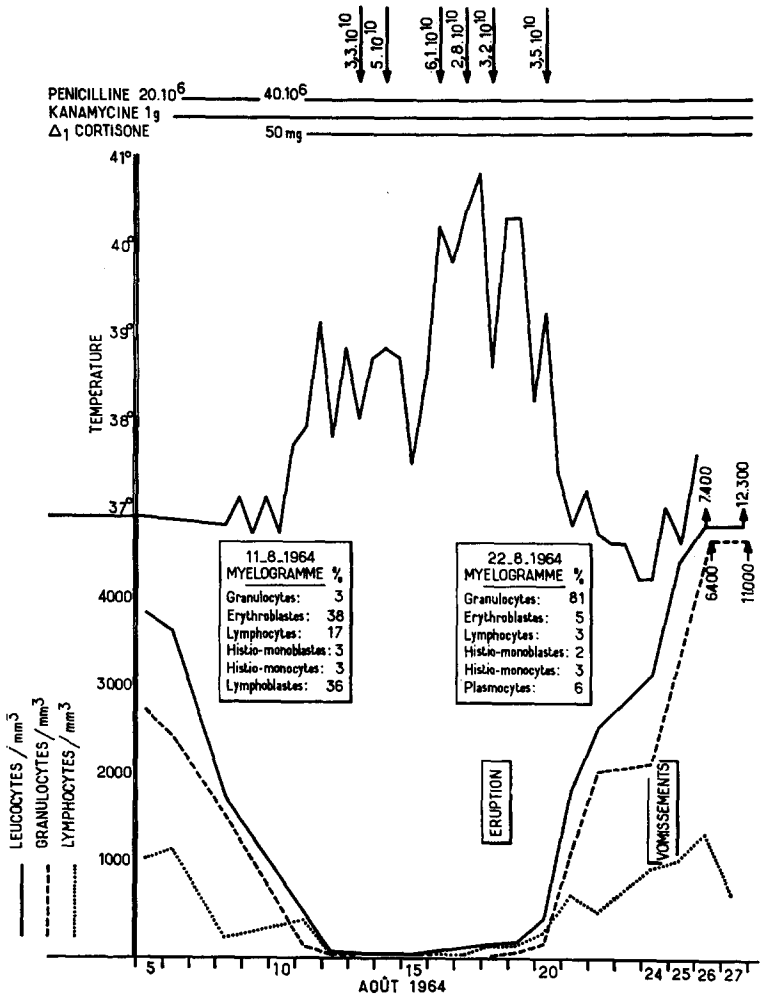


Fig. 17. *Evolution de la température et du taux des leucocytes dans le sang périphérique chez C.A. après transfusions de globules blancs.*

même temps qu'on instituait une corticothérapie (100 mg/jour de Δ-1-cortisone). L'apparition de nouvelles masses tumorales (sous-cutanée abdominale, sous-costale droite) au début de septembre entraîna l'administration intraveineuse quotidienne de cyclophosphamide (200 mg/jour). Le 7 septembre, le sang périphérique comptait 800 leucocytes, dont 400 polynucléaires par mm³; le 9 septembre s'affirmait une transformation leucémique aiguë: 1900 lymphoblastes par mm³ de sang, 400 polynucléaires. Il fut décidé d'injecter des globules blancs prélevés chez un sujet porteur de leucémie myéloïde chronique. Le patient reçut ainsi, les 8,9 et 10 septembre, $3,5 \cdot 10^{10}$ leucytes, soit au total 10^{11} leucocytes. Le 11 septembre, au quatrième jour après l'injection de globules blancs, le malade présenta une éruption généralisée, atteignant aussi bien le visage, le tronc (Fig. 19), les membres, que la paume des mains, faite de macules plus ou moins confluentes, rouge-violacé, chaudes, prurigineuses. En même temps, la température atteignait chez ce malade, jusque-là apyrétique, 38°5. Quatre jours plus tard, le patient mourait, après avoir été emmené par ses parents.

(e) P.T., garçon de 5 ans, était atteint de leucémie aiguë lymphoblastique depuis octobre 1963. Un traitement associant la Δ-1-cortisone (40 mg/jour) et la mercapto-6-purine (25 mg/jour) avait permis d'obtenir une rémission en janvier 1964. Pendant 2 mois, l'administration de mercapto-6-purine avait été poursuivie, et le malade était resté sans traitement d'avril à août 1964. La découverte d'une tumeur testiculaire bilatérale, dont l'examen cytologique montra une hyperplasie lymphocytaire, fit entreprendre une radiothérapie locale. Un myélogramme revéla la présence de quatre-vingt-dix lymphoblastes pour 100 éléments. Un traitement fut alors institué, associant la Δ-1-cortisone (40 mg/jour) à la Vincaleucoblastine (1 mg/jour).

Lorsque le malade arriva dans le service, le 24 septembre 1964, il était fébrile; l'examen bactériologique montra la présence dans la gorge de staphylocoques pathogènes et dans les urines de klebsielles. L'examen hématologique montrait dans le sang périphérique, par, mm³, 3.375.000 hématies, 44.000 thrombocytes et 400 leucocytes dont 100 polynucléaires. Le myélogramme montrait une moelle osseuse très pauvre, dans laquelle on comptait, pour 100 éléments, quarante-sept lymphoblastes, cinquante-trois lymphocytes et pas un seul élément granuleux. Une antibiothérapie intensive était instituée, mais ne parvenait pas à faire baisser la fièvre, qui dépassait 39°, l'état clinique de malade s'aggravant. Le surlendemain, l'hémogramme montrait, par mm³, 2.300.000 hématies, quatre-vingts lymphocytes; il n'était pas vu de polynucléaires. Le malade ayant déjà reçu plusieurs transfusions de sang lors de la première phase évolutive, et la nécessité de nouvelles

transfusions semblant s'imposer, on ne se trouvait plus retenu, chez ce patient, par le risque d'une éventuelle immunisation, et il fut décidé de lui injecter des leucocytes dans un but symptomatique. Des transfusions de globules blancs furent effectuées 6 jours de suite, du 27 septembre au 2 octobre 1964. Chaque transfusion apportait au patient environ, $2,5 \cdot 10$ leucocytes prélevés 6 mois plus tôt sur un sujet porteur de leucémie myéloïde chronique et conservés à −70° dans une suspension de glycérol à 15%. Le premier octobre, cinq jours après la première injection, l'enfant devint apyrétique. Le 4 octobre, trois jours plus tard, le sang périphérique contenait 1400 polynucléaires par mm^3; et le myélogramme, riche, montrait, pour 100 éléments: 66 granulocytes neutrophiles, 27 érythroblastes et 3 lymphoblastes. Le 6 octobre, soit au neuvième jour après la première transfusion de leucocytes, l'enfant présenta une éruption faite de petites macules congestives s'effaçant à la pression, non confluentes et siégeant essentiellement au niveau du thorax, de l'abdomen et des membres supérieurs. Cette éruption dura trois jours puis s'effaça le 9 octobre. L'hémogramme montrait alors, par mm^3: 3.180.000 hématies, 3400 polynucléaires, 1000 lymphocytes et 230.000 thrombocytes.

(f) M.J., âgée de 28 ans, était atteinte de maladie de Hodgkin depuis septembre 1962. Après avoir été traitée par cyclophosphamide, delta-1-cortisone, chlorambucyl, phénylbutazone et irradiation de 3000 r sur le médiastin, la malade entra dans le service en novembre 1964. La vitesse de sédimentation était alors très accélérée (115 mm à la première heure). L'examen radiographique des champs pulmonaires montrait des signes de lésions radiothérapiques. Un traitement par le chlorhydrate de méthyl-hydrazine fut entrepris début décembre, mais vite interrompu, après une quinzaine de jours, en raison de troubles psychiques. Une nouvelle cure chimiothérapique par la vincaleucoblastine fut commencée 10 jours plus tard, mais dut à son tour être interrompue après deux injections, en raison de désordres hématologiques. Le 14 janvier 1965, le sang périphérique de la malade contenait par mm^3: 140 leucocytes dont 60 polynucléaires et 30.000 plaquettes. Devant l'association à cette cytopénie d'un syndrome infectieux sans foyer décelable, on décida de transfuser des globules blancs à partir du 15 janvier. On injecta ainsi quotidiennement des leucocytes prélevés le jour même sur un sujet porteur de leucémie myéloïde chonique: la malade reçut ainsi du 15 au 20 janvier: $7,99 \cdot 10^{11}$ leucocytes, et le 21 janvier, 6 jours après la première transfusion, le sang périphérique comptait 9000 leucocytes par mm^3 dont 94% de granulocytes. Le 22 janvier apparaissait une éruption cutanée scarlatiforme qui devait s'accentuer les jours suivants pour atteindre son maximum le 26 janvier, presque entièrement généralisée, laissant quelques intervalles de peau saine au niveau du dos et (Fig. 20) et des cuisses, la peau était sèche, rugueuse, chaude, écarlate, lisse, non prurigineuse; un oedème infiltrait les doigts des deux mains: (Fig. 21): les conjonctives prérant une vasodilatation, la langue desquamait, présentant elle aussi un aspect scarlatiforme. L'examen biopsique de la peau montra une atrophie des couches cellulaires basales de l'épiderme, une hyperkératose avec de la parakératose locale, plusieurs follicules pileux plus ou moins distendus par un amas de kératine, des cellules dyskératosiques dispersées dans la couche de Malpighi, de nombreuses mitoses, une légère infiltration lymphocytaire de la partie supérieure du derme, pénétrant dans l'épiderme. Ces signes cutariés s'accompagnaient d'une fièvre élevée (39°–40°), de troubles digestifs importants: anorexie totale, selles pâteuses un peu décolorées, puis diarrhée liquide abondante. Le chiffre des globules blancs atteint dans le sang le 26 janvier: 12.300 par mm^3 dont 99% de granulocytes. Un examen caryotypique effectué au niveau de la moelle osseuse (Docteur de Grouchy) affirme la greffe des cellules injectées en montrant sur toutes les mitoses la présence de chromosome de Philadelphie, aussi bien dans les granulocytes (89%) que dans les érythroblastes (10%). L'état général de la malade continua à être bon, mais devant l'importance considérable du syndrome secondaire, on décida l'administration de cortisone, 150, puis 250 mg par jour, et d'améthoptérine selon un schéma pré-établi 0,5 mg par kg de poids le premier jour, puis 0,2 mg par kg tous les 2 jours.

Les jours suivants devait s'installer un syndrome ictérique qui devint franc le 29 janvier: les selles étaient décolorées, les urines contenaient des pigments biliaires, il existait une hépatomégalie, la bilirubinémie atteignait 85 mg par litre de plasma. Puis, alors que l'éruption cutanée s'atténuait et desquamait finement, l'ictère et la diarrhée persistaient. L'asthénie s'accentuait. Le 4 février, alors qu'il avait été administré en 9 jours 58,5 mg de méthotrexate, et qu'on donnait quotidiennement 50 mg de delta-1-cortisone, la sang périphérique contenait 24.400 leucocytes dont 97% de granulocytes; l'état général semblait s'améliorer, les selles devenaient moins liquides, plus pâteuses, la malade s'alimentait mieux, vomissait moins, l'ictère s'éclairait. Mais les tests de l'hémostase traduisaient le gravité de l'atteinte hépatique, le taux de facteur VII atteignait 9%, alors que paradoxalement (comme cela avait déjà noté au cours du syndrome secondaire, Tableau 15), on notait une hyperaccélérinémie: 126%.

Mais cette amélioration clinique ne dura pas. Le 8 février, la fatigue s'accentuait, la malade ne pouvait dormir, la diarrhée reprenait, plus abondante, jusqu'à atteindre, le 13 février, 2 l. par jour de selles liquides, foncées, non hémorragiques. Le 15 février, les selles devenaient melaeniques, les tests de l'hémostase, un moment améliorés grâce à l'administration de plasma et de PPSB (Centre National de Transfusion Sanguine), s'effondraient: temps de Quick à 5%: la protidémie atteignait 45 g par l., les gamma-globulines 4,1 g par l.; l'ictère fonçait à nouveau et c'était le tableau d'ictère grave; le 16 février apparaissaient des troubles psychiques: la malade, hébétée, désorientée, répondait mal aux questions. Et le 18, elle devait mourir dans un tableau d'atrophie jaune aiguë du foie que viendra prouver la vérification anatomique.

III. DISCUSSION

Nous ne tirerons de ces observations, pour les discuter, que les données concernant le syndrome secondaire, laissant délibérément de côté les conséquences directes de l'irradiation, qui ont fait l'objet d'autres études (Mathé *et al.* [1–4, 28]; Papiernik *et al.* [29]).

Tableau 16. *Résumé des principales manifestations du syndrome secondaire*

	Animal					Homme												
						Greffe de moelle									Transfusion de globules blancs					
Date d'apparition par rapport à la transfusion de moelle ou de leucocytes	Souris (7-8-9-10)	Rat (11-12)	Lapin (13-14-15)	Chien (16)	Singe (17-18)	D.V.	V.L.	E.D.	M.V.	B.B.	P.M.	B.F.	P.P.	D.F.	J.B.	G.M.	C.A.	C.C.	P.T.	M.J.
	20-30e j.	30e j.	20-30ej.	30 e j.	7-10e j.	45e j.	33e j.	5e j.	8e j.	8e j.	5e j.	7e j.	7e j.	8e j.	8e j.	8e j.	5e j.	4e j.	9e j.	Fe j.
Perte de poids	+	+	+	+	+	+	+	+	+	+	+	+	+	+						+
Asthénie						+	+	+	+	+	+	+	+	+	+					+
<Anorexie						+	+	+	+	+	+	+	+	+		+	+			+
Troubles <Nausées						+	+	+	+	+	+	+	+	+	+	+	+			+
digestifs: <vomissements						+	+	+	+	+	+	+	+	+		+				+
<diarrhée	+		+		+	+	+	+	+	+	+	+	+	+	+					+
Fièvre						+	+	+	+	+	+	+	+	++	+	+		+		+
Infections <Fungiques								+[1]	+[1]	+[1]		+[3]				+[1]	+[3]			
cliniques <bacteriènnes	+	+	+	+	+[2]	+	+[3]			+[4]			+[3]	+[3]		+[1]				
decelées: <virales								+[5]	+[6]	+[6]										
										+[7]										
Lesions cutanées (dyskératose, acanthose, infiltration lymphocytaire de l'épiderme)	+	+		+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+
Atteinte hépatique (nécrose cellulaire)	+	+	+	+	+			+	+	+			+	+	+					+
Atrophie lymphoïde	+	+	+	+	+	+	+	+	+	+	+	+	+	+						+

1. Candida albicans
2. Staphylocoque
3. Streptocoque
4. Bacille tuberculeux
5. Lésions de nécrose des surrénales avec inclusions nucléaires (maladie des inclusions cytomégaliques)
6. Lésions d'hépatite
7. Zona

A. *Réalité du syndrome secondaire après greffe de moelle osseuse allogénique chez l'Homme*

Parmi les vingt-trois malades que nous avons irradiés délibérément à des doses variables pour les soumettre à des transfusions de moelle osseuse (Mathé [20]; Mathé *et al.* [2–5, 23]) ou reçus après une irradiation accidentelle (Mathé *et al.* [1]), ou que nous avons irradiés avec Küss *et al.* [30] pour les conditionner en vue d'une transplantation allogénique de rein, ou avec Tubiana, Lalanne, Milliez et Lagrue (non publié) pour tenter de réduire l'évolutivité d'une maladie néphrotique sévère, parmi plus de 133 malades dont nos collègues, qui ont bien voulu se soumettre à notre enquête (Mathé [22]), ont mis à notre disposition les observations, seuls ont présenté des éléments de ce syndrome, dit secondaire, les sujets chez lesquels on a démontré la réalité d'une greffe des cellules hématopoïétiques, ou soupçonné fortement cette réalité sur des arguments indirects. Il convient de souligner, en particulier, que les patients irradiés aux plus fortes doses, et qui sont morts d'aplasie sans présenter de début de restauration (Mathé *et al.* [1–3]), ou qui ont été restaurés après une transfusion de moelle autologue ou isogénique, n'ont pas présenté ce syndrome.

Inversement, celui-ci ne complique pas obligatoirement la greffe des cellules médullaires transfusées: dans le cas des sujets irradiés accidentellement à Vinca (Yougoslavie), aucune manifestation clinique n'est survenue, bien que nous ayons montré que la restauration hématopoïétique fut assurée pendant 3 semaines par la greffe (Mathé *et al.* [1]); il faut remarquer cependant que les sujets ont présenté une lymphopénie qui est peut-être, comme nous le verrons plus loin, la modalité d'expression mineure du syndrome. En tous cas, l'absence ou la discrétion du syndrome secondaire s'explique probablement par le caractère transitoire de la greffe.

B. *Identité des manifestations compliquant les transfusions de leucocytes et du syndrome secondaire compliquant la greffe de moelle osseuse allogénique*

Les sujets ayant souffert de ces manifestations n'ont présenté que des symptômes connus pour faire partie du syndrome secondaire: anorexie, perte de poids, asthénie, fièvre, diarrhée, ictère, hépatomégalie, dermatose, les bases histologiques de ces derniers symptômes ne diffèrant pas de celles observées dans le syndrome secondaire. C'est ce que montre le Tableau 16.

C. *Identité du syndrome secondaire chez l'Homme et dans diverses espèces animales*

Ce même tableau montre aussi que cette symptomatologie ne diffère pas notablement de celle observée dans les diverses espèces animales qui ont été l'objet d'essais de greffe de moelle osseuse allogénique (Barnes *et al.* [7, 8]; Van Bekkum et Vos [9]; Balner [11]; Porter [13–15]; Thomas *et al.* [16]; De Vries *et al.* [17]). Les manifestations sont particulièrement proches chez l'Homme et chez le Singe (De Vries *et al.* [17]).

D. *Analyse des manifestations*

1. *Symptômes cliniques* — Un des symptômes les plus marquants est la perte de poids: celle-ci qui, dans le cas de la greffe de moelle osseuse, est généralement modérée pendant la phase de cytopénie due à l'irradiation, s'intensifie considérablement dès qu'apparaissent les premières manifestations du syndrome secondaire chronique, comme l'illustrent les cas n°1 (D.V.) et n°2 (V.L.). Dans le cas des syndromes secondaires aigus, on assiste à l'installation progressive et rapide d'une vériable cachexie avec fonte du panicule adipeux et des masses musculaires (observations n° 3, 4, 5).

Une asthénie accompagne l'amaigrissement: marquée dans les syndromes chroniques, elle a toujours été extrême dans les syndrômes aigus, empêchant le malade de se lever et même de s'asseoir sur son lit.

Perte de poids et asthénie relèvent certainement, au moins en partie, des troubles digestifs, notamment de l'anorexie. Celle-ci fut également intense dans le cas des syndromes secondaires chroniques, totale dans celui des syndromes aigus. Les nausées compliquèrent essentiellement les formes aiguës: elles furent très pénibles chez tous les patients, responsables en partie de l'absence d'alimentation, entrainant de nombreux vomissements, le plus souvent biliaires. Nausées et vomissements étaient souvent déclanchés par l'ingestion d'aliments et, chez B.B., par le moindre palper de la région hépatique. La diarrhée fut présente chez tous les sujets, différente selon que le syndrome était chronique ou aigu. Dans le premier cas, les selles étaient consistantes, mais d'un aspect anormal. Des examens macroscopiques donnèrent l'impression d'une absence de digestion globale pour toutes les catégories d'aliments. A l'examen microscopique, le fait le plus remarquable est l'absence totale d'éléments d'origine intestinale (débris épithéliaux, microbes, mucine . . . qui constituent habituellement les trois-quarts des selles normales). On note généralement l'absence de glaires, de pus, d'hématies, de leucocytes. On observe (surtout chez D.V.) de très nombreuses fibres musculaires, souvent intactes, une insuffisance de

digestion de l'amidon (totale chez D.V.), une absence complète de digestion de la cellulose digestible. Les corps gras semblent les seuls aliments digérés normalement: absence de graisses neutres; acides gras et savons normalement présents. Une des anomalies les plus précoces et importantes réside enfin dans la présence de bilirubine et l'absence de stercobiline. A noter qu'aucun germe pathogène n'a été mis en évidence et que la flore intestinale habituelle semble réduite à 20% de sa valeur normale. Ces examens grossiers suggèrent donc l'existence: (*a*) d'une insuffisance gastrique; (*b*) d'une capacité digestive très réduite pour les farineux et la cellulose; (*c*) d'une arrêt de la digestion au stade idéal; (*d*) d'une sidération des fonctions iléocoecales et coliques. Dans les cas aigus, la diarrhée consiste en émissions extrêmement fréquentes (jusqu'à 10 fois par jours) de selles liquides entraînant des désordres hydro-électrolytiques. D'odeur non fécale, ces selles se présentaient avec une masse de mucus dans laquelle flottaient quelques débris alimentaires; l'examen microscopique révélait une desquamation épithéliale massive.

Divers désordres hépatiques furent observés chez deux patients, M.V. et B.B. Le premier présenta un ictère révélé par une pigmentation des urines, s'exprimant encore par une jaunisse cutanée typique, se compliquant enfin de coma hépatique, avec ses manifestations neurologiques (agitation, tremblement, convulsions, délire, coma) et hémorragiques, liées, comme on le verra, à l'atteinte de certains facteurs de la coagulation, singulièrement la proconvertine (35% au 23e jour) (Tableau 15) associée à une thrombocytopénie. A noter que cette hypoconvertinémie du vingt-troisième jour se retrouve chez un autre patient qui présenta un syndrome secondaire aigu (E.D.): 35% au vingt-et-unième jour (Tableau 15). Le second malade présenta des douleurs de l'hypochondre droit, accompagnées de vomissements biliaires, et l'on palpait une hépatomégalie. Dans les deux cas, les épreuves hépatiques révélèrent des altérations importantes. Dans le cas de B.B., les manifestations hépatiques, cliniques et biologiques, s'atténuaient en même temps que les autres manifestations du syndrome secondaire, mais, lorsqu'on lui injecta des leucocytes du donneur dont la moelle était greffée, on assista à la reprise du syndrome secondaire, dominé par les désordres hépatiques, nausées, ictère, hépatomégalie.

Les lésions cutanées occupent une place importante parmi les manifestations du syndrome secondaire chez l'Homme; elles sont plus fréquentes que chez l'animal. Elles n'en diffèrent cependent pas notablement par leurs aspects qui peuvent être de deux types: (*a*) lésions dont l'allure suggère une origine infectieuse; (*b*) manifestations "inflammatoires" que rien ne permet d'attribuer à une infection.

Au premier type se rattachent les plaques infectieuses rappelant l'érysipèle, que nous avons observées chez l'un des deux malades atteints de syndrome secondaire chronique (V.L.) et les lésions rappelant une dermite staphyloccocique observée chez l'autre malade atteint de cette variété (D.V.). Ces deux "dermites" ont rapidement cédé à l'administration d'antibiotiques.

Tous les patients atteints de syndrome secondaire aigu après greffe de moelle osseuse ou transfusions de leucocytes ont, au contraire, présenté le second type de ces manifestations cutanées. Celles-ci ont commencé par une éruption rubéoliforme plus ou moins disséminée (Fig. 3) dont les taches ont conflué; comme celles-ci étaient légèrement surélevées, leur confluence a laissé apparaitre, sous la rougeur diffuse, l'infiltration oedémateuse, dure et laissant au malade une pénible sensation de cartonnage. Cette érythrodermie a bientôt desquamé, d'abord en fines plaques puis en plus ou moins larges lambeaux (Fig. 4).

La fièvre et les infections diverses ont enrichi ce tableau déjà fourni. La fièvre fut bien tolérée dans les syndromes secondaires chroniques et généralement très élevée dans les syndromes secondaires aigus. Si elle a toujours accompagné les diverses infections et a pris, de ce fait, une allure des plus variable, sa fréquence n'est pas conditionnée par elles. Elle est d'ailleurs apparue, chez la plupart des sujets, dès la transfusion de la moelle osseuse, alors qu'en règle générale, ceux-ci toléraient parfaitement la phase initiale de l'aplasie déterminée par l'irradiation, et demeuraient apyrétiques; les hémocultures, faites pendant cette phase fébrile qui a suivi les transfusions de moelle osseuse, furent, dans tous les cas, négatives.

Les infections rencontrées chez nos malades sont mentionnées au Tableau 16 et comparées à celles rencontrées dans d'autres espèces animales. On peut y voir qu'elles peuvent être infections mycosiques (singulièrement à candida albicans), responsables de la mort d'un malade, et particulièrement fréquentes, même si l'on administre à titre préventif un antifungique tel que la nystatine. On pourrait penser que cette fréquence élevée est liée au terrain particulièrement sensible à l'égard de ce micro-organisme que représentent les leucémiques.

Il est possible que ce terrain élève les chances d'une telle infection, mais, contre l'hypothèse de son rôle unique, il convient de signaler que chez un des sujets irradiés accidentellement à Vinca (Yougoslavie) (Jammet *et al.* [31]; Mathé *et al.* [1]), la seule manifestation clinique d'un syndrome secondaire après la greffe de moelle osseuse (qui n'a duré que trois semaines) fut une stomatite à candida albicans, l'expression de ce syndrome se limitant par ailleurs à une lymphopénie.

Les infections bactériennes sont également fréquentes, mais relativement faciles à contrôler. C'est ainsi que les dermites présentées au cours du syndrome secondaire chronique, d'origine staphylococcique chez D.V. et streptococcique chez V.L., ont cédé à l'administration d'antibiotiques. La miliaire tuberculeuse présentée par B.B. put être contrôlée par le traitement anti-tuberculeux. Cependant, les septicémies présentées vers le septième jour après l'injection de la moelle par J.L.C., P.P. et D.F., furent, à cause de la cytopénie périphérique, difficiles à combattre. Des exsanguino-transfusions quotidiennes systématiques permirent dans un cas (J.L.C.) de négativer les hémocultures et n'obtinrent qu'un effet malheureusement temporaire.

Par contre, les infections virales sont particulièrement redoutables. Elles ont été de trois types dans notre expérience: (*a*) varicelle, zona; (*b*) hépatite; (*c*) inclusions cytomégaliques.

La varicelle a pris d'emblée une allure évolutive marquée, le type hémorragique et s'est compliquée d'encéphalo-méningite mortelle en quelques jours. Il est vrai que le patient était soumis à un traitement par la Δ-l-cortisone (30 mg/jour) et que la varicelle est connue pour être très grave chez les sujets ainsi traités (Falliers [32]; Benatti [33]).

Le zona s'est également présenté sous une forme très sévère: les lésions cutanées furent très étendues et s'ulcérèrent; une méningo-encéphalite grave apparut, qui se compliqua d'hémiplégie, qui n'a été que lentement régressive. A noter aussi cependant qu'à cette période, le sujet était également sous corticothérapie.

L'hépatite est d'interprétation plus délicate, puisque le syndrome secondaire semble, par lui-même, capable de provoquer des lésions dans le foie. Il est cependant raisonnable d'attribuer à un virus les désordres hépatiques considérables présentés par M.V. Cette malade avait présenté, deux mois environ avant son irradiation, des anomalies des tests d'exploration hépatique (ceci 100 jours environ après des transfusions sanguines), qu'il y a tout lieu d'attribuer à une hépatite sérique latente. Il est donc possible que le syndrome secondaire ait favorisé le réveil de cette hépatite, ce qui n'est pas pour surprendre, puisque nous avons démontré que ce syndrome entraîne une perte de la mémoire immunitaire (Mathé *et al.* [34]). Rappelons la fréquence des infections virales au cours de syndrome secondaire chez le Singe (De Vries *et al.* [17]). Il semble donc que la maladie secondaire comporte une diminution considérable des défenses antivirales.

Enfin, les désordres hépatiques ont été au premier plan du syndrome secondaire présenté par la malade M.J. après transfusions de globules blancs; elle mourut dans un tableau d'ictère grave. Il est difficile d'affirmer qu'il puisse s'agir seulement de lésions hépatiques d'origine immunologique; il s'agit peutêtre d'une hépatite virale aggravée par le conflit immunologique.

2. *Désordres hématologiques*—Bien que caractéristiques et très proches de ceux observés chez l'animal (De Vries et Vos [35]; Rodriguez *et al.* [36]), les désordres hématologiques sont assez variables d'un malade à l'autre.

C'est ainsi que l'anémie n'est pas constante; elle est même relativement rare. Le test de Coombs est loin d'être fréquemment positif, comme cela a été constaté dans certaines espèces animales, singulièrement le Lapin (Porter [13]). Nous avons cependant observé chez un patient, des agglutinines froides (D.V.).

Aucun malade n'a jamais présenté de thrombocytopénie que l'on puisse attribuer au syndrome secondaire; on peut tout au plus noter un très fréquent retard de la restauration de la thrombocytose sanguine par rapport à la granulocytose.

Le taux des granulocytes est fréquemment augmenté de façon modérée, sans que l'on puisse en attribuer la raison au syndrome lui-même ou aux infections.

Les désordres lymphocytaires paraissent par contre les plus fréquents et plus les spécifiques; ils consistent le plus souvent en une lymphopénie intense ou modérée, et nous avons dit plus haut que plusieurs des sujets yougoslaves irradiés accidentellement avaient présenté une discrète lymphopénie en l'absence de tout syndrome secondaire clinique évident. Mais le désordre lymphocytaire peut consister en une hyperlymphocytose qui, en fait, est plus précoce que la lymphopénie. Ces deux aspects possibles

des anomalies de cette série sanguine correspondent en réalité à deux phases successives des lésions lymphoïdes, comme nous l'avons montré chez la Souris: une phase de prolifération, suivie d'une phase d'aplasie (Mathé et Amiel [37]), auxquelles correspondent d'ailleurs respectivement deux phases d'hyper- puis d'hypo-gammaglobulinémie.

La présence dans le sang de cellules hyperbasophiles et leur taux relativement élevé dans la moelle osseuse sont un symptôme également fréquent.

Nous avons enfin observé chez un malade (B.B.) une importante éosinophilie sanguine (jusqu'à 50% des leucocytes), et médullaire, au moment des plus intenses manifestations cliniques du syndrome secondaire. Cette éosinophilie a disparu en quelques jours après l'administration de Δ-1-cortisone.

Nous n'avons pas observé de cellules L.E., que nous avons attentivement recherchées avec F. Siguier.

3. *Aspects histologiques*—Une notion essentielle: la moelle est repeuplée ou en voie de restauration, tandis que, lorsque les sujets meurent d'aplasie myélo-lymphoïde due à l'irradiation et sans syndrome secondaire, la moelle est vide de cellules myéloïdes; on peut retrouver à l'examen histologique un excès de cellules hyperbasophiles. Le second signe important et essentiel pour le compréhension de la pathogénie du syndrome secondaire réside dans les infiltrations de l'épithélium de multiples tissus par des cellules du type lymphoïde. Dans les environs des cellules infiltrantes, on note diverses lésions dégénératives des cellules épithéliales. Il nous semble que ce phénomène traduit directement l'attaque, par les cellules du donneur, des tissus de l'hôte (Figs. 8, 9, 12, 18).

De tous les tissus, le tissu lymphoïde est celui qui présente les lésions les plus marquées. Celles-ci commencent par une phase d'hyperplasie, qui peut d'ailleurs aboutir à l'apparition de discrètes adénopathies, essentiellement spinales. Celles-ci sont dues à une prolifération d'éléments souvent hyperbasophiles et que le microscope électronique révèle très riches en ribosomes et dépourvus d'ergastoplasme (Fig. 15). Cet aspect est superposable à celui observé histologiquement par Mathé et Amiel [37]; Congdon et Goodman [38], et en microscopie électronique par Binet et Mathé [39] et Mathé *et al.* [40], dans le syndrome secondaire aigu provoqué chez la Souris par greffe semi-allogénique de cellules lymphoïdes, et chez le Singe traité avec moelle homologue (De Vries *et al.* [17]). Des éosinophiles peuvent infiltrer ces ganglions. La phase d'hyperplasie est suivie d'une atrophie secondaire. Les adénopathies disparaissent et les ganglions se réduisent ultérieurement à leur structure réticulo-endothéliale. Ils sont dépourvus de lymphocytes et de follicules germinatifs (Fig. 6).

La rate présente les mêmes lésions d'aplasie lymphoïde que les ganglions. Elle peut également présenter des nécroses, dont il est impossible actuellement de déterminer l'origine (infection, lésions vasculaires).

La lymphopénie concerne également les plaques de Peyer de l'intestin. Mais les lésions ne se limitent pas dans cet organe, au tissu lymphoïde: elles concernent surtout les cryptes, qui peuvent être atteintes de désintégration et de desquamation des cellules épithéliales, aboutissant à une disparition totale de l'épithélium, des cryptes et de la surface (Figs. 11 et 12).

Les lésions cutanées sont également très évocatrices. Elles consistent d'une part en infiltrats dermiques par des cellules mononucléées, singulièrement des histiomonocytes, ces infiltrats étant, en particulier, périvasculaires (Figs. 8, 9 et 18). Les cellules lymphoïdes envahissent l'épiderme et, dans les environs immédiats de ces cellules, on note des vacuolisations des cellules épithéliales et un type de nécrose caractéristique: la dyskératose (Figs. 1 et 18). Les autres lésions de l'épiderme: acanthose, hyperkératose et parakératose, semblent secondaires à l'infiltration lymphoïde et à la dégénérescence épithéliale.

Les lésions des autres tissus ou organes ne sont pas systématisées, mais variables de l'un à l'autre. C'est ainsi que le rein et le coeur sont généralement normaux, que le poumon est fréquemment atteint, mais de lésions infectieuses secondaires (pneumonie virale? pneumonie mycosique . . .). Le foie, par contre, semble atteint de façon plus spécifique: chez B.B., une hépatomégalie a accompagné le premier syndrome secondaire après la greffe de moelle osseuse; elle a disparu pour réapparaître au cours de la récidive du syndrome secondaire, provoquée, cette fois, par l'injection de leucocytes du donneur dont la greffe de moelle avait pris. Une ponction-biopsie a alors révélé la présence d'infiltrats à cellules mononucléaires, ressemblant à des histiomonocytes (Fig. 14), riches en ribosomes, comme l'a révélé la microscopie électronique (Fig. 15). L'absence d'une importante altération hépatocytaire suggère fortement qu'il ne s'agit pas d'une hépatite virale, mais bien d'un désordre immunitaire

traduisant la réaction du greffon contre l'hôte. Les infiltrats cellulaires ne résument cependant pas les lésions hépatiques possibles du syndrome secondaire; des cellules hépatiques nécrotiques dispersées dans le parenchyme et, moins fréquemment, des foyers de nécrose confluents peuvent être observés. Il faut souligner la fréquence de l'atteinte du foie au cours du syndrome secondaire, ce qui a d'ailleurs été observé chez l'animal, en particulier dans notre expérience (Mathé et Amiel [19]; Congdon et Kretchmar [41]).

4. *Désordres biochimiques* — Divers désordres biochimiques ont été enregistrés. Ils ne font, pour la plupart, que refléter les désordres cellulaires ou viscéraux déjà observés morphologiquement.

(*a*) Parmi les plus importants, s'inscrivent les désordres des immunoglobulines. Nous avons observé fréquemment une baisse d'intensité variable des trois immunoglobulines γ-1-A, γ-1-M et γ_2. Un des malades (D.V.) a cependant présenté une hyper-γ-globulinémie très importante (Tableau 4). Ces faits s'expliquent à la lumière d'une expérience que nous avons conduite chez la Souris, qui nous a montré que les désordres des γ-globulines évoluent, comme ceux du tissu lymphoïde (*vide supra*), en deux temps: une phase que caractérise un taux normal ou élevé, une de diminution (Amiel *et al.* 41 bis). Il est probable que la première correspond à la période maximale de réaction du greffon contre l'hôte, l'autre à l'aplasie lymphoïde séquelle.

(*b*) L'étude de divers enzymes sériques chez les malades a pu mettre en évidence d'importantes anomalies (Schwarzmann [42]). Celles-ci ont toujours été de pair avec des désordres hépatiques, et il est probable qu'elles ne font que les traduire. Le Tableau 12 donne un exemple des perturbations observées chez le malade B.B. Les taux de la lacticodéshydrogénase (LDH), de la transaminase glutamo-oxaloacétique (GOT), de la transaminase glutamo-pyruvique (GPT), de l'ornitine-carbamyl-transférase (OCT) et de l'aldolase sont notablement augmentés le 29 mai. On notera: (*a*) que le taux de la transaminase GOT est entre 3 et 4 fois supérieur à celui de la GPT, ce qui est à l'opposé de ce que l'on observe dans les hépatites virales, cette dissociation ne se retrouve toutefois pas lors du syndrome réactivé par les injections de globules blancs; (*b*) que l'augmentation de l'aldolasémie est, par contre, du même ordre que celle observée habituellement dans les hépatites; que l'augmentation du taux de l'OCT prouve la réalité de l'atteinte hépatique, cette enzyme n'existant que dans le foie.

L'étude de ces enzymes dans le sérum de souris radiochimères allogéniques a été tentée, mais rendue impossible par la constante hémolyse du sang après son prélèvement.

(*c*) Nous n'avons pas dosé dans le sérum la sérine et la glycine, dont Kretchmar et Congdon [43] ont trouvé les concentrations abaissées chez des souris chimères allogéniques; nous n'avons pu davantage mesurer dans les tissus les taux de ces substances (qu'ils trouvent diminuées dans le foie et les muscles) ni celui de l'acide aspartique (qu'ils trouvent augmenté dans le foie), ni ceux du glutathion, de la glutamine (qu'ils trouvent abaissés dans le foie), ni ceux de la β-alanine et de l'acide β-amino-isobutyrique (qu'ils trouvent élevés dans cet organe) (Kretchmar et Congdon [44, 45]).

Nous avons, par contre, étudié les concentrations de divers acides organiques dans le sang, et leur élimination dans l'urine, et n'avons trouvé aucune anomalie significative et constante (Tableau 12) . Une étude parallèlement menée chez la Souris (Nordmann *et al.* [46]) a révélé des modifications significatives du taux sanguin de l'acide l-malique et de la nicotinamide-adénine-dinucléotide (NAD). Si les taux sanguins de l'acide lactique et de l'acide pyruvique ne montrent pas, après greffe de moelle allogénique, de différences notables avec ceux de témoins, par contre, les deux acides α-céto-glutarique et l-malique, et le coenzyme NAD, voient leur taux nettement abaissé dans le sang d'animaux ayant reçu une greffe de cellules hématopoïétiques allogéniques. L'acide α-céto-glutarique voit également son taux diminué au cours d'une greffe isogénique. Si bien que l'acide l-malique et le coenzyme NAD signent, par leur abaissement, plus particulièrement le syndrome secondaire; il est à noter que cette étude a également noté une diminution très nette du NAD dans le foie des souris, à condition que le prélèvement soit précédé d'un jeune de 24 h. Le syndrome secondaire s'accompagne donc d'une perturbation de la synthèse ou d'un excès du catabolisme de ce coenzyme fondamental, perturbation qui pourrait être liée à un défaut d'ATP, substrat nécessaire à la biosynthèse du NAD.

E. *Aspects évolutifs*

Plusieurs aspects évolutifs du syndrome secondaire peuvent être distingués.

La forme tardive et bénigne est illustrée par les observations des patients D.V. et V.L. L'état du malade demeure satisfaisant pendant un mois après la restauration hématologique. Puis

apparaît un ensemble de manifestations dominées par l'aplasie lymphoïde et les infections. Les symptômes cliniques disparaissent au bout d'un mois, lorsque la lymphocytose se restaure.

Les formes précoces sont illustrées par les observations des malades E.D., M.V., B.B., M.J. Elles sont très sévères et caractérisées par l'association de deux types de symptômes: ceux qui, comme dans le cas précédent, sont liés à l'aplasie lymphoïde et ses conséquences infectieuses, et ceux qui témoignent plus directement de la réaction des cellules immunologiquement compétentes du greffon contre l'hôte, telles que l'érythrodermie et les atteintes hépatique et intestinale.

Ces formes précoces sont de deux types, selon que le syndrome prend son départ alors que la restauration myéloïde est accomplie (cas de E.D. et de M.J.), ou pendant que celle-ci s'accomplit, voire à son tout début (cas de B.B.). Les documents histologiques de ces derniers cas démontrent la réalité de ces formes, que les symptômes cliniques suggéraient. On sait, par ailleurs, que, chez la Souris, la réaction du greffon contre l'hôte si elle est induite par des cellules lymphoïdes, chez des animaux irradiés à dose létale ou 50% létale (suivie de transfusion de cellules médullaires) peut s'exprimer très précocement, avant la restauration myéloïde (Mathé et Amiel [37]). Ce phénomène de mort par syndrome secondaire aigu avant la restauration myéloïde peut même s'observer après simple transfusion de moelle osseuse allogénique chez des souris irradiées à dose létale; il suffit, pour cela, d'augmenter le nombre de cellules médullaires (Amiel *et al.* [22]). Le fait que le syndrome secondaire puisse être précoce au point de tuer tous les receveurs de moelle osseuse allogénique avant la restauration myéloïde, a également été démontré chez le Singe (De Vries *et al.* [17, 35]).

Il est possible que plusieurs des constants échecs de greffe de moelle allogénique mentionnés par diverses équipes après irradiation totale chez l'Homme (Thomas *et al.* [47]) aient concerné des cas compliqués de syndromes secondaires très précoces; c'est ce qui ressort en particulier à la lecture de compte-rendus de certains essais de Parra [48].

A noter, enfin, l'existence de *formes latentes*, que ne révèlent que la survenue d'une infection: c'est ainsi que l'enfant J.P.C., dont la restauration s'était faite facilement, et dont l'évolution se poursuivait sans manifestations de syndrome secondaire, est mort au troisième jour d'une varicelle; il est vrai que les stéroïdes surrénaliens suffisent à fragiliser les sujet à l'égard de cette affection [32, 33]. C'est ainsi que le patient B.B., alors que le syndrome secondaire induit par les transfusions de leucocytes semblait contrôlé, a fait un zona d'une sévérité exceptionnelle, sévérité que nous n'avons jusqu'ici rencontrée que chez des sujets atteints d'insuffisance lymphocytaire, parce que porteurs de leucémie lymphocytaire chronique ou de maladie de Hodgkin.

F. *Physiopathologie*

Le syndrome secondaire chez l'Homme pose trois problèmes physiopathologiques: (*a*) le processus qui l'induit; (*b*) le mécanisme des divers symptômes; (*c*) ses rapports avec les maladies diverses par auto-immunisation.

(*a*) Pour répondre à la première question, on dispose maintenant chez l'Homme des mêmes documents et arguments qui ont prouvé, chez l'animal (Van Bekkum *et al.* [49]; Denko *et al.* [50]; Mathé et Amiel [19, 51]), le rôle essentiel de la réaction du greffon contre l'hôte.

La responsabilité de l'irradiation est éliminée par le fait que: (*a*) un tel syndrome n'a jamais été observé après irradiation sublétale, même à des doses de 400 à 600 rads (Küss *et al.* [30]); (*b*) après irradiation létale, il n'a jamais été observé, après une transfusion de moelle osseuse autologue (Kurnick *et al.* [52, 53]; Mac Govern *et al.* [54]) ou isogénique (Atkinson *et al.* [55]; Thomas *et al.* [56], cas N.P.); (*c*) il n'a pas été rencontré davantage, après une telle irradiation, suivie de transfusion de moelle osseuse allogénique, lorsque la moelle ne présente aucun symptôme de restauration (Mathé *et al.* [2, 3, 57]); (*d*) ce syndrome peut être enfin provoqué par des transfusions de leucocytes, chez des malades non irradiés.

En faveur de la responsabilité de la réaction du greffon contre l'hôte, s'inscrivent trois arguments: (*a*) l'identité des lésions histologiques observées chez l'Homme à celles décrites chez l'animal (De Vries *et al.* [17, 35]); (*b*) la reproduction, chez B.B., du syndrome secondaire par injection de globules blancs du donneur de moelle osseuse qui a pris, et à l'égard duquel la tolérance spécifique est démontrée par une greffe de peau; (*c*) enfin la démonstration, toujours chez B.B., que l'individu chimère ne produit plus ses propres anticorps, mais ceux du greffon: la preuve en a été apportée par l'étude des groupes d'immunoglobulines Inv. (Tableau 17).

S'il est raisonnable d'attribuer à la réaction du greffon contre l'hôte la responsabilité essentielle du syndrome secondaire, il n'en reste pas moins possible que l'irradiation puisse ajouter à son action, en intensifiant telle ou

Tableau 17. *Phénotypes sériques du receveur et des donneurs chez BB.*

Receveur *avant*:	B.B.	Gm (a−b+x−e+) *Inv* (1+*a*+*b*+)
Donneurs:	H.B.	Gm (a−b+x−e+) Inv (1−a−b+)
	D.B.	Gm (a−b+x−e+) Inv (1−a−b+)
	M.B.	Gm (a−b+x−e+) Inv (1−a−b+)
	P.B.	Gm (a−b+x−e+) Inv (1−a−b+)
	F.B.	Gm (a−b+x−e+) Inv (1−a−b+)
Receveur *après*:	B.B.	Gm (a−b+x−e+) *Inv* (1−*a*−*b*+)

telle lésion, en particulier l'aplasie de la série lymphocytaire et la nécrose des cryptes intestinales; l'effet de la réaction du greffon contre l'hôte s'exerçant sur des tissus déjà affectés par l'irradiation, et alors que commence la restauration, est probablement plus marqué.

Le Tableau 16, représentant chez 14 malades la date d'apparition de diverses manifestations cliniques par rapport à la date de la transfusion de moelle osseuse ou de leucocytes, donne des résultats qui rejoignent ceux de Van Putten [58] quant à la date d'apparition du syndrome secondaire chez le Singe, du cinquième au dixième jour.

Le schéma de la Fig. 22 résume nos conceptions actuelles sur la physiopathologie du syndrome secondaire. La réaction du greffon contre l'hôte entraîne d'abord une prolifération de cellules à cytoplasme hyperbasophile, que l'on peut observer dans le tissu lymphoïde, qui entraîne diverses lésions dégénératives de l'épithélium, du derme, du foie et de l'intestin. Il y a également tout lieu de penser que cette réaction du greffon contre l'hôte est responsable de la dégénérescence des cryptes intestinales qui peut être produite chez la Souris, sans irradiation (Brocades Zaalberg [59]), et de l'anémie qui est liée à la production d'anticorps circulants par les cellules immunologiquement compétentes du greffon, comme cela a été démontré chez l'animal (Porter [13–15]).

Il est cependant probable que beaucoup des lésions observées ne sont pas spécifiques. C'est ainsi que l'aplasie lymphocytaire, par le défaut d'immunoglobulines qu'elle entraîne, comme nous l'avons montré chez la Souris (Amiel *et al.* [41 bis]) et comme le suggère l'observation de M.V. (Mathé *et al.* [3]), ainsi que par la perte de la mémoire immunitaire (Mathé *et al.* [34]), favorise les infections, comme peuvent encore le faire les lésions cutanées, lesquelles infections sont, à leur tour, à l'origine de diverses atteintes tissulaires ou viscérales (cutanées, intestinales, pulmonaires, hépatiques).

Parce que cet ensemble pathologique peut être considéré comme lié essentiellement à la réaction du greffon contre l'hôte, certains auteurs n'ont pas hésité à le considérer comme un modèle de "maladie par auto-anticorps" (Stastny *et al.* [60]), et à en déduire que la raison d'une telle affection réside dans une mutation d'un clone de cellules immunologiquement compétentes. La connaissance, maintenant approfondie du syndrome secondaire chez l'Homme, doit-elle conduire à soutenir ou à critiquer cette hypothèse? Si certaines lésions rappellent celles observées dans certaines maladies dont on a pu suggérer une origine auto-immunitaire sans toutefois en apporter la moindre preuve sérologique convaincante, l'infiltration cellulaire hépatique, qui rappelle celle de certaines hépatites chroniques (Schwartzman [61]), le syndrome cutané, qui rappelle d'une part le lupus érythémateux, d'autre part le pityriasis rubra de Hebra compliquant la leucémie lymphocytaire chronique (Miller [62]; Mathé et Seman [63]), si certains malades ont présenté une anémie avec auto-anticorps (D.V.), il faut bien reconnaître que l'on est surtout frappé par les différences entre ces symptômes et lésions du syndrome secondaire et les symptômes et lésions que l'on est aujourd'hui autorisé à attribuer à des auto-anticorps: on ne trouve le plus souvent pas d'anticorps anti-érythrocytaires; nous n'avons, avec F. Siguier, jamais mis en évidence de cellules L.E. chez nos malades ni chez des souris chimères allogéniques ; le rein et les vaisseaux ne sont presque jamais affectés dans le syndrome secondaire; l'aplasie lymphocytaire, son syndrome majeur, n'a jamais été signalé dans les maladies par auto-anticorps; il en est de même de l'hypo-γ-globulinémie. Il convient donc, pour le moins, de s'abstenir d'expliquer les "maladies par auto-anticorps" selon des mécanismes faisant appel à une analogie qui nous paraît loin d'être démontrée, avec le syndrome secondaire (Berger et Delarue [64]).

G. *Syndrome secondaire et effet anti-leucémique*

Si le syndrome secondaire est une conséquence néfaste de la réaction du greffon contre l'hôte, au point de représenter la principale pierre d'achoppement actuelle à l'usage de cette méthode au traitement des leucémies (Mathé [21]), on peut cependent attendre de cette réaction des effets bénéfiques, précisément dans le traitement de ces néoplasies.

Soit un sujet "A" porteur de cellules leucémiques "a", la leucémie étant éventuellement dûe à un virus "α"; une irradiation totale à la dose de 800 rads détruit un pourcentage élevé de cellules leucémiques et conditionne le sujet pour une greffe de moelle osseuse d'un sujet "B"; celle-ci restaure la moelle osseuse par la prolifération de ses cellules myéloïdes, et réalise une immunothérapie adoptive à l'égard de la leucémie, par la réaction de ses cellules immunologiquement compétentes à l'égard des cellules "a" d'une part, comme cela a été montré dans les leucémies (Barnes et Loutit [8]; De Vries et Vos [65]; Mathé et Bernard [2]; Mathé *et al.* [66]) et autres tumeurs greffées (Woodruff *et al.* [67]) et spontanées (Mathé et Bernard [68]; Mathé *et al.* [69]), d'autre part à l'égard du virus, comme nous l'avons montré sur la leucémie virale de Charlotte Friend (Mathé *et al.* [70]; Mathé *et al.* [71]): la réaction antivirale, non seulement exerce une action anti-leucémique (Mathé *et al.* [70]), mais peut encore éradiquer le virus dans le sang ou diminuer sa concentration (Mathé *et al.* [71]). Il n'est enfin pas déraisonnable d'espérer que, si la greffe a réussi à repeupler les tissus myéloïdes et lymphoïdes à 100% (Mathé *et al.* [53]) si elle est définitive et si le virus est spécifique d'individu ou de lignée, comme c'est le cas pour certaines leucémies de la Souris (Gross [73]; Friend [74]), le virus α ne sera pas capable d'induire une leucémie "b" chez cet individu devenu hématopoïétiquement "B".

Nos premiers résultats chez l'Homme sont encourageants à cet égard. Il convient de distinguer, d'une part, deux patients qui n'ont eu qu'une greffe de moelle osseuse transitoire, rejetée au bout de 3 mois, et, d'autre part, le sujet demeuré chimère à 100% durant 20 mois.

Chez les deux premiers patients, une rémission apparemment complète a été observée: le premier, dont la leucémie évoluait depuis 26 mois, et qui avait bénéficié de trois remissions dont les durées respectives avaient été de 12 mois, 5 mois et 3 semaines eux une survie de 12 mois, dont 9 mois de rémission complète; le second, dont la leucémie évoluait depuis 13 mois, et qui avait bénéficié d'une première rémission de 8 mois et d'une seconde rémission de 3 semaines, laissant alors la place à une rechute qui évoluait au moment où l'essai fut entrepris, eut une survie de 9 mois, dont 5 mois de rémission complète.

Le troisième malade, dont la leucémie évoluait depuis 20 mois, et qui avait bénéficié d'une première rémission de 11 mois et d'une seconde d'un mois, était en rémission complète 20 mois après l'irradiation, lorsqu'il mourut des suites d'une encéphalite zostérienne.

La comparaison des résultats obtenus chez les deux premiers malades, avec ceux enregistrés chez N.P. après greffe de moelle isogénique suivant la même irradiation totale, est en faveur de la réalité d'un effet immunitaire complémentaire à celui de l'irradiation.

Celui du patient B.B., souligne déjà cependant l'intérêt d'obtenir par une greffe définitive et totale, non seulement l'effet immunitaire, mais encore le changement de la constitution immunogénétique des tissus hématopoïétiques.

Un effet anti-leucémique également remarquable semble pouvoir être attribué aux transfusions de leucocytes; une rémission a été obtenue chez trois sur six des patients atteints de leucémie aiguë traités. Il s'agit certes de rémissions de courte durée (C.A.: 3 semaines, G.M.: 2 mois, P.T. est actuellement en rémission après 2 mois), mais apparemment complètes, les lymphoblastes disparaissant du sang et de la moelle osseuse, on note que si, dans tous les cas, un traitement par substance chimique se trouvait associé aux transfusions de leucocytes, la maladie se comportait antérieurement comme résistante à ce traitement. Un tel résultat n'a pas été obtenu par Freireich *et al.* [75–76]. De même, ces auteurs ne mentionnent pas de manifestations de syndrome secondaire, ce qui suggère que les transfusions de leucocytes qu'ils pratiquentnes' accompagnent pas, comme dans le cas de notre expérience, de réaction du greffon contre l'hôte. Or, la raison pourrait en résider dans le fait que ces auteurs administrent, en même temps que ces transfusions, du méthotrexate, qui est connu pour inhiber cette réaction (Uphoff [77]; Mathé *et al.* [66]; Thomas *et al.* [78]; Lochte *et al.* [79]).

H. *Les progrès dans le contrôle*

1. *Traitement curatif* — Le contrôle du syndrome secondaire chez l'Homme est de difficulté variable selon ses modalités d'expression.

C'est ainsi que les cas de syndromes tardifs apparus chez D.V. et V.L., dominés par quelques infections et des troubles digestifs

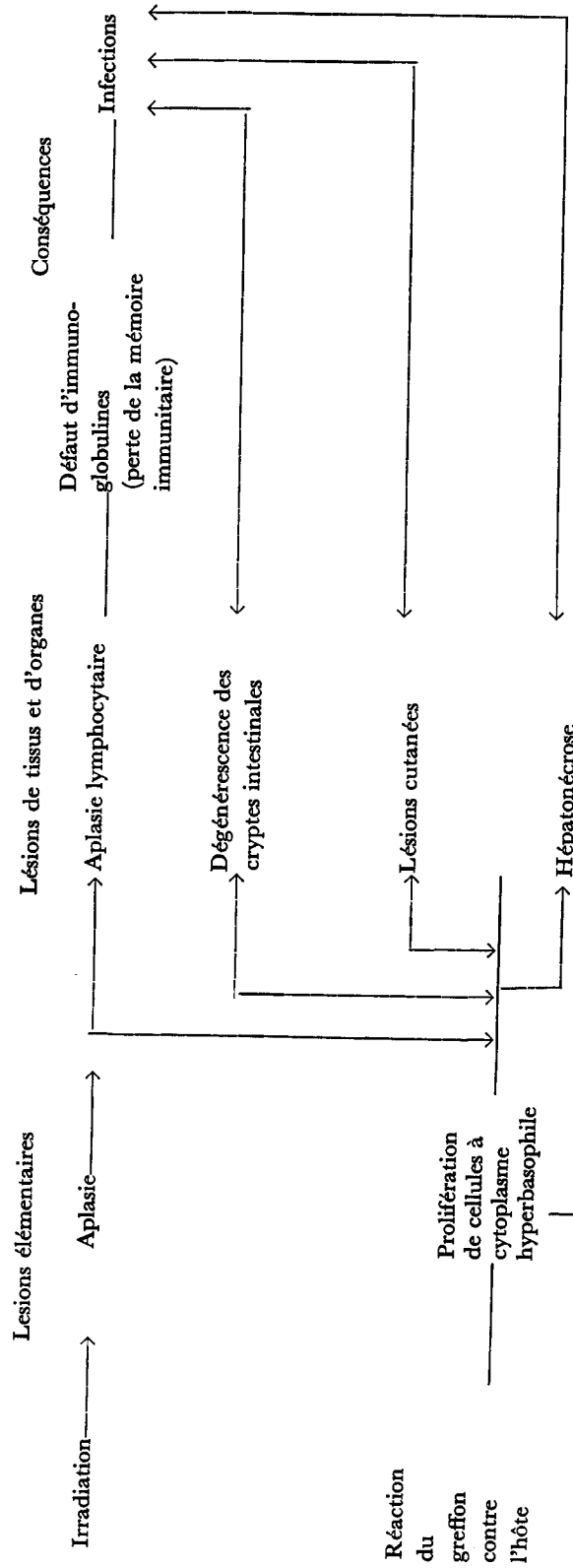


Fig. 22. Schéma de la physiopathologie du syndrome secondaire.

d'insuffisance sécrétoire et d'absorption, ont parfaitement répondu aux traitements symptomatiques, antibiotiques et préparations diverses de ferments digestifs.

Par contre, les cas de syndromes aigus ont été de controle difficile ou impossible: l'administration des antibiotiques indiqués par les examens microbiologiques ou à large spectre d'action, de γ-globulines, sont loin de parvenir toujours à dominer les infections. Dans le cadre physiopathologique, l'irradiation totale de 50 à 100 rads, les stéroïdes cortico-surrénaliens, l'acide ε-amino-caproïque (substance indiquée comme douée d'une action inhibitrice des réactions immunologiques (Gilette *et al.* [80]), mais qui ne s'est pas révélée, entre nos mains, active chez l'animal dans la réaction du greffon contre l'hôte (non publié), ont été, même donnés en association, inefficaces. Chez le patient B.B., le contrôle du syndrome secondaire, pourtant très sévère, semble cependant pouvoir être attribué en partie à la Δ-1-cortisone; une stricte corrélation existe, en effet, entre le début de son administration et la réduction à la normale de l'éosinophilie. Enfin, chez la malade N.P., la transfusion de moelle osseuse isogénique a également fait disparaître tous les symptômes que l'on pouvait attribuer au syndrome secondaire.

2. *Traitement préventif* — La conservation à 37°C, pendant 2 h, des cellules hématopoïètiques qui, chez la Souris, réduit considérablement la fréquence du syndrome secondaire sans diminuer celle de la restauration myéloïde après greffe de moelle osseuse allogénique (Mathé *et al.* [26]), phénomène qui est lié à une extrême sensibilité des cellules immunologiquement compétentes (Amiel et Mathé [27]), n'a été tentée qu'une fois, et, dans ce cas (N.P.), nous n'avons assisté à aucune restauration myéloïde. Il est donc possible que les cellules myéloïdes humaines ne se comportent pas, à cet égard, comme leurs équivalentes chez la Souris; une expérience plus étendue est cependant nécessaire. Toutefois, une transfusion de leucocytes conservés à −70°C a été suivie d'une élévation très nette du chiffre des granulocytes dans le sang périphérique (atteignant pendant 15 jours, 30.000 mm³) (C.A.) et ne s'est accompagnée que d'un syndrome secondaire très net mais transitoire; les cellules immunologiquement compétentes étant sensibles à ce genre d'agression (Schwarzenberg *et al.* [81]).

On peut attendre qu'un meilleur choix du donneur puisse réduire la fréquence et l'intensité du syndrome secondaire; des travaux sont en cours dans notre laboratoire, qui comparent la valeur, chez l'Homme: (*a*) de l'étude des antigènes leucocytaires selon la méthode de Colombani *et al.* [82]; (*b*) du test d'histocompatibilité indirecte que nous avons proposé (Mathé *et al.* [83]; Matsukura *et al.* [84]); (*c*) du test de Brent et Medawar [85], ce dernier inutilisable d'ailleurs dans le cas de la greffe de cellules hématopoïétiques chez des leucémiques, car il risque, d'une part, de transmettre aux donneurs sains des facteurs leucémigènes ou d'autres virus (de l'hépatite par exemple) et, d'autre part, d'immuniser ces donneurs, et partant, d'intensifier le syndrome secondaire. (*d*) enfin, le test de transformation *in vitro* des cellules lymphoïdes en cellules hyperbasophiles, proposé par Bach et Hirschborn [87].

Les premiers résultats de cette étude paraissent encourageants pour les deux premiers, décevants pour les deux derniers (Brézin *et al.* [86]).

Aussi attendons-nous beaucoup plus des deux méthodes suivantes: l'emploi de donneurs multiples et le traitement par l'améthoptérine.

L'emploi de donneurs multiples s'est avéré dans nos mains (Mathé *et al.* [88, 89]) très efficace chez la Souris, puisque, après la transfusion de la moelle osseuse de quatre donneurs, deux proches (mêmes gènes au locus H-2), deux éloignés (gènes différents au locus H-2), la fréquence des prises fut, pour un même nombre total de cellules, aussi élevée que celle obtenue avec le meilleur donneur, et la fréquence du syndrome secondaire aussi basse. L'étude de la tolérance spécifique liée au chimérisme a révélé que prenait la greffe du (ou des) donneur(s) le(s) plus proche(s) quant à l'histocompatibilité. Appliquant cette méthode chez l'Homme (patient B.B.), nous avons pu obtenir une greffe totale, portant sur le donneur que l'étude des antigènes leucocytaires et le test d'histocompatibilité avaient permis de considérer comme le plus proche du receveur, et le syndrome secondaire, qui fut certes sévère, put cependant être controlé. Un second essai chez l'Homme, au cours duquel fut transfusée la moelle de cinq donneurs, aboutit à une restauration rapide; le patient étant décédé secondairement de varicelle, il n'est pas possible d'en tirer des conclusions à tous les égards. Il est cependant remarquable de constater qu'aucune des manifestations du syndrome secondaire n'est apparue, et que l'état du patient était excellent lorque survint la varicelle (Fig. 13). Ce cas est d'interprétation complexe, car le sujet fut traité après la

transfusion de moelle osseuse par l'améthoptérine, divers travaux expérimentaux (Uphoff [77]; Mathé *et al.* [66]; Thomas *et al.* [78]) ayant montré que l'administration de ce produit après la greffe était capable de réduire la fréquence et la sévérité du syndrome secondaire. Il est donc possible que l'absence des manifestations habituelles de celui-ci relève de cette double tentative de traitement préventif: donneurs multiples, améthoptérine.

La malade M.J. qui présenta après greffe de leucocytes de leucémie myéloïde chronique, un syndrome secondaire aigu mortel avait été transfusée avec une quantité très importante de cellules granulocytaires ($7{,}99.10^{11}$, soit 160 fois plus que le nombre de cellules médullaires reçues par B.B.), provenant toutes de la même donneuse. L'administration d'améthoptérine ne parut dans ce cas efficace que durant 15 jours.

RÉSUMÉ

Quatorze observations de syndrome secondaire chez l'Homme, compliquant des transfusions de moelle osseuse allogénique après irradiation totale à dose létale, ou des transfusions de leucocytes, sont analysées.

Les signes cliniques (généraux, digestifs, hépatiques, cutanés, lymphoïdes, viscéraux divers), les signes hématologiques (anémie, lymphocytopénie, éosinophilie), les lésions histologiques (prolifération de cellules hypobasophile, aplasie lymphoïde, nécroses), les désordes biochimiques (anomalies des taux sériques des immunoglobulines et de diverse enzymes sériques) sont décrits et comparés à ceux observés dans diverses espèces animales.

Les données enregistrées chez l'Homme concourent à démontrer que le processus physiopathologique essentiel réside, comme chez l'animal, dans la réaction du greffon contre l'hôte.

Cette réaction, si elle est néfaste puisque responsable du syndrome secondaire, peut être utilisée dans un sens favorable en tant qu'immunothérapie adoptive de la leucémie: les résultats obtenus à cet égard chez trois patients sont encourageants; l'un d'entre eux, chimère hématopïétique totale (myéloïde et lymphoïde), était en parfait état hématologique mois 20 après.

Des rémissions apparemment complètes ont, par ailleurs, été obtenues après transfusion de leucocytes chez trois patients atteints de leucémie aiguë lymphoblastique sur six traités. Il est suggéré que cet effet antileucémique relève également de la réaction du greffon contre l'hôte.

La valeur des moyens employés pour contrôler le syndrome secondaire est analysée: il semble que l'utilisation de donneurs multiples et l'emploi d'améthopterine après la transfusion des cellules soient d'une réelle efficacité.

SUMMARY

Fourteen cases of secondary syndrome in Man, complicating transfusions of allogeneic bone marrow after total irradiation at lethal dose, or transfusions of leukocytes, are analysed.

The clinical signs (general, digestive, hepatic, cutaneous, lymphoid, various visceral), the haematological signs (anaemia, lymphocytopenia, eosinophilia), the histological lesions (hyperbasophilic cell proliferation, lymphoid aplasia, necroses), the biochemical disorders (anomalies of the serum immunoglobulin levels and of various serum enzymes) are described and compared with those observed in several animal species.

Data collected in Man contribute to prove that the essential physiopathologic process is to be found in the reaction of the graft to the host, as is the case in the animal.

This reaction, while unfavourable since it is the cause of the secondary syndrome, may nevertheless be used as immunotherapy in leukaemia: the results obtained in this respect in three patients are encouraging; one of them, a total haematopoietic chimera (myeloid and lymphoid) showed a normal haematological picture after 20 months.

Apparently complete remissions were obtained after leukocyte transfusions in 3 out of 6 patients with acute lymphoblastic leukaemia. It is suggested that this antileukaemic effect is also due to the reaction of the graft to the host.

The value of the means used to control the secondary syndrome is analysed: it seems that the use of multiple donors and of amethopterin after cell transfusions may be really effective.

ZUSAMMENFASSUNG

Es wird über vierzehn Beobachtungen des s.g.sekundären Syndroms bei dem Menschen, welches die, nach der, bei tödlicher Dosis durchgeführten, Ganzkörperbestrahlung erfolgte allogene Knochenmarkstransfusion, oder die Leukozytentransfusion erschwert, berichtet.

Die klinische Zeichen (allgemeine, oder von seiten des Verdauungstraktes, der Leber, der Haut, des lymphatischen Systems und der verschiedenen Eingeweide), die hämatologischen Veränderungen (Anämie, Lymphocytopenie, Eosinophilie), die histologischen Schäden (histiomonocytäre Proliferation, lymphatische Aplasie, Nekrosen), sowie die biochemischen Störungen (Abweichungen in der Konzentration der Immunoglobuline und der verschiedenen Enzyme im Serum) werden beschrieben und mit denen, in verschiedenen Tiergattungen beobachtet, verglichen.

Die bei dem Menschen festgestellten Angaben tragen dazu bei, um zu beweisen, dass der wesentliche patho-physiologische Vorgang, wie bei dem Tiere, in der Reaktion des Transplantates gegen den Wirt besteht.

Diese Reaktion, wenn auch schädlich, da sie für das sekundäre Syndrom verantwortlich ist, kann in einem günstigen Sinne als angepasste Immunotherapie der Leukämie verwendet werden; die diesbezüglichen, bei drei Patienten erreichten Resultate sind ermutigend: einer von ihnen, eine vollständige Blutschimäre, (sowohl myeloisch, wie auch lymphatisch), befand sich nach 20 Monaten in tadellosem hämatologischem Zustand.

Scheinbare vollständige Besserungen wurden nach Leukocytentransfusion bei drei, von sechs behandelten Kranken, die alle an akuter lymphoblastischer Leukämie litten, erreicht. Es wird hervorgehoben, dass diese antileukämische Wirkung ebenfalls in der Reaktion des Transplantates gegen den Wirt besteht.

Man untersucht den Wert der zur Kontrolle des sekundären Syndroms angewandten Mittel; es scheint, dass die Anwendung mehrerer Spender, sowie die Verabreichung von Amethopterin nach der Zelltransfusion wirklich wirksam ist.

BIBLIOGRAPHIE

1. G. MATHÉ, H. JAMMET, B. PENDIC, L. SCHWARZENBERG, J. F. DUPLAN, B. MAUPIN, R. LATARJET, M. J. LARRIEU, D. KALIC et Z. DJUKIC, Transfusions et greffes de moelle osseuse homologue chez des humains irradiés à forte dose accidentellement. *Rev. Franç. Etud. Clin. Biol.* **4,** 226 (1959).
2. G. MATHÉ, J. BERNARD, L. SCHWARZENBERG, M. J. LARRIEU, C. M. LALANNE, A. DUTREIX, P. DENOIX, J. SURMONT, V. SCHWARZMAN et B. CEOARA, Essai de traitement de sujets atteints de leucémie aiguë en rémission par l'irradiation totale suivie de transfusion de moelle osseuse homologue. *Rev. Franç. Etud. Clin. Biol.* **4,** 675 (1959).
3. G. MATHÉ, J. BERNARD, M. J. de VRIES, L. SCHWARZENBERG, M. J. LARRIEU, C. M. LALANNE, A. DUTREIX, J. L. AMIEL et J. SURMONT, Nouveaux essais de greffe de moelle osseuse homologue après irradiation totale chez des enfants atteints de leucémie aiguë en rémission. Le problème du syndrome secondaire chez l'Homme. *Rev. Hémat.* **15,** 115 (1960).
4. G. MATHÉ, J. L. AMIEL, L. SCHWARZENBERG, A. CATTAN et M. SCHNEIDER, Hematopoïetic chimera in man after allogenic (homologous) bone-marrow transplantation. Control of the secondary syndrome. *Brit. Med. J.* No. 5573, 1633 (1963).
5. G. MATHÉ, J. L. AMIEL, L. SCHWARZENBERG, A. CATTAN, M. SCHNEIDER, M. J. DE VRIES, M. TUBIANA, C. LALANNE, A. DUTREIX, J. L. BINET, M. PAPIERNIK, G. SEMAN, M. MATSUKURA, A. M. MERY, V. SCHWARZMAN et A. FLAISLER, Hematopoietic chimerism following allogenic bone marrow transplantation in man. *Blood* **25,** 179 (1965).

6. G. Mathé, J. L. Amiel, L. Schwarzenberg, A. Cattan et M. Schneider, Attempt and application of adoptive immunity in the treatment of acute leukemia. *Conf. Acute Leukaemia, Warrenton*, 1964. *Cancer Res.* Sous presse (1965).
7. D. W. H. Barnes, C. E. Ford, P. L. T. Ilbery, P. C. Koller et J. F. Loutit, Tissue transplantation in the radiation chimera. *J. Cell. Comp. Physiol.* **50,** (suppl. 1), 123 (1957).
8. D. W. H. Barnes et J. F. Loutit, Treatment of murine leukaemia with X-rays and homologous bone-marrow. *Brit. J. Haemat.* **3,** 241 (1957).
9. D. W. Van Bekkum et O. Vos, Immunological aspects of homo- and heterologous bone-marrow transplantation in irradiated animals. *J. Cell. Comp. Physiol.* **50,** 139 (1957).
10. L. J. Cole, R. M. Garver et J. P. Kurnick, Lethal graft-versus-host reaction induced in X-irradiated F1 hybrids by parental strain leukocytes. *Transplant. Bull.* **6,** 429 (1959).
11. H. Balner, Bone-marrow transplantation after whole-body irradiation. An experimental study on the rat. Un vol. Levisson, La Haye (1964).
12. H. Balner, M. J. de Vries et D. W. Van Bekkum, Secondary disease in rat radiation chimeras. *J. Nat. Cancer Inst.* **32,** 419 (1964).
13. K. A. Porter, Immune haemolysis in rabbit radiation chimaeras. *Brit. J. Exp. Path.* **41,** 72 (1960).
14. K. A. Porter, Graft-versus-host reactions in the rabbit. *Brit. J. Cancer* **14,** 66 (1960).
15. K. A. Porter, Immune hemolysis: a feature of secondary disease and runt disease in the rabbit. *Ann. N.Y. Acad. Sci.* **87,** 391 (1960).
16. E. D. Thomas, C. A. Ashley, H. L. Lochte, A. Jaretzki, O. D. Sahler et J. W. Ferrebee, Homografts of bone-marrow in dogs after lethal total-body irradiation. *Blood* **14,** 720 (1959).
17. M. J. de Vries, B. G. Crouch, L. M. Van Putten et D. W. Van Bekkum Pathologic changes in irradiated monkeys treated with bone-marrow. *J. Nat. Cancer Inst.* **27,** 67 (1961).
18. B. G. Crouch, L. M. Van Putten, D. W. Van Bekkum et M. J. de Vries, Treatment of total-body-X-irradiated monkeys with autologous and homologous bone-marrow. *J. Nat. Cancer Inst.* **27,** 53 (1961).
19. G. Mathé et J. L. Amiel, La Greffe. Aspects Histologiques et Cliniques. Un vol. Masson, Paris (1962).
20. G. Mathé, Transfusion et Greffe de Cellules myéloïdes Chez l'Homme. In: *Biological Problems of Grafting*, Un vol., p. 214, Blackwell, Oxford (1959).
21. G. Mathé, Secondary syndrome: a stumbling block in the treatment of leukaemia by whole-body irradiation and transfusion of allogenic hematopoietic cells. In: *Diagnosis and Treatment of Acute Radiation Injury*, Un vol., p. 191, O.M.S. Genève (1961).
22. G. Mathé, Irradiation totale et greffe de moelle osseuse chez l'Homme. In: *Cancer, Leucémie et Radiobiologie, Coll. francosoviétique*, Un vol., p. 86 Gauthier-Villars, Paris (1962).
23. G. Mathé, J. L. Amiel et L. Schwarzenberg, Treatment of total body irradiation in man. *N.Y. Acad. Sci.* **114,** 368 (1964).
24. L. Schwarzenberg, G. Mathé, J. de Grouchy, C. de Nava, M. J. de Vries, J. L. Amiel, A. Cattan, M. Schneider et J. R. Schlumberger, Les transfusions de globules blancs. *Israel J. Med. Sci.* Sous presse (1965).
25. C. M. Lalanne et J. Surmont, Une installation de télécobalthérapie pour irradiation totale. *Techn. Hosp.* **45,** 196 (1962).
26. G. Mathé, J. L. Amiel, L. Schwarzenberg et A. M. Mery, A method of reducing the incidence of the secondary syndrome in allogenic marrow transplantation. *Blood* **22,** 44 (1963).
27. J. L. Amiel et G. Mathé, A comparison of the sensitivity to storage at 37°C in tyrode solution of immunologically competent cells and of lymphoïd cells. *Nature, Lond.* **200,** 1224 (1963).
28. G. Mathé, J. L. Amiel et L. Schwarzenberg, *L'Aplasie Myélo-lymphoïde de l'Irradiation Totale*, Un vol. Gauthier-Villars, Paris (1965).
29. M. Papiernik, J. L. Amiel et G. Mathé, Anomalies chromosomiques aprés irradiation totale chez l'Homme. *C. R. Acad. Sci. Paris* **256,** 5232 (1953).

30. R. Küss, M. Legrain, G. Mathé, R. Nedey, M. Tubiana, C. M. Lalanne, L. Schwarzenberg, M. J. Larrieu, M. Maisonnet, F. Basset et P. Delaveau, Etude de quatre cas d'irradiation totale par le Cobalt radioactif (à des doses respectives de 250, 400 et 600 rads) préalable à une transplantation rénale allogénique *Rev. Franç. Etud. Clin. Biol.* **7,** 1028 (1962).
31. H. Jammet, G. Mathe, B. Pendic, J. E. Duplan, B. Maupin, R. Latarjet, D. Kalic, L. Schwarzenberg, Z. Djukic et J. Vigne, Etude de six cas d'irradiation totale aigue accidentelle. *Rev. Franç. Etud. Clin. Biol.* **4,** 210 (1959).
32. C. J. Falliers, S. G. Halberstein et S. C. Bukantz, Effect of rubeola, rubella, varicella, and a viral upper respiratory infection upon the severity of childhood asthma: with a note on the role of corticosteroids. *J. Allergy* **32,** 69 (1961).
33. C. Benatti et G. B. Cavazzuti, Su un caso di varicella emorragica ad esito infansto, insorta in patiente leucemico in corso di terapia cortisonica. *Clin. Pediat., Bologue* **45,** 496 (1963)
34. G. Mathé, J. L. Amiel et G. Daguet, Etudes des agglutinines sériques contre un antigène bactérien chez des radiochimères hématologiques immunisées contre l'antigène avant l'irradiation. *Nouv. Rev. Franc. Hémat.* **1,** 65 (1965).
35. M. J. de Vries et O. Vos, Delayed mortality of radiation chimeras: a pathological and hematological study. *J. Nat. Cancer. Inst.* **23,** 1403 (1959)
36. A. Rodriguez, G. Mathe, J. L. Amiel et J. Bernard, Etude hématologique du sang périphérique de radiochimères allogéniques et de radiochimères isogéniques. *Nouv. Rev. Franç. Hémat.* **1,** 219 (1961).
37. G. Mathe et J. L. Amiel, Aspects histologiques des lésions induites dans les organes hématopoïétiques par l'injection, à des hybrides F1 irradiés, de cellules ganglionnaires d'une lignée parentale. *Rev. Franç. Etud. Clin. Biol.* **5,** 20 (1960).
38. C. C. Congdon et J. W. Goodman, Changes in lymphatic tissues during foreign tissue transplantation, *Int. Symp. Tissue Transplantation* **181,** (1962).
39. J. L. Binet et G. Mathé, Etude en microscopie optique et électronique des "cellules immunologiquement compétentes" au cours des réactions de greffe, *C.R. Acad. Sci. Paris* **253,** 1852 (1961).
40. G. Mathé, J. L. Binet, G. Seman, J. L. Amiel et G. Daguet, Cellules immunologiquement compétentes. Immunité et tolérance. In: *La Tolérance Acquise et la Tolérance Naturelle à l'égard des Substances Antigéniques Définies* Un vol., p. 359. *Colloque CNRS* Paris (1963).,
41. C. C. Congdon et A. L. Kretchmar, Increased liver weight in bone marrow chimeras. *Exp. Molec. Path.* **2,** 277 (1963).
41. *bis.* J. L. Amiel, G. Mathé et M. Pays, Etude quantitative des γ-globulines sériques ches des souris irradieés et restaurées par des cellules hematopoïétiques isogéniques ou allogéniques. *Rev. Franç Etud. Clin. Biol.* **6.** 453 (1961).
42. V. Schwarzmann, N. Berthaux et F. Lapeyraque, Etudes de certaines enzymes au cours du syndrome secondaire qui complique la greffe de cellules hématopoïétiques allogéniques. *Colloque CNRS sur la Greffe de Cellules Hematoproiéyiques Allogéniques, Paris,* 1964, Un vol. CNRS, Paris (1965).
43. A. L. Kretchmar et C. C. Congdon, The levels of free aspartic acid, glycine and serine in tissues of bone-marrow chimeras. *Transplantation* **1,** 298 (1963).
44. A. L. Kretchmar et C. C. Congdon, Biochemical changes and increased liver weight in bone marrow chimeras. *Amer. J. Physiol.* **200,** 102 (1961).
45. A. L. Kretchmar et C. C. Congdon, Le problème métabolique dans la maladie secondaire. *Colloque CNRS sur la Greffe de Cellules Hématopoïétiques Allogéniques,* Paris (1964). (A paraître).
46. R. Nordmann, M. Arnaud et F. Lapeyraque, Etude de certains métabolites au cours du syndrome secondaire qui complique la greffe de cellul.es hématopoiétiques allogéniques. *Colloque CNRS sur la Greffe de Cellules Hématopoïétiques Allogéniques,* Paris 1964. Un vol., CNRS Paris (1965).
47. E. D. Thomas, E. C. Herman, W. B. Greenough, E. B. Hager, J. H. Cannon, O. D. Sahler et J. W. Ferrebee, Irradiation and marrow infusion in leukemia. Observations in 5 patients with acute leukemia treated by whole-body exposure of 1400 to 2000 rads and infusion of marrow. *Arch. Intern. Med.* **107,** 829 (1961).
48. D. J. Parra, Nuestras experiencias en las transfusiones de medula ossa. *Rev. Hémat. Hemot.* **2,** 116 (1962).
49. D. W. Van Bekkum, O. Vos et W. W. H. Weyzen, The pathogenesis of the secondary disease after foreign bone-marrow transplantation in X irradiated mice. *J. Nat. Cancer Inst.* **23,** 75 (1959).

50. J. D. Denko, E. L. Simmons et R. W. Wissler, The histopathology of delayed death in irradiated mice treated with homologous cells. *Radiat. Res.* **11,** 557 (1959).
51. G. Mathé et J. L. Amiel, Physiopathologie et contrôle du syndrome secondaire qui complique la greffe de cellules hématopoïétiques allogéniques. *Rev. Franç. Etud. Clin. Biol.* **8,** 537 (1963).
52. N. B. Kurnick, A. Montano, J. C. Gerdes et B. H. Feder, Preliminary observations on the treatment of post-irradiation hematopoietic depression in man by infusion of stored autogenous bone-marrow. *Ann. Intern. Med.* **49,** 973 (1958).
53. N. B. Kurnick, B. H. Feder, A. Montano, J. C. Gerdes et R. Nakamura, Some observations on the treatment of post-irradiation hematopoietic depression in man by the infusion of stored autogenous bone-marrow. *Ann. Intern. Med.* **51,** 1204 (1959).
54. J. J. Macgovern, Jr., P. S. Russel, L. Atkine et E. W. Webster, Treatment of terminal leukaemia relapse by total body irradiation and intravenous infusion of stored autologous bone-marrow. *New Engl. J. Med.* **260,** 675 (1959).
55. J. B. Atkinson, J. F. Mahoney, I. R. Schwartz et J. A. Hesch, Therapy of acute leukaemia by whole-body irradiation and bone-marrow transplantation from an identical twin. *Blood* **14,** 228 (1959).
56. E. D. Thomas, H. L. Lochte, J. H. Cannon, O. D. Sahler et J. W. Ferrebee, Supralethal whole-body irradiation and isologous marrow transplantation. *J. Clin. Invest.* **38,** 1709 (1959).
57. G. Mathé et J. Bernard, Essai de traitement de la leucémie L.1210 par l'irradiation X suivie de transfusion de cellules hématopoiétiques normales (isologues ou homologues, myéloïdes ou lymphoïdes) adultes ou embryonnaires. *Rev. Franç. Etud. Clin. Biol.* **4,** 442 (1959).
58. L. M. Van Putten, Bone-marrow transplantation in Rhesus monkey. In: *Bone Marrow Therapy and Protection in Irradiated Primates.* TNO, Rijswijk (1962).
59. O. Brocades-Zaalberg, La moelle osseuse des radio-chimères: son pouvoir de restaurer le tissu lymphoïde isogénique irradié. *Colloque CNRS sur la Greffe des Cellules Hématopiétiques Allogéniques*, Paris 1964, Un vol CNRS Paris (1965).
60. P. Stastny, V. A. Stembridge et M. Ziff, Homologous disease in the adult rat, a model for auto-immune disease. *J. Exp. Med.* **118,** 635 (1963).
61. V. Schwarzmann, L'hépatite chronique. *Rev. Franc. Etud. Clin. Biol.*, **10,** 28 (1965).
62. D. G. Miller, Les désordres immunologiques au cours des leucémies et des hématosarcomes. *Rev. Franç. Etud. Clin. Biol.* **7,** 1112 (1962).
63. G. Mathé et G. Seman, *Aspects Histologiques et Cytologiques des Leucémies et Hématosarcomes*, Un vol. Maloine, Paris (1963).
64. M. Berger et J. Delarue, Le syndrome secondaire représente-t-il un modèle d'étude des maladies dites auto-immunes? In: *Colloque de CNRS sur la Greffe des Cellules Hématopoiétiques Allogéniques.* Paris, 1964. Un vol., CNRS, Paris (1965).
65. M. J. de Vries et O. Vos, Treatment of mouse lymphosarcoma by total body irradiation and by injection of bone marrow and lymph node cells. *J. Nat. Cancer Inst.* **21,** 1117 (1958).
66. G. Mathé, J. L. Amiel et J. Niemetz, Greffe de moelle osseuse après irradiation totale chez des souris leucémiques suivie de l'administration d'un antimitotique pour réduire la fréquence du syndrome secondaire et ajouter à l'effet antileucémique. *C.R. Acad. Sci., Paris* **254,** 3603 (1962).
67. M. F. A. Woodruff, M. O. Symes et N. F. Anderson, The effect of intraperitoneal injection of thoracic duct lymphocytes from normal and immunized rats in mice inoculated with the Landschutz ascites tumor. *Brit. J. Cancer* **17,** 482 (1963).
68. G. Mathé et J. Bernard, Essai de traitement par l'irradiation X suivie de l'administration de cellules médullaires homologues, de souris AK atteintes de leucémie spontanée très avancée. *Bull. Cancer* **45,** 289 (1958).
69. G. Mathé, J. L. Amiel et J. Bernard, Traitement de souris AKR à l'âge de 6 mois par irradiation totale suivie de transfusion de cellules hématopoïetiques. Incidences respectives de la leucémie et du syndrome secondaire. *Bull. Cancer* **47,** 331 (1960).
70. G. Mathé, J. L. Amiel et C. Friend, Essai de traitement de la leucémie de C. Friend par la greffe de cellules hématopoiétiques de donneurs vaccinés contre le virus. *Bull. Cancer* **49,** 416 (1963).

71. G. MATHÉ et J. L. AMIEL, Réduction de la concentration plasmatique du virus leucémigène de Charlotte Friend par immunothérapie adoptive (greffe de moelle osseuse allogémique). *C. R. Acad. Sci., Paris*, **259**, 4408 (1964).
72. J. L. AMIEL, R. TENENBAUM, A. M. MERY, M. MATSUKURA et G. MATHÉ, Protection des animaux irradiés par la moelle osseuse allogénique selon le nombre de cellules. *Rev. Franç. Etud. Clin. Biol.* **7**, 970 (1962).
73. L. GROSS, *Oncogenic Viruses*, Un vol. Pergamon, Oxford (1961).
74. C. FRIEND, Cell-free transmission in adult Swiss mice of disease having the character of a leukaemia. *J. Exp. Med.* **105**, 307 (1957).
75. E. J. FREIREICH, R. M. LEVIN, J. WHANG, P. P. CARBONE, W. BRONSON et E. E. MORSE, The function and fate of transfused leukocytes from donors with myelocytic leukemia in leukopenic patients. *Ann. N.Y. Acad. Sci., Paris* **113**, 1081 (1964).
76. E. J. FREIREICH, R. M. LEVIN, J. WHANG, P. J. SCHMIDT et E. FREI III, Bone marrow homografts following homologous leukocytes transfusion in patients with acute leukemia. *Colloque CNRS sur la Greffe de Cellules Hématopoïétiques Allogéniques*, Paris 1964, Un vol. CNRS, Paris (1965).
77. D. E. UPHOFF, Alteration of homograft reaction by amethopterin in lethally irradiated mice treated with homologous marrow. *Proc. Soc. Exp. Biol. (N.Y.)* **99**, 651 (1958).
78. E. D. THOMAS, J. A. COLLINS, B. C. HERMAN et J. W. FERREBEE, Marrow transplantation in lethally irradiated dogs given methotrexate. *Blood* **19**, 217 (1962).
79. H. L. LOCHTE Jr., A. S. LEVY, D. M. GUENTHER, E. D. THOMAS et J. W. FERREBEE, Prevention of delayed foreign marrow reaction in lethally irradiated mice by early administration of methotrexate. *Nature, Lond.* **196**, 1110 (1962).
80. R. W. GILLETTE, A. FINDLEY et H. CONWAY, Effect on tumour homograft of treating host with antiproteolytic enzyme compounds. *Proc. Soc. Exp. Biol. N.Y.* **112**, 964 (1963).
81. L. SCHWARZENBERG, J. L. AMIEL, R. TENENBAUM et G. MATHÉ, Effets de la conservation à —70°C sur les pouvoirs myélo-restaurateur et inducteur de syndromes secondaires de la moelle osseuse allogénique. *Rev. Franc. Etud. Biol. Clin.* **8**, 783 (1963).
82. J. COLOMBANI, J. DAUSSET et J. PREAUX, Homogreffe de peau chez l'Homme en relation avec les iso-antigènes leucocytaires. *Nouv. Rev. Franç. Hémat.* **3**, 499 (1963).
83. G. MATHÉ et J. L. AMIEL, Haematological approach to modern grafting problem. *Brit. Med. J.* **2**, 527 (1964).
84. M. MATSUKURA, A. M. MERY, J. L. AMIEL et G. MATHÉ, Investigation on a test of histo-compatibility for allogenic grafts. II. A study on rabbits. *Transplantation* **1**, 61 (1963).
85. L. BRENT et P. B. MEDAWAR, Tissue transplantation. A new approach to the "typing" problem. *Brit. Med. J.* **2**, 269 (1963).
86. C. BREZIN, M. MATSUKURA, A. M. MERY et J. SORIEUL, Le choix du donneur. In: *Colloque CNRS sur la greffe des cellules hématopoïétiques allogéniques*, Paris, 1964, Un vol., CNRS, Paris (1965).
87. F. BACH et K. HIRSCHORN, Lymphocyte interaction. A potential histocompatibility test. *Science* **143**, 813 (1964).
88. G. MATHÉ, J. L. AMIEL, M. MATSUKURA et A. M. MERY, Restauration hématopoïétique de souris irradiées, par greffe de moelle osseuse allogénique de plusieurs donneurs de diverses lignées *C.R. Acad. Sci. Paris* **255**, 3840 (1962).
89. G. MATHÉ, J. L. AMIEL, M MATSUKURA et A. M. MERY, Restoration of the hematopoietic function in the irradiated mouse, with use of allogenic bone-marrow grafts from several donors of different strains. *Brit. J. Haemet.* **10**, 257 (1964).

Europ. J. Cancer Vol. 1, pp. 115–121. Pergamon Press 1965. Printed in Great Britain

The Transplantation of Chemically Induced Steroid Sensitive Mammary Tumours

D. C. WILLIAMS

The Research Department
The Marie Curie Memorial Foundation
Harestone Drive
Caterham, Surrey, England

HUGGINS and his coworkers [1, 2] have shown that a single feeding of one of a number of polycyclic aromatic hydrocarbons produces cancer of the breast in rats. Huggins and Yang [3] showed that 7,12-dimethyl(α)benzanthracene given in one single dose in oil produced 100% of tumours in Sprague Dawley rats of a suitable age. A number of other polycyclic hydrocarbons produced tumours of a similar type but only 2-aminophenanthrene appears to produce equally consistent results. The tumours are generally non-metastasising papillary adenocarcinomas of the breast but a proportion of benign fibroadenomas is also found. Huggins [4] also noticed that a certain proportion of tumours which were hormone responsive on induction eventually became autonomous and lost their steriod dependence.

Homotransplantation was successful in only 30 per cent of cases for the adenocarcinomas although 100 per cent takes were obtained in fibroadenomas. These carcinogens also appear to cause a certain degree of selective cellular necrosis in the mammary tissues and 7,12-dimethyl(α)benzanthracene has a specific necrotic effect on the adrenal cortex so that the involvement of adrenal hormones cannot be overlooked [5].

Mammary cancers of various types have been treated successfully by hormonal means in many differing hosts including man [6] but such treatment leads to remissions only and hardly ever effects the eventual outcome of the disease. Unlike prostatic cancer, which always appears to respond favourably to oestrogen treatment or androgen withdrawal by surgical means [7, 8], mammary tumours in man appear to respond in different ways to endocrine treatment. Thus a mammary tumour may be stimulated by androgens, oestrogens, both or neither and for this reason endocrine withdrawal by surgery or other means is often effective at least as a palliative measure.

Many attempts have been made to rationalize endocrine treatment of mammary cancer by the investigation of the actual chemical involvement of the hormones within the tumours [9, 10]. The difficulty in using chemically induced tumours for this purpose lies in the fact that no two tumours, even in the same animal, appear to respond in exactly the same way to steroid hormones. In the present work, therefore, specific tumours have been selected on the basis of their apparent response *in situ* and the selected tumour has been propagated by surgical transplantation. Tumours treated in this way appear to maintain their steroid sensitivity through a number of transplantations.

The objects of this work are: (1) The establishment of a surgical transplantation technique for selected chemically induced tumours. (2) The comparison of hormone response differences in tumours derived from

the same source. (3) The development of a sensitive biochemical tool for the investigation of the response to treatment of endocrine stimulated tumours.

METHODS

Female Sprague Dawley rats aged 8 weeks were treated by forced feeding of 7,12-dimethyl-(α)benzanthracene (20 mg in 1 ml oil). Although one treatment is usually sufficient this dose was repeated after 2 weeks and the animals were maintained on 41 B diet with an occasional complete vitamin supplement administered in the drinking water. Within about 2 months over 90 per cent of the treated animals developed mammary tumours, the majority having multiple tumours. There were also a small proportion of tumours of the head, mostly arising from the inner ear. These animals were killed as soon as the tumours were noticed although some of these animals had mammary tumours as well.

a. *The selection of chemically induced tumours*

Six dozen rats with small tumours were selected, the tumours were measured daily over a period of 10 days and a growth rate base-line was established for each individual tumour. In some cases several tumours were measured in the same animal. The tumour diameters were measured from six different aspects and from these the approximate volume and hence wet weight of the tumour was obtained. Only those tumours in which a steady growth rate could be established were used for future study. The animals were divided into four groups of twelve, each group containing an average twenty-six measurable tumours. The groups of animals were then injected (i.p.) with a daily dose of oestradiol-17β, testosterone and stilboestrol respectively, each in suspension in 10% ethanol (1 mg/ml). The fourth group was used for control purposes and these animals were injected daily with 10% ethanol for 10 days. The measurements of the tumours were continued over this period.

b. *The transplantation of selected tumours*

The skin of the host animal was shaved locally to the tumour and the skin was soaked in N/10 iodine solution containing 2% sodium iodide and 20% cetavalon (ICI Ltd., Wilmslow, Cheshire). The tumours were then removed and washed in sterile physiological saline. Each tumour was cut into small cubes of approximately 50 mg and the pieces stored in cold physiological saline in a covered sterile petri dish. The tumours were transplanted into groups of twenty-four female Sprague Dawley rats (approximately 200 g in weight) for initial test purposes. The transplantation was made in one of three ways.

1. *Subcutaneous transplants* — The skin was shaved in the axillary region and painted with the iodine preparation. A 13-gauge sterile trocar was used to implant one tumour fragment into each animal. In the case of tumours used for β-glucuronidase estimations, a number of tumour fragments were sometimes implanted into the same animal and the tumours removed in sequence as required. The trocar was removed and the incision closed with a single skin stitch. A fresh sterile trocar was used for each ten tumour fragments and each tumour was used within 30 min of removal from the host animal as suggested in the Cancer Chemotherapy Reports (Protocols for Screening, 1962).

2. *Attachment to the liver surface* — The animals were shaved over the xiphisternum and the area painted with the iodine solution. A medial skin incision was made over the xiphisternum and extending for about 1 cm/posterior to it. A similar incision was made in the body wall and connective tissue and the xiphisternum exposed. Gentle downward pressure just below the incision then caused one lobe of the liver to protrude. The fragment of tumour was then fixed to the outer ventral surface of the liver lobe with a single stitch. Attachment of the tumour to the posterior concave surface of one of the lobes resulted in slower growth of the tumour which was then completely surrounded by liver tissue. The incisions were then closed separately with braided silk satures, and the whole soaked in the iodine solution.

3. *Attachment to the surface of the spleen* — Tumour fragments were implanted in the spleen in a way similar to that described above. The initial incision was made near the lower pyloric end of the spleen and the tumour stitched on the ventral surface near its lower end. In some initial experiments the tumour was located on the concave visceral surface of the spleen which was then stitched around it. The tumour did not take as well as those not so intimately in contact with the spleen.

c. *The β-glucuronidase assay of tumours tissues* —

Tumours were removed from the animals and any obviously necrotic tissues removed. The tumour tissue was weighed and homogenised in

tris/HCl buffer (0·1 M, pH=4·5, 1 ml/g of tissue) using an Ultra-Turrax homogeniser for 30 sec with ice cooling. The homogenate was ultracentrifuged (105,000 x g for 1 hr) and the clear supernatant solution was removed and diluted approximately 50 times with buffer.

Protein concentration was determined in the extract by measurement of the absorbancies at 280 mμ and 260 mμ and calculations of the protein concentration made by the formula $1{\cdot}5 \times A_{280} - 0{\cdot}75 \times A_{260}$ = protein concentration in milligrams per ml. The extracts were diluted so that 1 ml of test solution contained about 1 mg of total protein.

The β-glucuronidase estimations were carried out by a modification of the method of Talalay *et al.* [11]. Phenolphthalein mono-β-glucuronic acid (0·05 g Sigma) was dissolved in 10 ml ethanol (freshly re-distilled from potassium hydroxide) and diluted to 100 ml with water. The ethanol stabilises the phenolpththalein glucuronic acid in solution.

Enzyme solution (1 ml) acetate buffer (1 ml, 0·1 M, p=H, 4·5) and substrate solution (1 ml) were incubated in a stoppered tube for 1 hr at 37° in a water bath. A blank containing no enzyme was also incubated. On removal from the waterbath, 1 ml of enzyme solution was added to the blank and glycine buffer (1 ml 0·4 M, pH=10·45) was added to each tube. The tubes were centrifuged at 600 ×g for 15 min and the duplicates read against the blank, using a Unicam SP.600 spectrophotometer at 550 mμ. The activity was expressed in units; 1 unit liberating 1 μg phenolphthalein per hour at 37°.

The scatter shown by the results of repeated estimations was small and the results were therefore not statistically analysed.

RESULTS AND DISCUSSION

a. *The effect of steroids on chemically induced tumours* in situ

Groups of animals with chemically induced tumours were treated with steroid hormones as described above. The response of individual tumours varied widely with treatment. The purpose of this experiment was to select the tumours which appeared to be most sensitive to endocrine treatment.

The method of choosing the most likely tumours for further work is illustrated by Fig. 1. This diagram shows the response in growth rate of three separate tumours on treating the animal with testosterone. Curve (a) is typical of a tumour which gives an increased growth rate on treatment with the steroid hormone. Curve (c) is typical of a tumour which is inhibited by the hormone and Curve (b) is apparently unaffected by the hormone.

By means of carefully selecting tumours which apparently were effected in some way by the different steriod hormones, tumours were obtained the growth rate of which could be increased by oestradiol and by testosterone respectively. Two further tumours which were inhibited by testosterone and oestradiol respectively and one which was inhibited by a mixture of both were also investigated. In all tumours so far examined, stilboestrol produced an

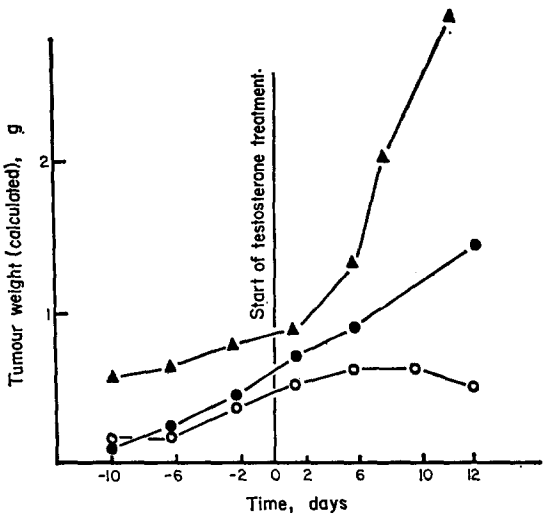


Fig. 1. *The effect of treating host animals daily with testosterone starting at day* 10 *after base-line measurement of tumours. Tumour weight is estimated from measurements of the tumour size.*

a. ▲–▲ *shows the growth rate of a tumour which is stimulated by testosterone;*

b. ●–● *shows the growth rate of a tumour which is not effected by testosterone;*

c. ○–○ *shows the growth rate of a tumour which is inhibited by testosterone.*

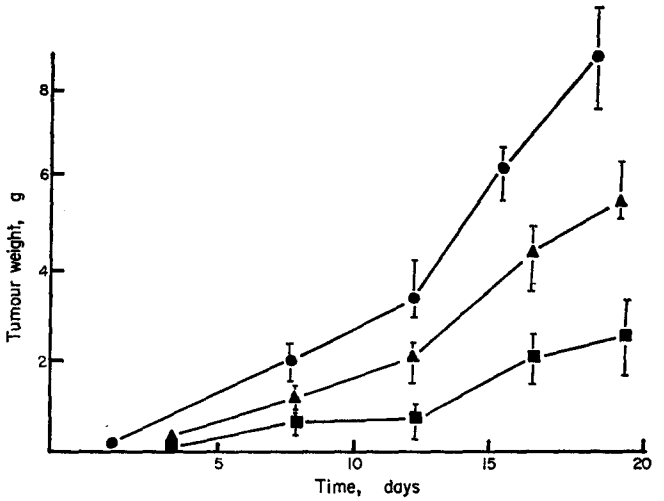


Fig. 2. The growth rate of a transplanted tumour at various sites in the rat.
a. ●–● *shows liver transplantation;*
b. ▲–▲ *shows spleen transplantation;*
c. ■–■ *shows subcutaneous transplantation.*
The experimental points each show the mean of six tumour weights and the maximum deviation is shown for each group.

inhibition of growth rate or had no effect on the rate of growth of the tumour.

b. *The rate of growth of transplanted tumours*

The growth rate of tumours transplanted in various sites was investigated. In these experiments the particular tumour was chosen because it was not strongly dependent upon either oestrogens or androgens. In this way it was hoped to avoid local response to the hormonal level of the host organ but this, of course, applies to oestradiol-17β and testosterone response only and it is difficult to predict which, if any, of the other hormones may have a controlling influence on the growth rate of the tumour at any particular site of implantation.

Subcutaneous transplantation of these tumours appears to be an unreliable method of propagation and only about 50 per cent of selected tumours could be transplanted in this way, even though the tumours had been originally selected for their solid texture and relatively fast growth rate. Further, subcutaneous transplants became necrotic much quicker than tumours transplanted in either of the other ways. This is presumably due to the more easily available blood supply in the latter cases. The relative rates of growth of transplanted tumours at different sites are shown in Fig. 2. These results are each the average of six tumour weights, weighed wet immediately on removal. It may be concluded from Fig. 2 that the best

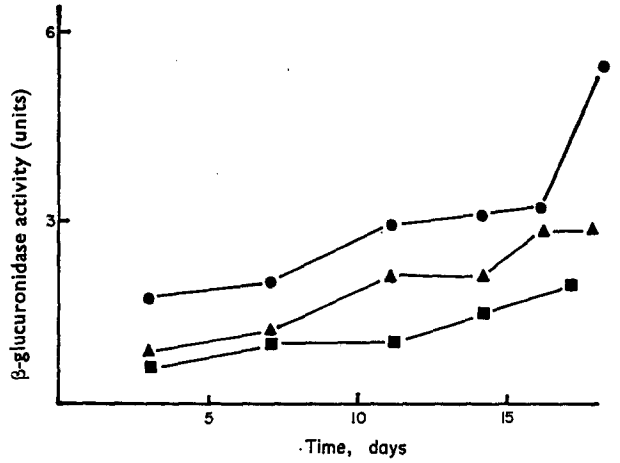


Fig. 3. The β-glucuronidase of the tumour transplants shown in Fig. 2 at various times after transplantation
a. ●–● *β-glucuronidase activity of the liver transplant;*
b. ▲–▲ *β-glucuronidase activity of the spleen transplant;*
c. ■–■ *β-glucuronidase activity of the subcutaneous transplant.*
The experimental points shown are the mean of twelve determinations obtained from six tumours. Each estimation being duplicated.

method for rapid growth of transplanted tumours is via the liver. Such tumours should also be more accessible to drug treatment, via the host's bloodstream, than subcutaneously transplanted tumours. The spleen transplants have the advantage of being more easily isolated which is especially advantageous for tumour perfusion work, so that both liver and spleen transplantations have been used in further investigations. The growth rate of all tumours was increased by such transplantation.

c. *The relationship between growth rate and β-glucuronidase activity*

The β-glucuronidase activity of the growing tumours, shown in Fig. 2 has been investigated and the results obtained are shown in Fig. 3. The β-glucuronidase levels appear to bear a close relationship to the actual rate of growth of the tumour. Although the treatment of the tumour with a β-glucuronidase inhibitor, which will be discussed elsewhere, remains a possibility, the use of β-glucuronidase, as a method of early diagnosis, would appear to be inapplicable to this type of tumour. There appears to be a delay between increase in tumour growth and the corresponding increase in β-glucuronidase activity.

d. *The effect of steroid hormones on selected tumours*

From the above experiments five tumours were selected for transplantation into liver and the other tumours were discarded. About 60 per cent of all tumours examined appeared to be dependent upon neither oestradiol-17β nor testosterone. The remaining tumours varied widely in growth rate and in the response of growth rate to steroid treatment. The five tumours were chosen because they had the following properties: (a) they were solid tumours composed of firm, apparently homogenous, tissue; (b) they could be easily transplanted into liver giving more than 90 per cent "takes"; (c) they exhibited a clear response to steriod treatment; and (d) they exhibited this same steroid response after at least three serial transplantations which indicates that this is a stable property of the tumour at least in early transplants. The latter effect is checked after every three serial transplantations since tumours have been known to change their steroid dependence during successive propagation. There was an increase in the proportion of "takes" with successive transplantations of tumours into liver or spleen. After six successive transplantations, only rarely did a transplant fail to grow.

The results obtained from the five tumour lines used are shown in Table 1. Each tumour was transplanted into the livers of thirty-six animals and these were divided into groups of twelve and treated with testosterone and oestradiol respectively (1 mg in 1 ml of 20% ethanol daily), one group being used as a control, by intra-peritoneal injection for 10 days.

The results obtained by the above technique are given in Table 1a. Tumour I exhibited an increased growth rate when treated with testosterone but oestradiol treatment made no difference to its growth rate. Tumour II would appear to exhibit the reverse effect and the growth of Tumour III was slowed by either of the hormones. Tumour IV exhibited decreased growth rate when treated with oestradiol and

Table 1.

Tumour	Control			Testosterone			Oestradiol		
	Average wt (g)	S.D.	Average β-gluc. activity	Average wt (g)	S.D.	Average β-gluc. activity	Average wt (g)	S.D.	Average β-gluc. activity
a. Intact animals									
I.	2·1	0·2	1·8	2·9*	0·3	2·7	2·0	0·3	2·1
II.	1·6	0·3	0·7	1·5	0·3	1·1	2·1*	0·3	1·8
III.	2·3	0·2	2·1	1·4†	0·3	2·0	1·9	0·3	1·1
IV.	1·9	0·3	1·6	2·4†	0·2	2·9	1·1*	0·2	0·6
V.	1·1	0·3	0·4	1·2	0·3	1·2	1·1	0·4	0·3
b. Castrates									
I.	2·5	0·3	1·7	3·8*	0·5	3·0	2·3	0·4	2·0
II.	1·8	0·3	1·0	1·9	0·4	1·1	2·6†	0·4	1·9

Figures represent averages of twelve estimations except in the case of tumour II in castrate animals which consisted of eleven animals per group. Intact animals were treated for 10 days with injections of the appropriate steriod and castrate animals for 12 days.

*Represents a probability less than 0·01.

†Represents a probability less than 0·05.

an increased growth rate with testosterone, and Tumour V was not responsive to either hormone.

The β-glucuronidase values bore a relationship to the rate of growth in the selected tumours but those treated with testosterone were somewhat higher and those treated with oestradiol somewhat lower than would be expected purely on a basis of growth rate alone. Much may depend on the effect of the hormone on the host tissue β-glucuronidase, which, in the case of liver, appears to vary considerably. depending on experimental conditions. Kerr *et al.* [12, 13] showed that, for example, estrone had a stimulatory effect on liver β-glucuronidase which was antagonised by testosterone. However, Fishman and Lipkind [14] showed that testosterone may also have a stimulatory effect on β-glucuronidase activity in certain tissues. The effect found in tumours is very much less than that obtained by these workers using liver extracts.

Somewhat similar results have been obtained from a parallel experiment in which the tumours have been transplanted into spleen, although in this case, transplantation of tumours appears rather more difficult and the effect of treatment with steroid hormones on spleen transplants is less marked than in liver. This may be due to the fact that circulating steroids are less easily available to spleen than to liver or there may be some direct effect of the hormones on the host organ as well as a direct action on the tumour. The relationship between tumour and host organ during steroid treatment is being examined further.

e. *The effect of tumour transplantation into castrated rats*

The experiments outlined for Tumours I and II were repeated in castrate female rats. Castration had been carried out 2 days prior to transplantation of tumours. The results of these experiments were essentially similar to those in the intact animal except that the effect of steroids was rather more marked. Treatment with either androgen or oestrogen was started immediately after castration and it was found that the number of "takes" in the implants was significantly greater in the steroid treated animals than in the controls. There was no evidence from the relatively small number of animals used as to the difference in "takes" between the two tumours. Tumours did not appear to be transplanted more easily in animals which were treated with the appropriate growth rate activating steroid. In general, the growth rate of tumours transplanted into castrate animals is somewhat slower than those in intact animals. The results obtained with castrate animals after daily treatment with steroids for 12 days after transplantation are shown in Table 1b. There appears to be no significant difference in β-glucuronidase levels due to the castration.

The technique described above for the selection and transplantation of tumours appears to be generally applicable to tumours which are obtained in mixed populations and the technique may be used to develop sensitive tumour lines for biochemical investigations into, for example, the action of chemical stimuli and anticancer drugs on tumours. Tumours should be checked after several transplantations to ensure that the specificity of the tumour does not change with transplantation. It appears likely that the results obtained by treating host animals with oestradiol-17β are more clearly defined when castrate animals are used. Presumably owing to the presence of endogenous oestrogen in the intact animals.

RÉSUMÉ

1. *Une méthode a été mise au point pour la sélection des tumeurs du rat, provoquées chimiquement.*

2. *Une méthode de transplantation des tumeurs a été décrite. Des tumeurs provoquées chimiquement peuvent être transplantées dans la rate et le foie, outre la transplantation sous-cutanée.*

3. *Les quantités de β glucoronidase sont mesurées dans les tumeurs transplantées.*

4. *L'effet du traitement aux stéroïdes de l'animal hôte, sur le taux de croissance des tumeurs sélectionnées, a été déterminé.*

5. *La transplantation de tumeurs chez des animaux castrés a également été investiguée.*

SUMMARY

1. *A method has been developed for the selection of chemically induced tumours in the rat.*
2. *A method of transplantation of tumours is described. Chemically induced tumours may be transplanted into spleen or liver in addition to subcutaneous transplantation.*
3. *β-Glucuronidase levels have been estimated in transplanted tumours.*
4. *The effect of steroid treatment of the host animal on the growth rate of selected tumours has been determined.*
5. *Transplantation of tumours into castrated animals has also been investigated.*

ZUSAMMENFASSUNG

1. *Es wurde eine Methode für die Auswahl chemisch herovrgerufener Geschwülste in der Ratte entwickelt.*
2. *Es wird eine Methode für Geschwulstüberpflanzung beschrieben. Neben der subcutanen Transplantation kann man chemisch hervorgerufene Geschwülste in die Milz, oder in die Leber überpflanzen.*
3. *In den überpflanzten Tumoren wurde der β-Glucuronidase-Spiegel bestimmt.*
4. *Es wurde die Wirkung der Steroidbehandlung des Wirtstieres auf die Wachstumsrate ausgewählter Geschwülste bestimmt.*
5. *Die Uberpflanzung von Tumoren auf kastrierte Tiere wurde ebenfalls untersucht.*

REFERENCES

1. C. Huggins, G. Briziarelli and N. Sutton, Rapid induction of mammary carcinoma in the rat and the influence of hormones on the tumours. *J. exp. Med.* **109,** 25 (1959).
2. C. Huggins, L. C. Grand and F. P. Brillantes, Mammary cancer induced by a single feeding of polynuclear hydrocarbons and its suppression. *Nature, Lond.* **189,** 204 (1961).
3. C. Huggins and N. C. Yang, Induction and extinction of mammary cancer. *Science* **137,** 3526 (1962).
4. C. Huggins, in: *Horizons in Biochemistry.* Edited by Kasha and Pulman. Academic, New York (1962).
5. C. Huggins and S. Morii, Selective adrenal necrosis and apoplexy induced by 7, 12-dimethylbenz(a)anthracene. *J. exp. Med.* **114,** 741 (1961).
6. C. Huggins, Control of cancers of man by endocrinologic methods. *Cancer Res.* **16,** 825 (1956).
7. C. Huggins and P. J. Clark, Quantitative studies of prostatic secretion. II. The effect of castration and of estrogen injection on the normal and on the hyperplastic prostate glands of dogs. *J. exp. Med.* **72,** 747 (1940).
8. C. Huggins and C. V. Hodges, Studies on prostatic cancer. I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *Cancer Res.* **1,** 293 (1941).
9. V. P. Hollander, H. Jonas and D. E. Smith, Estradiol-sensitive isocitric dehydrogenase in non-cancerous and cancerous human breast tissue. *Cancer* **11,** 803 (1958).
10. V. P. Hollander, D. E. Smith and T. E. Adamson, Studies on estrogen-sensitive transhydrogenase. The effect of estradiol-17β on α-ketoglutarate production in non-cancerous and cancerous human breast tissue. *Cancer* **12,** 135 (1959).
11. P. Talalay, W. H. Fishman and C. Huggins, Chromogenetic substracts II. Phenolphtalein glucuronic acid as substrate for the essay of glucuronise activity. *J. biol. Chem.* **166,** 757 (1946).
12. L. M. H. Kerr, J. C. Campbell and G. A. Levvy, β-glucoronidase as an index of growth in the uterus and other organs. *Biochem J.* **44,** 487 (1949).
13. L. M. H. Kerr, J. C. Campbell and G. A. Levvy, Further observations on changes in β-glucoronidase activity in the mouse. *Biochem J.* **46,** 278 (1950).
14. W. H. Fishman and J. B. Lipkind, Comparative ability of some steroids and their eaters to enhance the renal β-glucoronidase activity of mice. *J. Biol. Chem.* **232,** 729 (1958).

Europ. J. Cancer Vol. 1, pp. 123–124. Pergamon Press 1965. Printed in Great Britain

Note on a New Inbred Mouse-Strain GR/A

O. MÜHLBOCK

The Netherlands Cancer Institute, Amsterdam

IT CANNOT be emphasized too strongly how much the use of inbred strains of mice has contributed to cancer research. Inbred strains are invaluable in studies designed to evaluate the relative importance of hereditary, endogenous and environmental influences in cancer development. There are now known more than 100 different inbred strains of mice. The question then could be raised: Do we need more inbred strains? Without hesitation the answer must be in the affirmative. There are several reasons for the desirability of more inbred strains. Most of the inbred strains now in use all over the world originate from the United States, where the first inbred strains have been developed [1, 2]. As in the past only a very few centres developed inbred strains, many of the strains, even when they show how different characteristics have to a large extent the same genetic background. It would, therefore, be advisable to develop inbred strains in other parts of the world. With this reasoning in mind it was decided about 10 years ago to develop in our laboratory a series of new inbred strains of European origin. However as there was for a long time a quite extensive exchange of mice between the United States and Europe, it can never be stated with certainty that European mice have not been crossed with American ones.

The purpose of this note is to report on the establishment of a new albino European mouse-strain which shows some characteristic features which have not yet been reported for other strains and which may be, therefore, of importance for cancer research.

In 1955, Professor A. Grumbach from the Institute of Medical Microbiology in Zürich, Switzerland, offered us some breeding pairs of four strains, which he had used for studies with tuberculosis and which he wanted to discontinue. One of these strains was the strain "Grunder" [3] . From this name the symbol GR, which is proposed for the new strain, has been derived.

Although this strain had been inbred for some years in Zürich, brother-sister inbreeding has been continued for twenty more generations in our laboratory before the characteristics of this strain have been analysed.

The first observation which has been made was a high frequency of mammary carcinoma in female breeding mice. It was assumed that this high incidence was due to the presence of the mammary tumour agent (milk factor, Bittner Virus). Therefore the young were taken out of the uterus shortly before birth and fosternursed by a mother of the C_{57}BL strain, which is free of the mammary tumour agent. All subsequent observations were made with the descendants of this pair taken out of the uterus.

Again the most striking observation was a same frequency of mammary tumours in female breeding mice. It was thought that in spite of all precautions a contamination with the mammary tumour agent had occurred. The procedure was therefore repeated. A new line was established from a pair taken out of the uterus and fosternursed by a C_{57}BL-mother. Again the female descendants showed the same incidence of mammary carcinomas. It was thus obvious that either the agent is not present and the high incidence of these tumours is caused by other factors, or the agent is present but is transmitted from the parents to the young by other means than the milk.

The next experiment revealed the presence of a mammary tumour agent. Highly susceptible mice (♀ 0_{20} × ♂ DBA_f) F_1 [4] were fosternursed by

GR-mothers. They had a high percentage of mammary cancer late in life, whereas the control-mice had none. The agent therefore is transmitted by the mothers milk, but there are besides other means of transmission. This was proven by the classical cross-breeding test, with which Little [1] and Korteweg [5] discovered the maternal factor. The breeding female F_1 hybrids (♀ GR ×♂ C_{57}BL)F_1 and (♀ C_{57}BL ×♂ GR)F_1 showed the same high incidence of mammary tumours.

The GR-agent is apparently transmitted by the male as effectively as by the female. A direct transmission of the agent by the male to the offspring seems probable. This explains the failure of the former experiments to develop an agent-free subline of the GR-strain.

Preliminary results indicate that the agent can be also transmitted by cell-free extracts of mammary tumours.

The mammary tumours of the GR-strain and also those of both hybrids mentioned above show with the electron microscope the virus particles characteristic of the conventional Bittner virus (Benedetti, unpublished).

Only breeding or force bred females show a high incidence of mammary tumours early in life.

A documented publication of the observations on the GR-strain will be published *in extenso*.

The mammary tumours show a remarkable hormone dependence. They appear during pregnancy and disappear at the end of the pregnancy. They also show progression. A detailed study of the hormone responsiveness of these tumours by R. van Nie and P. J. Thung has been described [6].

RÉSUMÉ

Une nouvelle souche pure de souris (GR) a été isolée. Les femelles ayant été gestantes ont une très haute incidence de tumeurs mammaires qui réagissent fortement aux hormones.

Dans cette souche, l'agent de la tumeur mammaire peut être transmis par le lait (Facteur Lacté) mais aussi par d'autres voies. Ceci est un caractère particulier qui permet de différencier cette souche des autres lignées pures connues de souris.

SUMMARY

A new inbred mouse -strain GR has been developed. Breeding females have a high incidence of mammary tumors, which are highly hormone responsive. A mammary tumour agent is present in this strain, which can be transmitted by the milk but also by other pathways. In this respect this new strain is different from other known inbred strains.

ZUSAMMENFASSUNG

Uber die Entwicklung eines neuen eingezüchteten Mäuse-Stammes wird berichtet. Zuchtweibchen haben eine hohe Rate an Mamma-Tumoren, die hormonabhängig sind. Dieser Stamm ist Träger eines Mamma-Tumor-Agens, das mit der Muttermilch aber auch auf anderem Wege übertragen werden kann. In dieser Hinsicht ist dieser neue Stamm verschieden von anderen bisher bekannten Stämmen.

REFERENCES

1. C. C. Little, in: *Biology of the Laboratory Mouse.* Edited by G. D. Snell. Blakiston, Philadelphia (1941).
2. L. C. Strong, The origin of some inbred mice. *Cancer Res.* **2,** 531 (1942).
3. A. Grumbach and H. Beer, Tuberculosis experiments in genetically known mice. Survival rates of 2 homozygous strains of mice and F-1 generation of their hybrids 4. *Acta genet. (Basel)* **12,** 103 (1962).
4. O. Muhlbock, Biological studies on the mammary tumor agent in different strains of mice. *Acta Un. Int. Cancr.* **XII,** 665 (1956).
5. R. Korteweg, Proefondervindelijk onderzoeking aangaande erfelijkheid van kanker. *Ned. T. Geneesk.* **78,** 240 (1934).
6. R. van Nie and P. J. Thung, Responsiveness of mouse mammary tumours to pregnancy. *Europ. J. Cancer* **1,** 41 (1965).

Europ. J. Cancer Vol. 1, pp. 125–133. Pergamon Press 1965. Printed in Great Britain

Transplantation Characteristics and Response to Chemotherapy of a Murine Adrenal Tumor*

S. R. HUMPHREYS†, G. SATO‡ and A. GOLDIN†

†*Drug Evaluation Branch, Cancer Chemotherapy National Service Center*
National Cancer Institute, Bethesda, Maryland
and
‡*Graduate Department of Biochemistry*
Brandeis University, Waltham, Massachusetts

INTRODUCTION

TRANSPLANTABLE hormone secreting tumors of mice are relatively rare. Few of them retain their ability to perform the functions of the differentiated tissue of origin after a number of transplant generations. Recently Buonassisi, *et al.* (1) reported on an adrenal hormone secreting tumor, initially obtained by Furth, which has maintained its functional ability after both *in vivo* and *in vitro* transplantation for approximately 15 years. The origin, pathology and the original *in vivo* transplantation characteristics of this tumor have been described (2–4).

In the present study the current *in vivo* transplantation characteristics of this adrenal tumor are described and chemotherapy studies are presented with methotrexate, 6-mercaptopurine, Cytoxan, and 1,3-bis(2-chloroethyl)-1-nitrosourea.

MATERIALS AND METHODS

The adrenal tumor was received as a large subcutaneous growth in the right inguinal region of an LAF_1 "J" hybrid male mouse. Since then it has been carried by monthly S.C. transplantation of a tumor brei diluted in n-saline. All experimental tumor inocula have been expressed as percent concentration of tumor brei. The tumor brei was suspended in n-saline and injected S.C. into the right inguinal area at a volume of 0·2 ml per mouse into the inguinal region.

LAF_1 male hybrid mice obtained from three separate sources and representing a number of different sublines of parental stocks were employed in these experiments and for carrying the tumor line. The source of the mice are indicated in the Tables and text as "B," "H," and "J."*

The mice were approximately 4 weeks of age when received from the supplier, and were used in experiments when 8–12 weeks of age. They were maintained in plastic cages with cedar shaving bedding and were fed Purina Laboratory Chow and water *ad libitum*.

Immunization of the mice was accomplished by I.P. injection into the left lower quadrant of the abdomen of 0·2 ml of a 50 per cent con-

*Publication No. 338 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Mass. Supported in part through U.S. Public Health Service research grant C-4123.

*"B" mice were obtained from the Battelle Memorial Institute, 505 King Avenue, Columbus, Ohio; "H" mice from Hall Laboratory, University of Kansas, Lawrence, Kansas; and "J" mice from Jackson Memorial Laboratory, Bar Harbor, Maine.

centration of tumor suspension in n-saline which had been exposed to X-irradiation* at standard temperature and pressure. The X-irradiation of the tumor cells was delivered at the rate of 1000r/1·92 min using 2 tubes spaced 25 cm from center of target, filtered with 0·25 mm Cu and 0·55 mm Al. The immunization inoculum was injected 7 days prior to the challenge with viable tumor cells.

The drugs employed in these experiments were supplied by the Cancer Chemotherapy National Service Center (CCNSC) and are designated in the text and tables by common name or abbreviation. All drug solutions were prepared and stored in brown glass serum bottles and when not in use were stored in a refrigerator at approximately 5° centigrade. Methotrexate (MTX) CCNSC No. 740 was dissolved and diluted in 2 per cent $NaHCO_3$ solution. Amounts sufficient for a period of up to 2 weeks were prepared at one time. 6-mercaptopurine (6-MP) CCNSC No. 755 was dissolved in dilute NaOH and neutralized to the point of precipitation with dilute HCl and diluted with H_2O and used for periods up to 2 weeks.

Cyclophosphamide (Cytoxan) CCNSC No. 26,271 was dissolved in n-saline for periods of up to 2 weeks. Urea, 1,3-bis(2-chloroethyl)-1-nitroso-: (BCNU), CCNSC No. 409,962 was prepared fresh daily in ion free sterile distilled H_2O. All drug injections were given S.C. in the loose skin of the back in a volume of 0·01 ml/g of body weight.

Surgery was performed under aseptic conditions using nembutal sodium (Abbott) (80 mg/kg/injected I.P.) as the anesthetic. An incision was made in the area of the tumor, the major blood vessels were clamped off, and, employing blunt dissection, the tumor was separated from the surrounding tissues and removed. The wound was closed and clamped with metal surgical clips.

Randomization procedures were employed in implantation of tumor, distribution of the mice into groups and in the treatment of the mice.

*We wish to acknowledge the services of Mr. Henry Meyers in administering the X-irradiation.

RESULTS

Transplantation characteristics

The initial retransplantation of this adrenal tumor from the LAF_1"J" mice in which it was received into LAF_1"B" mice resulted in less than 100 per cent tumor takes; therefore, studies on the transplantability of this tumor were undertaken. LAF_1 male mice from three different breeding colonies (J,H,B) were obtained and implanted S.C. with concentrations of tumor brei ranging from 50 per cent to 0·5 per cent. Normal mice from each of the three suppliers developed tumors which grew and killed at approximately the same rate for equivalent tumor inocula (Tables 1 and 2).

Table 1. *Comparison of growth of adrenal tumor in* "J" *and* "H" *lines of* LAF_1 *normal, total body* X-*irradiated, and immunized mice.*

Gr.	Mouse line	Condition	S. C. tumor				S. C. tumor		
			Tumor inoculum (% conc.)	Takes total	Tumor dia. day 30 (av. mm)	Day of death	Surv. day 77	Inoc. day 77 (% conc.)	Surv. day 137
1	J	Normal	50	3/3	25	39, 43, 43	0		
			5	3/3	16	46, 46, 59	0		
			0·5	2/2	8	57, 67, —	0		
2	H	Normal	50	3/3	18	33, 43, 45	0		
			5	3/3	15	41, 50, 55	0		
			0·5	2/2	8	61, 68, —	0		
3	J	Pre-immunized I.P.	50	2/3	0	55, 64, —	1	50	0
			5	0/3	0	— — —	3	50	0
			0·5	0/2	0	— — —	2	50	0
4	H	Pre-immunized I.P.	50	2/3	0	81, 111 —*	3	50	0
			5	0/3	0	— — —	3	50	0
			0·5	0/2	0	— — —	2	50	0
5	J	X-rayed	50	3/3	17	39, 39, 45	0		
			5	3/3	10	47, 51, 54	0		
			0·5	2/2	10	42, 65, —	0		
6	H	X-rayed	50	2/2	16	48, 51, —	0		
			5	3/3	14	45, 46, 51	0		
			0·5	1/1	—	63, — —	0		

*Two of the three mice in this group had S.C. tumor on day 77 when they received the second S.C. tumor inoculum.

Table 2. Growth of adrenal tumor in normal LAF$_1$ male "H" line mice compared to growth in normal, X-irradiated or immunized "B" line mice.

Gr.	Mouse line	Condition	Tumor inoculum (%)	S. C. takes total	Tumor dia day 30 (av. mm)	Day of death			S. C. tumor free surv. day 90
1	H	Normal	50	3/3	17·6	38	>43	>43*	0
			10	3/3	14·3	44	>43	87	0
			2	3/3	8·3	54	60	65	0
2	B	Normal	50	3/3	14·3	40	44	52	0
			10	3/3	10·0	46	50	67	0
			2	3/3	9·3	52	57	62	0
3	B	X-rayed†	50	3/3	19·0	32	35	37	0
			10	3/3	11·3	49	52	59	0
			2	3/3	10·3	53	54	56	0
4	B	Pre-immunized‡ I.P.	50	0/3	0	—	—	—	3/3
			10	§0/3	0	53	58	—	1/3
			2	§0/3	0	57	—	—	2/3

*On day 43 three mice which were in near terminal condition were sacrificed for pathological examination.
†400r.
‡10,000r
§These mice did not have S.C. tumors but developed I.P. tumors, presumably from the initial immunization inoculum.

Suppression of the immune response of the mice by pre-X-irradiation of the mice with 400r total body irradiation had little or no effect. X-rayed mice and normal mice from all three sources showed no appreciable difference in the number of tumor takes, tumor growth, or survival time for equivalent tumor inocula (Tables 1 and 2).

Mice pre-immunized by I.P. injection of X-irradiated (5000r) tumor cells a week before they were challenged S.C. with various concentrations of viable tumor inocula either exhibited no tumor growth or slower growth for a given inoculum as compared to non-immunized mice. At the 50 per cent concentration level of inoculum, normal mice from both the "J" and "H" stocks of mice all developed tumor and died at about the same time. With this same overwhelming concentration (50 per cent) of inoculum, 2/3 of the immune mice also developed tumor. However, the "J" stock mice died on about the sixtieth day but the "H" stock mice survived somewhat longer, to day 88 and 111 respectively. None of the immune mice developed tumor from the diluted (5 per cent, 0·5 per cent) inocula (Table 1). The immunity to S.C. tumor challenge was not permanent. On day 77 post tumor inoculation all of the surviving immunized mice both with and without S.C. tumors were challenged with a second S.C. tumor inoculum. All of the mice developed tumor at the site of the second inoculum and died within 60 days following the second challenge (Table 1).

Pre-immunized "B" stock mice showed immunity against a viable S.C. tumor inoculum (Table 2). None of the mice developed S.C. tumors. However, three out of nine of the immunized mice died on the 53–58 day and at autopsy were found to have intraperitoneal tumors. On the nintieth day, the six surviving mice which showed no S.C. tumor growth were sacrificed and four of these were also found to have intraperitoneal tumors. Apparently the initial immunization inoculum contained some viable cells which persisted and grew in the abdominal cavity in the presence of sufficient immunity to prevent the growth of a later viable S.C. inoculum (Table 2).

An additional experiment was conducted (Table 3) in which the tumor cells employed in the immunization inoculum received three dose levels of X-ray. Nearly all (13/14) of the mice injected I.P. with tumor cells X-irradiated with 2000r developed I.P. tumors whereas only 3/14 which received the 4000r inoculum and 1/15 which received the 6000r inoculum developed I.P. tumors.

All nine of the non-immunized mice challenged with the S.C. inoculum of viable tumor cells developed S.C. tumors at the site of inoculation, whereas only16/29 of the pre-immunized mice developed S.C. tumors. Of the thirteen mice which were immune to the S.C. tumor inoculation, four mice were found to have I.P. tumors at autopsy by day 60 (Table 3). This experiment confirmed the observation that immunity to a second tumor inoculum can exist in the presence of pre-existing cells from a

Table 3. Effect of X-irradiated adrenal tumor cells injected I.P. upon the subsequent growth of a S.C. inoculum of viable tumor cells given 7 days later.

Gr.	X-rayed inoculum I.P. day (−7)	I.P. tumor day 60	Viable inoculum S.C. day 0	S.C. tumor day 60	Mice with I.P. tumors immune to S.C. inoculum
1	6000r	0/5	50%	4/5	
2	6000r	0/5	5%	2/5	
3	6000r	1/5	0	—	
4	4000r	0/5	50%	4/5	
5	4000r	2/5	5%	1/5	1
6	4000r	1/4	0	—	
7	2000r	4/5	50%	3/5	1
8	2000r	4/4	5%	2/4	2
9	2000r	5/5	0	—	
10	None	—	50%	5/5	
11	None	—	5%	4/4	

LAF_1 ♂ "B" mice were used in this experiment.

Table 4. Daily chemotherapy of adrenal tumor with MTX, 6-MP, Cytoxan.

Gr.	Mice group	Drug	Daily dose day 19 or 25* (mg/kg)	Day 39 Surv. total	Day 39 Wt. changes	Day 39 Av. tumor size mm av. dia.	Survival time Med. day	Survival time Range (days)
1	7	MTX	1·0	5/7	+0·8	26·4	43	37–46
2	7		0·7	7/7	+2·0	25·9	44	44–49
3	6		0·49	6/7	+2·0	25·2	45	40–50
4	7	6-MP	70	3/7	−4·3	16·3	38	32–41
5	7		49	6/7	−2·0	17·2	44	34–52
6	7		34	7/7	−2·3	18·5	43	41–53
7	7	Cytoxan	70	5/6	−2·0	15·8	44	32–49
8	6		49	5/6	0·0	20·2	44	34–50
9	6		34	5/6	−2·0	18·8	45	38–51
10	6	None	—	6/6	+3·0	24·5	44	41–51

*LAF_1 ♂ "B" mice were used in this experiment.

Chemotherapy was commenced when the implanted tumors were palpable (8mm diameter). Four mice in each group were started on day 19. In the remainder treatment was started on day 25.

previous inoculum (I.P.) which initiated the immune reaction.

Where there were large amounts of tumor growing intraperitoneally the tumors were scattered throughout the abdomen and attached to various organs. This growth was accompanied by the presence of bloody ascitic fluid. Most of the cells in the ascitic fluid were erythrocytes and polymorphonuclear neutrophils but no identifiable tumor cells were seen. Mice which had small intraperitoneal tumors had no ascitic fluid and the tumor growth was almost invariably attached to the kidney. The kidney, or its surrounding tissues, may provide a particularly favorable environment for the maintenance of these adrenal tumor cells.

Response to chemotherapy

Daily treatment with MTX, 6-MP or cytoxan was ineffective in increasing the median survival time of mice with palpable S.C. adrenal tumors (Table 4). Although none of the drugs elicited a significant increase in survival time, 6-MP and Cytoxan did cause inhibition of tumor growth. However, this was accompanied by some toxicity as indicated by the body weight change of the animals. Methotrexate did not inhibit tumor growth. In a second experiment with Methotrexate, Cytoxan and 6-mercaptopurine a combination of surgery and chemotherapy was employed (Table 5). Surgical removal of the local tumor mass was performed on the twenty-seventh day following tumor inoculation and a limited (7 day) course of therapy was initiated on the twenty-eighth day. Untreated mice with tumor died with a median survial time of 50 days post tumor implantation. Surgical removal of the local tumor mass on day 27 increased the survival time of the mice to a median of 64 days.

Table 5. *Daily chemotherapy with* MTX, 6-MP *and Cytoxan.*

Gr.	Tumor inoc. day 0	Mice group	Surgery day 27	Drug daily day 28–34	Dose S.C. (mg/kg/day)	Day of death med.	Day of death range	Regrowth of surg. removed tumors day 60	Growth of retransplants of pooled surg. removed tumor* day 60 (inoc. day 27)
1	5%	6	—	—	—	50	41–57		
2	5%	7	—	MTX	1·0	44	41–57		
3	5%	7	—	6-MP	70·0	46	35–57		
4	5%	7	—	Cytoxan	70·0	48	43–64		
5	5%	4	+	—	—	64	59–76	3/3	1/3
6	5%	4	+	MTX	1·0	64	58–70	3/3	3/3
7	5%	3	+	6-MP	70·0	69	65–75	3/3	0/3
8	5%	4	+	Cytoxan	70·0	69	65–77	4/4	0/4
9	None	6	—	None	—	—	—	—	6/6

	Challenge					
	Condition	Tumor inoc. % day 0	Takes total	Day of death		
A	Normal	50	3/3	34	39	42
		5	3/3	41	43	46
		0·5	3/3	63	66	74
B	Immunized	50	2/3	58	60	—
		5	0/3	—	—	—
		0·5	0/3	—	—	—

*5% tumor inoculum prepared from pooled specimens of surgically removed tumors and injected S.C. into a tumor free area on opposite side of the mice immediately after surgery on day 27.

Chemotherapy alone with MTX (1·0 mg/kg/day), 6-MP(70 mg/kg/day) or Cytoxan (70 mg/kg/day) administered S.C. decreased the median survival time of the tumorous mice to 44, 46, 48 days respectively indicating that the drugs were somewhat toxic in the tumor bearing animals. Surgical removal of the tumor mass apparently permitted greater tolerance to therapy. Mice given MTX treatment post-operatively showed no increase in survival time as compared to those which received only surgery; however they did live appreciably longer than untreated tumorous mice or tumor mice which received only MTX treatment. Cytoxan and 6-MP administered post-operatively gave a slight increase in survival time as compared to mice which received only surgery, but a marked increase in life span compared to untreated control mice or mice receiving chemotherapy alone. The same tumor inocula in concentrations of 50 per cent, and the experimental inoculum of 5 per cent and even 0·5 per cent caused tumor growth and death in all non-pretreated mice. Mice immunized against the tumor by pre-inoculation of X-rayed (10,000r) tumor cells failed to grow tumors from the 0·5 per cent or 5 per cent viable tumor inoculum; however the 50 per cent tumor inoculum apparently overwhelmed the immunity and 2/3 mice developed tumors and died (Table 5, gr A & B).

Regrowth of the surgically removed tumor at the site of operation occurred in all of the animals, including those given chemotherapy (Table 5). In this experiment a tumor cell suspension was prepared from pooled surgically removed tumors and immediately injected back into a tumor free area of the same mice. This "simulated metastasis" grew in only 1/3 mice which received no post-operative chemotherapy. It grew in 3/3 mice which received MTX treatment post-operatively, in 0/3 which received 6-MP and 0/4 which received Cytoxan. The same inoculum produced tumors in 6/6 untreated control mice.

These observations suggest that an immune response similar to that observed following pretreatment with X-irradiated tumor cells (Table 5, gr A & B) may occur following growth and surgical removal of the mass of tumor. This may be sufficient to prevent metastatic growth in tumor free areas but not

prevent the growth of established tumor cells at the surgical site. Post-operative treatment with MTX may have suppressed or inhibited the development of immunity without exerting any appreciable anti-tumor effect since all of the animals developed tumors at the site of the "simulated metastasis."

The complete prevention of the growth of simulated metastasis by post-operative administration of Cytoxan or 6-MP may be attributable to anti-tumor effectiveness of these drugs or the failure of these drugs to suppress the immune response or a combination of the two.

BCNU administered S.C. in large single doses to mice with established 21-day-old subcutaneous tumors inhibited tumor growth and increased the survival time of the animals (Table 6). A dose of 100 mg/kg of BCNU was lethal to all the mice within a period of 5 days. A dose of 50 mg/kg almost completely inhibited tumor growth for a period of 20 days and approximately doubled the median survival time as compared to untreated mice. A dose of 25 mg/kg also caused significant tumor inhibition and increase in survival time.

In a second experiment with BCNU the

Table 6. Chemotherapy of adrenal tumor with large single doses of BCNU.

Gr.	Mice group	Dose mg/kg/sc day 21	Av. tumor size (mm) day 21	Av. tumor size (mm) day 40	Survival time med.	Survival time range
1	7	100	7·9	—	25	25–26
2	7	50	7·6	9·9	>79*	71–91
3	7	25	7·8	14·1	61	48–76
4	8	0	8·1	22·4	46	36–53

*On day 79, three of the four remaining mice in this group sacrificed for gross pathological examination.

Table 7. Single and multiple dose chemotherapy and post-operation chemotherapy with BCNU *of mice with adrenal tumors.*

Gr.	Pretreatment day 18	Drug	Dose	Days	Day of death med.	Day of death range	Tumor size av. diam. (mm) day 19	day 45	day 74	Post operation tumor regrowth "Met"*	
1	—	—	—	—	45	37–51	8·4	25·6	—		
2	—	BCNU	50	19	74	34–83	8·4	13·4	25·0		
3	—	BCNU	50	19, 34, 48	104	78–114	8·6	14·1	14·5		
4	0+MET†	BCNU	50	19	91	34–121	—	—	—	10/10	0/10
5	MET controls	None	—	—	93		—	—	—	—	8/10

*MET="Artificial metastasis" created by reimplantation of a tumor cell mash from the surgically removed tumor. The LAF_1 ♂ mice used in this experiment were obtained from "H". Ten mice per group.
†0=surgical operation.

Table 8. Effect of BCNU *treatment on the blood and adrenals of mice with adrenal tumors.*

	Untreated day 19	Untreated day 41	BCNU* treated day 41	BCNU* treated day 71
WBC/mm³	14,900	3,500	5,300	5,500
	15,200	6,100	5,500	10,300
	14,600	5,100	4,400	11,700
Ht%	64	18	53	19
	47	34	49	22
	62	36	46	18
Plasma color index (I.I.)	±	±	+++	+
	±	±	+++	+
	±	±	+++	+
Adrenal	normal	atrophy	normal	atrophy

*Single dose of BCNU (50 mg/kg).

effects of a single large dose, multiple doses and post-operative chemotherapy were investigated (Table 7). Some preliminary hematological and pathological data were also obtained (Table 8). LAF_1"H" mice with 19-day-old adrenal tumor implants averaging 8·4 mm in dia. were treated with a single dose of BCNU (50 mg/kg S.C.). This dose of BCNU caused an average maximum body weight loss of 3·9 g/mouse or 14·3 per cent of the initial body weight and 1/10 toxic deaths on the fifth day after treatment. There was marked inhibition of tumor growth and the median survival time was increased from 45 days in controls to 74 days in the treated group.

In another group of mice three doses of BCNU (50 mg/kg) given at 14-day intervals caused 2/10 deaths but minimal weight loss in surviviors, and the mice had a median survival time of 104 days (Table 7).

In a third group, each tumor was removed surgically, a cell suspension prepared from a fragment and retransplanted into the same host and also into a normal control mouse. Post-operative treatment with a single dose of BCNU 50 mg/kg S.C. was administered on the following day. Tumors regrew in all of the mice at the site of the surgical operation but their median survival time was increased from 45 days to 91 days. None of the "artificial metastases" grew in the mice treated with BCNU. Of the normal untreated mice which served as controls for the "artificial metastases" inocula 8/10 developed tumor (Table 7).

Untreated and BCNU (50 mg/kg) treated tumor mice from the same experiment (see Table 7) were sacrificed for hematological and pathological examination. Untreated tumor mice on day 19 were essentially normal except for the growth of the local tumor. The average volume of packed R.B.C. (Ht.) was 58 per cent, the average W.B.C. was 14,900/mm^3 and the plasma color index (I.I.) was normal. Gross examination of the internal organs revealed nothing abnormal (Table 8).

On day 41 when tumor growth in the untreated mice had approached a maximum and the mice were dying, they were anemic (Ht. 29 per cent average), leukopenic (W.B.C. 4950/mm^3 av) and their plasma color index was normal. Gross examination of the internal organs revealed some enlargement and congestion of liver and spleen, and atrophy and/or marked cavernous congestion of the adrenals (Table 8). At the same time (day 41) mice which had been treated with BCNU had normal Ht. (49 per cent average), low W.B.C. (5100/mm^3 av) and an unusually high plasma color index. The gross autopsy findings were normal except for the presence of the small tumors at the site of tumor inoculation.

On day 71 when the BCNU treated mice had large tumors and were in terminal condition their W.B.C. had risen to an average of 9183, their Ht. had fallen to an average of 20 per cent and their color index had returned to nearly normal. As in terminal untreated controls there was enlargement and congestion of liver and spleen and the adrenals were atrophied or showed marked cavernous congestion (Table 8).*

DISCUSSION

The irradiation induced transplantable adrenal tumor employed in these experiments still retains most of its original characteristics including the ability to produce adrenal steroid hormone and to cause adrenal atrophy in the host (1–4). However, although the tumor was highly malignant initially (4), it is now antigenic to the LAF_1 hybrid mice in which it originated and no longer appears to metastasize.

The antigenicity of the adrenal tumor is not surprising since the tumor has a long transplantation history. Genetic drift in one or both of the inbred parent strains as well as in the tumor cell population itself could undoubtedly account for the observed antigenicity. Cognizance should be taken of any tumor cell antigenicity in chemotherapy since even small antigenic differences may affect both the host and the therapeutic effect. Hybrid CDF_1 mice with advanced leukemia LI210 have survived following therapy with 3',5'-dichloromethrotrexate (DCM) and were found to be immune to reinoculation of the same tumor (5). Also, C57/Bl mice have been cured of adenocarcinoma Ca755 tumor with a short course of 6-MP treatment alone and in post-operative treatment and were found to be immune to subsequent reinoculation (6).

That immunity to a second tumor inoculum can exist in the presence of surviving cells was demonstrated in two instances (a) following intraperitoneal immunization with X-irradiated tumor cells where a portion of the cells remained viable (b) following growth and surgery of an initial implant. These observations are similar to and in support of a previous observation made in leukemia L1210 mice treated with 3',5'-dichloromethotrexate (DCM). Leukemic mice

*We are indebted to Dr. Thelma Dunn and Mr. Arleigh Green for the pathological examination of these animals. A more detailed description of the gross and microscopic pathology will be reported elsewhere.

undergoing treatment with DCM were refractory to the growth of a DCM resistant tumor inoculation although the original tumor cells were still present. The resistant tumor inoculum grew well in normal mice receiving the same dosage of DCM (7). The immunological relationships demonstrated in these experiments undoubtedly influence the growth rate of these tumors, the degree of metastasis and the effect of various chemotherapeutic agents.

Of the drugs tested, BCNU was the most effective in inhibiting the growth of mice with established adrenal tumors. It produced definite inhibition of tumor growth, delayed adrenal atrophy and yielded a substantial increase in life span of the mice. Cytoxan and 6-mercaptopurine inhibited the growth of the adrenal tumor but failed to increase survival time. Methotrexate did not appear to exert any therapeutic effect against this tumor. In preliminary experiments actinomycin D* and the hormone influencing drugs ACTH* o,p'DDD* or Metopirone* were ineffective in retarding tumor growth or increasing survival time of mice with established adrenal tumor. The current studies indicate the feasibility of undertaking chemotherapeutic studies with functional tumors such as the transplantable adrenal tumor employed here. Further studies on the *in vivo* and *in vitro* relationship of drugs to the growth and hormone secreting ability of normal and cancerous adrenal cells and other hormone producing tissues are in progress.

*Actinomycin D, NSC No. 3,053; ACTH—corticotropin: "ACTHAR" injection (Armour); Ethane, 2-(0-chloroethyl) - 2-(p-chlorophenyl)-1-1-dichloro-, o,p'DDD), NSC No. 38,721; 2-methyl-1,2-di-3-pyridyl-1-propanone: "Metopirone" (Ciba SU-4885).

RÉSUMÉ

Une lignée de cellules tumorales de la souris, secrétant une hormone surrénalienne, et qui a été transmise pendant environ 15 *ans,* in vivo *et* in vitro, *a été réétudiée au point de vue de la possibilité de la transplanter et de son histo-compatibilité* in vivo *chez des souris hybrides LAF*$_1$, *desquelles elle provenait.*

Les greffes de tumeurs ont montré une croissance de 100%, *sans aucune régression, chez des souris de trois sources. Cependant, l'immunuté à une seconde implantation de tumeur a été démontrée en présence d'une croissance de cellules tumorales après pré-immunisation par des cellues tumorales irradiées aux rayons X, mais encore viables, et également après ablation chirurgicule d'une grosse tumeur implantée.*

La chimiothérapie à la 6-mercaptopurine ou au cyclophosphamide retarda la croissance de la tumeur, mais ne put augmenter, de façon significative, le temps de survie. Le Méthotrexate ne se montra pas actif. L'urée 1,3-*bis* (2-*chloroéthyl*)-1-*nitriso*-(*BCNU*) *s'est montrée très efficace pour réduire la taille de la tumeur, retarder le développement de l'anémie, de la leucopénie et de l'atrophie surrénalienne, ainsi que pour augmenter le temps de survie des souris porteuses de tumeur.*

SUMMARY

A murine adrenal hormone secreting tumor cell line which has been carried for approximately 15 *years* in vivo *and* in vitro *was recharacterized for* in vivo *transplantability and histo-compatibility in LAF*$_1$ *hybrid mice in which it originated.*

Tumor transplants gave 100% *growth with no regressions in mice from three suppliers. However, immunity to a second tumor implantation was demonstrated in the presence of growing tumor cells following pre-immunization with x-irradiated but still viable tumor cells and also following surgical removal of a large established tumor.*

*Chemotherapy with 6-mercaptopurine or Cytoxan retarded tumor growth but failed to significantly increase survival time. Methotrexate was inactive in this system. Urea,*1,3-*bis* (2-*chloroethyl*)-1-*nitroso*-(*BCNU*) *was very effective in reducing the size of the tumor, delaying the development of anemia, leukopenia, and adrenal atrophy, and increasing the survival time of tumor mice.*

ZUSAMMENFASSUNG

Ein Mäusetumorzellstamm, welcher ein Nebennierenhormon absonderte und während ungefähr 15 *Jahren* in vivo *und* in vitro *fortgepflanzt werden konnte, wurde auf seine*

in vivo — *Uebertragbarkeit und histologische Verträglichkeit in LAF_1 hybriden Mäusen, von denen die ursprüngliche Geschwulst adstammte, überprüft.*

Die Tumortransplantate gingen in 100% *der Mäuse an, welche von drei verschiedenen Quellen geliefert worden waren. Andererseits konnte eine Immunität einer zweiten Geschwulstimplantation gegenüber festgestellt werden und zwar entweder in der Gegenwart von wachsenden Tumorzellen nach vorheriger Immunisation mit röntgenbestrahlten aber noch lebensfähigen Tumorzellen oder nach chirurgischer Entfernung von umfangreichen angegangenen Geschwulsten.*

Chemotherapie mit 6-Mercaptopurin oder mit Cytoxan verlangsamte das Tumorwachstum, blieb aber ohne bedeutenden Einfluss auf die Ueberlebenszeit. Methotrexat war unwirksam bei dieser Versuchsanordnung. 1,3-bis (2-chloroethyl)-1-nitroso-Harnstoff (BCNU) war sehr wirksam in Bezug auf die Abnahme der Geschwultsgrösse, verzögerte das Auftreten der Anämie, der Leukopenie und der Nebennierenatrophie und verlängerte gleichzeitig die Ueberlebenszeit der geschwulsttragenden Mäuse.

REFERENCES

1. V. Buonassisi, G. Sato and A. I. Cohen, Hormone-producing cultures of adrenal and pituitary tumor origin. *Proc. Nat. Acad. Sci.* **48,** 1184–1190 (1962).
2. A. I. Cohen, J. Furth and R. F. Buffett, Histologic and physiologic characteristics of hormone-secreting transplantable adrenal tumors in mice and rats. *Amer. J. Path.* **33,** 631–651 (1957).
3. J. Furth, Hormone production in endocrine tumors. In: *Ciba Foundation Colloquia on Endocrinology* **12,** p. 3 (Edited by G. E. W. Wolstenholme and Maeve O'Connor) Little, Brown, Boston.
4. J. Furth, In: *Recent Progress in Hormone Research* **2,** p. 22 (Edited by Gregory Pincus) Academic, New York. (1955).
5. A. Goldin and S. R. Humphreys, Studies of immunity in mice surviving systemic leukemia L1210. *J. Nat. Cancer Inst.* **24,** 283–300 (1960).
6. M. A. Chirigos, J. Colsky, S. R. Humphreys, J. P. Glynn and A. Goldin, Evaluation of surgery and chemotherapy in the treatment of mouse mammary adenocarcinoma 755. *Cancer Chemother. Rep.* **22,** 49–53 (1962).
7. A. Goldin, S. R. Humphreys, G. O. Chapman, J. M. Venditti and M. A. Chirigos, Augmentation of therapeutic efficacy of 3′,5′-dichloroamethopterin against an antifolic resistant variant of leukemia (L1210-M46R) in mice. *Cancer Res.* **20,** 1066–71 (1960).

Europ. J. Cancer Vol. 1, pp. 135–136. Pergamon Press 1965. Printed in Great Britain

The Isolation of Herpes Virus from Burkitt Tumours

P. J. SIMONS and M. G. R. ROSS

Imperial Cancer Research Fund
Burtonhole Lane
Mill Hill, London, N.W.7
Imperial Cancer Research Fund
East African Virus Research Unit
Entebbe, Uganda

IT HAS been suggested that the peculiar geographical distribution of the Burkitt lymphoma could be explained on the basis of a viral aetiology [1]. In December 1961, a unit from the Imperial Cancer Research Fund joined the staff of the East African Virus Research Institute, Entebbe, Uganda, in an investigation of this possibility. Most of the work described in this paper was carried out in close collaboration with the staff of that Institute [2, 3].

METHODS AND MATERIAL

Fresh tumour material was collected from the operating theatre in phosphate buffered saline (PBS) containing penicillin, streptomycin and Mycostatin. The tumour was kept at 4°C for varying periods, then finely minced, washed in PBS and a few fragments inoculated into tubes of primary hamster kidney (BHK) cells or early passage human foetal lung (HL) cells. The BHK cells were prepared by trypsinization of kidney tissue from 3 to 5 day old hamsters, and grown in Hanks balanced salt solution containing lactalbumin hydrolysate and calf serum. HL cells were prepared and maintained as described by Hayflick and Moorhead [4].

RESULTS

The first virus isolate was obtained from an extract of a tumour biopsy (CAN63) which had been stored in a mechanical freezer at −60°C for 5 days. Forty-eight hours after the inoculation of the extract into BHK cultures, groups of round, refractile cells could be seen increasing in number to involve the whole culture. Uninoculated control cultures showed no such changes. Passage of medium from infected cultures onto fresh cultures produced similar changes after 24 hr incubation. Dr. R. R. Dourmashkin, Imperial Cancer Research Fund, examined infected cultures in the electron microscope and showed the presence of an agent that resembled herpes virus. Stained preparations of such cultures showed round, dense cells mixed with giant cells containing many nuclei.

Two rabbits were scarified on the cornea with infective passage material. Three days later an exudate appeared and a marked kerato-conjuctivitis rapidly developed. Eight days later one of the rabbits was found moribund. The same virus was isolated from the lesion.

The characterization of the virus as herpes virus was confirmed by means of cross neutralization tests. An antiserum was prepared against a strain of herpes virus isolated in mice from material collected from a clinically typical herpetic skin lesion. The CAN63 isolate produced plaques on confluent BHK cells after 3 days incubation under agar, and this technique was used for the neutralization tests. Complement-fixation tests carried out by the staff of the East African Virus Research Institute with commercially obtained (Microbiological Associates Inc.) herpes simplex control antigen

and hyperimmune guinea pig serum provided further confirmation.

Ten days after the original biopsy a further biopsy was obtained from the same patient and inoculated into BHK tissue cultures. A virus identified as herpes simplex virus was isolated from this biopsy.

A total of fifty biopsies and the material from one post-mortem examination have been tested. Of the fifty biopsies, thirteen have been inoculated onto HL and BHK cells and the remaining thirty-seven on BHK cells only. These cultures were maintained for up to 7 weeks and the medium was then passed to fresh cultures. In addition six samples of bone marrow, seven throat swabs and six samples of urine from lymphoma patients have been inoculated into tissue cultures.

A total of five isolates, each identified as herpes simplex virus, was obtained from the biopsies, all from jaw tumours. Throat swabs were obtained from three of the five patients whose tumours contained herpes simplex virus. Two out of these three throat swabs were positive for herpes simplex virus. This means that at least two of the patients were actively secreting herpes virus at the time the biopsies were taken. Four throat swabs from other lymphoma cases did not yield any virus isolates.

It has been shown that young children can secrete herpes virus into the mouth or stools without showing signs of disease. [5] This finding was confirmed in Uganda. Throat swabs were collected from normal African children in 1 ml of PBS containing 2% calf serum and antibiotics and 0·1 ml of the swab washings inoculated into each tube of BHK cells. The cultures were examined daily and those which were normal were discarded after 7 days incubation. Under these conditions eight herpes virus isolations were obtained from 104 children aged between 1–4 years. One isolation was obtained from forty-six children aged between 6–14 years.

CONCLUSIONS

The ubiquitous nature of herpes simplex virus and the fact that African children can secrete the virus into the mouth, suggests that herpes virus is probably not involved in the aetiology of the Burkitt tumour. Further work will show whether the reported isolations of other viruses [6–8] from this tumour are significant or not.

REFERENCES

1. D. Burkitt, A tumour syndrome affecting children in Tropical Africa. *Postgrad. Med. J.* **38,** 71 (1962).
2. J. P. Woodall and A. J. Haddow, E. *Afr. Virus Res. Inst. Rep.* p. 30 (1962).
3. P. J. Simons and M. G. R. Ross, *Ann. Rep. Imp. Cancer Res. Fund,* p. 48 (1963).
4. L. Hayflick and P. S. Moorhead, The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25,** 585 (1961).
5. G. J. Buddingh, D. I. Schrum, J. C. Lanier and D. J. Gindey, Studies on the natural history of herpes simplex infections. *Pediatrics* **11,** 595 (1953).
6. G. Dalldorf and F. Bergamini, *Unidentified filterable agents isolated from African* children with malignant lymphoma. *Proc. nat. Acad. Sci. (Wash.)* **51,** 263 (1964).
7. M. A. Epstein, B. G. Achong and Y. M. Barr, Virus particles in cultured lymphoblasts from Burkitts lymphoma. *Lancet* **ii,** 702 (1964).
8. T. M. Bell, A. Massie, M. G. R. Ross and M. C. Williams, Isolation of a reovirus from a case of Burkitts lymphomas. *Brit. Med. J.* **i,** 1212 (1964).

Europ. J. Cancer Vol. 1, pp. 137–140. Pergamon Press 1965. Printed in Great Britain

The Isolation in Mice of Strains of Herpes Virus from Burkitt Tumours

J. P. WOODALL, M. C. WILLIAMS,
D. I. H. SIMPSON and A. J. HADDOW
East African Virus Research Institute
Entebbe, Uganda

THE BURKITT tumour is characterized by a peculiar anatomical, geographical and age distribution, and was first described as a jaw sarcoma of African children [1]. A comprehensive review of the subject and its implications has appeared elsewhere [2]. Since 1960, when it was first recognized that the climatic and geographic distribution of the tumour was such as to suggest a viral aetiology [3–5], this Institute has been actively investigating it. It was assumed that if an arbovirus were the aetiological agent, it would produce an encephalitis in inoculated laboratory animals after an incubation period measured in days, rather than tumours after a period of months. Standard techniques for the isolation of arboviruses in a wide range of laboratory animals were therefore used on tumour material, beginning in February 1961.

METHODS AND MATERIAL

Biopsy

Tumour material was kept at 4°C for short periods or frozen at below −50°C for longer periods. Biopsy CAN63 was taken on 18th June 1962 from the left maxilla of a 10-year-old girl with a 2-week history (Burkitt's Case No. J.161) under care of Dr. D. A. Hyslop, Arua Government Hospital, West Nile District, Uganda.

Mice

Newborn albino Swiss mice derived from the stock of Carworth Farms, New York ,were used in litters of five.

Inoculation

After storage for 48 hr at 4°C in a modified Eagles' medium, the tumour material was strained off and triturated in a pestle and mortar with sterile powdered glass and heat-inactivated normal guinea-pig serum to make an approximate 10% suspension. After light centrifugation 0·03 ml of the supernate was inoculated into each mouse (0·01 ml intracerebrally, intraperitoneally and subcutaneously).

RESULTS

In June 1962, newborn mice inoculated with a suspension of the ninth biopsy processed (CAN63) died with encephalitic symptoms. The biopsy material had been collected from D. P. Burkitt by a team from the Imperial Cancer Research Fund (ICRF), working at this Institute since December 1961 in close collaboration with us, and they subsequently isolated the virus in tissue culture from the same material [6].

The virus was reisolated in mice, and shown to be ether sensitive. It was passed through a porcelain Selas filter (max. pore size 850 mμ) by courtesy of the ICRF team, and subsequently through gradocol membranes of APD 680 and 410 but not 210 mμ. Adult mice showed no regular pattern of mortality after either intracerebral or intraperitoneal inoculation. Suckling mice inoculated in the footpad had dermal lesions and paralysis. Infant hamsters inoculated by our ICRF colleagues with our mouse brain virus died after 4–5 days and we

reisolated the virus in mice from the hamster brains. We also succeeded in passing the virus through the brain of an African field rat, *Arvicanthis abyssinicus* (Ruppell), and producing a trace of viraemia on the sixth post-inoculation day in a rhesus monkey inoculated intravenously. The virus produced lesions on the scarified cornea of a rabbit (carried out in collaboration with the ICRF team), and also produced plaques in chick embryo fibroblast monolayer cultures without previous adaption to the tissue culture system.

When electron microscopy by Dr. R. R. Dourmashkin, ICRF, showed that the agent resembled herpesvirus [6], a strain of virus (MHK) was obtained by isolation in mice from material collected from a clinically typical herpetic skin lesion by Dr. M. H. King (Dept. of Microbiology, Makerere University College Medical School). This strain was tested against the CAN63 isolate in a suckling mouse intracerebral neutralization test, with the results shown in Table 1.

TABLE 1

Virus	Titre in normal mouse ser.	Antiserum: MHK Pass 1–2 mouse ser. 2 inocs.	Antiserum: CAN 63 Pass 12 mouse ser. 6 inocs.
MHK Pass 2	5·2	1·8*	2·7
CAN 63 Pass 29	5·6	1·5	2·7

*Neutralization index: titres are expressed as log LD50 and the neutralization index is the difference between the control titre in the presence of the test serum.

Subsequently a herpesvirus complement fixing antigen and hyperimmune guinea-pig antiserum were obtained from Microbiological Associates Inc. (U.S.A.) and tested against CAN63, MHK and CAN98 (a later isolate from another Burkitt tumour biopsy). The results are shown in Table 2.

TABLE 2

Antiserum	Antigen: U.S.A.	CAN 63	CAN 98	MHK
Herpes U.S.A. hyperimmune guinea-pig	256*/2	512/2	512/2	256/1
CAN 63 hyperimmune mouse	8/2	32/1	16/1	8/1

*numerator = reciprocal of dilution of serum at endpoint. denominator = reciprocal of optimal dilution of antigen.

After biopsy CAN63, a further seventy-one biopsies were processed by us up to the end of 1963; sixty-seven of these were confirmed histologically as Burkitt tumours by Dr. D. H. Wright (Dept. of Pathology, Makerere University College Medical School). Five of the confirmed, but none of the unconfirmed tumours yielded agents in mice which behaved identically to CAN63 and were identified by neutralization, and in some cases complement fixation tests, as herpes virus. All were from jaw tumours with the exception of CAN98 from an ovarian tumour; this strain was the only one not isolated in parallel in tissue culture by the ICRF team, and not reisolated by us, and, as the biopsy was inoculated on the same day as both MHK and CAN63 passage material, it is possible that contamination occurred.

To discover whether herpes virus might be oncogenic in the embryonic animal, pregnant mice and a pregnant rhesus monkey were inoculated intravenously with the CAN63 strain; all produced apparently normal offspring. Attempts to obtain transmission of the strain after feeding or inoculation of three species of mosquito failed. In mouse protection tests, three different serum specimens from the donor of the CAN63 biopsy, taken at intervals, were protective, as were 13/18 sera from other Burkitt tumour cases, 8/8 from the parents of these cases and 18/18 from other cancer patients. Among the Burkitt tumour cases there was no obvious difference in the sex ratio or presenting site (jaw or elsewhere) of the protective and non-protective serum donors. Further protection tests showed that 13/43 European, 11/12 Asian and 126/130 African residents of East Africa, 12/15 natives of Papua, 8/10 wild baboons and 5/8 wild vervet monkeys from East Africa were immune.

DISCUSSION

The cumulative incidence of the Burkitt tumour [7] suggests an attack rate in children comparable to that of herpes virus. The lack of demonstrable immunity in some cases may be due to a failure in immune response such as occurs in a proportion of patients with other forms of lymphoma [8]. If carcinogenesis is a two-stage process [9], then herpes virus could be the initiating agent of the Burkitt tumour, provided that the promoting agent were a climatically or geographically limited factor.

On the other hand, the presence of herpes virus in jaw tumours could obviously be the result of concurrent oral infection, and in

visceral tumours of systemic spread such as is known to occur [10]. Children with acute lymphatic leukaemia secrete more herpes virus than controls [11], and the same may be true of Burkitt tumour cases [6]. Thus in the absence of further evidence, the presence of herpes virus in these tumours cannot be regarded as significant.

RÉSUME

1. *Six isolements de virus herpétique ont été faits chez des souris nouveau-nées à partir de* 76 *biopsies de tumeur de Burkitt.*

2. *Toutes les biopsies renfermant le virus provenaient de tumeurs du maxillaire, à l'exception d'une seule qui provenait d'une tumeur ovarienne, mais la validité de cet isolement est douteuse.*

3. *Les lignées de virus furent identifiées par des tests de neutralisation, certains étant confirmés par des tests de fixation de complément.*

4. *Soixante-dix pour cent seulement des sérums des cas de tumeur de Burkitt testés montrèrent de l'immunité au virus herpétique.*

5. *Des tentatives faites pour obtenir la transmission par les moustiques échouèrent.*

6. *En conclusion, le virus herpétique était présent dans ces tumeurs comme virus passager, bien qu'une liaison étiologique ne soit pas totalement exclue.*

SUMMARY

1. *Six isolations of herpes virus have been made in newborn mice from seventy-six biopsies of Burkitt tumours.*

2. *All the biopsies yielding virus were from jaw tumours with the exception of one which was from an ovarian biopsy, but the validity of that isolation is doubtful.*

3. *The strains of virus were identified by neutralization tests, some being confirmed by complement fixation tests.*

4. *Only* 70 *per cent of sera from Burkitt tumour cases tested were immune to herpes virus*

5. *Attempts to obtain mosquito transmission of the virus failed.*

6. *It is concluded that herpes virus was present in these tumours as a passenger, although an aetiological connection is not entirely excluded.*

ZUSAMMENFASSUNG

1. *In sechs Fällen konnte in neugeborenen Mäusen das Herpesvirus isoliert werden bei Ueberprüfungen von* 76 *Biopsieen von Burkitt-Tumoren.*

2. *Alle Biopsieen, aus welchen das Virus isoliert wurde, stammten aus Unterkiefergeschwulsten mit Ausnahme einer Eierstocksbiopsie, aber die Gültigkeit dieser Isolierung ist zweifelhaft.*

3. *Die Virusstämme wurden durch Neutralisationsteste identifiziert und verschiedene Male durch Komplementbindungsteste bestätigt.*

4. *Nur* 70% *der Seren stammend von Burtkittgeschwulstfällen waren dem Herpesvirus gegenüber immun.*

5. *Versuche das Virus durch Moskitos zu übertragen, scheiterten.*

6. *Es wird der Schluss gezogen, dass das Herpesvirus in diesen Tumoren nur mitgeschleppt wird, obwohl ein ätiologischer Zusammenhang nicht vollkommen ausgeschlossen werden kann.*

REFERENCES

1. D. Burkitt, A sarcoma involving the jaw in African children. *Brit. J. Surg.* **46,** 218 (1958).
2. D. Burkitt, A lymphoma syndrome in Tropical Africa. *Int. Rev. Exp. Path.* **2,** 67 (1963).
3. G. T. O'Connor, Malignant lymphoma in African children. *Cancer* **14,** 270 (1961).
4. D. Burkitt and J. N. P. Davies, Lymphoma syndrome in Uganda and Tropical Africa. *Med. Press* **245,** 367 (1961).
5. *E. Afr. Virus Res. Inst. Rep.* 1960–1, 1961–2, 1962–3, Nos. 11–13, Nairobi.
6. P. J. Simons and M. G. R. Ross, The isolation of herpes virus from Burkitt tumours. *Europ. J. Cancer* **1,** 135–136 (1965).
7. A. J. Haddow, Age incidence in Burkitt's lymphoma syndrome. *E. Afr. Med. J.* **41,** 1 (1964).
8. S. Saslow, H. N. Carlisle and B. Bouroncle, Antibody response in hematologic patients. *Proc. Soc. exp. Biol. N.Y.* **106,** 654 (1961).
9. C. Andrewes, Tumour viruses and virus tumours. *Brit. med. J.* **1,** 653 (1964).
10. T. F. McNair Scott, The herpes virus group. In *Viral and Rickettsial Infections of Man* (Edited by T. M. Rivers and F. L. Horsfall) 3rd ed. Pitman, London.
11. K. Cook, quoted by R. J. Huebner, Group Discussions: Addendum on Cancer Causation. *Ciba Foundation Symposium on Tumour Viruses of Murine Origin,* p. 407 Churchill, London (1961).

Europ. J. Cancer Vol. 1, pp. 141–145. Pergamon Press 1965. Printed in Great Britain

Effect of Triazolium Derivatives on the Growth of Tissue Cultured Cells

L. MORASCA

Istituto di Ricerche Farmacologiche "Mario Negri"
Via Eritrea, 62-Milano, Italy
and
European Group of Cancer Chemotherapy (GECA)

SEARCHING for new chemical structures provided with antitumoural activity it was considered interesting to study a small series of triazolium derivatives synthesized by Runti and Nisi [1] reported in the Table 1 as compounds T_2–T_5.

In addition to the five triazolium derivatives 4-anilino-1-phenyl-1 H-1,2,4 triazolium chloride (T_1); 1-*p* tolyl-4-*p* toluidin-1 H-1,2,4, triazolium (4) chloride (T_2); 1 *m* tolyl-4-*m* toluidin 1 H-1,2,4, triazolium (4) chloride (T_3); 1-*o*-tolyl 4-*o* toluidin, 1-H 1,2,4, triazolium chloride (T_4); 1-methyl-4-methylamino-1-H-1,2,4 triazolium bromide (T_5); the effect of 1-phenyl-1-H-1,2,4 triazole (T_6); 1,5 diphenyl-formazan (T_7); and N (anilino) -N-(N formyl-anilino) formamidine (T_8) was also studied (see Table 1).

The possible antitumoural effect of these compounds was studied *in vitro* by evaluating the growth of human cell strains. Some active compounds were studied also as inhibitors of mitosis and of DNA synthesis by H^3-thymidine. In addition morphological changes occurring in the cells damaged by the compounds under study will be described.

MATERIALS AND METHODS

Most of the experiments have been performed on monolayer KB cell strain (human nasopharyngeal carcinoma) cultivated in a modified Eagle's medium additioned of 10% calf serum [2]. Human Amnion cells have also been used in one set of experiments.

Evaluation of growth

A method already described was used [2].

Fifty thousand cells in 1 ml of medium were inoculated in each test tube in order to obtain a monolayer growth.

Forty-eight hours after the inoculum a sample was treated with 0·25% trypsin and counted by an electronic cell counter (Coulter Counter mod. D) as the base line of the experiment.

At the same time the medium was changed with a fresh medium containing the drug under study. Forty-eight hours after this treatment the cells were again trypsinized in order to perform another cell counting.

The number of cells and the standard error in each experimental group are represented as a percentage of the controls.

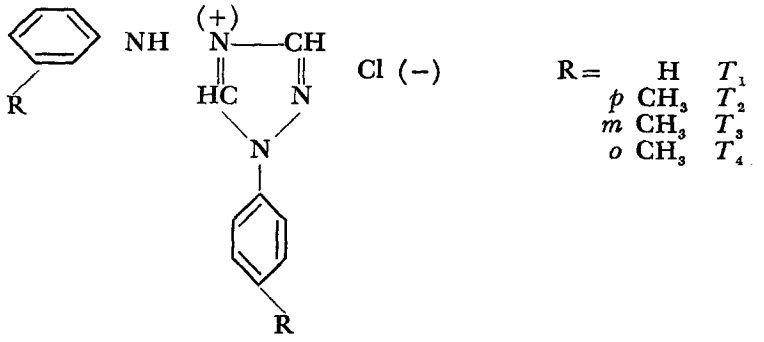
Mitotic index

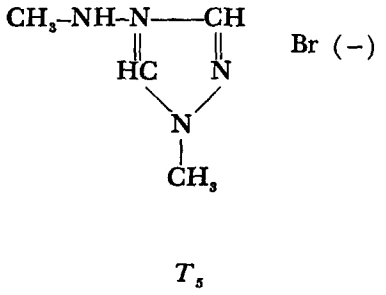
Other KB cells were cultivated on coverslips in Leighton tubes. After the treatment with the compounds under study the cells were fixed in ethanol–acetic acid (3:1) and stained with hemalum. The number of mitosis found on 1000-cell counts was recorded as the mitotic index.

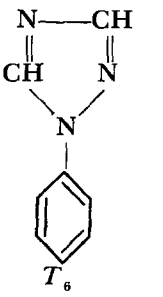
H^3—*tymidine incorporation*

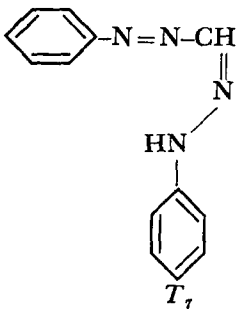
Coverslip cultures in Leighton tubes during logarithmic growth were treated with 1 μg/ml of H_3-thymidine (obtained from the Schwartz Bioresearch Inc., Orangeburg, N.Y.) for 5 min and then rapidly fixed in ethanol acetic acid (3:1) according to a technique previously des-

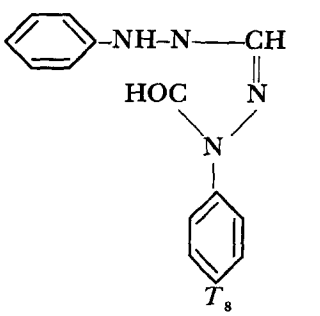
Table 1. *Chemical structures of the compounds under study*











cribed [3]. After hydrolysis in 1 N HCl at 60°C for 8 min cultures were stained by Feulgen and covered with an AR 10 Kodak stripping film.

After 2 weeks of exposure autoradiographic emulsions were processed in a D 19 Kodak developer as recommended by the manufacturer.

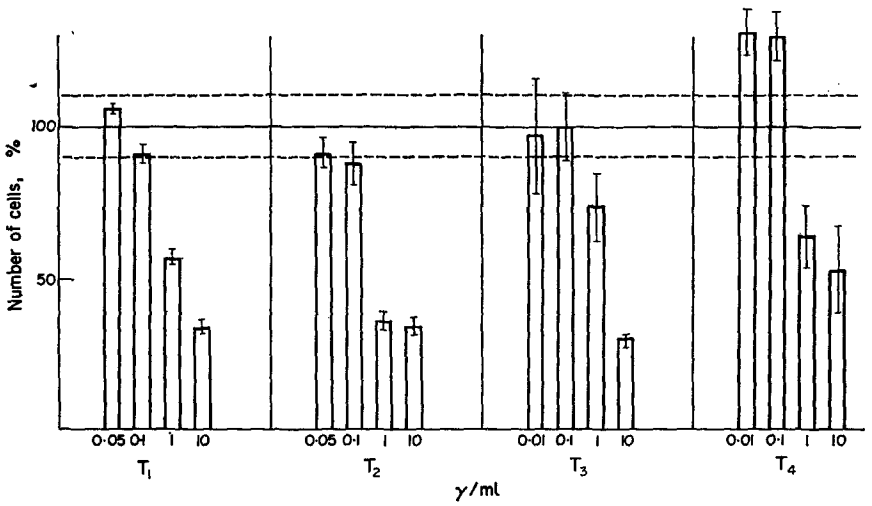


Fig. 1. *Effect of compounds* T_1–T_4 *on the number of* KB *cells after* 48 hr *treatment. The vertical bars represent the S.E. of the mean.* (*Controls*=100)

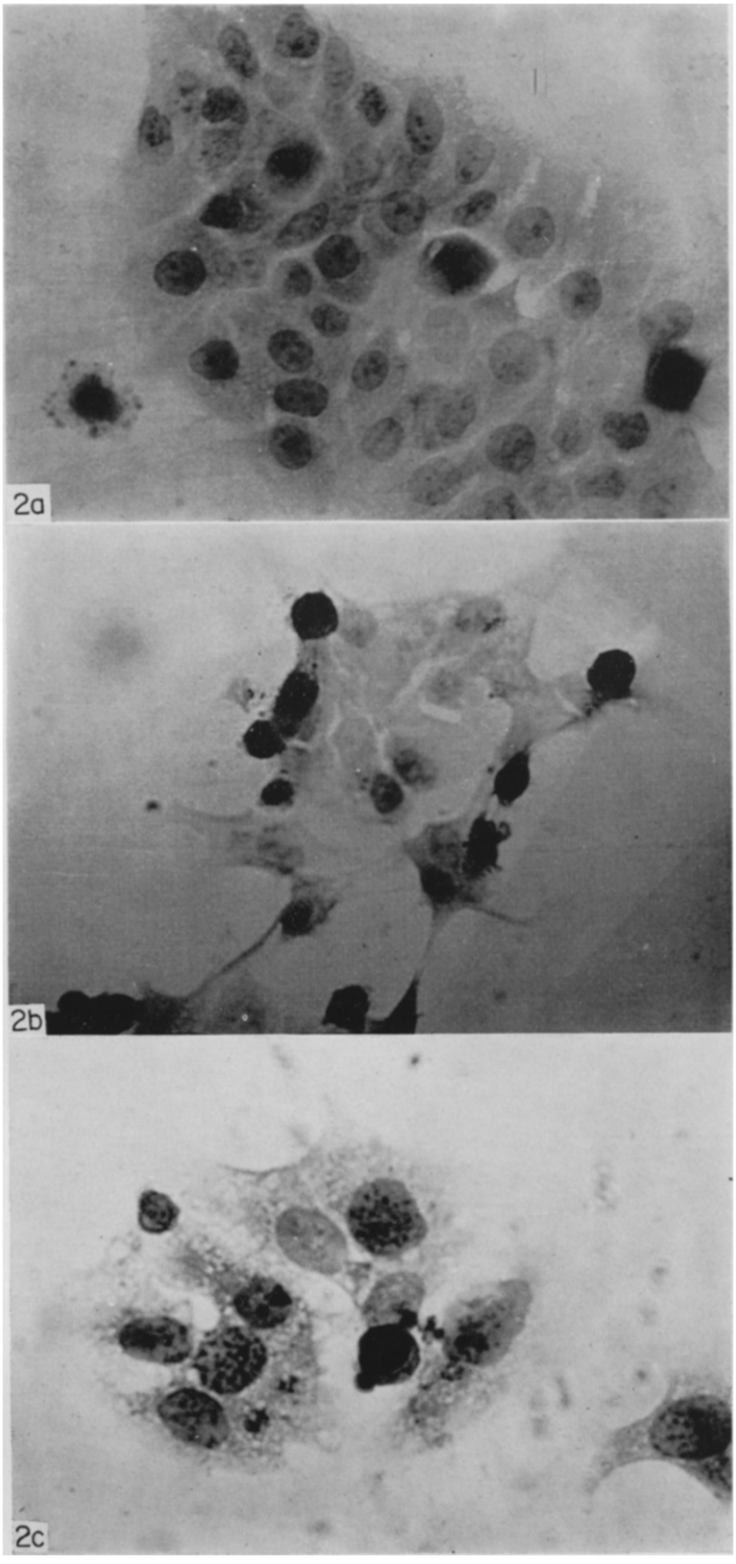


Fig. 2.

a. *Control* ×900.

b. T_1 1 µg/ml *after* 10 hr; *cellular contraction* ×900.

c. T_1 1 µg/ml *after* 40 hr; *fragmentation of chromatin* ×1200.

(to face p. 142)
Refer to p. 144

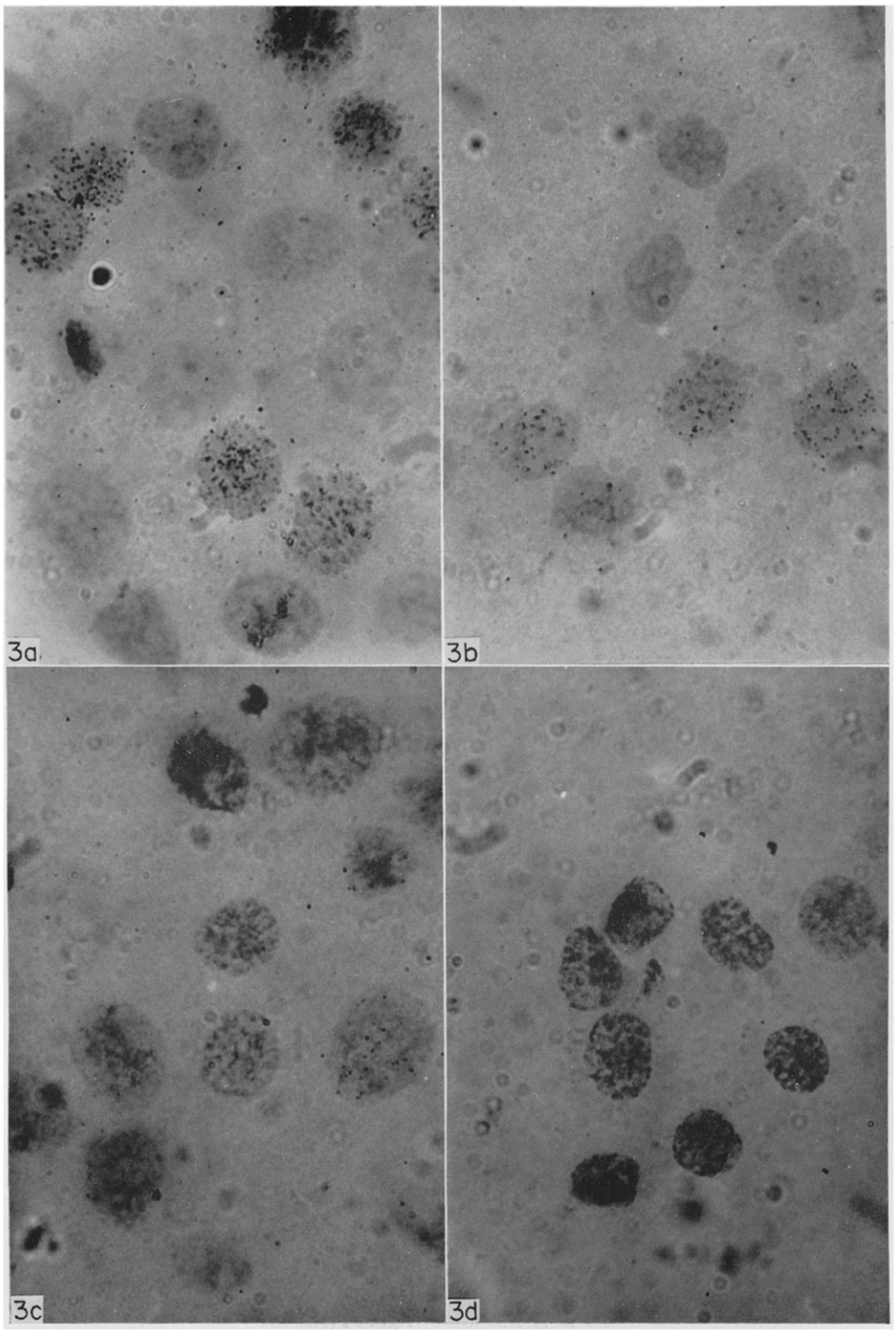


Fig. 3. *Autoradiographs of cells stained with Feulgen after hydrolysis* $\times 1725$.

a. *Control: heavy density of radioautographic grains;*

b. T_2 5 μg/ml *after* 5 hr: *nuclei are not so heavily labelled;*

c. T_2 5 μg/ml *after* 10 hr: *very few nuclei labelled with few grains; chromatin is fragmented;*

d. T_2 5 μg/ml *after* 20 hr: *no labelled nuclei are present; chromatin fragmentation is evident.*

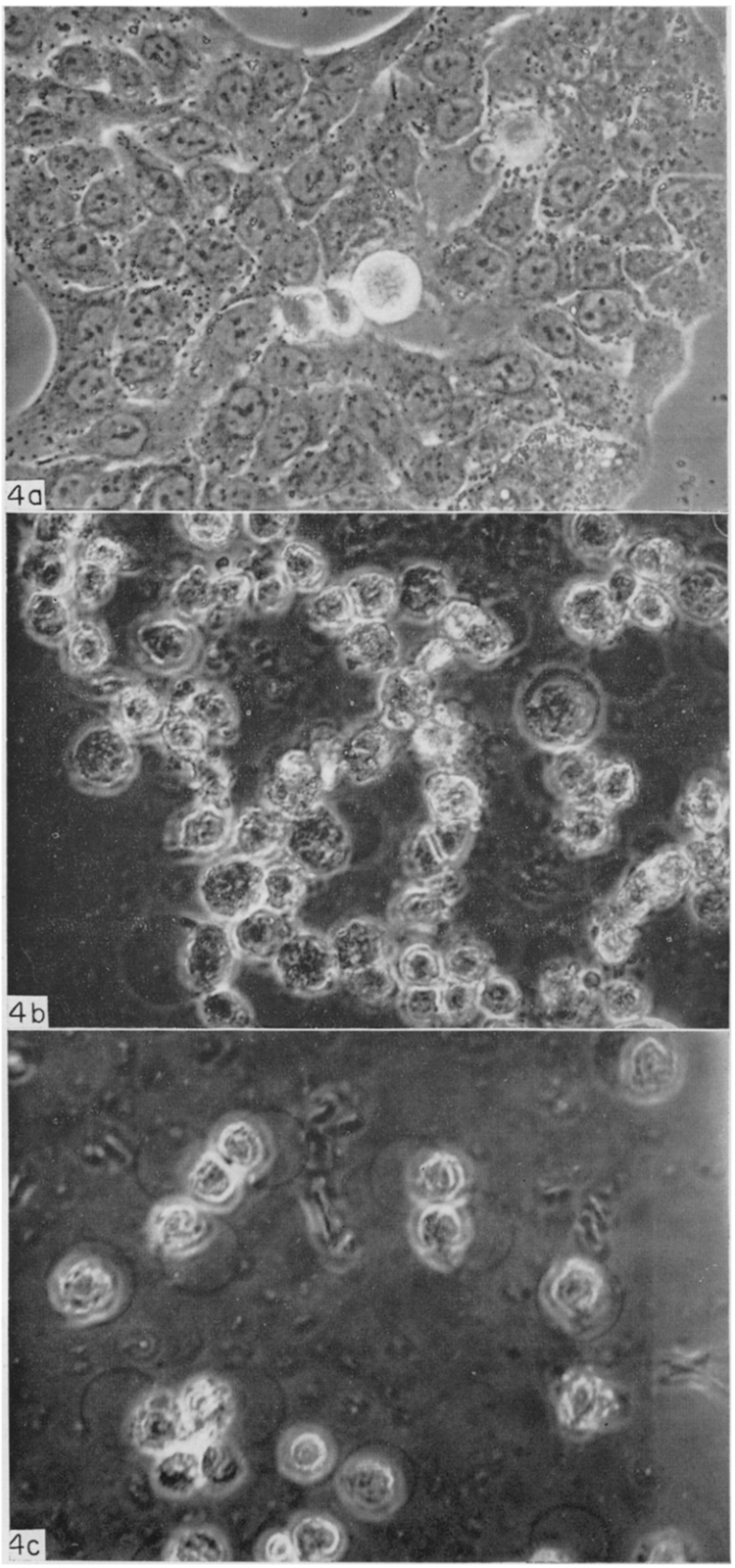


Fig. 4. *Phase contrast pictures.* ×510.

a. control;

b. T_2 5 μg/ml *after* 20 hr;

c. T_2 5 μg/ml *after* 10 hr *treatment plus* 24 hr *recovery.*

Table 2. Effect of compounds T_1–T_4 *on mitotic index of* KB *strain*

Compound (μg/ml)		Mitoses (% cells) after treatment (hr)				
		1	5	10	20	40
Fresh medium		2·6±0·05	2·2±0·05	2·1±0·05	2·5±0·05	1·8±0·04
T_1	0·1	n.d.	2·3	2·2	2·1	3·2
T_1	1·0	2·0	1·2	0·7	0·4	0·6
T_1	5·0	2·2	0·8	n.d.	0	n.d.
T_2	0·1	2·3	2·1	2·5	1·9	1·8
T_2	1·0	2·0	0·8	0·8	0·4	0·2
T_2	5·0	2·3	0·9	0	n.d	n.d.
T_3	5·0	0·7	n.d.	0·6	n.d.	n.d.
T_4	5·0	0·9	0	n.d.	n.d.	n.d.

n.d. = not determined
The standard error was always less than ± 0·05 for each figure reported in the Table.

*Morphological studies**

Microphotographic pictures and time lapse films were taken on the KB strain of cells during logarithmic growth in Rose's chambers [4].

Treatments were performed in thermostatic room by using prewarmed medium.

RESULTS

Effect on cell growth

Figure 1 shows the inhibition of growth produced by various doses of the compounds T_1–T_4, found effective in a preliminary test.

The data presented for compounds T_1 and T_2 are the mean of three experiments while the data for T_3 and T_4 are taken from only one experiment.

The four compounds are all effective at 1 μg/ml as growth inhibitors of the KB cells.

Activity of related compounds

1-methyl-4-methylamino-1H-1,2,4triazolium bromide (T_5); 1-phenyl-1H-1,2,4-triazole (T_6); N-(anilino)-N-(formylanilino)-formamidine (T_7); and 1·5-diphenylformazan (T_8) have been also tested for growth inhibition properties, but even at a dose of 100 μg/ml they did not show any toxicity for the cells.

The loss of the quaternary ammonium for T_6, T_7 and T_8 resulted also in the loss of the antiproliferative properties. However other compounds with a quaternary nitrogen, such as succinylcholine chloride and hexamethonium bromide tested on the same system did not show any effect. The substitution of the phenyl with methyl groups (compound T_5) resulted also in the disappearance of the activity.

Effect on mitotic index

Determinations of the mitotic index were carried out at various times after the addition to the medium of two concentrations (0·1 and 1 μg/ml) of T_1 and T_2.

Table 2 shows that a decrease of the number of cells in mitosis requires the contact of T_1 or T_2 with the cells for a period of at least 5 hr. Compounds T_3 and T_4 appear instead to act more rapidly.

Effect on DNA *synthesis*

The uptake of H^3-thymidine in the described experimental conditions was taken as an indication of the DNA synthesis.

The percentage of labelled nuclei is reported in Table 3.

Table 3. Effect of compounds T_1–T_4 *on the incorporation of* H^3*-thymidine into* DNA (KB *strain*)

Compound (5 μg/ml)	% labelled nuclei (hr)			
	1	5	10	20
Fresh medium	39	40	41	38
T_1	34	28	n.d.	0
T_2	41	26	0	1
T_3	29	20	8	0
T_4	28	n.d.	11	n.d.

n.d. = not determined
The S.E. was always less than 2 per cent of the figures reported in the Table.

There is a sharp decrease evident in the number of labelled cells beginning from 5 hr after contact of the compounds T_1, T_2, T_3 and T_4 with the cells. Again compounds T_3 and T_4 already showed activity after 1 hr of contact. The number of radioactive grains was greatly reduced, although no quantitative data are reported here.

Morphological observations

The first morphological sign of cell damage appears with a dose of 1 μg/ml of T_1 or T_2 after

*The films have been performed at the Department of Embryology and Experimental Morphology, University of Milan (Director Prof. V. G. Leone).

Table 4. Summary of the morphological effects induced by compounds T_1 *and* T_2

Effect	Concentration (μg/ml)	Delay (hr)
Inhibition of DNA synthesis	5	5–10
Chromatin fragmentation	1	20
Chromatin fragmentation	5	10
Mitotic index decrease	1	5–10
Cell contraction	1	10 (partial)
Cell contraction	5	7 (partial)
Cell contraction	10	5 (total)
Cell contraction	50	3 (total)

10 hr of contact. Cytoplasmic contraction (see Fig. 2) is the abnormality commonly found in a small number of cells when most of the culture looks normal. After 20 and 40 hr of treatment at the same concentration few cells show fragmentation of chromatin.

In the autoradiographic experiments 10 hr after application of 5 μg/ml of T_1, T_2, T_3 or T_4 the fragmentation of chromatin was evident in a large number of cells (see Fig. 3). In a phase contrast "time lapse film" a rapid contraction of the whole colony occurred after 5 hr of treatment with 10 μg/ml of T_2 and the process of contraction lasted about 3 hr after which the colony was apparently dead.

In other films with lower doses (1 and 5 μg/ml of T_2) the contraction was limited to a small number of cells (see Fig. 4). Using a strain of Human Amnion cells the cytoplasmic contraction was evident 5 hr after the addition of 5 μg/ml of T_2 in part of the culture. Increasing the concentration of T_2 up to 50 μg/ml the cytoplasmic contraction was present in the whole culture.

CONCLUSIONS

The triazolium derivatives T_1–T_4 show a clear inhibition on the growth of human cancer cells cultivated *in vitro*. Human amniotic cells are however also sensitive to these compounds.

A triazole derivative (T_6) as well as two compounds (T_7 and T_8) where the triazole ring was open, lost almost completely the inhibiting effect. The simple presence of a quaternary nitrogen does not explain the antimitotic activity because two other ammonium derivatives (succinylcholine and hexamethonium) and a triazolium derivative (compound T_5) do not show inhibition of the growth of KB cells.

The active compounds decrease the number of mitoses and prevent the incorporation of labelled thymidine into DNA. The two effects were however observed at different concentration and required a certain delay.

In addition the compound T_2, as well as the others, exerts a number of morphological effects including cytoplasmic contraction and chromatin fragmentation depending upon the concentration and the time of contact.

The reported results suggest other investigation to establish the possible value of triazolium derivatives in cancer chemotherapy.

Acknowledgements—I wish to acknowledge the helpful criticism and support by Prof, S. Garattini and V. G. Leone as well as the technical assistance of Dr. C. Rainisio.

RÉSUMÉ

Un groupe de dérivés de triazolium, comprenant le chlorure de triazolium 4-*anilino*-1-*phényl*-1*H*-1,2,4 (T_1), *le chlorure de triazolium* 1-p-*tolyl*-4-p-*toluidine*-1 *H*-1,2,4 (T_2), *le chlorure de triazolium* 1 m-*tolyl*-4-m-*toluidine*-1 *H*-1,2,4 (T_3), *et le chlorure de triazolium* 1-o-*tolyl* 4-o-*toluidine*, 1-*H*-1,2,4 (T_4), *inhibent la croissance de culture de cellules tissulaires d'origine néoplasique, à des concentrations comprises entre* 1 *et* 5 μg *par ml. Ces composés inhibent la mitose, empêchent l'incorporation de thymidine*-H_3, *et provoquent des changements morphologiques consistant en une contraction cytoplasmique et en une fragmentation de la chromatine.*

SUMMARY

A group of triazolium derivatives including 4-*anilino*-1-*phenyl*-1*H*-1,2,4 *triazolium chloride* (T_1); 1-p *tolyl*-4-p *toluidin* -1 *H*-1,2,4 *triazolium chloride* (T_2); 1-m *tolyl*-4-m-*toluidin* 1-*H*-1,2,4 *triazolium chloride* (T_3) *and* 1-o *tolyl* 4-o-*toluidin*, 1-*H*

1,2,4 *triazolium chloride* (T_4) *inhibits the growth of tissue cultured cells of neoplastic origin at concentrations ranging between* 1 *and* 5 μg/ml. *These compounds inhibit mitosis, prevent uptake of* H_3*-thymidine and induce morphological changes consisting of cytoplasmic contraction and chromatin fragmentation.*

ZUSAMMENFASSUNG

Einige Triazolderivaten, namentlich das 4-*Anilino*-1-*phenyl*-1-*H*-1,2,4-*triazol-chlorid* (T_1), *das* 1-p-*Tolyl*-4-p-*toluidin*-1-*H*-1,2,4-*triazol-chlorid*(T_2), *das* 1-m-*Tolyl*-4-m-*toluidin*-1-*H*-1,2,4-*triazol-chlorid* (T_2) *und das* 1-o-*Tolyl*-4-o-*toluidin*-1-*H*-1,2,4-*triazol-chlorid* (T_4), *hemmen in Konzentrationen zwischen* 1 *und* 5 μg/ml *das Wachstum der Gewebekultur von Zellen neoplastischen Ursprungs. Diese Verbindungen hemmen die Zellteilung, verhindern den Einbau des* H_3*-markierten Thymidins und rufen morphologische Veränderungen, durch Schrumpfung des Cytoplasmas und Zerbrockelung des Chromatins gekennzeichnet, hervor.*

REFERENCES

1. C. Runti and C. Nisi, Reactions between orthoesters and organic nitrogen compounds—VI. Arylhydrazines. *J. Med. Chem.* **7,** 814 (1964).
2. L. Morasca and A. Leonardi, *Rev. Franc. Etud. Clin. Biol.* In press.
3. J. E. Sisken, Methods for measuring the length of the mitotic cycle and the timing of DNA synthesis for mammalian cells in culture. In: *Method in Cell Physiology* **1** p. 387. Edited by D. M. Prescott. Academic, New York (1964).
4. G. G. Rose, A separable and multipurpose Tissue Culture chamber. *Tex. Rep. Biol. Med.* **12,** 1074 (1954).

Europ. J. Cancer Vol. 1, pp. 147–151. Pergamon Press 1965. Printed in Great Britain

Studies on the Anti-tumour Activity of Aliphatic Aldehydes—II

Effects on Survival of Yoshida Ascites Hepatoma-bearing Rats

E. CIARANFI, L. LORETI, A. BORGHETTI and G. G. GUIDOTTI

Institute of General Pathology and C.N.R. Centre for Research in Cell Pathology, University of Milano, Italy

INTRODUCTION

PREVIOUS studies in this Laboratory [1–3] showed that a number of aliphatic aldehydes depress amino acid incorporation into protein of normal and neoplastic tissues *in vitro* and that this inhibition occurs without substantial impairment of the mechanisms of aerobic or anaerobic energy production [4]. In a preceding paper of this series [5] the hypothesis has been advanced that aliphatic aldehydes condense with endogenous cysteine, thus depriving cells of this thio-amino acid. The resulting inhibition of protein synthesis could then explain the encouraging results reported by Furst *et al.* [6], French and Freedlander [7], Evans [8], Warburg *et al.* [9] and Bruns *et al.* [10] on the survival of leukemia and Ehrlich ascites tumour-injected mice upon treatment with aliphatic aldehydes.

In the present work, we report results of survival experiments performed on rats injected with Yoshida ascites hepatoma cells and subsequently treated with DL-*erythro,threo*-α,β-dihydroxybutyraldehyde. This aldehyde was selected since it has been shown to depress markedly protein synthesis at concentrations which have only a minor effect on tissue respiration and glycolysis [5] and on the basis of its low toxicity when injected intraperitoneally or fed *per os* to Wistar rats [11]. DL-Glyceraldehyde, which inhibits glycolysis besides protein synthesis [4, 12] was used for comparison.

METHODS

Wistar albino rats of either sex, weighing 160–180 g were used in these experiments. They were maintained throughout on a diet of commercial rat pellets supplied *ad libitum* and ordinary tap water.

Unless otherwise specified, the groups of animals were given intraperitoneal inoculations of approximately 10^7 cells from Yoshida hepatoma (AH-130) maintained in ascitic form by intraperitoneal transplantation. Rats injected in this way invariably die within 14 days.

Preliminary experiments showed that intraperitoneally injected aliphatic aldehydes disappear almost completely from the ascitic fluid in a very short time (Fig. 1). Drugs were then administered every 4 hr for a total of seven injections, starting 1 to 8 days after tumour inoculation. Aldehydes were dissolved in saline to a final osmolarity of 0·30; the solutions were sterilized by filtration and kept refrigerated until used. Every injection consisted of approximately 2 ml of a 0·14 M aldehyde solution (0·15 mmole/100 g body weight).

Chromatographically pure DL-*erythro,threo*-α,β-dihydroxybutyraldehyde (3-methyl-glyceraldehyde) was supplied by Laboratori Farmitalia, Milano, Italy; DL-glyceraldehyde was purchased from Mann Research Laboratories, New York, U.S.A.

In some experiments, the number of tumour cells per ml of ascitic fluid was evaluated after aldehyde injection by counts in a standard

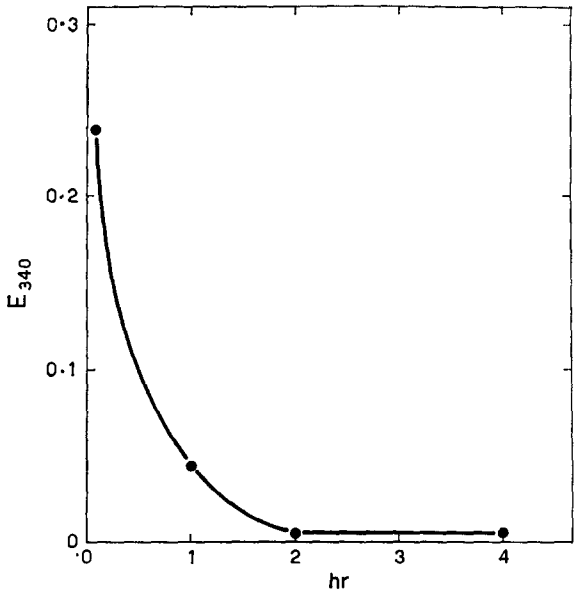


Fig. 1. Disappearance of DL-*glyceraldehyde from the ascitic fluid of Yoshida ascites hepatoma-bearing rats. Rats with an ascitic fluid volume of about* 40 *ml were injected with glyceraldehyde (intraperitoneal concentration after the injection, approximately* 10 *mM). Five minutes before and* 5 *min,* 1, 2 *and* 4 *hr after the injection,* 2 *ml of ascitic fluid were withdrawn, centrifuged, deproteinized and assayed for glyceraldehyde with the method of* Tsao *and* Schwartz [22], *based on the reaction between* α*-hydroxycarbonyl compounds and nicotinamide-adenine dinucleotide to form* 340 mμ*-absorbing addition compounds.*

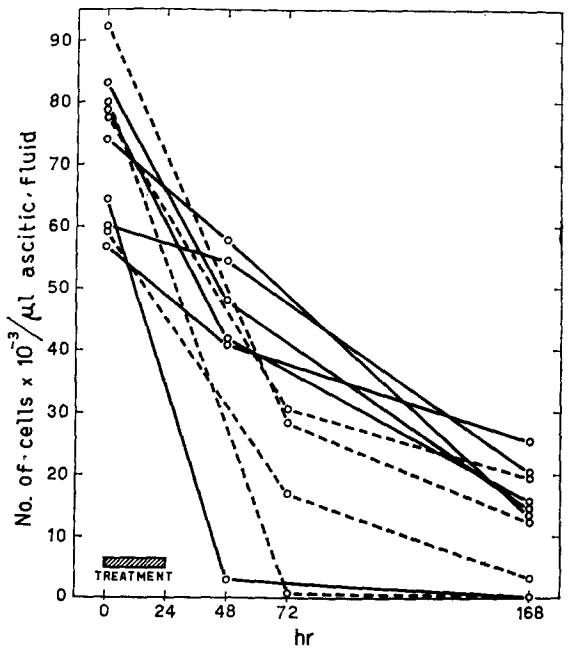


Fig. 2. Effects of DL-erythro,threo-α, β*-dihydroxybutyraldehyde on cell concentration in the ascitic fluid of Yoshida ascites hepatoma-bearing rats. Rats were inoculated with approximately* 10^7 *cells of ascites hepatoma. A* 24*-hr treatment with aldehyde (shaded bar) began* 8 *days later. Cell counts were performed just before the first aldehyde injection and* 1, 2 *and* 6 *days after the end of the treatment.*

Table 1. *Effects of* DL-erythro,threo- α-, β-*dihydroxybutyraldehyde and* DL-*glyceraldehyde on survival of Yoshida ascites hepatoma-bearing rats*

Compound	Total dose* (mg/100 g body wt)	Time elapsed between tumour inoculation and treatment initiation (hr)	No. rats	Indefinite survivors†	Mortality Ascitic tumour (within 3 weeks)	Solid tumour (within 8 weeks)	Non-neoplastic ascites (within 12 weeks)
Nil	—	—	42	0	39	3	0
Dihydroxybutyraldehyde	110	24	39	37	0	1	1
Dihydroxybutyraldehyde	110	144	41	23	9	1	8
Glyceraldehyde	95	144	18	8	1	6	3

*The drug was administered intraperitoneally every 4 hr for a total of seven injections.
†Alive and healthy after 5 months.

blood counting chamber; their viability was controlled by a nigrosin-permeability method [13] and morphological changes were followed on smears stained with May Grünwald-Giemsa and Feulgen-Light green

RESULTS

When rats bearing a well developed ascitic tumour were injected with dihydroxybutyraldehyde, the number of cells in the ascitic fluid decreased as shown in Fig. 2. Nigrosin-permeability tests and smears examination indicated that counts at the intervals following aldehyde treatment included a number of dead cells and cells and large in volume. In these animals, a The observation of the peritoneal surface revealed the presence of a fibrin-like membrane which consisted of dead cells, cell debris and amorphous materials. This finding was particularly apparent in animals treated with aldehydes 7 or more days after tumour inoculation, *i.e.*, when their ascitic fluid was rich in cells and large in volume. In these animals, a subsequent process of organization led to the formation of a thick membrane spread over the whole peritoneal surface which inhibited fluid reabsorption. As a consequence, the rats, although free of neoplastic lesions, normally died within 20–30 days in complete starvation with ascitic fluid volumes as high as 200–500 ml. Therefore, in subsequent experiments, the administration of aldehyde was started 6 days after tumour inoculation or earlier.

The results of these experiments are presented in Table 1. Dihydroxybutyraldehyde injections started 24 hr after tumour transplantation were the most effective. When the treatment began, 6 days after tumour inoculation, only half of the treated animals survived and approximately 25 per cent died for ascitic or solid tumours. With DL-glyceraldehyde the results were similar except for an increased mortality with solid tumours and decreased number of ascitic hepatomas. The number of animals (per cent) died with non-neoplastic ascites was similar with either aldehyde.

Identical doses of aldehyde given to normal rats were well tolerated, except for a transient, pronounced modification of the animal behaviour (reduction of activity, apathy, confusion) observable in rats injected with DL-glyceraldehyde.

DISCUSSION

The low toxicity of aliphatic aldehydes such as glycolaldehyde and glyceraldehyde administered by several routes to animals and man has been recognized for a long time [14, 15] and probably reflects their rapid disappearance either by utilization [16, 17] or by condensation with different compounds [5, 12, 18, 19]. Our results indicate that dihydroxybutyraldehyde is also well tolerated and even less toxic than DL-glyceraldehyde as far as behavioural change is concerned. This finding is understandable if one considers the specific inhibitory effect of L-glyceraldehyde on hexokinase [12] and the prominence of glucose metabolism in nervous tissue.

The question then arises as to the mechanism by which these aldehydes display their anti-tumour activity. In preceding papers we showed that aliphatic aldehydes inhibit protein synthesis *in vitro* [4] possibly through a condensation with intracellular cysteine [5]. The resulting thiazolidine-4-carboxylic acids are likely to be reconverted into thio-amino acids in the living animal [20], this occurrence being more efficient in normal than in neoplastic tissues [21]. If this is the case, aldehydes

injected in the peritoneal cavity could display a more direct effect on tumour ascites cells than on other tissues. In this regard, the marked urinary retention which occurs during aldehydes treatment [11, 15] would increase their differential effect on normal and neoplastic cells. Moreover, a mechanism of this sort could be in agreement with the observation of Warburg *et al.* [9] that no immunity against tumour cells develops in aldehyde-treated animals.

It is recognized, of course, that in the present study, the anti-tumour effect of aldehydes is limited to an ascitic form, easily accessible to the drug. A large number of ascitic and solid tumours are now under investigation by techniques which will attempt to increase the aldehyde effect on neoplastic tissues taking advantage either of the anatomical situation (distrectual circulations) or of the different permeability characteristics of normal and neoplastic cells toward aldehyde derivatives.

Acknowledgements — The authors are indebted to Prof. B. Camerino of the Laboratori Farmitalia, Milano, Italy, for the generous supply of dihydroxybutyraldehyde.

RÉSUMÉ

L'aldéhyde DL-érythro,thréo-α,β-*dihydroxybutyrique et l'aldéhyde* DL-*glycérique montrent une évidente activité carcinostatique et carcinolytique à l'égard de l'hépatome ascite de Yoshida du rat.*

Les effets des aldéhydes sur la survie des animaux et les effets secondaires dépendent du temps écoulé entre l'inoculation de la tumeur et le traitement avec l'aldéhyde. On a observé que l'aldéhyde glycérique est un peu moins bien toléré que l'aldéhyde dihydroxybutyrique, à la même concentration molaire.

L'activité anticancéreuse des aldéhydes aliphatiques est discutée.

SUMMARY

DL-Erythro,threo-α,β-*dihydroxybutyraldehyde and* DL-*glyceraldehyde have been shown to display a marked carcinostatic and carcinolytic activity against established Yoshida ascites hepatoma in rat.*

Their effects on rat survival and side effects were dependent upon the time elapsed between tumour inoculation and aldehyde administration. Glyceraldehyde was slightly less tolerated than dihydroxybutyraldehyde on a molar basis.

The anti-tumour activity of aliphatic aldehydes is discussed.

ZUSAMMENFASSUNG

Es ist gezeigtworden, dass DL-Erythro,threo-α,β-*dihydroxybutyraldehyd und* DL-*Glycerinaldehyd eine bedeutende karzinostatische und karzinolytische Aktivität auf das Yoshida Ascites-Hepatom der Ratte ausüben.*

Ihre Wirkung auf das Uberleben der Tiere und die Nebenwirkungen waren von der Zeit abhängig, welche zwischen der Einimpfung der Tumorzellen und der Aldehyde-Verabreichung verflossen war. Auf molarer Basis wurde das Glycerinaldehyd etwas weniger gut ertragen als das Dihydroxybutyraldehyd.

Es wird die anti-tumorale Wirkung der aliphatischen Aldehyden besprochen.

REFERENCES

1. E. Ciaranfi and A. Fonnesu, Sui rapporti fra metabolismo energetico e incorporazione di aminoacidi nelle proteine delle cellule di tumori-ascite. *Atti Accad. Lincei. Rend.* **32,** 835 (1962).
2. G. G. Guidotti, La gliceraldeide, un nuovo inibitore delle sintesi proteiche. *Tumori* **49,** 303 (1963).
3. G. G. Guidotti, L. Loreti and E. Ciaranfi, Inibizione delle sintesi proteiche nei tessuti neoplastici. Effetto delle aldeidiali fatiche. *Atti. Soc. Ital. Cancerol.* **5,** 406 (1964).

4. G. G. Guidotti, A. Fonnesu and E. Ciaranfi, Inhibition of amino acid incorporation into protein of Yoshida ascites hepatoma cells by glyceraldehyde. *Cancer Res.* **24,** 900 (1964).
5. G. G. Guidotti, L. Loreti and E. Ciaranfi, Studies on the anti-tumour activity of aliphatic aldehydes — I. The mechanism of inhibition of amino acid incorporation into protein of Yoshida ascites hepatoma cells. *Europ. J. Cancer* **1,** 23 (1965).
6. A. Furst, B. L. Freedlander, F. A. French, H. Gross and D. Demsher, β-Ethoxy-α-ketobutyraldehyde (kethoxal) as a carcinostatic agent in mouse tumours. *Proc. Amer. Ass. Cancer Res.* **2,** 204 (1957).
7. F. A. French and B. L. Freedlander, Carcinostatic action of polycarbonyl compounds and their derivatives — I. 3-Ethoxy-2-ketobutyraldehyde and related compounds. *Cancer Res.* **18,** 172 (1958).
8. J. S. Evans, quoted by H. G. Petering, H. H. Buskirk and G. E. Underwood, The anti-tumor activity of 2-keto-3-ethoxybutyraldehyde bis (thio-semicarbazone) and related compounds. *Cancer Res.* **24,** 367 (1964).
9. O. Warburg, K. Gawehn, A. W. Geissler and S. Lorenz, Über Heilung von Mäuse-Ascites-Krebs durch D- und L-Glycerinaldehyd. *Z.klin. Chem.* **1,** 175 (1963).
10. G. Bruns, W. Jungstand and H. Knöll, Zur Wirkung des DL-Glycerinaldehyds auf das Ehrlich-Ascites-Carcinom der weissen Maus. *Naturwissenschaften* **51,** 560 (1964).
11. C. Bertazzoli, T. Chieli, I. Dell'Oro, G. Ferni and E. Turolla, Laboratori Farmitalia, Milano, Italy (Personal communication).
12. H. A. Lardy, V. D. Wiebelhaus and K. M. Mann, The mechanism by which glyceraldehyde inhibits glycolysis. *J. biol. Chem.* **187,** 325 (1950).
13. J. P. Kaltenbach, M. H. Kaltenbach and W. B. Lyons, Nigrosin as a dye for differentiating live and dead ascites cells. *Exp. Cell Res.* **15,** 112 (1958).
14. W. D. Sansum and R. T. Woodyatt, Studies on the theory of diabetes — III. Glycollic aldehyde in phlorhizinized dogs. *J. biol. Chem.* **17,** 521 (1914).
15. W. D. Sansum and R. T. Woodyatt, Studies on the theory of diabetes — VI. The behavior of DL-glyceric aldehyde in the normal and diabetic organism. *J. biol. Chem.* **24,** 327 (1916).
16. B. Friedmann, H. W. Levin and S. Weinhouse, Metabolism of glycolaldehyde in the rat. *J. biol. Chem.* **221,** 665 (1956).
17. B. R. Landau and W. Merlevede, Initial reactions in the metabolism of D- and L-glyceraldehyde by rat liver. *J. biol. Chem.* **238,** 861 (1963).
18. M. P. Schubert, Compounds of thiol acids with aldehydes. *J. biol. Chem.* **114,** 341 (1936)
19. E. V. Ellington, J. Mellanby and D. H. Williamson, The non-enzymic condensation of acetoacetate and glyoxylate: An explanation for the anti-ketogenic effect of glycolaldehyde and related compounds. *Biochem. J.* **91,** 352 (1964)
20. C. G. Mackenzie and J. Harris, N-formylcysteine synthesis in mitochondria from formaldehyde and L-cysteine via thiazolidine-carboxylic acid. *J. biol. Chem.* **227,** 393 (1957).
21. C. B. Rossi, L. Loreti and G. G. Guidotti, Metabolism of thiazolidine carboxylic acids in neoplastic tissues. *Fed. Europ. Biochem. Soc., Vienna* 1965, Abstracts, p. 133.
22. M. U. Tsao and E. L. Schwartz, A simple method for estimating dihydroxyacetone (or glyceraldehyde). *Analyt. Biochem.* **3,** 448 (1962).

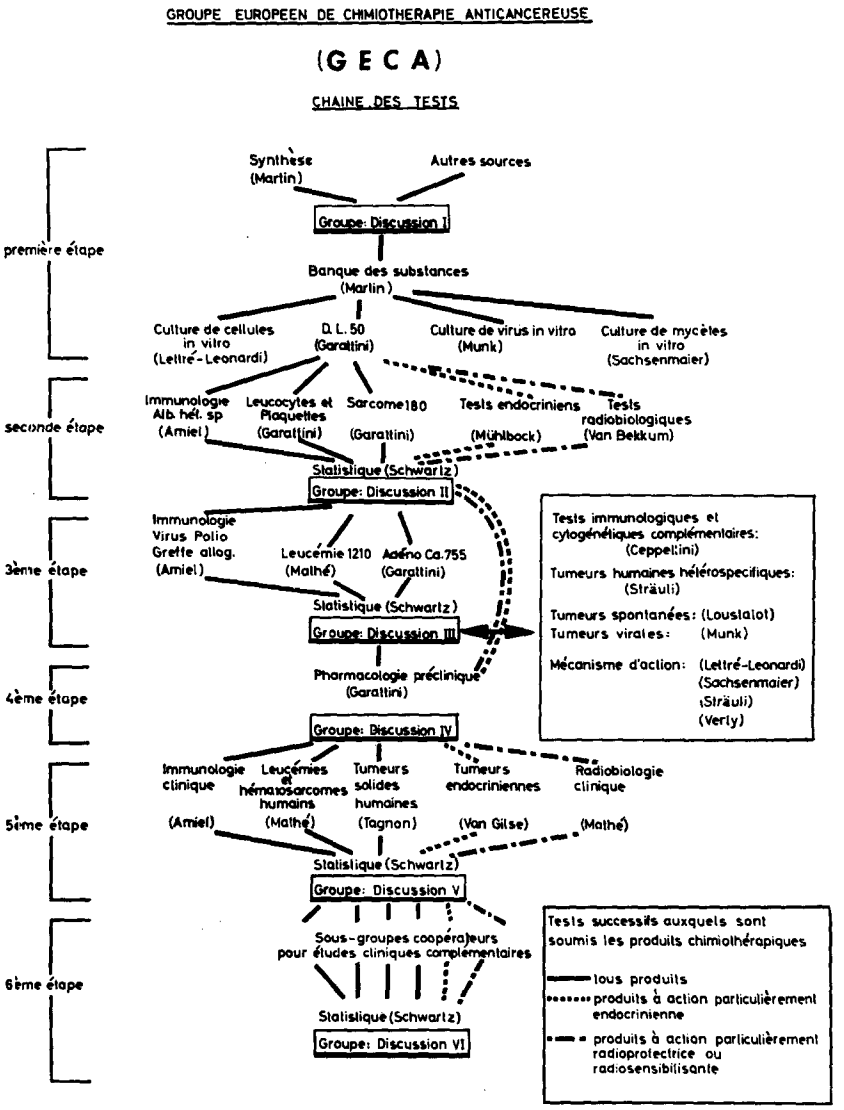
Europ. J. Cancer Vol. 1, pp. 153–154. Pergamon Press 1965. Printed in Great Britain

Communication

Groupe Européen de Chimiothérapie Anticancéreuse G.E.C.A.*

Le Groupe Européen de Chimiothérapie Anticancéreuse a été constitué en 1963 pour conduire, développer, coordonner et stimuler, en Europe, les recherches sur la chimiothérapie du cancer, et les problèmes qui y sont liés. C'est un groupe de travail réunissant des chercheurs — un par discipline — des différents pays du Marché Commun et de Suisse. Les disciplines représentées vont de la chimie

*Siège Social: Institut J. Bordet — 1 rue Héger-Bordet, Bruxelles (Belgique).
Secrétariat: P. Sträuli, Institut de Pathologie — Schmelzbergstrasze, Zürich (Suisse).



organique à la statisitique, en passant par la biologie moléculaire, la pharmacologie, l'immunologie, la génétique, la virologie, la chimiothérapie des leucémies et des tumeurs solides etc. Les membres se réunissent une fois par mois et ils ont établi un programme d'études s'étendant de la préparation des substances aux essais cliniques chez l'Homme. Le schéma indique les différentes étapes franchies par un produit nouveau avant d'aboutir à l'évaluation clinique.

Plusieurs groupes coopérateurs ont été créés pour faciliter cette dernière étape. Leur but est d'étudier rapidement et valablement l'effet thérapeutique d'un produit donné, en conduisant, selon des critères strictement définis, des études cliniques poursuivies en commun dans des institutions de plusieurs pays. Le matériel clinique ainsi réuni permet de tester, en un temps relativement court, une substance donnée dans une variété de cancer trop rare pour que le spécialiste du G.E.C.A. puisse assurer à lui seul un nombre suffisant d'essais. Il permet aussi de réaliser des études comparatives (essais contrôlés) nécessitant un grand nombre de malades.

Les groupes suivants sont déjà constitués et ont réalisé, ou sont en train de réaliser, un certain nombre d'études cliniques de ce genre: cancer du sein, cancer broncho-pulmonaire, radiobiologie clinique, leucémies et hématosarcomes, tumeurs cérébrales. D'autres groupes sont en voie de formation: cancer de la prostate, choriocarcinome, tumeurs des enfants.

Plusieurs études réalisées par ces groupes paraîtront dans le Journal Européen de Cancérologie.

Membres du Groupe Européen de Chimiothérapie Anticancéreuse:

AMIEL, Jean-Louis, *Paris*
CEPELLINI, Ruggero, *Turin*
GARATTINI, Silvio, *Milan*
LEONARDI, Alfredo, *Milan*
LETTRE, Hans, *Heidelberg*
LOUSTALOT, Prosper, *Bâle*
MARTIN, Richard, *Bruxelles*
MATHÉ, Georges, *Paris*
MÜHLBOCK, Otto, *Amsterdam*
MUNK, Klaus, *Heidelberg*
SACHSENMAIER, Wilhelm, *Heidelberg*
SCHWARTZ, Daniel, *Paris*
STRÄULI, Peter, *Zürich*
TAGNON, Henri, *Bruxelles*
THOMAS, René, *Bruxelles*
VAN BEKKUM, Dirk, *Leyden*
VAN GILSE, Henriette, *Rotterdam*
VERLY, Walter, *Liège*

Announcement

Letters to the Editor

Beginning with No. 3, the European Journal of Cancer will accept "letters to the Editor" for prompt publication. The length of these short communications should not exceed 750 words including title, authors, bibliography and acknowledgements. They should furnish sufficient data to support their conclusions and conform to the usual standards of the Journal.

Lettres à l'Editeur

A partir du n°3, le Journal Européen de Cancérologie acceptera, en vue d'une publication rapide, de courtes notes appelées "lettres à l'Editeur". Leur longueur ne doit pas dépasser 750 mots, y compris le titre, les noms d'auteurs, la bibliographie et les autres mentions. Elles devront fournir les données expérimentales ou d'observation nécessaires pour étayer leurs conclusions et se conformer aux instructions applicables aux articles ordinaires.

Briefe an den Herausgeber

Beginnend mit der 3. Folge wird die "Europäische Zeitschrift für Cancerologie" "Briefe an den Herausgeber" für baldige Veröffentlichung annehmen.

Der Umfang dieser kurzen Mitteilungen sollte 750 Worte nicht überschreiten, einschliesslich Ueberschrift, Autoren, Bibliographie und andere Anmerkungen. Sie sollten genügend Unterlagen enthalten, um die Schlussfolgerungen zu untermauern und allen übrigen Anforderungen der Zeitschrift entsprechen.

organique à la statisitique, en passant par la biologie moléculaire, la pharmacologie, l'immunologie, la génétique, la virologie, la chimiothérapie des leucémies et des tumeurs solides etc. Les membres se réunissent une fois par mois et ils ont établi un programme d'études s'étendant de la préparation des substances aux essais cliniques chez l'Homme. Le schéma indique les différentes étapes franchies par un produit nouveau avant d'aboutir à l'évaluation clinique.

Plusieurs groupes coopérateurs ont été créés pour faciliter cette dernière étape. Leur but est d'étudier rapidement et valablement l'effet thérapeutique d'un produit donné, en conduisant, selon des critères strictement définis, des études cliniques poursuivies en commun dans des institutions de plusieurs pays. Le matériel clinique ainsi réuni permet de tester, en un temps relativement court, une substance donnée dans une variété de cancer trop rare pour que le spécialiste du G.E.C.A. puisse assurer à lui seul un nombre suffisant d'essais. Il permet aussi de réaliser des études comparatives (essais contrôlés) nécessitant un grand nombre de malades.

Les groupes suivants sont déjà constitués et ont réalisé, ou sont en train de réaliser, un certain nombre d'études cliniques de ce genre: cancer du sein, cancer broncho-pulmonaire, radiobiologie clinique, leucémies et hématosarcomes, tumeurs cérébrales. D'autres groupes sont en voie de formation: cancer de la prostate, choriocarcinome, tumeurs des enfants.

Plusieurs études réalisées par ces groupes paraîtront dans le Journal Européen de Cancérologie.

Membres du Groupe Européen de Chimiothérapie Anticancéreuse:

AMIEL, Jean-Louis, *Paris*
CEPELLINI, Ruggero, *Turin*
GARATTINI, Silvio, *Milan*
LEONARDI, Alfredo, *Milan*
LETTRE, Hans, *Heidelberg*
LOUSTALOT, Prosper, *Bâle*
MARTIN, Richard, *Bruxelles*
MATHÉ, Georges, *Paris*
MÜHLBOCK, Otto, *Amsterdam*
MUNK, Klaus, *Heidelberg*
SACHSENMAIER, Wilhelm, *Heidelberg*
SCHWARTZ, Daniel, *Paris*
STRÄULI, Peter, *Zürich*
TAGNON, Henri, *Bruxelles*
THOMAS, René, *Bruxelles*
VAN BEKKUM, Dirk, *Leyden*
VAN GILSE, Henriette, *Rotterdam*
VERLY, Walter, *Liège*

Announcement

Letters to the Editor

Beginning with No. 3, the European Journal of Cancer will accept "letters to the Editor" for prompt publication. The length of these short communications should not exceed 750 words including title, authors, bibliography and acknowledgements. They should furnish sufficient data to support their conclusions and conform to the usual standards of the Journal.

Lettres à l'Editeur

A partir du n°3, le Journal Européen de Cancérologie acceptera, en vue d'une publication rapide, de courtes notes appelées "lettres à l'Editeur". Leur longueur ne doit pas dépasser 750 mots, y compris le titre, les noms d'auteurs, la bibliographie et les autres mentions. Elles devront fournir les données expérimentales ou d'observation nécessaires pour étayer leurs conclusions et se conformer aux instructions applicables aux articles ordinaires.

Briefe an den Herausgeber

Beginnend mit der 3. Folge wird die "Europäische Zeitschrift für Cancerologie" "Briefe an den Herausgeber" für baldige Veröffentlichung annehmen.

Der Umfang dieser kurzen Mitteilungen sollte 750 Worte nicht überschreiten, einschliesslich Ueberschrift, Autoren, Bibliographie und andere Anmerkungen. Sie sollten genügend Unterlagen enthalten, um die Schlussfolgerungen zu untermauern und allen übrigen Anforderungen der Zeitschrift entsprechen.

Europ. J. Cancer Vol. 1, p. 155. Pergamon Press 1965. Printed in Great Britain

Symposium International sur la Caractérisation Biochimique des Tumeurs Humaines

INTRODUCTION

Du 26 au 29 mai 1965, sous les auspices du "Comité Européen pour l'étude des tumeurs humaines" (European Committee for human tumour investigation), un symposium a eu lieu à Royaumont. Dans cette vieille abbaye, à quelques kilomètres de Paris, au milieu d'un parc à la beauté sévère mais accueillante, une centaine de chercheurs ont exposé les résultats de leurs travaux et participé à des discussions souvent passionnées.

Ainsi s'est trouvé confirmé l'avènement de cette nouvelle branche de la biochimie cancérologique, celle qui s'occupe des tumeurs humaines. Cet avènement consacre plus définitivement l'union, dans la recherche cancérologique, de la clinique et des sciences biochimiques, cette union que chacun sait nécessaire mais qui ne se réalise pas sans effort. C'est maintenant chose faite. Les cliniciens du cancer vont se tourner vers la biochimie au seuil de laquelle les accueilleront de plus en plus volontiers les hommes de laboratoire qui ne connaissaient pas les malades. C'est cette collaboration déjà largement établie, comme le montre la réunion de Royaumont, qu'il convient de saluer comme une promesse de progrès décisifs en cancérologie.

C'est pourquoi, le Journal Européen de Cancérologie est heureux de pouvoir publier les travaux qui ont été exposés au cours de ce symposium. Ils présentent, dans leur unité essentielle définie par le titre de la réunion, une diversité suffisante pour satisfaire les intérêts variés des lecteurs de ce journal.

Nos remerciements sont dus au Professeur F. Bergel, modérateur ou animateur suivant les circonstances, des séances scientifiques, et dont l'autorité souriante est reconnue de tous; ils sont dus également au Docteur W. Davis et à ses collaborateurs, responsables de l'organisation impeccable du symposium. Je tiens à remercier les auteurs des manuscrits, qui ont accepté avec bonne grâce, et respecté, des délais de livraison des manuscrits incroyablement courts, grâce à quoi nous voyons paraître ces articles cinq mois après leur présentation publique. Mais cette diligence n'aurait pas été efficace sans le dévouement de Mademoiselle Leroy, secrétaire du bureau de rédaction, et l'entière collaboration de la firme Pergamon où nous remercions surtout MM. G. F. Richards et R. J. Coleman.

Il a malheureusement été impossible d'inclure tous les articles présentés à Royaumont dans le double numéro. Un petit nombre de manuscrits, arrivés avec retard, seront publiés dans le numéro de janvier 1966, où ils seront identifiés par une note au bas de la page.

H. J. TAGNON

Europ. J. Cancer Vol. 1, pp. 157–159. Pergamon Press 1965. Printed in Great Britain

International Symposium on the Biological Characterisation of Human Tumours

Introduction to the Symposium

F. BERGEL, *Guest Editor*

IT IS a very great pleasure for me to open the two-day Symposium on the Biological (which includes molecular) Characterisation of Human Tumours, which is a collective noun embracing all forms of neoplasms, reticuloses and leukaemias. There is no doubt that we who are interested in this field of medical research have a very definite *present* in form of this meeting and its contributions. Whether we shall have a strong *future* depends to some extent on this our conference and its repercussions.

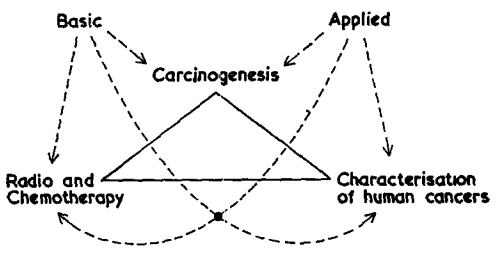


Fig. 1.

Our *past* is relatively short. It begins, I venture to say, in 1960 in Louvain on the occasion of a UNESCO meeting "Biological Approaches to Cancer Chemotherapy," when during a coffee break Professor de Duve talked to some of us about the desirability to revive and intensify work on the characterisation of human cancers; in other words, to add to the two main subjects of cancer research, carcinogenesis and therapy, in the widest sense, a third to form a triangle with fundamental and applied versions (Fig. 1).

Of course, nobody ignored and ignores the fact, certainly not during two subsequent meetings, one in Madison (organised by C. Heidelberger) in 1960, and the other in the following year at the Chester Beatty Research Institute (organised by W. Davis and L. A. Elson), that very useful and interesting older work with human material had been done. However, as it is well known to all of you, it was and still is to a large extent of a diagnostic nature or, if more basic, it often came to a sudden end. What were the reasons? They are:

1st: the quality, quantity and "uniformity" of human samples were on the whole inferior to those from animals.
2nd: often the only techniques available at that time did not come up to requirements.
3rd: open and secret hopes existed that species similarities were greater than dissimilarities.
4th: human patients while sources for biopsies and operation samples cannot be subjects of experimentation.

So why is there, in spite of this, a case for renewed and new efforts to use human material for serious and reliable research work? The positive answer arises, briefly expressed, from the following points which all of you must have often considered:

1. The problems of quantity, including statistical assessments, and quality, such as histologically identifiable cellularity, could be tackled and slowly resolved.

2. Techniques in all fields have been considerably improved over the last few years, and refined, and new ones added.
3. There do exist species differences between man and animals with respect to cancer, just as there are species specificities; (e.g. croton-oil as co-carcinogen, according to Shubik, acts only in mice; the stomach of rats and mice is histo-anatomically different from that of man).
4. These differences and specificities extend to cancer therapies, and drug-produced side reactions.
5. Apart from prevention and treatment, there could exist fundamental differences in biological and biochemical properties between normal and abnormal tissues.

Assuming all this to be true and acceptable as premises, whoever decides on working in this field will have to debate, sort out and make provision for two principal items and for two inherent main difficulties. The former refer to right conditions under which any investigation is carried out and a clear-cut and not over-ambitious programme, the latter to the number of good samples available in any given study and to comparative cellularity.

Conditions — Of all approaches to the cancer problem, this kind of research demands more than any other close and planned co-operation, because, as it was just pointed out and as everybody knows, the complications are manifold and can only be resolved by closing the ranks for mutual assistance. This could be done on an international and national level, and *must* be done internally in each Institute or cancer laboratory. As you know, there exists an as yet informal European Committee for Human Tumour Investigation with increasing representation from all countries and links with America. Several meetings of working groups have taken place. Meanwhile (I hope among others) a British-Irish Committee has been formed which issues information sheets from time to time and is arranging (Secretary — R. W. Baldwin, and host — R. A. Q. O'Meara) a meeting in Dublin in September. I hope sincerely that if this symposium establishes beyond doubt the soundness and timeliness of the whole subject matter, then formation of further national associations and international recognition of our endeavours in some shape ought to follow. But at this moment I am perhaps putting the cart before the horse. Yet every kind of new direction or emphasis in any sort of research had to overcome small and large prejudices of people representing often a majority, whether it turned on the solar system, the antiphlogiston theory of oxidation, spatial configuration of molecules, existence of bacteria, cellular concepts or vitamins. (Table 1): Most of you must have come to the conclusion that internally some kind of project team or "task force" had or has to be created. I venture to say that different places have their own special type or degree of difficulties, somewhere at the beginning, in the middle or at the end of the line between surgeon or physician via distributor, histopathologist-haematologist, to the workers in the laboratories. Often personality questions play a part, or the absence or presence of a common technical language and knowledge hinders or helps. Sometimes one's histopathologist-morbid anatomist is overwhelmed with diagnostic work for the hospital side or one's collector or distributor is challenged by storage and preservation problems of tissue samples.

The Programme — Our symposium will demonstrate how varied this can be (Table 2): At a very early stage of recent developments I put this schedule forward. It comprises nearly everything possible. Naturally each laboratory or Institute must decide on what to concentrate, maybe only on one or two aspects. Whether the emphasis is on biological or biochemical, fundamental or applied matters, it is most wise to link any work in the laboratory with prevailing interests in the hospital or hospitals to which it is attached or with which one is on friendly and colleaguial terms.

Now to the major difficulties, numbers and cellular quality of samples. The papers to be presented during this meeting will each propose

Table 1. Project teams

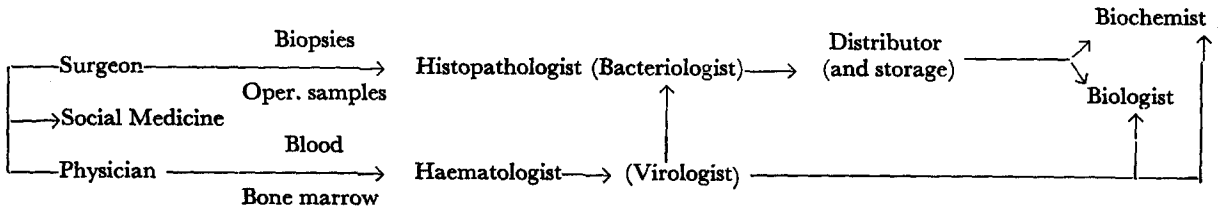
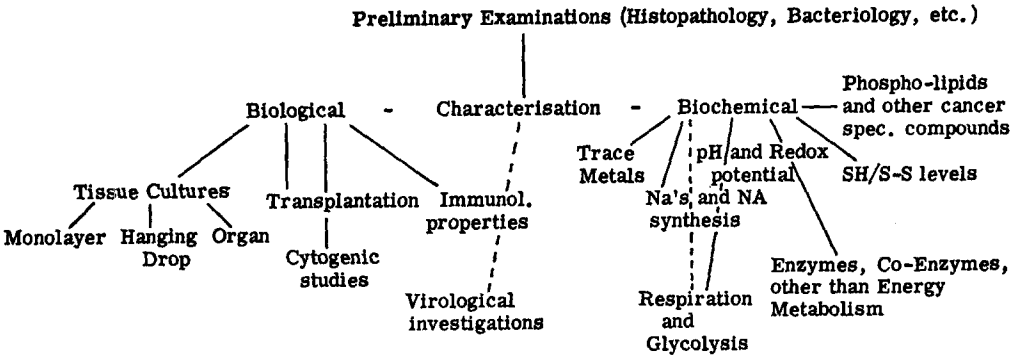


Table 2.

Possible programme of investigations



individual solutions and attempt generally applicable ones. Obviously relative measurements and selection and estimation, not necessarily quantitised, of patterns, will overcome or avoid initial difficulties with baseline and significance calculations. I think most of you will agree that close collaboration with an enthusiastic research histopathologist is of greatest importance and will solve, at least partly, some of the problems connected with comparative cellularity. Indeed this constant morphological check of work with solid tumours is a condition sine qua non for sustained success. Only those of us who work with blood fractions or excreta are not dependent on methods established over long periods for diagnostic means. Under any circumstances the development of micro- and ultramicro techniques will increasingly help in the betterment of everybody's results, and, maybe, open up new approaches.

This, Ladies and Gentlemen, is some of the background of our subject, some of its difficulties and potentialities, most of it known to this assembly. I am convinced that in coming together we are doing something useful for fundamental knowledge and for the patient, and not just having "another conference."

I do not wish to give the impression that anyone interested in utilising human material in his or her researches wants to do this to the exclusion of mice, rats or any other animal. On the contrary, wherever necessary, everybody is or will use animal cells, tissues or whole animals as well. On the other hand one is disinclined to succumb to any restrictive working philosophy of some workers who wish to carry out their investigations in a "Mousoleum." I think we can accept, at least to a large extent this statement printed some time ago in the Times:

"In cancer the proper study of mankind is man."

My best wishes go with all of you.

Europ. J. Cancer Vol. 1, pp. 161–172. Pergamon Press 1965. Printed in Great Britain

Glycolytic Enzymes in Human Tumors:

Difficulties, Methods and Results

G. E. BOXER

Merck Sharp & Dohme Research Laboratories,
Division of Merck & Co., Inc., Rahway, New Jersey, U.S.A.

PROFESSOR BERGEL was kind enough to leave the choice of title of this talk open which permits me not only to present some of the results we have obtained in the area of glycolytic enzymes, but also to use these to discuss some of the difficulties which can be anticipated in studies of enzyme activities of malignant tissue in any area of metabolism. In general, it is essential to define the purpose of such studies rather carefully since the difficulties and ambiguities encountered still leave the question open whether the necessary effort is commensurate with the results one can expect.

Table 1. *Purpose.*

1.	Validity of extrapolation from rodent to man of 'exploitable' metabolic differences between malignant and normal tissue.
2.	'Enzymological anatomy.'
3.	Choice of appropriate chemotherapeutic agent for a patient's tumor.
4.	Demonstration of metabolic effects of chemotherapy.

Table 1 lists what we would consider the main purpose of enzymic studies on human tissues. The fact that we are really quite successful in treating rodent neoplasms by chemotherapy needs no further discussion. Unfortunately, the extrapolation from mouse to man is not equally successful. Although this extrapolation fails for a number of reasons, some of these undoubtedly reflect metabolic differences between species. In the glycolytic area, such differences were not striking, but it might be said parenthetically that we encountered such significant species differences in some phases of nucleoside metabolism.

The second point is really directly dependent on the first one. If one looks for exploitable differences between human normal and malignant tissues, quite obviously information on normal tissues is of equal importance. While there is a large amount of information available on a variety of enzymes in body fluids of man, the information on enzymic makeup of human tissues is quite sporadic. Necessarily such studies will yield data on the enzymic characteristics of normal tissues that might be of interest in areas other than cancer research.

It is to be hoped that the third and fourth points will become of importance in the future but, at the moment, no data are reported on these aspects. It should be added that information on point one will always be a prerequisite whenever we shall be able to consider the last two points in the clinical laboratory.

Table 2. *Choice of metabolic area.*

1.	Relevance to malignancy.
2.	Experience.
3.	Reliable methodology.
4.	Stability of enzymes.

The choice of the metabolic area (Table 2) to be studied is, of course, clearly defined by one's own scientific interests. In view of the lack of knowledge of distinguishing character-

istics of tumour metabolism, onemight cynically say that most areas are equally relevant or irrelevant, but certain metabolic phenomena have a more direct relevance, at least to the rapid growth of malignant tissues.

The listing of experience and reliable methodology may be presumptuous or platitudinous, but it should be emphasized that, although the difficulties encountered in enzymological investigations of rodent tissues are quite considerable, these difficulties are multiplied if one works with unreproducible bits of surgical biopsies. The collection of odd measurements without the background of carefully worked out methodology in animal systems is liable to lead to useless, or what is worse, misleading data.

The fourth point on stability of enzymes will vary in importance depending on the facilities available. It is a serious limiting factor in our laboratory which is not part of a clinical research unit. The data discussed are thus restricted to enzymes that have clearly defined stability in the frozen state. But even under the most favorable circumstances where an enzyme analytical laboratory is physically next to the surgical area, there are problems that are dictated by surgical necessity. With the exception of punch biopsies, the surgical material is obtained from seriously ill patients who have been prepared for surgery and are under anesthesia. Although the patients are nearly always receiving glucose infusions, they are certainly not in a normal nutritional state. Surgical procedures usually involve anoxic conditions of the resected material and careful inspection and dissection of specimens for surgical pathology takes some time before a representative sample can finally be frozen in dry ice. Under these conditions, studies on the stability of the enzyme under investigation are an essential prerequisite for meaningful work on human tissues. It also means that a whole series of quite interesting investigations simply cannot be done.

In view of the points listed in Table 2, the data reported here are on the metabolic area of glycolytic enzymes, although we have gone into related areas of metabolism.

The collection and storage of material, the histological samples taken, and the methodology of enzymic assays employed with all the necessary precautions and controls have been described in detail [1–3]. In essence, they are an adaptation of the methodology of Bücher [4] and his group and Wu and Racker [5]. It should be emphasized that such measurements provide data on the *potential* capacities of each enzyme under optimal conditions of substrates and cofactors. Such data can, therefore, only provide upper, limiting values on the *potential* of metabolite flow through a pathway, but they do *not* give information on the actual metabolite flow through the pathway *in vivo*.

Point four will vary in importance depending on the logistics of obtaining the sample and on the enzyme assayed. Data to show stability of the enzymes under discussion under our conditions are presented below.

For most specimens in this study, the surgical pathologist in the operation room selected a block of tissue, normal or malignant, weighing between 100–500 mg, that was macroscopically as homogeneous as possible and immediately immersed it into crushed dry ice. Slices of tissue adjacent to the ones used for enzyme determinations were fixed and stained by H and E unless special stains were advisable. It is a sad fact of life that the histological sections usually lag behind the enzyme assays from 1 to 3 days. As many as half of the specimens of some tissues turn out to be so inhomogeneous that the enzymic data must be set aside since no sensible correlation with a predominant tissue element can be made.

Table 3. *Histology.*

1. Malignant cells	5. Necrosis
2. Connective tissue	6. Normal tissue features
3. Lipid	7. State of preservation
4. Blood	8. Diffuse infiltration

An estimate of the constituents listed in Table 3 is obtained by visual observation on multiple fields of the three sections and is recorded as per cent in digits of tens only. Attempts to be more precise proved to be extremely laborious and did not yield more usable information. For example, we used planimetry of areas of malignant cells in photographs of these fields. This is an extremely time-consuming procedure and to our surprise the results did not significantly vary from the estimates made visually on the same fields by the same skilled pathologist.

In the case of malignant tissue, we do not use data on specimens that contain less than 30 per cent maligant cells, provided — and this is an important proviso — that most of the other material is connective tissue. Sufficient data are available to show that connective tissue has such a low specific activity of glycolytic enzymes that it only dilutes the high activity of these enzymes in the malignant cells. This diluent effect becomes unimportant if one primarily

examines the activity of these enzymes relative to each other, as we mostly do.

Connective tissue can also be estimated chemically as hydroxy-proline-containing collagen, but again our experience has been that the considerable additional work necessary does not improve significantly the estimate that can be obtained by visual observation. The same is true for lipid, but in this case we generally have to set the results aside if the sample contains more than 10 per cent fat. Fat tissue has a quite distinct glycolytic enzyme pattern of its own [1, 2] which can seriously distort the picture if it is not taken into account. On the other hand, it is usually not too difficult to obtain specimens that are nearly free of fat.

Blood content of the surgical specimens is usually quite low. Previously published data indicated [2, 6] that the normally occurring blood content does not distort the glycolytic enzyme patterns, except in such organs as the spleen or the thyroid.

The remaining histological features, if present to more than 10 per cent, usually mean that the enzymic data must be put aside. Although every effort is made by the surgical pathologist to avoid necrotic areas in the small frozen specimen, there are still samples that contain prohibitively large areas of necrosis. Necrotic areas are by no means devoid of glycolytic enzymes, but not all enzymes deteriorate at equal rates. For example, GAPDH is drastically reduced while most of the other enzymes are still quite normal. Actually the disproportion between GAPDH and certain other glycolytic enzymes might be useful as an enzymic measure of the presence of extensive necrosis.

Normal tissue features do not make too much of a difference since, with one or two exceptions, most glycolytic enzymes are quantitatively not very different in malignant tissues. Diffuse infiltration of the tissue by inflammatory or lymphocytic elements makes the specimen unsuited for sensible interpretation of the enzyme data.

The inhomogeneity of human biopsy samples makes the frame of reference for enzyme determinations a difficult choice. In Table 4 are listed a number of such reference parameters that have been used successfully in studies involving animal tissues. Only points one and eight are discussed in detail here, but it should be pointed out that in a series of nearly fifty neoplasms and normal tissues we have determined many of these parameters simultaneously. This rather tedious effort can be summarized by saying that it was not worth all the labour involved. Although really any one of these frames of reference can be used when comparing a series of one given type of normal tissue, they become useless and sometimes misleading in comparing a tumor with its normal tissue of origin. Wet weight which is extremely simply and reasonably accurately measured proved to be most useful. It is essential anyway in preparing a proper homogenate and gave in our experience as much or as little information as any of the other parameters but involved no extra work.

Table 4. Frame of reference.

1. Wet weight	5. RNA
2. Dry weight	6. Protein
3. Lipid free dry weight	7. Number of cells
4. DNA	8. Reference enzyme

As will be illustrated shortly with experimental data, the use of one or more enzyme activities as a reference actually has given us some of the most useful information. Even from a theoretical point of view, enzyme activities relative to each other should give the physiologically important information of the direction of metabolite flow, while the relatively lowest activity should give an indication of the limiting enzyme in a chain of reactions.

Table 5. Stability of glycolytic enzymes

1. Before freezing
2. During frozen storage
3. In homogenates

Table 5 lists factors that have to be considered in establishing enzyme stability. Each of these three points has to be studied separately.

Data on the stability of glycolytic enzymes under conditions to be expected during surgical removal have been reported [1]*. They can be summarized by saying that a tissue can be kept cut off from blood supply for periods of up to 6 hr at room temperature before being frozen in dry ice without significant change in enzyme activities. Particularly surprising was the stability of GAPDH in the

*The following abbreviations are used: ALD, aldolase; ENO, enolase; FDPase, fructose-1,6-diphosphatase; GAPDH, glyceraldehyde phosphate dehydrogenase; GK, glucokinase; GPDH, α-glycerolphosphate dehydrogenase; G-6-PDH, glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase; C-MDH, extra-mitochondrial malate dehydrogenase; M-MDH, mitochondrial malate dehydrogenase; PFK, phosphofructokinase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; P-Gluc-M, phosphoglucomutase; PK, pyruvate kinase.

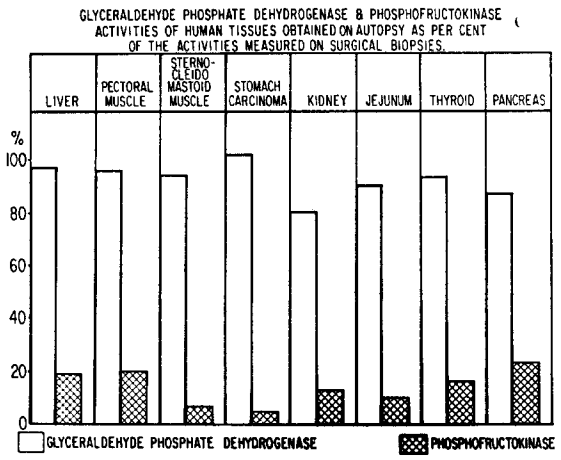


Fig. 1. *Comparative stability of glyceraldehyde phosphate dehydrogenase and phosphofructokinase in tissues at room temperature.*

whole tissue. PFK was not included in these determinations at that time which was unfortunate since, as demonstrated in Fig. 1, this is the most labile enzyme *prior* to freezing.

Figure 1 illustrates data on samples where comparison was possible — of course not on the same patient — between values obtained on surgical biopsies and on autopsies performed within 4 hr. The ordinate is the average percentage of activity found in an autopsy sample compared to the average activity found in an immediately frozen surgical biopsy. At a glance it is clear that PFK activity in the autopsy samples has fallen to about 20 per cent of the values found in the surgical biopsies. On the other hand, GAPDH which is considered the most labile enzyme of the glycolytic pathway, held up quite well under these conditions.

Similarly data for two rare instances where for surgical reasons some intestinal tissue was removed immediately after laparotomy, and a major resection was completed 80 min later yielding another uninvolved piece of tissue from the same patient have been reported [3]. The enzyme activities in the two paired samples were quite similar with the exception of PFK. Subsequent work established that the loss of PFK during 80 min prior to freezing was quite real.

The question of enzyme stability prior to immersion of the sample in dry ice is of considerable importance as far as data on normal, or better, uninvolved tissue are concerned. While it is not too difficult to obtain surgical biopsies of tumor tissue quite rapidly, it is nearly impossible to obtain samples of uninvolved tissues of many internal organs. Autopsy material has to be used predominantly in these instances and stability of enzyme activity after death then becomes a decisive

Table 6. *Long-term stability of glycolytic enzymes in human liver stored at* −20°C.

Time of storage	Enzyme activities (u/g)							
	GAPDH	LDH	GPDH	ALD	PGK	PK	ENO	FDPase
1 week	72	210	17	4·4	59	18	23	3·6
7 weeks	78	180	16	5·0	68	19	32	3·4
23 weeks	73	182	19	6·0	65	18	34	2·8
71 weeks	81	248	20	5·9	55	23	37	3·0

Table 7. *Enzyme activities of glycolytic enzymes in human hepatoma stored at* −20°C.

Time of storage	Enzyme activities (u/g)						
	GK	ALD	GAPDH	PGK	PGM	PK	LDH
1 week	1·2	2·3	47	47	31	26	64
75 weeks	1·3	2·2	57	32	37	18	44

factor. Our experience can be summarized by saying that many detailed studies have indicated that all glycolytic enzymes, with the exception of PFK, are stable if autopsy is performed within 4 hr after death. The one striking exception is PFK.

Preliminary results of the stability of glycolytic enzymes under conditions of prolonged storage in the frozen state have been reported [1, 2]. Table 6 documents stability of glycolytic enzyme activities during storage at −20°C. A human liver obtained on autopsy was stored in the deep-freeze and samples were assayed at the indicated times. PFK is not included since a good deal of activity had been lost prior to autopsy, but it should be mentioned that once the tissue was frozen, PFK stayed constant throughout this whole period.

Table 7 presents similar data for a human hepatoma, indicating stability for the same enzymes in the corresponding malignant tissue. A whole series of investigations of other tissues and their malignant counterparts produced results in essential agreement with those illustrated for liver in Tables 6 and 7. One exception was encountered with samples of stomach, either normal or malignant, where activity was lost on long term storage in the frozen state, undoubtedly due to peptic activity. The loss of enzyme activity was insignificant, however, if the samples were stored frozen for less than one week.

Table 8. *Enzyme activities after storage of* 30,000 × *G rat liver supernatant fractions.*

Enzyme	Initial activity	Activity after 18 hr	
		at 4°C	at −20°C
Glyceraldehyde phosphate dehydrogenase	59	17	27
Lactate dehydrogenase	136	129	131
Glycerolphosphate dehydrogenase	55	44	45
Pyruvate kinase	19	4	12

Enzyme activity is expressed as u/g wet weight of tissue

Once a tissue has been homogenized, it is essential to proceed with the assays immediately. Activities are lost from supernatants of homogenates even if they are kept frozen at −20°C. Not all the enzymes decay at the same rate in the homogenates. Under these conditions GAPDH is the most labile enzyme and is, therefore, always assayed immediately after homogenization. The order of enzyme determination indicated in our publications takes account of the differential decay of the various activities and permits comfortable time limits to perform duplicate assays and the necessary controls [1].

Table 9. *Enzyme activities of human stomach supernatant fraction stored in liquid nitrogen.*

Enzyme	Initial activity	Activity after 48 hr Storage in liquid nitrogen
Glyceraldehyde phosphate dehydrogenase	47	47
Pyruvate kinase	37	47
Phosphofructokinase	3·2	2·0
Enolase	8·1	8·7
Glucokinase	2·1	2·0
Lactate dehydrogenase	40	41

Enzyme activity is expressed as u/g wet weight of tissue

If it should become essential to store homogenates for at least short periods of time prior to assay, this is possible by freezing at liquid nitrogen temperature. Table 9 presents an illustrative example, taking the worst possible tissue, human stomach. At least for 48 hr there was no significant loss of any of these activities, again with the possible exception of PFK.

Methodology and difficulties in selecting and storing of tissues have been discussed in considerable detail because of the fact that enzyme assays always yield numerical answers. We feel rather strongly that a great deal of work is essential to prove that the numbers obtained have at least limited biological meaning.

The remainder of the discussion is concerned with the application of the methodology outlined to the investigation of clinical biopsy material. The two basic approaches outlined in Table 10 will be discussed

Table 10. *Basic approaches.*

1. Comparison of malignant tissue to 'uninvolved' tissue of 'origin' of the same patient.
2. Comparison of a series of related malignancies to a series of 'uninvolved tissues of origin.'

The first approach is undoubtedly the more logical one, and it is also the one that will have to be used when we ultimately want to study the effect of chemotherapeutic agents on specific metabolic reactions. It is also technically the most difficult approach. The quotes in Table 10 underline the difficulties. Although the surgeon tries to resect as far as possible within areas of uninvolved tissues, histologic examination occasionally indicates that the tissue is partially infiltrated by malignant cells but more frequently the so-called uninvolved tissue is found to be heavily infiltrated by

inflammatory or lymphoid tissue. It is thus not sensible to use these specimens as normal counterpart. As far as the tissue of origin is concerned, it sometimes is actually not available on surgery or, for example, in the case of the intestinal mucosa is only a small part of the excised specimen. Nevertheless, it is in some instances possible to obtain tissues for valid comparisons.

The second approach involves collection of a reasonable number of data on malignant tissues and comparing them to an approximately equal number of specimens of uninvolved tissue not from the same patient or not even necessarily from a tumor-bearing patient. Unavoidably this approach introduces a number of new ambiguities but it is, after all, the approach that is taken so frequently in studies of the metabolic reactions of rodent tumors, although the normal rodent population is, of course, genetically more homogeneous than the human one available for study.

Finally, there are many instances where it is essentially impossible to obtain uninvolved tissues for sensible comparisons. We have found this to be the case for mammary adenocarcinomata. So-called normal mammary gland tissue, particularly post-menopausally, is so heavily infiltrated with fat that the patterns observed are usually those of fatty tissue. Benign adenomata probably afford the best tissue for comparison. Similar difficulties are encountered in obtaining viable uterine mucosa, or normal prostatic or brain tissue. We simply have not included such tumors in our studies, except to demonstrate qualitatively the deletion of one enzyme. Comparative metabolic studies on many tissues are at the moment both practically and ethically impossible. Ultra-micro-methodology is available for very small samples but these raise considerable questions on the statistical significance of data obtained on single clumps of cells.

The first approach of Table 10 is illustrated on a case of an adenocarcinoma of the descending colon in Fig. 2.

In this instance the tumor was resected with a large section of uninvolved colon, and it was possible to obtain by dissection clean samples of mucosa and serosa and, of course, the whole gut wall. Absolute activities, that is units per g/wt, are plotted on the ordinate; but it is necessary to use a logarithmic scale, since the absolute activities span about three orders of magnitude. On the abscissa are listed the various glycolytic enzymes in their metabolic sequence.

The most striking feature is the fact the pattern for the tumor and the tissue of origin and its various parts are essentially very similar. True, the absolute activities of the glycoltyic enzymes are higher in the tumor — and this is generally true for most tumors, but the over-all pattern is quite similar. Therefore, if one arbitrarily sets one of the enzyme activities equal to 100, the relative activities for the other enzymes will be more or less constant, whether we compare the tumor with the colon, the colon mucosa or even the serosa. For this reason we have generally stressed the usefulness of recording of enzyme activities relative to each other. We usually use GAPDH as our reference

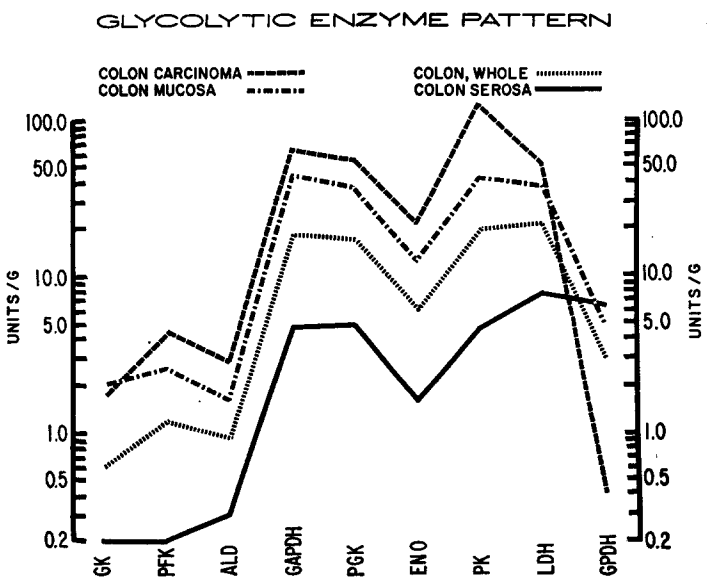


Fig. 2. Patterns of glycolytic enzymes in a colon carcinoma, and parts of the uninvolved colon of the same patient.

enzyme and many of the data are reported in this fashion.

The very high absolute activity of the tumor tissue and the very low activity of the serosa are undoubtedly reflections of their respective cellularity. It is obvious, however, that the relative enzyme activities are still essentially the same.

From a metabolic point of view, the absolute activities will determine the potential for metabolite flow, but it will be the relative activities that are determining rate limiting steps or direct metabolite flow through alternate pathways.

Another phenomenon rather well documented for rodent tissues is also clearly demonstrated here for a human tissue, namely the so-called constant proportion enzymes [7–10]. Note that the proportions of GAPDH, PGK, PGM, ENO, PK and LDH rather closely approximate one to one regardless of their absolute values. The somewhat lower activity for ENO within this group is also quite typical. The members of the constant proportion group vary somewhat in different tissues. LDH, for example, does not fall into this group in many tissues and PK is quite variable in hepatomas.

The relative constancy of the enzyme pattern in tumor and normal tissue makes the one exception all the more striking. This enzyme is GPDH. In spite of the fact that all the other enzymes are substantially higher in the tumor than in the mucosa, GPDH is fifteen times lower in the tumor. The deletion of GPDH, which has been observed in most rodent tumors in a number of laboratories [4, 11] and our own [7], is thus also found in human tumours. We have described the possible role of this deletion in the phenomenon of aerobic glycolysis of rodent tumors [12], and subsequent data document this deletion in essentially all human tumours that we have studied.

The relatively high level of GPDH in the serosa we feel to be real. Serosa necessarily contains some fatty tissue and, in our experience, high GPDH activity on a relative basis is a hallmark of fat tissue [1, 2].

The same relations are demonstrated in Table 11 in three more cases of carcinomata of the colon and the uninvolved colon of the same patient. The first set of figures for each case (abs.) are the ratios of the absolute activity in the tumor to those of the normal tissue. The enzymes of the glycolytic pathway are about two to five times as active in the tumor than in the uninvolved colon.

The second set of figures for each case (rel.) give the same ratios for the relative activities. In this instance we have set the activity of glucokinase arbitrarily as one in both tumor and normal tissue. It is quite clear that the relative pattern of these enzymes in tumor and normal tissues are essentially the same. GPDH was not included in this tabulation intended to demonstrate the constancy of the over-all pattern. In each instance the GPDH activity was more than 20 times lower in the tumor than in the normal tissue. PFK activity was unusually low in case 1. In view of the known instability of this enzyme before freezing this is considered to be an experimental artifact.

Table 11. *Ratio of enzyme activities in colon carcinomas to uninvolved colon.*

Enzyme	Case 1		Case 2		Case 3	
	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.
GK	2·9	1·0	1·7	1·0	4·3	1·0
PFK	0·7*	0·3*	1·8	1·1	5·0	1·2
ALD	3·3	1·2	1·6	1·0	4·1	0·9
GAPDH	2·1	0·7	1·9	1·1	5·1	1·2
PGK	2·0	0·8	1·6	1·0	4·6	1·1
ENO	1·9	0·7	2·2	1·3	3·7	0·9
PK	2·3	1·2	1·9	1·3	5·9	1·3
LDH	3·3	1·2	2·2	1·3	5·4	1·3
Average	2·2	1·0	1·9	1·1	4·7	1·1

Table 12 presents similar data for three cases of bronchogenic carcinomata of the lung compared to uninvolved lung. Again the first set of figures (abs.) indicates a three to five times higher absolute activity in the tumor. The second set of figures again shows that the relative pattern of these enzymes was, however, the same in both tumor and lung. GPDH again violates this pattern being in all cases more than 15 times lower in the tumor than in the normal tissue. Case 2 illustrates another preservation artifact of PFK. In this instance

Table 12. *Ratio of enzyme activities in lung carcinoma to uninvolved lung.*

Enzyme	Case 1		Case 2		Case 3	
	Abs.	Rel.	Abs.	Rel.	Abs.	Rel
GK	2·8	1·1	2·8	1·0	2·0	0·6
PFK	1·9	0·7	39·0*	14·0*	2·8	0·9
ALD	3·4	1·3	2·6	1·0	7·1	2·2
GAPDH	2·6	1·0	2·7	1·0	3·2	1·0
PGK	4·8	1·8	2·6	1·0	4·4	1·4
ENO	4·1	1·6	4·1	1·5	4·6	1·4
PK	4·1	1·5	2·1	0·8	3·8	1·2
LDH	3·8	1·5	2·8	1·0	7·6	1·3
Average	3·4	1·3	2·8	1·0	4·4	1·2

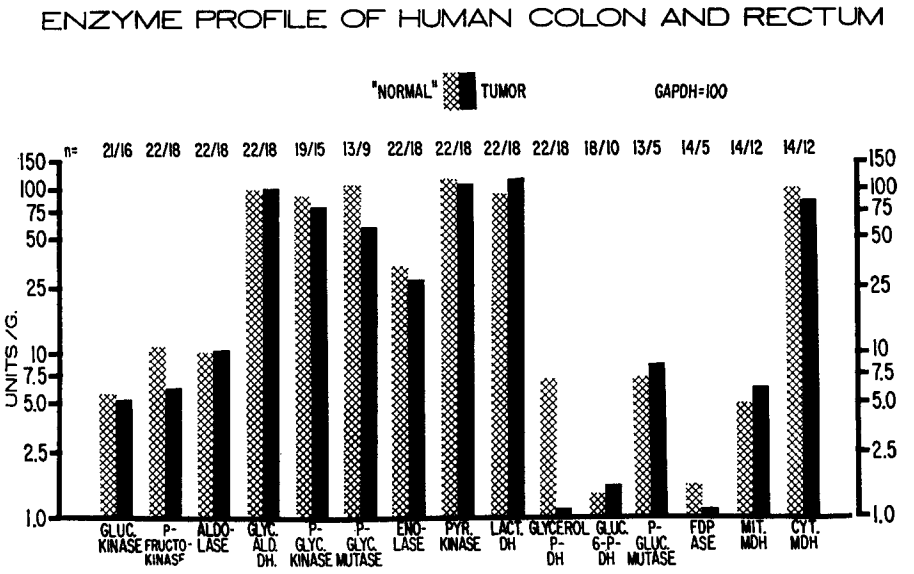


Fig. 3. Average pattern of glycolytic enzymes in specimens of 'normal' human colon and rectum and of corresponding carcinomas. Number of specimens included in each average is indicated on top of each bar. The activity of glyceraldehyde phosphate dehydrogenase (GAPDH) was arbitrarily set at 100 for each specimen, and all other activities were expressed relative to the GAPDH activity.

the normal tissue apparently lost activity leading to an unusually high ratio.

The second approach indicated in Table 10 is illustrated in Fig. 3. This is a graphic summary of data on tumours of the lower digestive tract which, to a large extent, have been previously published with numerical details [3]. The ordinate, on a logarithmic scale, indicates for each case the ratio of absolute enzyme activity to the absolute enzyme activity of GAPDH arbitrarily set at 100. The numbers on top of the bars indicate the number of samples averaged in this summary. The abscissa lists the enzymes of the glycolytic pathway augmented by some enzymes directly connected to the glycolytic pathway.

The bar graph again clearly demonstrated on an average pattern the constant proportion group of enzymes from GAPDH to LDH, that has been illustrated on an individual basis in Fig. 2. The somewhat lower activity of enolase in this group is quite typical.

On the average, the relative activities of glucokinase is similar in the normal and malignant tissues. This is somewhat in contrast to rodent tumours whose higher glucokinase activities have been frequently reported [13, 14].

The data for phosphofructokinase are somewhat problematic. On the average the relative activity of this enzyme in the tumour is only half of that in the normal tissue and this difference is statistically highly significant. The question whether it is metabolically significant is somewhat harder to answer. As stressed before, PFK is the only enzyme that tends to lose activity before freezing. In each case included in Fig. 3 the tumor tissues were excised rapidly and immediately frozen. In general, it was the normal tissue that was exposed to room temperature somewhat longer and should, therefore, be subject to enzyme loss, but on the contrary the values are higher in the normal tissue. We, therefore, tend to accept this difference as real but with slight reservations.

This is not purely an analytical question. Actually, from their absolute activities, either glucokinase or phosphofructokinase could be a rate limiting step in the glycolysis of these tissues. There is considerable evidence from animal tumor data that PFK is the rate limiting step in glycolysis and, in addition, it is subject to a number of allosteric influences [15–17]. Although we have never been able to show these allosteric effects in homogenates, Wu has very recently been able to bring some evidence that they might be operative in tumor cells [18].

The very low activity of glycerolphosphate dehydrogenase in the malignant tissues is clearly indicated in Fig. 3. Of direct metabolic significance is the ratio of the activities of LDH to GPDH. These enzymes are both acceptors of reducing equivalents from DPNH produced in the GAPDH reaction, and we have previously discussed the potential importance of this ratio in the phenomenon of aerobic glycolysis [13]. A comparison of the height of the bars for these two enzymes for the two types of tissue clearly indicates that this ratio is much

higher in the tumors than in the normal tissue. On the average the LDH/GPDH ratio is about six times higher in the tumor than in the normal tissue. Qualitatively and quantitatively, this abnormality is in full agreement with the findings in rodent tumors.

The remaining enzymes are of some interest. Phosphoglucomutase is involved in glycogen catabolism. Glycogen is found in the intestinal mucosa but not usually in the tumors. The relative enzyme activity is, however, the same in both types of tissue. Deletion of phosphoglucomutase has been described by Weber in rodent hepatomas [19–21].

FDPase is a curious case. The deletion of this enzyme in rodent hepatomas has been described by Weber [20, 22] and has been confirmed by us and others. Its physiological role in liver in reversal of glycolytic pathway is undoubted. We have found low levels of this enzyme in many human tissues and have found it to be quite low or absent in the corresponding malignant tissues. We hesitate to ascribe any metabolic significance to this observation, since reversal of the glycolytic pathway is probably not a major pathway in any tissue except the liver.

No significant differences between normal and malignant tissues have been found for glucose-6-phosphate dehydrogenase and either the cytoplasmic or mitochondrial malic dehydrogenases.

Similar data have been accumulated for malignancies from a number of tissues of origin; but since in essence they show the same relations that are described for the colon and rectum in Fig. 3, they are not illustrated with more sets of numbers or bar graphs. Most of these data are in the process of being published *in extenso*.

We have also investigated the question of metabolic similarity or dissimilarity between the primary tumor and its metastases. Frequently it is not an easy matter to obtain samples of a primary tumor and its metastases, that are of comparable cellularity and free of necrosis. For these technical reasons, squamous cell carcinomas of the head and neck region predominate in our data, but there are also examples of tumors of internal organs. The over-all results indicate that with respect to the glycolytic enzymes and the related enzymes indicated in Fig. 3, primary tumors and their metastases show no significant differences. This holds true with respect to the constant proportion enzymes, the very low or absent GPDH and FDPase activity, and the decreased activity of PFK.

Acknowledgments — The data presented in this talk were gathered with the collaboration and experimental skill of Dr. Carl E. Shonk. We are indebted to Drs. Bernard J. Koven and Hisashi Majima of Seton Hall Medical School, Jersey City, N.J. for providing us with the tissues and the careful clinical analysis of the material. We are particularly grateful for the pathological judgment of Dr. Ritsu N. Arison who has seen to it that enzymological enthusiasm was kept on the straight and narrow path of morphological realities. This investigation was supported by the Cancer Chemotherapy National Service Center, National Cancer Institute, under the National Institutes of Health, Contract No. PH-43-64-883.

RESUME

L'auteur a mis au point une méthodologie pour l'étude des enzymes glycolytiques, applicable à des fragments de tissus normaux et cancéreux, prélevés chirurgicalement. Il montre que les données recueillies ont une signification biologique, pour autant que soient respectées certaines conditions de prélèvement et de conservation des échantillons.

Le poids frais de l'échantillon était le cadre de référence le plus simple et le plus généralement applicable. Il est apparu que le rapport d'activité relative des différentes enzymes était le plus propre à donner des informations valables. Ces rapports étaient essentiellement indépendants de la cellularité de l'échantillon.

Une analyse histologique détaillée des coupes adjacentes aux fragments utilisés pour l'analyse enzymatique est essentielle. Il n'est pas rare que le manque d'homogénéité histologique des biopsies empêche toute interprétation valable des données enzymatiques.

En général, l'activité enzymatique du tissu malin, sur la base du poids humide, est deux à cinq fois plus élevée que l'activité du tissu normal correspondant ou du tissu d'origine non envahi. Des groupes d'enzymes glycolytiques, présentés dans une proposition constante, sont clairement mis en évidence dans les tissus normaux, comme dans les tissus cancéreux.

De façon relative, et en rapport avec l'existence de ces proportions constantes, les activités de la plupart des enzymes étaient tout à fait semblables, dans les deux types de

tissus. Seule la glycérophosphate-déshydrogénase fut trouvée abaissée de façon substantielle, ou absente, dans le tissu cancéreux. En moyenne, la différence était de un à dix. Cette observation est en complet accord avec les données provenant des études sur les tumeurs des rongeurs. L'activité de la phosphofructokinase, dans les tumeurs, était la moitié de celle des tissus normaux correspondants. Cette observation tend à confirmer la conclusion, tirée des études chez l'animal, que cette enzyme pourrait être le facteur limitatif de la glycolyse, également dans les tumeurs humaines.

Dans d'autres études, l'activité des enzymes glycolytiques fut comparée, dans la tumeur primitive et dans les métastases. Elle était très voisine dans les deux cas.

En dehors des délétions observées dans le processus glycolytique, nous pensons que la détermination de certaines enzymes glycolytiques, — particulièrement la phosphofructokinase, la GAPHD et l'une ou l'autre enzyme en proportion constante—, est capable de donner des informations très importantes sur l'état de conservation du tissu. La mesure de l'activité de ces enzymes dans le matériel de biopsie peut aussi donner un excellent cadre de référence pour la mesure d'activité des enzymes dans d'autres domaines du métabolisme.

SUMMARY

We have developed methodology for the assay of glycolytic enzymes that can be applied to surgical biopsies of normal and malignant tissues and have shown that the data obtained have biological meaning under proper conditions of collecting and storing of samples.

Wet weight of sample proved to be the simplest and most generally applicable frame of reference. We feel strongly that the activity ratio of enzymes relative to each other has given the most meaningful information. These ratios are essentially independent of cellularity of specimen.

A thorough histological analysis of sections adjacent to the pieces used for enzymic analysis is essential. Not infrequently, the histological inhomogeneity of the biopsies precludes any sensible interpretation of the enzymic data.

In general, enzymic activity of the malignant tissue on a wet weight basis is two to five times higher than the corresponding normal or uninvolved tissue of origin. Groups of glycolytic enzymes present in a constant proportion are clearly evident in normal as well as malignant tissues.

On a relative basis, due to the existence of constant proportions, the activities of most of the enzymes in the two types of tissues are similar. Only glycerolphosphate dehydrogenase has been found to be substantially lower or absent in the malignant tissue. On the average the difference is about ten-fold. This finding is in complete agreement with data on rodent tumors. Phosphofructokinase in tumors was found to be only half as active as in the corresponding normal tissue. This observation tends to support the conclusion from animal tumors that this enzyme might be the rate limiting step in glycolysis of human tumors too.

In another series, the glycolytic enzyme profiles of primary tumors and their metastases were compared. Activities were found to be very similar in both tissues.

Besides the deletions observed in the glycolytic pathway, we feel that determination of some of the glycolytic enzymes, specifically phosphofructokinase, GAPDH and one of the other constant proportion enzymes can give considerable information about the state of preservation of the tissue. Measurements of these enzymes in biopsy material also provides an excellent frame of reference for measurements of activities of enzymes or whole pathways in other areas of metabolism.

ZUSAMMENFASSUNG

Die Autoren haben eine Methode für die Bestimmung von glykolytischen Enzymen entwickelt, welche auf chirurgisches Biopsie-material stammend von normalen und bösartigen Geweben angewandt werden kann. Sie haben ausserdem zeigen können, dass die auf diese Weise erhaltenen Ergebnisse in der Tat biologische Geschehnisse wiederspiegeln, sofern das Material regelrecht gesammelt und aufbewahrt wird.

Das Feuchgtewicht scheint der einfachste und allgemein verwertbare Masstab zu sein. Die Verfasser sind von der Tatsache überzeugt, dass das Verchältnis der verschiedenen

Enzyme zueinander die wichtigsten Aufklärungen zeitigt. Dieses Verhältnis is von dem Zellgehalt der Proben unabhängig.

Eine genaue histologische Untersuchung der Gewebsabschnitte nächst denen, welche auf ihren Enzymgehalt analysiert werden, ist absolut unumgänglich. Es ist nicht selten, dass das Biopsiematerial histologisch so ungleichförmig ist, dass es jede Auswertung der Resultate ausschliesst.

Im Allgemeinen ist die enzymatische Aktivität von bösartigen Geweben, basierend auf dem Feuchtgewicht, etwa 2 bis 5 Mal höher als die von entsprechenden normalen Geweben. Konstante Proportionsgruppen von glycolytischen Enzymen finden sich sowohl in normalen- als auch bösartigen Geweben vor.

Relativ gesehen sind die Aktivitäten der meisten Enzyme in den beiden Gewebsarten gleichwertig in Folge der konstanten Proportionen innerhalb der Gewebe. Nur die Glycerol-phosphat-dehydrogenase ist in den bösartigen Geweben bedeutend vermindert oder gar abwesend. Durchschnittlich besteht hier eine etwa zehnfache Differenz. Diese Feststellung stimmt genau mit der bei Nagetiertumoren gemachten überein. Die Phosphofructokinase in Tumoren war nur etwa halb so aktiv wie in normalem Gewebe. Diese Beobachtung scheint den Schluss zu rechtfertigen, dass dieses Enzym die Geschwindigkeit der Glykolyse auch in menschlichen Tumoren begrenzt.

In einer anderen Untersuchungsreihe wurde der Gesamtaspekt der glykolytischen Enzyme in den Primärtumoren und in ihren Metastasen verglichen. Der Aktivitätsgrad stellt sich als sehr vergleichbar in beiden Geweben heraus.

Bestimmung der Aktivität einzelner glycolytischer Enzyme scheint den Verfassern wichtig zu sein, nicht nur um das Ausfallen gewisser Enzyme zu beweisen, sondern auch um einen Einblick in die Erhaltung des Gewebes zu gewinnen. Messungen der Phosphofructokinase, der GAPDH und eines der anderen konstanten Proportionsenzyme, eignen sich für diesen Zweck. Messung dieser Enzyme in dem Biopsiematerial gibt auch einen ausgezeichneten Masstab fur Bestimmungen von Enzymaktivitäten oder ganzer Abbauwege in anderen Gebieten des Stoffwechsels.

REFERENCES

1. C. E. Shonk and G. E. Boxer, Enzyme patterns in human tissues. I. Methods for the determination of glycolytic enzymes. *Cancer Res.* **24,** 709–721 (1964).
2. C. E. Shonk, B. Koven, H. Majima and G. E. Boxer, Enzyme patterns in human tissues. II. Glycolytic enzyme patterns in non-malignant human tissues. *Cancer Res.* **24,** 722–731 (1964).
3. C. E. Shonk, R. N. Arison, B. J. Koven, H. Majima and G. E. Boxer, Enzyme patterns in human tissues. III. Glycolytic enzymes in normal and malignant tissues of the colon and rectum. *Cancer Res.* **25,** 206–213 (1965).
4. A. Delbrück, H. Schimassek, K. Bartsch und Th. Bücher, Enzym-Verteilungsmuster in einigen Organen und in experimentellen Tumoren der Ratte und der Maus. *Biochem. Z.* **331,** 297–311 (1959).
5. R. Wu and E. Racker, Regulatory mechanisms in carbohydrate metabolism. III. Limiting factors in glycolysis of ascites tumor cells. *J. Biol. Chem.* **234,** 1029–1035 (1959).
6. E. Schmidt and F. W. Schmidt, Enzym-Muster menschlicher Gewebe. *Klin. Wochschr.* **38,** 957–962 (1960).
7. G. E. Boxer and C. E. Shonk, Low levels of soluble DPN-linked α-glycerophosphate dehydrogenase in tumours. *Cancer Res.* **20,** 85–91 (1960).
8. D. Pette and Th. Bücher, Proportionskonstante Gruppen in Beziehung zur Differenzierung der Enzymaktivitätsmuster von Skelett-Muskeln des Kaninchens. Hoppe-Seylers *Z. Physiol. Chem.* **331,** 180–195 (1963).
9. D. Pette, M. Ulingengerg, and Th. Bücher, Comparable and specific proportions in the mitochondrial enzyme activity pattern. *Biochem. Biophys. Res. Comm.* **7,** 425–429 (1962).
10. D. Pette, W. Luh and Th. Bücher, A constant-proportion group in the enzyme activity pattern of the embden-Meyerhof chain. *Biochem. Biophys. Res. Comm.* **7,** 419–424 (1962).

11. B. Sacktor and A. B. Dick, α-Glycerophosphate and lactate dehydrogenase activities of the hematopoietic cells from leukemic mice. *Cancer Res.* **20,** 1408–1412 (1960).
12. G. E. Boxer and T. M. Devlin, Pathways of intracellular Hydrogen Transport. *Science* **134,** 1495–1501 (1961).
13. Y. C. Lin, J. C. Elwood, A. Rosado, H. P. Morris and S. Weinhouse, Glucose metabolism in a low glycolyzing tumor, the Morris hepatoma 5123. *Nature, Lond.* **195,** 153–155 (1962).
14. J. C. Elwood, Y. C. Lin, V. J. Cristofalo, S. Weinhouse and H. P. Morris, Glucose utilization in homogenates of the Morris Hepatoma 5123 and related tumors. *Cancer Res.* **23,** 906–913 (1963).
15. J. V. Passonneau and O. H. Lowry, Phosphofructokinase and the Pasteur Effect. *Biochem. Biophys. Res. Comm.* **7,** 10–15 (1962).
16. J. V. Passonneau and O. H. Lowry, P-Fructokinase and the control of the citric acid cycle. *Biochem. Biophys. Res. Comm.* **13,** 372–379 (1963).
17. T. E. Mansour, Studies on heart phosphofructokinase: purification, inhibition and activation. *J. Biol. Chem.* **238,** 2285–2292 (1963).
18. R. Wu, H. Power and D. Hamerman, Rate-limiting factors in glycolysis and transport of inorganic phosphate (Pi) in DBAH tumor, DBAG tumor, Novikoff hepatoma, and Novikoff ascites tumor. *Cancer Res.* In press (1965).
19. G. Weber, G. Banerjee and H. P. Morris. Comparative biochemistry of hepatomas. I. Carbohydrate enzymes in Morris hepatoma 5123. *Cancer Res.* **21,** 933–937 (1961).
20. G. Weber and A. Cantero, Fructose-1, 6-diphosphatase and lactic dehydrogenase activity in hepatoma and in control human and animal tissues. *Cancer Res.* **19,** 763–768 (1959).
21. G. Weber and H. P. Morris, Comparative biochemistry of hepatomas. III. Carbohydrate enzymes in liver tumors of different growth rates. *Cancer Res.* **23,** 987–994 (1963).
22. G. Weber and J. Ashmore, Absent fructose-1,6-diphosphatase activity in hepatoma. *Exp. Cell. Res.* **14,** 226–228 (1958).

Europ. J. Cancer Vol. 1, pp. 173–181. Pergamon Press 1965. Printed in Great Britain

Evidence of Immunological Reactions to Autochthonous Cancer in Man

CHESTER M. SOUTHAM

Sloan-Kettering Institute for Cancer Research and Memorial and James Ewing Hospitals, New York City, New York

No INCONTROVERTIBLE evidence of an immunological reaction against autochthonous cancer in man has yet been published, nor will the reader find such evidence in this report. However, the data to be presented here are consistent with such an interpretation and hence should be of interest since there is little evidence of any kind on this most important question.

In experimental animals, in contrast to man, recent studies have provided very convincing evidence that resistance to autochthonous cancer does occur and can be augmented by experimental procedures. Up to date reviews of these animal studies [1, 2] and of clinical studies [3] are available, so no attempt will be made to review the literature in this report.

The studies are presented in five categories: 1. Quantitative studies of human cancer autotransplantation; 2. Studies of cancer cell antigens; 3. Studies of the immunologic responsiveness of cancer patients to various antigenic stimuli; 4. The relationship of cancer autotransplantability and the recipient's immunological status; and 5. Studies on the possibility of lymphocyte-associated resistance against autochthonous human cancer. Some of these data which have been or will be reported elsewhere in detail are presented here in summary form.

AUTOTRANSPLANTATION STUDIES

If there is any resistance to the continuing growth of cancer after the neoplastic cell has appeared, it should result in the retardation or prevention of growth of the patient's own cancer cells. Since this question of host resistance to autochthonous cancer is the most basic of all problems in cancer immunology, it seemed mandatory to investigate it as a prerequisite to any more detailed inquiry. To this end we studied the autotransplantability of serial doses of tumor cells in patients with non-resectable cancer.

It seems obvious that if there is any kind of resistance to cancer it is not of high degree—at least not in those patients who do have clinical cancer. (And, of course, patients with incurable cancer are the only patients in whom such autologous implantation studies could be done without risk.) Therefore, it seemed that a quantitative method of study that would detect rather small degrees of resistance would be necessary.

Our method was to prepare fresh single cell suspensions from surgical specimens of human tumors by mincing the tissue with scissors or scalpels in a balanced salt solution. After removing residual chunks of tissue by allowing them to sediment out and by filtering through a single layer of surgical gauze, the resulting suspension, which consisted of single cells or occasional small clusters of cells, was counted in a hemocytometer. The volume was then adjusted to yield the highest possible cell concentration, and serial ten-fold dilutions were prepared therefrom. At least four dilutions of this suspension were autotransplanted subcutaneously on the anterior thigh and these sites were observed for development of nodules

This work has been supported in part by grants from the American Cancer Society (T-229) and U.S. Public Health Service, National Institutes of Health (CY 3215).

Table 1. *Growth of quantitated subcutaneous cancer autotransplants in patients with incurable cancer. All patients with a "take" are included, but "no takes" are excluded unless followed for at least* 8 *weeks. (Condensed from data being prepared for full publication.)*

Number of cells inoculated	Epidermoid Ca. (31 patients)	Adenoca (59 patients)	Sarcoma (5 patients)	All types (95 patients)
100,000,000	—	100%	100%	100%
10,000,000	60%	78%	75%	73%
1,000,000	39%	49%	60%	46%
100,000	14%	23%	0	19%
10,000	0	0	0	0
1,000	0	0	0	0

as long as the patient survived. Because these studies were done only in patients with non-resectable cancer many of the patients had only a very short survival time. However, evaluation of our total data has shown that an observation period of 8 weeks is sufficient for the detection of more than 90 per cent of nodules that will develop. Consequently, implants which produced no palpable nodules by 8 weeks are considered as negative. (Patients who lived less than 8 weeks were considered not evaluatable and were excluded from the data unless nodules had already developed.)

The results (Table 1) indicate clearly that it requires a rather large number of live cancer cells to establish an autotransplant. Obviously the cells in these suspensions were not all cancer cells and the preparation of the suspension probably caused death of some of the cancer cells. It seems reasonable, however (based on study of histologic sections of the tumor tissue, smears of the cell suspension and tests of cell viability with eosin-y or nigrisin stain) that at least 10 per cent of the cells in the suspension were live cancer cells. However, even if this estimate should be over optimistic it does not alter the fact that a very large number of cancer cells was required for autotransplantation in most patients, and thus it may be concluded that there is some kind of resistance to the implantation and propagation of cancer cells. This resistance is not necessarily a specific immunologic phenomenon, but our objective at this stage of our studies was merely to determine whether any type of resistance exists.

Further studies with the cancer autotransplantation technique will be presented when we consider the mechanism of this resistance but we will turn now to the question of cancer antigens.

CANCER CELL ANTIGENS

If there is any resistance to cancer, and if that resistance does act through specific immunologic mechanisms, there must be antigens in cancer cells which are not present in normal tissues of the same person (or, if present, are not susceptible to effective immunologic attack because of their location or concentration). Such cancer-specific antigens can be sought by *in vitro* or *in vivo* methods of detecting antigen-antibody reactions.

Autoantibody studies

Dr. Cyril Toker attempted to demonstrate autoantibodies against cancer in the serum of fifteen tumor patients (unpublished data from this laboratory and the Department of Pathology, Memorial Hospital). He used a modification of the tanned erythrocyte agglutination technique of Boyden [4, 5] with the supernatant fluid of fresh homogenates of the patient's own tumor as the test antigen. In each case a 'control' antigen was also prepared from the non-neoplastic tissue of origin of the tumor. Only one of these fifteen patients showed a clear agglutination reaction with the tumor tissue extract with negative reaction in all controls including the normal tissue control, but this positive reaction occurred only at a serum dilution of 1:5. This patient had Whartin's tumor—a benign salivary gland adenoma characterized by a prominent lymphoid component. Another patient showed a somewhat doubtful pattern of agglutination with the autologous cancer antigen and none with the normal control antigen, but this too was at a titer of only 1:5. This patient had a papillo-cystic parotid tumor (a tumor of very low grade malignancy). The other thirteen patients gave negative results. Usually these were completely negative with both cancer and

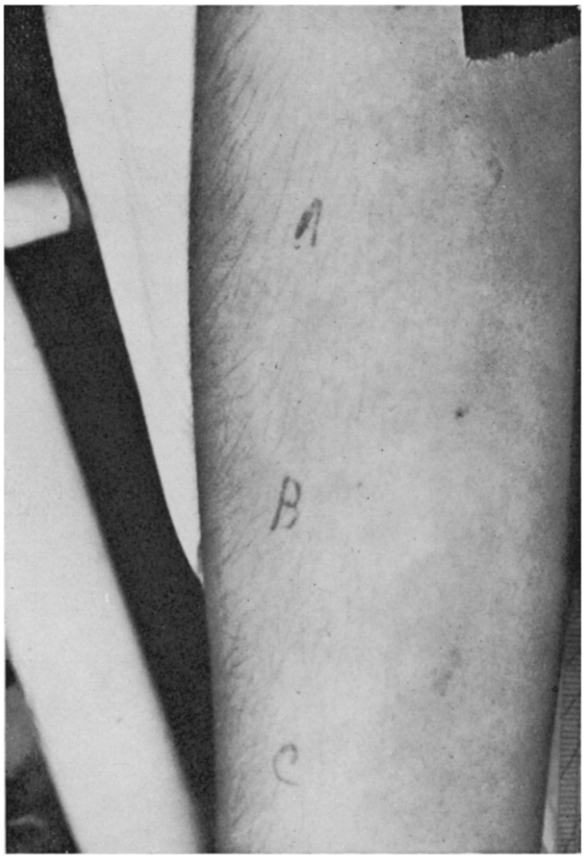


Fig. 1. *Skin test reactions* 20 *min after intradermal injections of autogenous leukocyte extracts* (*see text for patient's history*). *The first irradiated preparation* (*proximal test site*) *gave an erythematous flare about* 5 *cm in dia. The second irradiated preparation gave an almost identical reaction* (*distal test site*). *An unirradiated aliquot of the second preparation was injected at the middle site and gave a positive reaction but only* 3 *cm in dia.*

(to face p. 174)
Refer to p. 176



Fig. 2. Homotransplant nodules in thigh of a patient with advanced cancer. The obvious nodule is the site where 2 million HEp 2 cells were implanted 10 weeks earlier. At the areas outlined by dots, smaller subcutaneous transplant nodules could be palpated.

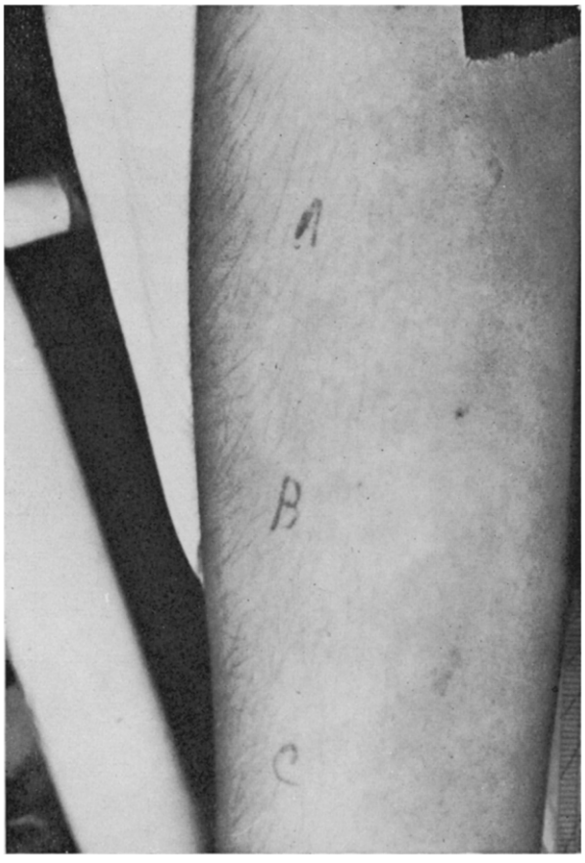


Fig. 1. *Skin test reactions* 20 *min after intradermal injections of autogenous leukocyte extracts* (*see text for patient's history*). *The first irradiated preparation* (*proximal test site*) *gave an erythematous flare about* 5 *cm in dia. The second irradiated preparation gave an almost identical reaction* (*distal test site*). *An unirradiated aliquot of the second preparation was injected at the middle site and gave a positive reaction but only* 3 *cm in dia.*

(to face p. 174)
Refer to p. 176

normal antigens, but two were classified as negative because of equal agglutination with both normal and control antigens at low serum dilutions. These thirteen included one patient with a benign lymphoepithelial lesion of the parotid, seven adenocarcinomas of the large bowel, one gastric carcinoma, three epidermoid carcinomas of the oral cavity, and one osteogenic sarcoma.

In addition to the serum studies, fresh extracts were prepared from regional lymph nodes of three of the cancer patients and spleen of one. The supernatant of these lymphoid tissue extracts was tested at a dilution of 1: 10 in the same manner as the sera. All four were negative at the lowest dilution.

Thus, by this technique in this small series of patients, there was no significant evidence for the presence of autoantibodies against cancer.

Autoimmune reactions to autogenous leukocyte extract

Currently Dr. Arthur G. Levin and I are studying the effect of administering *autogenous* leukemic cell extract to patients with acute leukemia when they are in hematologic *remission*. For these studies the leukemic cells are harvested from peripheral blood or bone marrow before treatment is started, and stored at −70°C until the patient enters remission as result of chemotherapy. The patient's leukemic cells are then thawed and homogenized in a Virtis grinder to disrupt cells and nuclei. The clear supernatant from this homogenate is then irradiated with ultraviolet light and administered to the patient. The first four or five injections are usually given intravenously at one week intervals and thereafter they are given intramuscularly until the material is all gone or the patient relapses.

This clinical study is based on mouse leukemia studies by Latarjet [6] which showed a suppressive effect on mouse leukemia when a similar extract of leukemic mouse spleen was administered during the incubation period of virus-induced leukemia. It is thought that the antileukemic effect might result from either viral interference, as suggested by Latarjet, or perhaps from specific immunization against the leukemia virus or leukemia-specific cellular antigens.

It is not yet possible to make any evaluation of the therapeutic efficacy of these clinical studies (or of similar studies which are in process in France). However, we have observed that the recipients of these autologous leukemic cell extracts develop positive skin tests which, we believe, indicates the development of an immunologic reaction against antigens in the leukemic cell extracts.

All patients skin-tested with the autologous leukemic cell extracts shortly after entering hematological remission had no reaction, but the six patients who have been studied to date all developed positive skin reactions of the immediate wheel and flare type when retested after four or more injections.

The most dramatic evidence of an autoimmune reaction occurred in a 20 year old girl with acute leukemia who was the first patient to receive this kind of treatment. On first hospital admission she had a leukocyte count of approximately 300,000/mm^3 and the extract was prepared from peripheral blood leukocytes. She was treated with hydrocortisone and 6 mercaptopurine and was soon in complete hematologic remission as judged by peripheral blood, bone marrow, and physical examination. She was then given the first injection of her UV-irradiated autologous leukocyte extract. This and three succeeding weekly intravenous injections caused no reaction, but immediately after the fifth injection she felt weak and dizzy (while still in the supine position). Her pulse became rapid and thready. (Her blood pressure was not taken at this time.) Her skin became clammy and beads of sweat appeared on her forehead. There was no respiration difficulty. She did not lose consciousness. The hypotension and tachycardia lasted only about 2 min. Within 10 min she felt normal and went home without difficulty. After this episode the injections were given intramuscularly instead of intravenously without further difficulty. An intradermal skin test was done 1 week after the episode of shock and showed a typical immediate hypersensitivity type of reaction. Maximum reaction occurred between 8 and 20 min after injection when there was induration over one cm in diameter and a surrounding erythema totaling about 5 cm in dia.

The patient remained in remission for another four months and then had bone marrow evidence of leukemic relapse. At this same time her skin tests became less positive. When readmitted to the hospital and treated with cytosine arabinoside her skin tests became negative. A second leukemic cell preparation was obtained during this period of relapse and some of the extract was not irradiated.

The patient went into a second complete hematological remission which is still maintained (12 months after onset of disease) and her skin tests have again become positive to both the first and the second irradiated autogenous

Table 2. Reaction of human sera with tissue-cultured human cancer cells, as measured by an antiglobulin consumption technique. Data condensed from Ref. 8. Numbers indicate numbers of persons.

Serum donors		Number	Reaction*		
Diagnosis	Mean age		Insignificant	Moderate	High
Healthy adults	34	75	68%	28%	4%
Cancer patients	46	8	0%	87%	13%
Sick, not cancer	61	16	6%	25%	69%

*Reactions are graded according to the number of tubes (two-fold dilutions) by which the titer of an anti-human globulin serum is lowered after it has reacted with the tissue cultured cells which have previously been incubated with a test serum. Insignificant is 0–2 tubes, moderate is 3 or 4 tubes, high is 5 or more tubes.

leukemic cell extracts. There is also a distinctly positive, but smaller, skin reaction to the unirradiated extract (Fig. 1).

We interpret the reactions in this patient and the other five patients as specific immune reactions to the autologous leukemic cell extracts. We have not yet had the opportunity to compare extracts from normal autologous leukocytes. It seems probable that the UV-irradiation has caused a chemical alteration in the extract which has increased its antigenicity, but it appears that the resulting immune reaction occurs with unirradiated as well as the irradiated material.

Isoantibody studies

Another type of data which is pertinent to this discussion is serological studies of cancer patients and other persons for isoantibodies against antigens of various human cancer cells. (We have carried out such studies in conjunction with homotransplantation studies, but the following data refer only to serum from persons who have *not* received homotransplants.)

Using antigens from tissue-cultured cancer lines coated onto tanned erythrocytes, our results [4, 5, 7] have been uniformly negative, even at serum dilutions of 1: 5 or 1: 10. These studies include sera from approximately sixty healthy persons and sixty cancer patients.

However, using a modification of the antiglobulin consumption technique, with live tissue-cultured cancer cells as the antigen, we found [8] that firm adsorption of serum globulin to the cancer cells occurred more frequently and in greater amounts with serum from cancer patients than with serum from healthy controls, and that sera from old people with non-neoplastic diseases gave even greater reactions (Table 2).

There is no evidence to indicate, or even suggest, that this reaction is cancer-specific, in fact we have no evidence that the adsorbed globulin is true antibody. If it should prove to be antibody, it would have to be assumed that such antibodies develop with increasing age even in persons who do not develop clinical cancer.

IMMUNOLOGICAL CAPABILITY OF CANCER PATIENTS

If there is a host resistance against cancer, it must obviously have failed in those people who develop clinically evident cancer, and especially in those patients who had reached the stage of advanced metastatic cancer. It seems pertinent then, when considering the possibility of immunologic reactions to cancer, to investigate the general immunological capability of cancer patients.

Over the past ten years this question has been investigated with the help of several coworkers and the generalization now seems permissible that cancer patients—even those who are within three or four weeks of death—show no evidence of an inability to produce serum antibody [4, 7, 9, 10] and no depression of total serum complement or the second component of complement [11, 12]. (This statement does not apply to patients with lymphomatous neoplasms such as lymphosarcoma, chronic lymphatic leukemia and multiple myeloma, or to patients with profound lymphoid depression from chemotherapy or radiotherapy.)

On the other hand, there is clear evidence of a major impairment of immunologic reactions of the type thought to be mediated by lymphoid cells in a large percentage of patients with advanced carcinoma. The bases for this statement are summarized in Table 3 which is

Table 3. Cellular response mechanisms in healthy persons, cancer patients, and patients with non-neoplastic diseases.

	Homograft* regression by 4 weeks		Positive DNFB test		Positive tuberculin test		Macrophage response† 50% at 24 hr	
Healthy	17/17	100%	12/16	75%	6/13	46%	29/30	97%
Cancer	17/30	57%	10/44	23%	8/27	30%	4/83	5%
Noncancer	18/19	95%	14/19	74%	13/18	72%	51/53	96%
Lymphoma	0/3	0%	2/19	11%	2/18	11%	No data	

*Homografts of cancer cell lines HEp 2, HEp 3, and RPMI-41.
†For data see Ref. 14.

reproduced from Ref [13]. The frequency of reaction to the tuberculin test, the induction and expression of sensitivity to dinitrofluorobenzene (DNFB), and the speed of rejection of homografts of subcutaneously transplanted human cancer cell lines are all depressed. (The low rate of tuberculin reactions in the healthy control series reflects the generally lower exposure of young Americans to this disease, rather than a poor immunologic responsiveness.) The ability to mobilize macrophages following a non-antigenic skin irritation (mechanical abrasion) is also impaired [14, 15].

The most striking of these defects is the poor homograft rejection reaction. This is well illustrated by the nodules illustrated in Figs 2 and 3 which developed in a 22 year old woman with widespread epidermoid cervix cancer. This patient received no anticancer chemotherapy at any time. She received 5400 r Co^{60} teletherapy to the pelvic tumor, which was completed 13 weeks before the homotransplant was injected. Her total leukocyte count was normal at all times but she had a persistent lymphopenia (10% to 26%) following the irradiation. Blood urea nitrogen was normal throughout the course of the homotransplantation study. Subcutaneous homotransplants of 2 million cells of the lines HEp 2 and RPMI-41 produced nodules that reached maximum size at 8 weeks and changed but slightly in the next two weeks at which time the first photograph was taken; thereafter they regressed rapidly until completely rejected by 16 weeks. This behaviour is in marked contrast to the prompt rejection which would be expected of a homograft and which has occurred in every normal recipient of such homotransplants. This phenomenon has been described more fully elsewhere [16, 17] and further reports are planned.

CORRELATION BETWEEN AUTOTRANSPLANTABILITY AND IMMUNOLOGICAL STATUS

If there is in fact some form of immunologic resistance to autochthonous cancer in man, and if there is some deficiency of the immune capability of cancer patients which is related to their disease *per se* (that is, not to treatment), the possibility is immediately apparent that these two things may be more than coincidence. They may be causally related. To prove a causal relationship is often difficult or impossible but as a first step one should inquire how closely correlated the two phenomena may be.

To this end we have carried out *simultaneous* studies of quantitated cancer autotransplants as a criterion of cancer resistance and tissue culture homotransplants as a measure of immunologic capability, in a group of cancer patients, to test this correlation in individual subjects. The results are summarized in Table 4 which is modified from another publication [18]. Patients who had defective immunologic responsiveness, as judged by delayed rejection of the homotransplant, had a greater percentage of takes of their cancer autotransplants at all inoculum levels from 10^7 to 10^5 cells. At 10^8 all autotransplants grew regardless of immunologic status, and at 10^4 none grew regardless of immunologic status.

These data demonstrate that there is a greater probability that a patient's own cancer will implant and grow if his immunologic capability is depressed. Considering the crudeness of quantitation that these techniques allow and the narrow range of clinical conditions which can be studied, it is perhaps surprising, and certainly encouraging, that even this much evidence of correlation was demonstrated, for if this is a reflection of host resistance to cancer it implies that some degree of resistance persists even in patients with far advanced

Table 4. Percentage of "takes" of quantitated cancer autotransplants in thirty three patients with normal, and twenty patients with impaired immunologic capacity. Immunologic status was measured by promptness of rejection of homografts of tissue cultured cell lines.*

Number of tumor cells transplanted	Growth of autotransplants in patients with:	
	Normal immunologic capacity†	Impaired immunologic capacity
100,000,000	100%	100%
10,000,000	65%	100%
1,000,000	31%	68%
100,000	6%	30%
10,000	0%	0%

*Cell lines used for homotransplantation were HEp 2, HEp 3, or RPMI-41. Some of the patients received two of these cell lines simultaneously.

†Normal rejection is defined as that seen in normal subjects, i.e., maximum size of transplant nodules is reached by 3 weeks or earlier and regression is complete or nearly so by 6 weeks. Delayed rejection is defined as continual growth or persistence at maximum size for at least 6 weeks before regression is apparent by palpation. Intermediate reactions are excluded from this tabulation.

disease. It must be recognized, however, that there is still no proof that the correlation is causal rather than coincidental, nor that the immunologic status is the cause rather than a result of aggressive cancer growth.

EFFECT OF AUTOLOGOUS LEUKOCYTES ON CANCER AUTOTRANSPLANTS

If there is resistance to cancer autotransplantation which is correlated with the efficiency of homograft rejection, and if homograft rejection serves as a measure of cellular mechanisms of specific immunologic reaction, it is natural as a next step to consider the possibility that the postulated resistance to cancer is effected via cellular mechanisms.

As a crude test of this hypothesis we have undertaken studies of the effect of adding autologous leukocytes to autologous cancer cell suspensions in order to observe the effect of the leukocytes upon autotransplantability. (We have used leukocytes from the peripheral blood as a convenient source of lymphocytes, and the leukocyte preparations were usually 60 per cent or more lymphocytes. However, we do not actually know that the results obtained are attributable to lymphocytes or to other less numerous types of leukocytes.)

These studies will be published in detail elsewhere and therefore are summarized only briefly in this report.

The number of leukocytes has been about 10 million mixed with each dilution of cancer cells. Evaluation has been based on size and time of appearance of autotransplant nodules in the series that was mixed with leukocytes, in comparison with the series containing no added leukocytes. Of the seventy three patients studied in this way, forty two had growth of autotransplants at some site and hence the effect of the added leukocytes could be evaluated. Of these, 50 per cent showed evidence of inhibition of the cancer cells by the admixed autologous leukocytes. In most of these (31 per cent of the total) the minimum number of cells required to produce an autotransplant nodule was increased ten-fold in the presence of leukocytes. This corresponds to an inhibition of 90 per cent of the cancer cells.

In some studies the patient's undiluted plasma was mixed with the cancer cells as a further basis for comparison. This autologous plasma was less frequently inhibitory than the autologous leukocytes (33 per cent showed some inhibition and 19 per cent showed a 90 per cent inhibition) but it is significant that every patient whose plasma caused inhibition of their cancer cells also showed inhibition by autologous leukocytes. This suggests that plasma might contain anticancer antibody in some cancer patients, but that cell-associated immunologic mechanisms are more effective.

In nine evaluatable patients leukocytes from a different donor were admixed with the autologous cancer cells for further comparison with the autologous leukocyte mixtures. Three of the nine showed some inhibition of the cancer autotransplant by the homologous leukocytes, and one of these showed a 90 per cent inhibition,

but in three other patients the homologous leukocytes enhanced growth of the cancer autotransplants. (With autologous leukocytes enhancement occurred in only one of the forty two evaluatable patients.)

It seems safe to conclude that autologous leukocytes have an inhibitory effect on autologous cancer. However, it obviously cannot be concluded that the inhibitory effect is due to specific immunologic mechanisms, since the number of studies with homologous leukocytes, is so small and because homologous leukocytes are probably not biologically satisfactory as a control material.

Although the limitations of these studies must be recognized, it is possible that the results do reflect a lymphocyte-mediated anticancer effect and that the results with plasma indicate similar but less effective humoral defense mechanism.

Acknowledgements—Many collaborators have participated in these studies and the author wishes to acknowledge particularly Dr. Miki Aizawa, Dr. Richard Brooks, Dr. Alexander Brunschwig, Dr. Quirino Dizon, Dr. Charles A. Doan, Dr. Tetsuo Itoh, D. Arthur Levin, Dr. Nobuyuki Tanigaki and Dr. Cyril Toker.

SUMMARY AND CONCLUSION

The question of immunologic reactions against autochthonous human cancer was investigated using the techniques of autotransplantation, homotransplantation, serology and skin tests. Due to the biological complexity of cancer and the restrictions of clinical research, the data seldom permit unequivocal conclusions but all of the results are consistent with the hypothesis that there are host defenses against autochthonous cancer and that such defenses may act upon cancer-specific antigens and may work through cellular mechanisms.

RESUME

Le problème des réactions immunologiques contre le cancer humain autochtone a été étudié en utilisant les techniques d'autogreffe et d'homogreffe, des techniques sérologiques ainsi que des tests cutanés. Les résultats sont présentés en cinq sections.

1. *L'autogreffe de suspensions graduées de cellules cancéreuses produit des nodules au lieu d'inoculation de doses massives de cellules. La proportion de nodules en croissance manifeste a été de* 100 *pour cent avec* 10^8 *cellules, de* 73 *pour cent avec* 10^7, 46 *pour cent avec* 10^6 *et* 19 *pour cent avec* 10^5 *cellules. On n'a jamais remarqué de croissance avec* 10^4 *ou moins de cellules. Il semble donc qu'il existe une résistance vis-à-vis de l'implantation de cancer autochtone.*

2. *Les études sérologiques utilisant la technique de Boyden n'ont pas démontré d'auto-anticorps contre le tissu tumoral propre du malade. Elles n'ont pas mis en évidence non plus d'iso-anticorps, vis-à-vis de lignées cellulaires cultivées* in vitro. *Les sérums des malades cancéreux et des patients avec maladies chroniques non néoplasiques ont montré, plus fréquemment que les sérums témoins normaux, l'absorption de globuline sérique sur des cellules cultivées* in vitro. *Ceci a été déterminé par une technique de déficit d'antiglobuline. Cependant, on n'a pas pu démontrer que cela correspondait à une réaction antigène-anticorps. Après administration des extraits irradiés de cellules leucémiques autologues à des malades leucémiques en rémission hématologique, les* 6 *malades traités développaient des réactions cutanées positives, à la suite d'injection intradermique de l'extrait.*

3. *Les malades cancéreux avaient souvent des réactions immunologiques déficientes de type cellulaire. Cela a été démontré par un développement déficient de l'hypersensibilité de type retardé, après l'application à la peau de dinitrofluorobenzène (DNFB) et par des délais prolongés de rejet des homogreffes de cellules cancéreuses cultivées* in vitro.

4. *Une comparaison des homogreffes et des autogreffes, chez le même malade cancéreux, a montré que l'autogreffe réussissait avec un plus petit nombre de cellules chez les malades avec mécanismes immunologiques déficients.*

5. *L'adjonction de leucocytes autologues aux suspensions de cellules cancéreuses réduisait la prise de l'autogreffe chez* 21 *malades, sur un ensemble de* 42.

Ces résultats traduisent la nature complexe des phénomènes étudiés. Ils sont cependant en accord avec l'hypothèse de l'existence de mécanismes de défense chez l'hôte contre le cancer autochtone. Ces défenses peuvent agir sur des antigènes spécifiques propres aux tumeurs malignes, par l'intermédiaire de réactions immunologiques de type cellulaire.

ZUSAMMENFASSUNG

Die Frage immunologischer Reaktionen gegen autochthonen menschlichen Krebs wurde mittels Autotransplantation, Homotransplantation, Serologie und Hautreaktionen untersucht. Die Ergenbisse werden in 5 *Abteilungen präsentiert.*

1. *Autotransplantate von serienweise verdünnten Krebszellsuspensionen ergaben Knötchen an den Stellen an welchen eine grosse Anzahl von Zellen injiziert wurde. Prozentual war das Angehen der Transplantate* 100% *an den Stellen welche* 10^8 *Zellen empfingen,* 73% *an denen mit* 10^7, 46% *mit* 10^6 *und* 19% *mit* 10^5 *Zellen. Inocula mit* 10^4 *Zellen oder weniger wuchsen nie. Somit scheint in irgendeiner Weise eine Abwehr gegenüber der Einpflanzung und dem Wachstum von autochthonem Krebs zu bestehen.*

2. *Serologische Untersuchungen mittels der Boydenschen Methode ergaben keine Anzeichen für Autoantikörper gegen das eigene Krebsgewebe der Patienten, sowie Isoantikörper gegen in Gewebskulturen gezüchteten Krebszell-linien. Nach der Antiglobulinkonsumtionsmethode zeigten Sera von Krebspatienten und von Patienten mit chronischen nichtneoplastischen Krankheiten häufiger eine Adsorption von Serumglobulin an in Gewebskultur gezüchtete Krebszellen als Sera von gesunden Menschen, Beweise von Antigen-Antikörper Reaktionen wurden aber nicht gefunden. Nach der Behandlung von akuten Leukämiepatienten in haematologischer Remission mit bestrahltem Extrakt der eigenen Leukämiezellen, entwickelten alle* 6 *Patienten eine positive Hautreaktion nach intradermaler Injektion des autogenen Extraktes.*

3. *Krebspatienten zeigten häufig verminderte immunologische Reaktionen der zellvermittelten Art; dies wurde durch die mangelhafte Ausbildung der verzögerten Uberempfindlichkeitsreaktion gegen eine standardisierte Applikation von Dinitroflurobenzol (DNFB) auf der Haut, sowie durch das verzögerte Abstossen der Homotransplantate von in Gewebskultur gezüchteten Krebszellen nachgewiesen.*

4. *Vergleiche von gleichzeitigen Homotransplantaten und Autotransplantaten an den gleichen Krebspatienten zeigten, dass der eigene Krebs mit weniger Zellen in Patienten, welche immunologische Schäden aufwiesen, verpflanzt werden konnte.*

5. *Beimischung von autogenen Leukozyten zu Krebszellsuspensionen reduzierte die Autotransplantabilität des Krebses in* 21 *der* 42 *auswertbaren Patienten.*

Die Resultate dieser Untersuchungen erlauben keine eindeutigen Schlüsse; sie sind aber vereinbar mit der Hypothese, dass körpereigene Abwehrkräfte gegen autochthonen Krebs bestehen und dass diese Abwehr auf krebsspezifische Antigene und über zelluläre Immunmechanismen agieren könnte.

REFERENCES

1. L. J. Old and E. A. Boyse, Immunology of experimental tumors. *Ann. Rev. Med.* **15,** 167–186 (1964).
2. E. D. Day, *The Immunochemistry of Cancer.* Charles C. Thomas, Springfield, 170 pp. (1965).
3. C. M. Southam, Applications of immunology to clinical cancer — Past attempts and future possibilities. *Cancer Res.* **21,** 1302–1316 (1961).
4. M. Aizawa and C. M. Southam, Serum antibodies following homotransplantation of human cancer cells. *Ann. N.Y. Acad. Sci.* **87,** 293–307 (1960).
5. T. Itoh and C. M. Southam, Isoantibodies to human cancer cells in healthy recipients of cancer homotransplants. *J. Immunol.* **91,** 469–483 (1963).
6. R. Latarjet, Action inhibitrice d'extraits leucémiques isologues irradiés sur la leucémogenèse spontanée de la souris AkR. *Annls. Institut Pasteur* **107,** 1–26 (1964).
7. T. Itoh and C. M. Southam, Isoantibodies to human cancer cells in cancer patients following cancer homotransplants. *J. Immunol.* **93,** 926–936 (1964).
8. N. Tanigaki and C. M. Southam, Use of an indirect antiglobulin consumption test to detect isoantibodies following human cell homotransplants. (Submitted for publication).
9. C. M. Southam and A. E. Moore, Anti-virus antibody studies following induced infection of man with West Nile, Ilheus, and other viruses. *J. Immunol.* **72,** 446–462 (1954).

10. C. M. SOUTHAM and E. L. GREENE, Clinical application of the hemagglutination inhibition test for West Nile virus antibodies. *J. Infect. Dis.* **102,** 174–178 (1958).
11. C. M. SOUTHAM and Y. GOLDSMITH, Effect of nitrogen mustard on serum complement *in vitro* and in patients with neoplastic disease. *Proc. Soc. Exper. Biol. & Med.* **76,** 430–432 (1951).
12. C. M. SOUTHAM and A. H. SIEGEL, Second component of complement in cancer patients. Manuscript in preparation.
13. A. G. LEVIN, E. F. MCDONOUGH, Jr., D. G. MILLER and C. M. SOUTHAM, Delayed hypersensitivity response to DNFB in sick and healthy persons. *Ann. N.Y. Acad. Sci.* **120,** 400–409 (1964).
14. Q. DIZON and C. M. SOUTHAM, Abnormal cellular response to skin abrasion in cancer patients. *Cancer* **16,** 1288–1292 (1963).
15. H. S. GOLDSMITH, A. G. LEVIN and C. M. SOUTHAM, A study of cellular response in cancer patients by qualitative and quantitative Rebuck tests. *Surgical Forum*, in press (1965).
16. C. M. SOUTHAM, A. E. MOORE and C. P. RHOADS, Homotransplantation of human cell lines. *Science* **125,** 158–160 (1957).
17. A. G. LEVIN, D. B. CUSTODIO, E. E. MANDEL and C. M. SOUTHAM, Rejection of cancer homotransplants by patients with debilitating non-neoplastic diseases. *Ann. N.Y. Acad. Sci.* **120,** 410–423 (1964).
18. A. BRUNSCHWIG, C. M. SOUTHAM and A. G. LEVIN, Clinical experiments on host resistance to cancer. *Annls. Surg.*, in press (1965).

Europ. J. Cancer Vol. 1, pp. 183–188. Pergamon Press 1965. Printed in Great Britain

Viruses and Tumours

R. J. C. HARRIS

Division of Experimental Biology and Virology,
Imperial Cancer Research Fund,
Mill Hill, London, N.W.7.

INTRODUCTION

DESPITE the fact that viruses have been known for nearly 60 years to play a causative role in animal cancer — spontaneous, as well as induced — the final step, isolation of an infective agent, has yet to be taken for any example of the human disease. I want, today, to consider briefly some of the characteristics of virus-cell interaction in the aetiology of animal cancers and the application of such data, if any, to the search for viruses, or virus-like agents, especially for leukaemia and the Burkitt tumour.

VIRUS-CELL INTERACTION IN THE PRODUCTION OF ANIMAL TUMOURS

Viruses containing RNA

In general, and we shall consider the exceptions later, the animal tumour viruses which contain RNA multiply in the malignant cells which they induce. This means that they can be visualized in the cells by electron-microscopy, separated from cellular material by a number of methods, and inoculated into test animals, usually newborn, to reproduce the disease. Koch's postulate is thus fulfilled.

Most of these viruses are completed in the cytoplasm of the cell, usually at a membrane, and incorporate host cell antigens into their structure. In addition, the infected cell may manufacture soluble complement-fixing antigens of a non-virion nature and also transplantation-type antigens (non-virion neo-antigens) which can be detected in certain circumstances by graft-rejection phenomena in suitably immunized hosts.

This is a simplified picture and some of the exceptions are of interest for our comparative purposes.

(a) The virus itself may not be the sole determinant of the tumour. The induction of mammary cancer in mice, for example, requires three factors, genetic, hormonal and viral, each of which may determine the outcome.

The murine leukaemia agents may be transmitted vertically from mother to offspring but neonatal thymectomy reduces the incidence of the disease.

There are genetic factors associated with the induction of chicken sarcomas in different strains of fowls by Rous sarcoma agents.

(b) One strain of RSV — RSV(B) — is said to be 'defective' in the sense that, whereas it can transform normal fowl cells into malignant cells, these are unable to achieve synthesis of new virus without the intervention of another virus — RAV — which is normally present as a contaminant virus in laboratory stocks of the former. RAV has structural and biological properties similar to those of the 'defective' RSV(B), but it cannot be excluded that other, unrelated, viruses could act as 'helpers.'

The way in which an RNA virus brings about an apparently genetic change in the transformed cell population is not yet clear. Some observers [1] believe that a complementary DNA is synthesized by the transformed cell and that this becomes incorporated into the genome of the malignant cells.

Viruses containing DNA

Although DNA-containing tumour viruses have been known since the discoveries of SHOPE in the mid-1930s, it was the discovery of

polyoma virus in 1950 and the application of *in vitro* methods to its study that has lead to the very substantial progress in this field.

Whereas the RNA viruses usually multiply in the transformed cells, the DNA viruses usually do not. Correspondingly they cannot readily be visualized by electron-microscopy in the tumour cells and their presence has to be inferred from other evidence. It is generally believed that the viral DNA is incorporated directly into the cell genome with the consequence that the host cell is transformed and new, non-virion antigens appear — soluble, complement-fixing and structural transplantation. *In vitro*, of course, in conditions in which virus multiplies rapidly, virion antigen is also synthesized and it is by no means excluded that, in tumours, occasional cells lyse and liberate very small amounts of virus.

In SV40 virus-induced tumours of hamsters the capacity of the malignant cells to liberate virus in association *in vitro* with sensitive normal cells has been called "induction by association" [2, 3]. A similar phenomenon has been recorded for some rat tumours induced by the Schmidt–Ruppin strain of Rous sarcoma virus [4, 5] but the question whether this represents the 'induction' of viral synthesis *de novo* or the 'amplification' of otherwise undetectable amounts of virus produced 'spontaneously' remains to be resolved, This is an important question for the study of human tumours under similar conditions *in vitro*.

The complement-fixing, and transplantation antigens, appear to be specific to the virus, or related groups of viruses responsible for their production. Even after many transplant generations the tumours induced in hamsters by polyoma, or by SV-40 viruses betray their viral origin by reason of these 'new' antigens — complement-fixing or transplantation, or both — which the malignant cells still carry.

It has been comparatively easy, therefore, to screen large numbers of human tumours and sera for complement-fixing antigens (or antibodies) for a variety of viruses — those such as adenoviruses which are known to be oncogenic for animals, and those which, at present, are only under suspicion. The results have invariably been disappointingly negative.

Difficulties in interpretation

It might not be inappropriate at this point to consider some of the difficulties in the interpretation of results obtained in these ways before considering leukaemia and the Burkitt tumour in more detail.

First, considerable cross-reaction in complement-fixation tests for 'specific tumour antigens' have been encountered in studies of SV-40, polyoma and non-viral (F.Sa3) hamster tumours [6] and the greatest care is therefore required in the conduct of the tests.

Second, SV-40 and human wart virus appear to induce cross-reacting transplantation antigens *in vitro* [7].

Third, viral hybridization may complicate the whole picture. In brief, adenovirus 7 (which had been passaged twenty-two times in monkey cells containing SV-40 virus but had been freed from SV-40 subsequently) produces the virion antigens of adenovirus 7 but the non-virion neo-antigen of SV-40 [8]. Type 3 adenovirus may also be hybridized but it remains to be seen to what extent, e.g. 'non-oncogenic' (for animals) DNA virus can 'rescue' oncogenic DNA from (malignant) cells and become oncogenic hybrids. If the hybrids can transform cells without being further reproduced their detection may be extremely difficult. In this connection it has been reported that SV-40 virus DNA can transform cells *in vitro* but these cells may show neither specific complement-fixing nor transplantation antigen [9].

Fourth, the relationship of the two types of non-virion neo-antigens needs to be clarified. Most observers agree that they are different and one possibility is that the soluble antigens represent new enzyme moieties induced in the transformed cell for the production of viral DNA.

Fifth, the superinfection of transformed cells by viruses or other organisms may again lead to difficulties in the interpretation both of electron-microscope and of immunological data. It must surely be obligatory for those who are looking for aetiological agents for malignancies in man to specify the steps they have taken to exclude the contribution of contaminating, or superinfecting, organisms to their results. This is especially true for those who purport to have found "tumour specific antigens" in such malignant cells. Resistance of tumour cells to viral superinfection is not a criterion for their own viral transformation, and one suspects that many of the cells, or cell lines, or even culture media used for association *in vitro* with cancerous tissues (or extracts of them) of man harbour more than one active or latent, inducible virus or virus-like agent with which to surprise the investigator, whether he uses electron microscopy, density–gradient centrifugation, or animal inoculation — or all three.

Sixth, and last, we know that virus-induced animal tumours, transplanted in normal hosts, may lose, or appear to lose, their induced transplantation antigens [5, 10, 11]. Whether this is loss, or masking, remains to be seen but the essential point to grasp is that most types of cancer in man have many years in which to 'progress' and, by the time they have become clinically apparent, the cells may bear slight relationship in biological properties to those with which the tumour began.

Viruses and leukaemia in man

I cannot repeat each time the desiderata which I have given above but I would ask you to bear them in mind while I briefly review progress in two related fields — leukaemia and the Burkitt lymphoma.

Leukaemia — Great interest was aroused by the discovery of so-called "clusters" of leukaemia in children and the subsequent suggestion that those of Niles, Ill. and Orange, Texas, were associated with congenital heart disease [12]. Some 15 per cent of very young children with such heart malformations showed cytopathic agents in their kidneys [13].

Electron-microscopists have seen a number of different agents associated with leukaemia — (i) Myxovirus-like and 178 mμ in diameter in the sera of acute leukaemic children (138 mμ for those with infectious mononucleosis) [14]. Some 3 per cent of normal children (but not normal adults) showed similar particles [15] and it has been suggested that these are platelet breakdown products. (ii) Eight out of fifty-six acute leukaemics showed 80–90 mμ particles (with 1 or 2 membranes) in their blood. These particles had tails when prepared in PTA [16]. (iii) Nuclear(orcytoplasmic)particles(45–70mμ) were found in eight out of thirty patients with acute granulocytic leukaemia [17] and (iv) virus-like particles (similar to murine leukaemia viruses) have been "seen" in bovine and human milk [18].

Attempts to show that leukaemic cells induced antibodies in the same patient were unsuccessful in one series [19] but, in a different approach [20] fluorescein-labelled rabbit antisera to leukaemic human plasma reacted with the bone-marrow and blood of forty-one out of seventy-two patients with leukaemia — but not with those of twenty-five controls. The same antiserum reacted with 50 per cent of the cells cultured by Epstein from a Burkitt lymphoma. Labelled rabbit antiserum against Rauscher's leukaemia virus behaved in the same way in both tests.

Isolation experiments from leukaemic bone-marrow or sera were originally believed to have demonstrated a virus, cytopathic for human embryo kidney cells in culture [21, 22]. Subsequent work has demonstrated that this is most probably a cytopathic mycoplasma (P.P.L.O.) [23, 24] unrelated serologically to those hitherto described in man. Some observers believe that a virus may also play a role in this complex [25] but mycoplasma have now been isolated directly in agar culture from leukaemia bone-marrow [26] or visualized with the electron-microscope [27].

Whether these agents are 'drivers' or merely 'passengers' remains to be determined, but it is of some interest in this connection that eighteen out of twenty-two mycoplasma strains tested by SCHMIDT *et al.* [28] blocked or destroyed the I receptors of normal red blood cells *in vitro* and that, whereas less than 0·1 per cent of normal individuals are I-negative, thirty-eight out of 124 leukaemic individuals were found to be so.

Burkitt lymphoma — I shall assume, for reasons of space and time, that you all know the background to this story and the reasons why it appeared to be worthwhile to conduct a search for a transmissible (probably insect-transmissible) agent. The epidemiological situation has now changed and I must comment on this before I describe the most recent attempts at virus isolation. Although the geographical distribution of the disease within Africa remains much as Burkitt has postulated — and it has also been seen in other rain-forest areas such as New Guinea — exceptions have been described both in the age distribution and the finding of cases in children living above the 5,000 ft. contour[29].

O'Conor [30] has stressed that there are only two differences between African (Burkitt) and European or American lymphomas — the unusally high frequency in Africa and the predilection for the bones of the jaw and face, and both he [31] and Dorfman [32] have recently described cases in the files, respectively, of the Armed Forces Institute of Pathology in Washington and the Department of Pathology, Washington University School of Medicine, St. Louis, Miss., U.S.A.

Direct virus isolation studies from the tumours have revealed — (i) Herpes simplex virus [33, 34] — but the normal population secrete this virus, too; (ii) reovirus III [35] — again a virus with a very wide distribution; and (iii) a number of unidentified viruses [36] — which may, indeed, be mycoplasmas.

Cultures of lymphoblasts from Burkitt tumours have revealed herpes-like viruses in

electron-microscopic examination of the cells [37, 38]. This virus was not Herpes simplex but cell cultures carrying it appeared to show viral interference [39]. The probable identity of this as cytomegalic inclusion disease virus has been suggested by Huebner [40]. This is a member of the herpes virus group, has similar size and biological properties, is wide-spread (especially where socio–economic conditions are poor) and has a particular affinity for salivary glands [41].

There has also been a claim that the disease could be transmitted to young African green monkeys by direct inoculation of biopsy material [42]. However, two separate groups of investigators [43, 44] claim that the lesions seen in these monkeys have also been seen in untreated 'normal' African green monkeys kept in captivity and the relationship to the inoculum is therefore questioned.

Dalldorf [45] believes that the significant fact about the Burkitt lymphoma is its geographical association with malaria and the role of any, or all, of the agents so far described remain to be determined. They might all be passengers, one or other could be the aetiological agent but, even so, might not act alone but in association with other factors which *per se* might be geographically determined.

Finally, a few comments on aspects of the search for viruses as a cause of human tumours.

I am impressed by the absence of tumours in hamsters which have received inoculation of very many different viruses of human origin [46]. Only the adenovirus group provided oncogenic strains.

The herpes virus group is, however, of equal interest — and must be more closely investigated. The association with the Burkitt lymphoma may not be just coincidence. We should recall first that herpes simplex has been implicated in carcinoma of the lip [47]; second, that these viruses can cause chromosome breakage in infected cells *in vitro* — herpes zoster also acts like colchicine on human embryo lung [48] — and third, that they (and other viruses [49]) can act synergistically with chemical carcinogens in the induction of papillomas in mice [50].

The possible intervention of adenoviruses in the aetiology of cancer in man is being throughly investigated especially with reference to cancer of the respiratory tract. It would be a mistake to exclude the myxoviruses from this consideration. Kotin and Wiseley [51] employed influenza viruses combined with a synthetic Los Angeles 'smog' to produce adenocarcinomas of the lung in mice. PR8 virus transforms mouse kidney cells in culture [52] and Harris and Negroni [53] have obtained malignant lung cancers in mice exposed to aerosols of influenza viruses alone during our investigations of a possible synergism between cigarette smoke and influenza virus in the aetiology of lung cancer in man.

REFERENCES

1. H. M. Temin, Homology between RNA from Rous sarcoma virus and DNA from Rous sarcoma virus-infected cells. *Proc. Nat. Acad. Sci.* (*Wash.*) **52,** 323–329 (1964).
2. P. Gerber and R. L. Kirchstein, SV-40-induced ependymomas in newborn hamsters. *Virology* **18,** 582–588 (1962).
3. A. B. Sabin and M. A. Koch, Evidence of continuous transmission of noninfectious SV-40 viral genome in most or all SV-40 hamster tumour cells. *Proc. Nat. Acad. Sci.* (*Wash.*) **49,** 304–311 (1963).
4. J. Svoboda, P. Chyle, D. Simkovic and I. Hilgert, Demonstration of the absence of infectious Rous virus in rat tumor XC, whose structurally intact cells produce Rous sarcoma when transferred to chicks. *Folia biol.* (*Krakow*) **9,** 77–81 (1963).
5. R. J. C. Harris and F. C. Chesterman, Growth of Rous sarcoma in rats, ferrets and hamsters. *Nat. Cancer Inst. Monogr.* No. 17. *Avian Tumor Viruses*, pp. 321–335 (1964).
6. A. B. Sabin, *Symp. Specific Tumour Antigens.* Sukhumi, U.S.S.R. (1965).
7. J. L. Melnick and F. Rapp, Possible relationship between two primate papovaviruses, human wart and simian SV-40. *J. Nat. Cancer Inst.* **34,** 529–534 (1965).
8. W. P. Rowe and S. G. Baum, Evidence for a possible genetic hybrid between adenovirus type 7 and SV-40 virus. *Proc. Nat. Acad. Sci.* (*Wash.*) **52,** 1340–1347 (1964).
9. R. J. Huebner, *Symp. Specific Tumour Antigens.* Sukjhumi, U.S.S.R. (1965).
10. G. I. Deichman, *ibid.*
11. R. J. C. Harris, *ibid.*

12. C. W. Heath, M. D. Manning and L. Zelkowitz, Case clusters in the occurence of leukaemia and congenital malformations. *Lancet* **II,** 136–137 (1964).
13. M. Benyesh-Melnick, H. J. Rosenberg and B. Watson, Viruses in cell cultures of kidneys of children with congenital heart malformations and other diseases. *Proc. Soc. Exp. Biol. (N.Y.)* **117,** 452–459 (1964).
14. K. O. Smith, M. Benyesh-Melnick and D. J. Fernbach, Studies on human leukemia. II. Structure and quantitation of myxo-virus-like particles associated with human leukemia. *J. Nat. Cancer Inst.* **33,** 557–570 (1964).
15. M. Benyesh-Melnick, K. O. Smith, and D J. Fernbach, Studies on human leukemia. III Electron microscopic findings in children with acute leukemia and in children with infectious mononucleosis. *J. Nat. Cancer Inst.* **33,** 571–579 (1964).
16. G. H. Porter, A. J. Dalton, J. B. Moloney and E. Z. Mitchell, Association of electron-dense particles with human acute leukemia. *J. Nat. Cancer Inst.* **33,** 547–556 (1964).
17. A. Graffi, D. Bierwolf, L. Baumbach, H. Blankenhagel, R. Widmaier and A. Randt, Virus particles in the cells of myeloid leukaemia of man. *Dtsch. Gesundh. Wes.* **19,** 1576–1580 (1964).
18. E. M. Jensen and G. Schidlovsky, Sub-microscopic particles in normal bovine and human milks: A preliminary morphological survey. *J. Nat. Cancer Inst.* **33,** 1029–1053 (1964).
19. F. Rapp, J. L. Melnick, T. Kitahara and R. Sheppard, Search for virus-induced antigens in human tumors using the SV-40 hamster system as a model. *Proc. Soc. Exp. Biol. (N.Y.)* **118,** 573–576 (1965).
20. M. A. Fink, R. A. Malmgren, F. J. Rauscher, H. C. Orr and M. Kyron, Application of immunofluorescence to the study of human leukemia. *J. Nat. Cancer Inst.* **33,** 581–588 (1964).
21. G. Negroni, Isolation of viruses from leukaemic patients. *Brit. Med. J.* **I,** 927–929 (1964).
22. D. R. Inman, D. A. Woods and G. Negroni, Electron microscopy of virus particles in cell cultures inoculated with passage fluid from human leukaemic bone-marrow. *Brit. Med. J.* **I,** 929–931 (1964).
23. N. R. Grist and R. J. Fallon, Isolation of viruses from leukaemic patients. *Brit. Med. J.* **II,** 1263 (1964).
24. A. J. Girardi, L. Hayflick, A. M. Lewis and N. L. Somerson, Recovery of mycoplasmas in the study of human leukaemia and other malignancies. *Nature, Lond.* **205,** 188–189 (1965).
25. W. H. Murphy, D. Furtado and E. Plata, Possible association between leukemia in children and virus-like agents. *J. Amer. Med. Ass.* **191,** 110–115 (1965).
26. L. Hayflick and H. Koprowski, Direct agar isolation of mycoplasmas from human leukaemic bone-marrow. *Nature, (Lond.)* **205,** 713–714 (1965).
27. L. Dmochowski, C. E. Grey, D. A. Dreyer, J. A. Sykes, P. L. Langford and H. G. Taylor, Mycoplasma (Pleuropneumonia-like organisms [PPLO]) and human leukemia. *Med. Rec. (Houston)* **57,** 563–568 (1964).
28. P. J. Schmidt, M. F. Barile and M. H. McGinnis, Mycoplasma (pleuropneumonia-like organisms) and blood group I; Associations with neoplastic disease. *Nature (Lond.)* **205,** 371–372 (1965).
29. A. G. Khan, The multifocal lymphoma syndrome in African children in Kenya. *J. Laryng.* **78,** 480–498 (1964).
30. G. T. O'Conor, Significant aspects of childhood lymphoma in Africa. *Cancer Res.* **23,** 1514–1518 (1964).
31. G. T. O'Conor, H. Rappaport and E. B. Smith, Childhood lymphoma resembling "Burkitt tumor" in the United States. *Cancer* **18,** 411–417 (1965).
32. R. F. Dorfman, Childhood lymphosarcoma in St. Louis, Missouri, clinically and histologically resembling Burkitt's tumor. *Cancer* **18,** 418–430 (1965).
33. P. J. Simons and M. G. R. Ross, Epidemic lymphoma in children in Africa. *A. R. Imp. Cancer Res. Fd.*, pp. 27–28 (1962).
34. J. P. Woodall and A. J. Haddow, Isolation of an agent from a case of Burkitt's lymphoma syndrome. *E. Afr. Virus Res. Inst. Rept.*, pp. 30–31 (1962).
35. T. M. Bell, A. Massie, M. G. R. Ross and M. C. Williams, Isolation of a reovirus from a case of Burkitt's lymphoma. *Brit. Med. J.* **I,** 1212–1213 (1964).
36. G. Dalldorf and F. Bergamini, Unidentified, filtrable agents isolated from African children with malignant lymphomas. *Proc. Nat. Acad. Sci. (Wash.)* **51,** 263–265 (1964).

37. M. A. Epstein, B. G. Achong and Y. M. Barr, Virus particles in cultured lymphoblasts from Burkitt's lymphoma. *Lancet* **I,** 702–703 (1964).
38. S. E. Stewart, E. Lovelace, J. J. Whang and V. A. Ngu, Burkitt Tumor: Tissue culture culture, cytogenetic and virus studies. *J. Nat. Cancer Inst.* **34,** 319–327 (1965).
39. G. Henle and W. Henle, Evidence for a persistent viral infection in a cell line derived from Burkitt's lymphoma. *J. Bact.* **89,** 252–258 (1965).
40. R. J. Huebner, *personal communication* (1965).
41. C. H. Andrewes, *Viruses of Vertebrates*, pp. 236–238 (1964).
42. M. A. Epstein, J. P. Woodall and A. D. Thomson, Lymphoblastic lymphoma in bone-marrow of African green monkeys (*Cercopithecus aethiops*) inoculated with biopsy material from a child with Burkitt's lymphoma. *Lancet* **II,** 288–290 (1964).
43. D. H. Wright and T. M. Bell, Bone disease in African green monkeys. *Lancet* **II,** 969–970 (1964).
44. B. A. Lapin, *Personal communication* (1965).
45. G. Dalldorf, C. A. Linsell, F. E. Barnhart and R. Martyn, An epidemiological approach to the lymphomas of African children and Burkitt's sarcoma of the jaws. *Perspect. Biol. Med.* **7,** 435–449 (1964).
46. A. J. Girardi, V. M. Larson and M. R. Hilleman, Further tests in hamsters for oncogenic quality of ordinary viruses and *mycoplasma*, with correlative Review. *Proc. Soc. Exp. Biol. (N.Y.)* **118,** 173–179 (1965.)
47. A. Kvasnicka, Relationship between *Herpes simplex* and lip carcinoma. IV Selected cases. *Neoplasma* **12,** 61–70 (1965).
48. M. Benyesh-Melnick, H. F. Stich, F. Rapp and T. C. Hsu, Viruses and mammalian chromosomes. III Effect of *Herpes zoster* virus on human embryonal lung cultures. *Proc. Soc. Exp. Biol. (N.Y.)* **117,** 546–549 (1964).
49. C. M. Martin, Virus-carcinogen interaction. *Bact. Rev.* **28,** 480–489 (1964.)
50. S. Tanaka and C. M. Southam, Joint action of *Herpes simplex* virus and 3-methylcholanthrene in production of papillomas in mice. *J. Nat. Cancer Inst.* **34,** 441–451 (1965.)
51. P. Kotin and D. V. Wisely, Production of lung cancer in mice by inhalation exposure to influenza virus and aerosols of hydrocarbons. *Prog. Exp. Tumor Res. (Basel)* **3,** 186–215 (1963).
52. C. Leuchtenberger, R. Leuchtenberger, T. Brunner, D. Norlin and S. Weiss, Transformation induced by PR8 influenza virus in primary cultures of mouse kidney and bronchus and production of malignant kidney tumors in mice by subcultures. *Proc. Nat. Acad. Sci. (Wash.)* **53,** 694–701 (1965).
53. R. J. C. Harris and G. Negroni, Lung cancer and smoking. *A. R. Imp. Cancer Res. Fd.*, p. 53 (1964).

Europ. J. Cancer Vol. 1, pp. 189–194. Pergamon Press 1965. Printed in Great Britain

Some Aspects of Sulphydryl Metabolism in Granulocytic Leukaemia

K. R. HARRAP

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London, S.W.3.

INTRODUCTION

THE INTEREST in the metabolism of sulphydryl compounds described in this communication arises from previous studies associated with the response of patients (CGL*) to busulphan therapy [1]. Many patients in the later stages of this disease exhibit the well known blast-cell crisis, which is accompanied by (though not necessarily associated with) an increasing resistance to busulphan therapy. In attempting to understand the biochemistry of this process it is necessary to identify some biochemical property which might be expected to change in accordance with both the progress of the disease and the reaction of the latter to therapy. The task of identifying such a property is eased somewhat by the literature implicating abnormalities of sulphur metabolism in leukaemia [2–4], and by the work of Roberts and Warwick, who have demonstrated that busulphan reacts *in vivo* in animals with sulphydryl-containing compounds [5]. Furthermore, Calcutt and Connors have shown that the response of certain transplantable tumours in the mouse to treatment with an alkylating agent (to which class of compounds busulphan belongs) may be related to the ratio of protein-free thiol to protein-bound thiol [6].

Measurement of the levels of protein-free sulphydryl compounds in the blood cells of patients (CGL) might be expected therefore to reflect the progression of the disease and the response of the patient to treatment with busulphan. Soluble disulphide has also been measured, since a change in the free sulphydryl-disulphide ratio found normally in healthy tissues may be a more intrinsic indication of metabolic abnormality than changes in gross sulphydryl concentration [1].

Previous studies [1] have indicated that oxidised glutathione can occur in the blood cells of patients suffering from chronic granulocytic leukaemia, and the results are summarised in Fig. 1 [7]. In the present communication these studies are extended to include a survey of patients suffering from acute granulocytic leukaemia.

The oxidation and reduction of glutathione in the erythrocyte is mediated by the system shown in Fig. 2: the present paper also describes an attempt to account for the presence of oxidised glutathione in the erythrocyte in terms of this enzyme system.

*Abbreviations to be used in this paper: CGL — chronic granulocytic leukaemia; AGL — acute granulocytic leukaemia; GSH — reduced glutathione; GSSG — oxidised glutathione; SH — sulphydryl; SS — disulphide; NADP — nicotinamide adenine dinucleotide phosphate; NADPH — reduced nicotinamide adenine dinucleotide phosphate; EDTA — ethylene-diamine-tetra-acetic acid (disodium salt); G6P — glucose 6 phosphate; 6PG — 6 phosphogluconate; R5P — ribulose-5 phosphate.

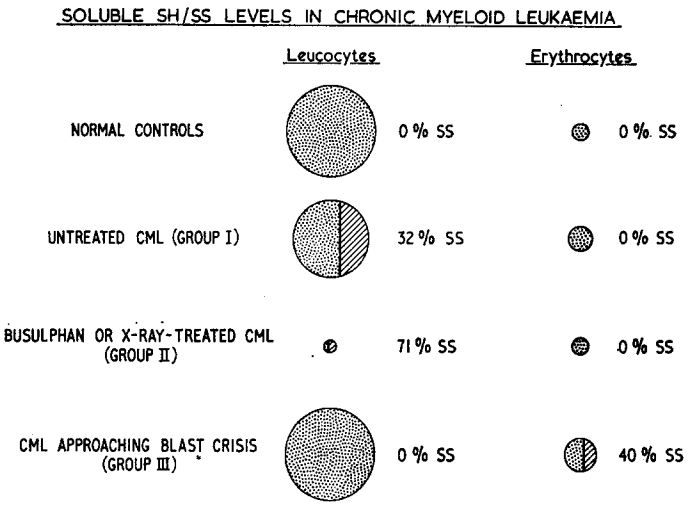


Fig. 1. *Soluble SH/SS levels in chronic myelogenous leukaemia. The circumference of the circles is proportional to the total SH content of the cells (normal cells: erythrocytes* 0·18 *μmoles/10⁹ cells, leucocytes* 1·1 *μmole/10⁹ cells). Patients fall into three groups as shown.*

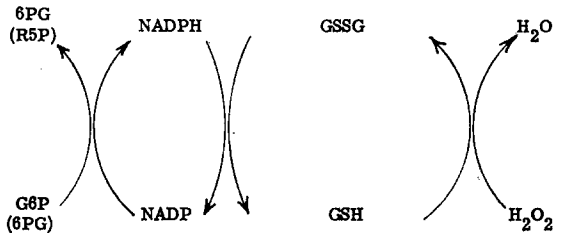


Fig. 2. *Enzyme system responsible for the oxidation and reduction of glutathione within the erythrocyte: Enzymes: glutathione reductase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione peroxidase.*

METHODS

Chemicals were obtained from Hopkin and Williams Ltd. or B.D.H. Ltd.: AnalaR grades were used where available. GSH and GSSG were obtained from Sigma Chemical Company; NADP and NADPH from Boehringer; G6P and 6PG from Wessex Biochemicals Ltd., Bournemouth, England; 5,5'-dithiobis- (2-nitro benzoic acid) from Aldrich Chemical Co. Inc.

Venous blood was collected and fractionated as described previously [1]. Erythrocyte haemolysates were prepared by centrifuging whole blood at 350 g for 10 min at 0°C, removing the plasma and buffy coat, and washing the packed red cells twice with ice-cold physiological saline. The washed cells were then diluted to a measured volume with a further aliquot of saline, counted, removed from suspension by centrifugation at 350 g, and haemolysed by adding ice-cold water until the haemolysate volume was equivalent approximately to that of the blood originally taken. The haemolysate was then allowed to stand at 0°C for 30 min before removing the stroma by centrifugation at 6 500 g (0°C) for 30 min. The haemolysates were stored for at least 24 hr at −40°C before the enzyme assays were performed.

Enzyme assays

All enzyme assays were carried out in 0·05M triethanolamine-HCl buffer pH 7·6 [8], and at a temperature of 25°C (except for glutathione peroxidase (GSHP) which was assayed at 0°C).

Glutathione reductase (GSSGR) was determined according to Scott *et al.* [9], and glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) by the method of Shonk and Boxer [8]. Glutathione peroxidase (GSHP) was assayed by a modification of the method of Mills [10] as follows: To 1 ml GSH ($4{\cdot}0\times10^{-3}$M in 0·05M triethanolamine-HCl buffer pH 7·6, 0·006M with respect to EDTA) was added 3·1 ml triethanolamine buffer pH 7·6 and 0·1 ml sodium azide (0·31M), followed by 0·3 ml haemolysate. The solution was equilibrated at 0°C for 5 min, whereafter the enzyme reaction was started by adding 0·5 ml of $4{\cdot}0\times10^{-3}$M hydrogen peroxide in triethanolamine buffer (0·05M, pH 7·6). One millilitre samples were removed at 0, 3, 6, 9 min intervals and added rapidly to a separate tube containing 2 ml isobutanol and 4 ml 2·5% metaphosphoric acid [11]. These tubes were shaken thoroughly, centrifuged at approximately 400g for 10 min, the supernatant organic layer aspirated off, and residual GSH estimated in 1 ml aliquots of the aqueous layer by the colorimetric method described below.

Table 1. *Occurrence of oxidized thiol in the blood cells of patients: AGL.*

Patient	Cellular thiol content (μmoles SH/10^9 cells) Erythrocytes		Leucocytes		Total WBC	Early granulocytes (%)	Blasts (%)
	Total thiol	Oxidized form (%)	Total thiol	Oxdized form (%)			
C.L.	0·15	0	0·74	0	1,550	8·0	2·5
P.D.	0·32	9	0·48	58	6,750	0	0
D.H.	0·28	21	0·40	50	3,800	6·5	2·0
H.P.	0·45	16	None detected		4,500	2·0	0·5
M.S.	0·22	10	0·03	100	280,000	0	95·5
R.G.	0·21	0	0·06	0	23,700	18	58
W.L. (CGL in "blast crisis")	0·34	18	0·9	0	66,000	1	79

Units of enzyme activity

GSSGR: μmoles NADPH oxidized per minute per 10^9 cells.

G6PD and 6PGD: μmoles NADP reduced per minute per 10^9 cells.

GSHP: mμmoles GSH oxidized per minute per 10^9 cells.

GSSG and GSH were estimated as described previously, or by a modification of the colorimetric method of Beutler *et al.* [12] as follows: to 1 ml of the erythrocyte suspension in isotonic saline was added 9·0 ml ice-cold 3% sulphosalicylic acid. After 20 min at 0°C the protein precipitate was removed by centrifugation or filtration. Reduced glutathione was determined by adding 1 ml of the filtrate to 4·0 ml of 5,5'-dithiobis-(2-nitrobenzoic acid) $-0{\cdot}5\times 10^{-3}$M in 1·0M phosphate buffer pH 7·0 (10^{-3}M with respect to EDTA). The optical density of the solution was recorded at 412 mμ and compared with appropriate standards. GSSG was determined by a similar colorimetric procedure following electrolytic reduction [1]. In order to obtain consistent results it was found necessary to rinse all glassware before use with the buffered reagent, and to measure the optical density of the solutions within 3 min of colour development.

RESULTS

The distribution of soluble thiol (SH) and disulphide (SS) between the erythrocyte and leucoctyte fractions from a series of patients (AGL) is shown in Table 1. In Table 2 are shown the results of SH/SS determinations in the erythrocytes of a further series of patients suffering from granulocytic leukaemia. The analyses in Tables 1 and 2 were carried out on freshly fractionated material. It has already been shown, in a group of 11 healthy controls, that the mean erythrocyte SH content was $0{\cdot}20\pm0{\cdot}02$ μmoles/10^8 cells: No GSSG present [1].

Tables 3 and 4 respectively list the results of enzyme assays carried out on the two series of patients in Tables 1 and 2. Haemolysates from the acute leukaemia patients in Table 1 were stored (at −40°C) for a longer period than the haemolysates of the second series of patients in Table 2, but are compared with a set of controls taken at the same time as the patient blood

Table 2. *Occurrence of oxidized thiol in the erythrocytes of patients: CGL, AGL.*

Patient	Diagnosis	Erythrocyte thiol content (μmoles SH/10^9 cells)		Total WBC	Early granulocytes (%)	Blasts (%)
		Total thiol	Oxidized form (%)			
S.H.	AGL	0·44	15	5,700	0	0
F.B.	AGL	0·21	0	2,200	6	5
M.P.	AGL	0·33	0	3,900	7	0
E.I.	CGL	0·33	21	2,100	4	21
H.C.	CGL	0·17	0	40,000	50	10
A.C.	CGL	0·27	0	14,400	6	2
G.J.	CGL	0·23	0	7,600	5	0·5
J.S.	CGL	0·28	7	11,200	8·5	0·5

Table 3. Enzyme assays on erythrocyte haemolysates from patients: AGL (cf. Table 1 for SH/SS analysis and blood data)

	GSSGR (μmoles NADPH oxidized per min per 10^9 cells)	G6PD (μmoles NADP reduced per min per 10^9 cells)	6PGD (μmoles NADP reduced per min per 10^9 cells)	GSHP (mμmoles GSH oxidized per min per 10^9 cells)
Patients				
C.L.	0·030	0·011	0·028	1·03
P.D.*	0·170	0·029	0·044	8·20
D.H.*	0·070	0·034	0·039	15·60
H.P.*	0·118	0·025	0·068	17·90
M.S.*	0·087	0·015	0·032	15·50
R.G.	0·100	0·023	0·030	1·88
W.L.*	0·100	0·022	0·037	8·60
Means	0·096	0·023	0·039	9·81
Standard deviation	0·018	0·007	0·013	6·27
Controls				
A	0·065	0·015	0·055	11·10
B	0·089	0·016	0·030	8·99
C	0·096	0·016	0·037	8·36
D	0·053	0·016	0·024	3·55
E	0·071	0·012	0·019	8·15
F	0·080	0·028	0·061	24·60
G	0·072	0·033	0·061	14·10
H	0·067	0·013	0·034	
Means	0·074	0·018	0·040	11·26
Standard deviation	0·013	0·007	0·015	2·14
Comparison of means ("t" test)	$P>0·3$	$P>0·3$	$P>0·9$	$P>0·9$

*Erythrocytes contain GSSG (cf. Table 1).

Table 4. Enzyme assays on erythrocyte haemolysates from patients: AGL, CGL (cf. Table 2 for SH/SS analysis and blood data)

	GSSGR (μmoles NADPH oxidized per min per 10^9 cells)	G6PD (μmoles NADP reduced per min per 10^9 cells)	6PGD (μmoles NADP reduced per min per 10^9 cells)	GSHP (mμmoles GSH oxidized per min per 10^9 cells)
Patients				
E.I. (CGL)*	0·167	0·200	0·181	5·96
H.C. (CGL)	0·125	0·250	0·120	14·00
A.C. (CGL)	0·048	0·066	0·090	4·35
G.J. (CGL)	0·108	0·110	0·120	7·57
J.S. (CGL)*	0·103	0·160	0·090	8·26
S.H. (AGL)*	0·136	0·270	0·024	18·30
F.B. (AGL)	0·122	0·130	0·130	6·00
M.P. (AGL)	0·173	0·170	0·140	12·70
Means	0·123	0·170	0·112	9·64
Standard deviation	0·023	0·064	0·021	4·54
Controls				
J.B.	0·101	0·180	0·140	17·20
P.W.	0·099	0·160	0·110	9·58
M.W.	0·112	0·180	0·110	13·20
L.H.	0·060	0·140	0·090	11·70
K.H.	0·087	0·070	0·110	5·08
Means	0·091	0·150	0·110	11·35
Standard deviation	0·017	0·023	0·014	4·02
Comparison of means ("t" test)	$P>0·02$	$P>0·5$	$P>0·8$	$P>0·5$

*Erythrocytes contain GSSG (cf. Table II).

samples. The assays recorded in Table 4 were performed after only 24 hr storage at −40°C and are compared with controls taken at the same time as the patient blood samples, and stored similarly. Comparison of the control group in Table 3 with that of Table 4 reveals that GSHP and GSSGR are stable under these storage conditions: G6PD and 6PGD are less stable.

Table 5. Patient erythrocyte enzyme activities: expressed as percentage of mean control values.

	GSSGR	G6PD	6PGD	GSHP
	Erythrocytes containing GSSG			
P.D.	230	160	110	73
D.H.	95	189	98	140
H.P.	159	139	170	159
M.S.	116	83	80	138
W.L.	135	122	93	76
E.I.	183	133	163	53
J.S.	113	107	82	73
S.H.	149	180	22	161
	Erythrocytes not containing GSSG			
	GSSGR	G6PD	6PGD	GSHP
C.L.	41	61	70	9
R.G.	135	128	75	11
H.C.	137	167	109	123
A.C.	53	44	82	38
G.J.	119	73	109	67
F.B.	134	87	118	53
M.P.	190	113	127	112

In Table 5 the enzyme activity figures in patients' erythrocytes are expressed as a percentage of the corresponding mean control values, and are tabulated in two groups according to whether or not GSSG was found in the erythrocytes.

DISCUSSION

From an inspection of Tables 1 and 2 it is apparent that patients suffering from AGL do not fall into such clearly defined categories, in terms of the distribution of disulphide between the cell fractions, as has been found with CGL [1]. Also there would seem to be a tendency for the total erythrocyte thiol content to be raised in those erythrocytes containing GSSG.

The activities of the enzymes in the two groups of patients listed in Tables 3 and 4 do not differ significantly from those of the corresponding control groups.

In Table 5 the relative enzyme activities of erythrocyte preparations from cells containing GSSG are compared with those from cells containing only GSH. Only in one patient (SH), whose erythrocytes contained GSSG, did the relative activities of any of the reduced enzyme, studied fall below the control values: in all other cases the enzyme activities were comparable with, or greater than, the mean control values. On the other hand, with patients whose erythrocytes contained only GSH, there were several cases where the activities of the erythrocyte enzymes fell below the mean control values (CL, RG, AC, GJ, EB).

It would seem therefore that the appearance of GSSG in the erythrocytes of those patients reported here cannot be attributed to impairment of the apo-enzymes under study: the enzyme assay methods used here for GSSGR, G6PD, 6PGD have, of course, only measured apo-enzyme levels (excess cofactor has been added during assay), and it is feasible that impairment of holoenzyme function *in vivo* could exist as a result of cofactor deficiency. This aspect of the problem is under study.

Acknowledgements — The author is grateful to Prof. F. Bergel, F.R.S. and Prof. A. Haddow, F.R.S. for their continued interest and encouragement in this work. He also wishes to thank Dr. Sidney Farber (Childrens Cancer Research Foundation, Boston) for his kindness in providing a grant and laboratory facilities while some of the work described herein was carried out, and Dr. W. C. Maloney (Tufts Haematology Laboratory, Boston City Hospital) for referring patient material for study. Thanks are also due to the consultant staff of the Royal Marsden Hospital for referring patient material for study, and to Mr. R. Burch for technical assistance. The work has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and the British Empire Cancer Campaign for Research, and by the Public Health Service Research Grant No. CA-03188-08 from the National Cancer Institute, U.S. Public Health Service.

RESUME

L'auteur a étudié la répartition de composés solubles sulfhydryl et disulfure entre les fractions leucocytaires et érythrocytaires, chez sept malades atteints de leucémie myéloblastique aiguë.

On a essayé d'expliquer la présence de glutathion oxydé dans les érythrocytes de malades (leucémie myéloblastique aiguë, leucémie myéloïde chronique) en fonction de l'intégrité du système enzymatique responsable de l'oxydation et de la réduction du glutathion. En conclusion, la fonction des apo-enzymes en cause n'est pas diminuée.

SUMMARY

The distribution of soluble sulphydryl and disulphide compounds between the leucocyte and erythrocyte fractions of a group of patients (AGL) has been investigated.

An attempt has been made to account for the presence of oxidised glutathione in the erythrocytes of patients (AGL, CGL) in terms of the integrity of the enzyme system responsible for oxidation and reduction of glutathione. It is concluded that the function of the apo-enzymes involved was not impaired.

ZUSAMMENFASSUNG

Die Verteilung von löslichen Sulfhydryl- und Disulfidverbindungen zwischen den roten und den weissen Blutkörperchen wurde bei einer Gruppe von Patienten mit akuter Granulozytenleukämie (AGL) untersucht.

Es wurde versucht die Anwesenheit von oxydiertem Glutathion in den Erythrozyten ovn Patienten mit akuter und chronischer Granulozytenleukämie durch die Unversehrtheit des Enzymsystems, welches für die Oxydation und für die Reduktion des Glutathions verantwortlich ist, zu erklären. Aus den Ergebnissen wurde der Schluss gezogen, dass die Funktion der in Frage kommenden Apoenzyme nicht beeinträchtigt ist.

REFERENCES

1. K. R. Harrap and D. E. M. Speed, Some biochemical aspects of leukaemias: the appearance of a soluble disulphide in the blood in chronic granulocytic leukaemia. *Brit. J. Cancer* **18,** 809 (1964).
2. A. N. Contopoulas, and H. H. Anderson, Sulphydryl content of blood cells in dyscrasias. *J. Lab. Clin. Med.* **36,** 929 (1950).
3. J. White, G. B. Mider and W. E. Hoston, Effect of amino acids on the induction of leukaemia in mice. *J. Nat. Cancer Inst.* **4,** 409 (1943).
4. A. S. Weisberger and B. L. Levine, Incorporation of radioactive L-cystine by normal and leukaemic leukocytes *in vivo*. *Blood* **9,** 1082 (1954).
5. J. J. Roberts and G. P. Warwick, The mode of action of alkylating agents. II. Studies on the metabolism of Myleran. The reaction of Myleran with some naturally occurring thiols *in vitro*. *Biochem. Pharmacol.* **6** 205 (1961). J. J. Roberts and G. P. Warwick, The mode of action of alkylating agents. III. The formation of 3-hydroxytetrahydrothiophene-1:1-dioxide from 1:4 dimethanesulphonyloxybutane (Myleran), S-β-L-alanyltetrahydrothiophenium mesylate, tetrahydrothiophene, and tetrahydrothiophene-1:1-dioxide in the rat, rabbit and mouse. *Biochem. Pharmacol.* **6,** 217 (1961).
6. G. Calcutt and T. A. Connors, Tumour sulphydryl levels and sensitivity to the nitrogen mustard Merophan. *Biochem. Pharmacol.* **12,** 838 (1963).
7. F. Bergel and K. R. Harrap, Future possibilities for the development of treatments of leukaemia. In: *Conf. on Obstacles to the Control of Acute Leukaemia,* Warrenton, Virginia, U.S.A. *Cancer Res.* (1965) In press.
8. C. E. Shonk and G. E. Boxer, Enzyme patterns in human tissues. I. Methods for the determination of glycolytic enzymes. *Cancer Res.* **24,** 709 (1964).
9. E. M. Scott, I. W. Duncan and V. Ekstrand, Purification and properties of glutathione reductase of human erythrocytes. *J. Biol. Chem.* **238,** 3928 (1963).
10. G. C. Mills, The purification and properties of glutathione peroxidase of erythrocytes. *J. Biol. Chem.* **234,** 502 (1959).
11. G. Cohen and P. Hochstein, Glutathione peroxidase: the primary agent for the elimination of hydrogen peroxide in erythrocytes. *Biochemistry* **2,** 1420 (1963).
12. E. Beutler, O. Duron and B. M. Kelly, Improved method for the determination of blood glutathione. *J. Lab. Clin. Med.* **61,** 882 (1963).

Europ. J. Cancer Vol. 1, pp. 195–197. Pergamon Press 1965. Printed in Great Britain

Fluorimetric Enzymatic Assay of Metabolites in Small Amounts of Tissues or Cells, with Special Reference to the Estimation of Adenosin-phosphates

J. FREI and H. ALEYASSINE

Laboratoire Central, Hôpital Cantonal, Lausanne, Switzerland

THE STUDY of the cellular energetic metabolism can be approached in three different complementary manners:

1. By the estimation of multi-enzyme systems such as glycolysis, tricarboxylic acid cycle, oxygen consumption, or oxidative phosphorylation.
2. By measurement of well defined enzyme activities belonging to the multi-enzyme systems mentioned.
3. By the estimation of the concentration of metabolites implicated in the same multi-enzyme or enzyme systems.

The estimation of enzyme and multi-enzyme activities gives the metabolic potential of a cell or a tissue. Generally these kind of dosages do not present problems at the micro- or even at the "ultramicro" analytical levels, especially in the use of substrates labelled with radioisotopes. In certain cases it is even possible to carry out these measurements on single cells.

The estimation of metabolites gives the possibility to study a "metabolic state" after the complete interruption of any metabolic processes — if possible *in vivo* or after incubation of cell suspensions (by very rapid deep freeze or deproteinisation). The estimation of metabolites requires much more senstive methods than those needed for the measurement of enzyme activities, since some metabolite levels are very low. The hepatic levels of some compounds involved in the energetic metabolism are recorded in Table 1. For the specific enzymatic estimation of metabolites in liver biopsy fragments of the order of 10 mg, the optical test of Warburg is usable only for compounds present at a relative high level (lactate, ADP, ATP). Nevertheless it is possible with this amount of tissue to measure oxygen consumption, oxidative phosphorylation as well as several enzyme activities [3, 4].

Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
G-6-PDH	glucose-6-phosphatedehydrogenase
HK	hexokinase
LDH	lactic dehydrogenase
NAD, NADH	nicotin adenin dinucleotide, oxidized and reduced form
NADP, NADPH	nicotin adenin dinucleotide phosphate, oxidized and reduced form
PK	pyruvate kinase

Table 1. *Level in the liver of some metabolites involved in the energetic metabolism*

Metabolite	Level 10^{-3}µm/mg fresh tissue	Metabolite	Level 10^{-3}µm/mg fresh tissue
glucose-6-phosphate*	0·511	i-citrate*	0·029
fructose-6-phosphate*	0·156	malate*	0·615
fructose-1,6-diphosphate*	0·031	oxalacetate*	0·011
dihydroxyacetone-phosphate*	0·052	ATP*	4·040
glycerol-1-phosphate*	0·342	ADP*	1·250
3-phosphoglyceric acid*	0·528	NAD†	0·628
phosphoenolpyruvate*	0·163	NADH†	0·252
pyruvate*	0·116	NADP†	0·115
lactate*	1·110	NADPH†	0·502

*from Hohorst, Kreutz and Bücher [1].
†from Lowry [2].

Generally when the level of a metabolite is low and/or if one has available only small amounts of tissue, it is necessary to use specific enzymatic reactions in which the phosphopyridine nucleotides (PPN) are involved and estimated by fluorimetric measurements (Table 2) of the reduced forms. The measured fluorescence is about 100 times more sensitive than the extinction of these compounds measured at 340 nm. The oxidized forms of these coenzymes can be made fluorescent in a strong alkali medium (or by treatment with a ketone) [5, 6]; in this case the sensitivity is still about 10 times greater. One can use in enzymatic fluorimetry the same enzyme reactions as in the optical test of Warburg. If the concentrations to be measured are particularly low (e.g. for the estimation of the PPN), or if it is question of quantitative histochemistry it may be necessary to use enzymatic cycling which has been worked out by Lowry *et al.* [2]. Alternatively the bioluminescence techniques with luciferase permit the estimation of very small amounts of ATP or related substrates by enzyme systems [7].

Table 2. *Sensitivity of some enzymatic techniques for metabolite estimation*

	Approximate limit of sensitivity of µmoles
Systems with PPN [2]	
Optical test at 340 nm	10^{-3}
Direct fluorimetry	10^{-5}
Indirect fluorimetry	10^{-6}
Enzymatic cycling	10^{-9} — 10^{-11}
Bioluminescence	
Luciferase [5]	10^{-7}

Table 3. ADP *and* ATP *levels of leucoctyes expressed in* 10^{-3}µm/10^6 *cells. Values in brackets: standard deviation of the mean*

Type of leucocytes	ATP	ADP	ATP/ADP
Human blood (normal subjects)	6·25 (0·43)	0·697 (0·103)	9·0
Peritoneal exsudates of guinea pigs	1·59 (0·083)	0·237 (0·013)	6·7

ESTIMATION OF THE ADENOSINE PYROPHOSPHATES IN LEUCOCYTES

During our investigations concerning the energetic metabolism of the leucocytes, the ADP and ATP levels of these cells were estimated by direct fluorimetric techniques [8, 9]; in the case of ATP we measured the appearance of fluorescence of the NADPH formed by the system:

$$\text{ATP} + \text{glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{glucose-6-phosphate}$$

$$\text{Glucose-6-phosphate} + \text{NADP} \xrightarrow{\text{G-6-PDH}} \text{6-phosphogluconate} + \text{NADPH}$$

For the estimation of ADP we measured the decrease of the fluorescence of the NADH utilised in the system:

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{PK}} \text{ATP} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}$$

RESULTS

In Table 3, the levels of the adenosine pyrophosphates of leucocytes isolated from human blood and from peritoneal exsudates of guinea pigs [9, 10] are reported.

We were also able to show that the chemical energy of the white cells is produced essentially by the glycolytic system. The inhibitors of this process (deoxyglucose, monoiodacetate, fluoride) cause a decrease of the ATP level during incubation of leucocytes; in contrast inhibitors of the respiratory chain (cyanide, azide) affect the ATP level to a much smaller extent [11].

Acknowledgement — We are very grateful to the Fonds National Suisse de la Recherche Scientifique for their financial help.

RESUME

Les pyrophosphates d'adénosine (ADP et ATP) peuvent être dosés spécifiquement à l'aide de tests enzymatiques-fluorimétriques très sensibles. Il est ainsi possible de déterminer ces métabolites dans des fragments de tissu de l'ordre de 10 μg *à* 10 *mg.*

SUMMARY

Adenosin-pyrophosphates (ADP and ATP) can be specifically measured with great sensitivity by using enzymatic fluorimetry. By this method, it is possible to determine these metabolites in fragments of tissue ranging from 10 μg *to* 10 *mg.*

ZUSAMMENFASSUNG

Die Adenosin Pyrophosphate (ADP und ATP) können spezifisch mit hoch empfindlichen enzymatischen-fluorimetrischen Methoden bestimmt werden. So ist es möglich den Gehalt dieser Metabolite in Gewebsstücken in der Grössersordnung von 10 μg *bis* 10 *mg zu ermitteln.*

REFERENCES

1. H. J. Hohorst, F. H. Kreutz and Th. Bücher, Uber Metabolitgehalte und Metabolit-Konzentrationen in der Leber der Ratte. *Biochem. Z.* **332,** 18–46 (1959).
2. O. H. Lowry, J. V. Passonneau, D. W. Schulz and M. Rock, The measurement of pyridine nucleotides by enzymatic cycling. *J. Biol. Chem.* **236,** 2746–2755 (1961).
3. J. Frei and H. Ryser, Méthodes microanalytiques appliquées à l'étude biochimique du tissu hépatique humain. *Helv. Physiol. Pharmacol. Acta* **15,** C62–C64 (1957).
4. H. Ryser and J. Frei, Enzymic studies in small amounts of human tissue with the help of microanalytical methods. III. Energetic metabolism of cirrhotic and precirrhotic liver tissue removed by needle biopsy. *Clin. Chim. Acta* **3,** 486–493 (1958).
5. O. H. Lowry, N. R. Roberts and J. I. Kapphahn, The fluorometric measurement of pyridine nucleotides. *J. Biol. Chem.* **224,** 1047–1064 (1957).
6. N. O. Kaplan, S. P. Colowick and C. C. Barnes, Effect of alkali on diphosphopyridine nucleotide. *J. Biol. Chem.* **191,** 461 (1951).
7. E. Tal, S. Dikstein and F. G. Sulman, ATP determination with the Tricarb Scintillation Counter. *Experientia* **20,** 652 (1964).
8. P. Greengard, Bestimmung durch enzymatische Fluorimetrie; in: H. U. Bergmeyer, *Methoden der Enzymatischen Analyse*, p. 573. Verlag Chemie, Weinheim (1962).
9. J. Frei, Métabolisme énergétique du leucocyte. I. Détermination enzymatique par fluorimétrie des di- et triphosphates d'adénosine des leucocytes péritonéaux du cobaye. *Enzymol. Biol. Clin.* **2,** 175–187 (1962/63).
10. J. Frei, Unpublished data.
11. H. Aleyassine and J. Frei, Origine de l'énergie chimique du polynucléaire neutrophile d'exsudats péritonéaux de cobayes. *Helv. Physiol. Pharmacol. Acta* **22,** C6–C7 (1964).

Europ. J. Cancer Vol. 1, pp. 199–202. Pergamon Press 1965. Printed in Great Britain

On the Structure and Bioligical Effects of a Newly-discovered Cytotoxic Polypeptide in Tumor Fluid*

B. SYLVÉN and BO HOLMBERG

The Cancer Research Division of Radiumhemmet, Karolinska Institute, Stockholm 60, *Sweden*

INCUBATION of Chang or strain L cells in interstitial fluid from Walker rat tumors or cell-free Ehrlich-Landschütz diploid (ELD) ascites tumor fluid was found to be deleterious to these cells [1, 2]. No effect could be found on tumor cells incubated under identical conditions. The first sign of cell injury was inhibition of pseudopodia formation followed by loss of cellular adhesion to glass surfaces and cessation of growth. Later on, this led to complete cytolysis. Such translocation experiments were used as a test method for the cytotoxic activity.

The cytotoxic factor is a *diffusible polypeptide* partly occurring in a free state in fresh tumor fluids. It is completely different from the so-called "toxohormone" of Nakahara and Fukuoka [3, 4].

Table 1. *The cytotoxic polypeptide has been prepared at a large scale and analysed from ascites fluid and pleural exudates from the following tumor types.*

Ehrlich-Landschütz (ELD) *mouse* ascites carcinoma		
Yoshida *rat* sarcoma, ascites form		
Advanced human cases (mostly terminal) with the following diagnoses:		
	(patients)	
Ca. abdominis	2	(not specified)
Ca. ovarii	13	
Ca. corp. uteri	1	
Ca. mam. gland	1	(pleural effusion)
Colon carcinoma	2	
Hypernephroma	1	
Reticulum cell sarcoma	2	(retroperitoneal)
Carcinosis pleurae	1	
Ca. bronchial.	1	
Adenocarc. stomach	1	

MATERIALS AND METHODS

Active fractions have been prepared from the following types of tumor fluids (Table 1). All human cases selected were without fever and clinical signs of bacterial infections.

Preparation of dialysates — The cells were spun down and tumor fluid was dialysed over night through commercial Viscose tubing against ten times its volume of ion-free water.

Biochemical analysis — Other analytical work of a conventional type has included fractionation studies, qualitative biochemical tests, demonstration of free-SH groups, paper chromatography, activation and inactivation studies [3].

Preliminary data on the amino acid sequence are under way using the PTC method of Edman

Bioassay — The cytotoxic activity of the various fractions was routinely assayed by the cell growth inhibiting and/or cell injuring effect on suspension cultures of strain L cells, clone 929, following one single addition of the test sample.

*Supported by institutional grants from the Swedish Cancer Society, the King Gustaf Jubilee Fund, the Cancer Society of Stockholm and the Damon Runyon Memorial Fund.

RESULTS

Isolation and purification

The cytotoxic factor contained in crude dialysates was isolated according to Table 2. The amount of biuret-positive material recovered during the gel filtration corresponds roughly to 75 per cent of the amount applied to the column. In sufficiently desalted preparations the activity in all cases studied was connected with a biuret-positive peak appearing in the same sequence of tubes eluted by the 0·050 M phosphate buffer representing between 20 and 30 per cent of the total biuret-positive material applied to the column. This material could not be subdivided by further column chromatography and did not contain ninhydrin positive impurities. Thus, the present chromatographic procedure led to the isolation of a possibly homogeneous biuret-positive fraction carrying all the cytotoxic activity contained in the crude dialysates [3].

Table 2. Preparation of the cytotoxic polypeptide by chromatography.

1. Centrifugation 15 min at 3500 rev/min.
2. Dialysis against 5 times its vol. of ion-free water.
3. Freeze-drying.
4. Gel filtration through Sephadex G-25. Ion-free water.
5. Fractionation from DEAE Sephadex A-25 in OH-cycle. Eluants: ion-free water, 0·050 M phosphate buffer pH 7·0 and 0·100 M phosphate buffer + 0·1 M NaCl.

The first method of chromatographic isolation of the cytotoxic polypeptide is tedious, involving considerable losses. A more elegant method has been developed by which the pure polypeptide is obtained as a crystalline mercury complex directly from the dialysate (data to be published).

Composition of the cytotoxic fraction

Qualitative amino acid analysis demonstrated the presence of eight amino acids only (Fig. 1). The N-terminal amino acid was in all cases Gly. So far, the same qualitative composition of active fractions was found in all preparations irrespective of their origin from human, rat or mouse materials.

No steriods, cholesterol, bilirubin, polysaccharides or nucleotides were co-present [3].

The UV-absorption curves showed a peptide material containing aromatic amino acids. The UV-absorption at acid and alkaline pH's corroborated that tyrosine was the only aromatic amino acid.

Thus, the cytotoxic activity seemed to be due to a pure polypeptide in which intact tyrosine and free-SH groups are essential for full activity.

Parts of the amino acid sequence of the polypeptide from human tumor cases are shown in Fig. 2. In all cases a rather unique configuration of Tyrosine — Cysteine — Tyrosine was noted. This seems to represent the most vulnerable part of the molecule. We are under the impression that the selective biological activity resides in this region.

The particle size has been estimated to about 3500 (unpublished).

Stability

The polypeptide retained an appreciable activity after even 30 min at 100°C but was easily oxidized in air. Some hours irradiation by UV-light abolished both the cytotoxic effect and the original tyrosine absorption. The joint results suggest a fairly good stability in the dark and cold, at neutral pH's, during absence of air and possibly in the presence of some -SH containing substance, such as cysteine.

Biological assays

Intraperitoneal injections of large amounts of this polypeptide did not depress the catalase activity of normal mouse liver within three days. Similarly there was no rise in the LDH activity of normal mouse plasma following injection of purified material.

The rapidly appearing primary effect on cells growing on glass first led us to believe that the active material might react with negative charges at the cell surface [4]. Careful free cell electrophoresis performed by Dr. Ambrose and Dr. Forrester, however, demonstrated that addition of polypeptide did not diminish the electrophoretic cell mobility. We have presently no clear explanation why the polypeptide

Fig. 2. Preliminary data on the amino acid sequence (c.p. text). The arrow denotes where the polypeptide is split by alpha chymotrypsin.

Gly — Cys — Ala — ..... — Val — Tyr — ↓ — Cys — Tyr —
— Arg — Glut? — ..... — Leu

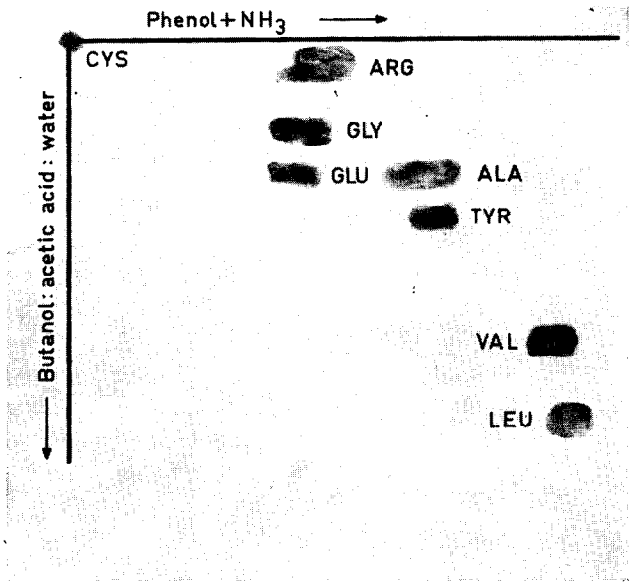


Fig. 1. *The cytotoxic polypeptide contained eight amino acids shown in this chromatography diagram.*

(to face p. 200)

effectively inhibits membrane movement and cell locomotion. The primary results on cell growth curves following one time addition of polypeptide material to normally growing strain L cells in suspension cultures have been verified many times [1, 4]. It is not easy to explain why these cells, following the addition of minute amounts of polypeptide, seem to be killed at such a late date. Holmberg has also added polypeptide material at various stages of synchronously growing cell populations. The preliminary findings suggest that the polypeptide blocks some essential synthetic process during the pre-mitotic stage. This problem necessitates further detailed study.

We might further mention preliminary isotope data indicating that the life-span of red blood cells becomes shortened with about 15–20 per cent following short-time treatment with this polypeptide. This might help to explain the anemia associated with the later stages of tumor disease [4]. The usual small amount of added factor deleterious to normal cells has not damaged tumor cells tested. It might, however, be possible that tumor cells also become damaged if larger amounts of factor material are supplied.

Enough material has not yet been available for a careful *in vivo* study of the cytotoxic effects in mice.

Origin

The assumption is justified that this particular polypeptide must be derived from a larger protein molecule — "a common denominator protein" — which seems to have certain generic and structural characteristics in common in all species studied. Histones seem not to be implicated since they lack cysteine. The peptide seems to be produced or liberated from tumor cells particularly during stages of autolysis and necrosis. The peptide is stable as long as it is still incorporated into this unknown protein.

Speculations regarding its local and systemic effects

The results so far obtained call attention to the pathophysiological role and clinical consequences of the effects of very special cytotoxic substances liberated during protein degradation. The following suggestions might appropriately be advanced:

1. Polypeptide liberated from solid tumors might be responsible for damage of adjacent stromal cells of the host [5, 6]. Polypeptide liberated into the interstitial fluid phase ought to affect and kill normal fibroblasts and histiocytes in the same manner as it kills strain L and other cells during *in vitro* tests.

2. In advanced tumor cases the presence of excess amounts of this polypeptide are found in the blood plasma and may, at a distance from the tumor, change different kinds of host cells as to their normal permeability and selectively block vital synthetic processes. It is reasonable to assume that the anemia, bone marrow changes, anorexia and several symptoms constituting part of general cachexia and so-called "metabolic death" to some extent could be explained by liberation of this polypeptide. The different amounts of polypeptide liberation and time of onset of such systemic effects may vary with different types of tumors, degrees of malignancy, amount and onset of necrosis, etc. The slowly growing scirrhous tumors form a group not yet investigated where necrotic changes and distant systemic effects seem less prominent. We have so far only investigated *terminal stages of rapidly growing and rapidly necrotizing highly malignant tumors*, and therefore present results only refer to such cases.

In future, it might be possible to design specific antidotes or analogues for the protection of the host organism against this cytotoxic effect; in this way a new basis of special chemotherapy could be envisaged.

RESUME

Les auteurs présentent de nouvelles données expérimentales sur l'isolement et la composition d'un polypeptide cytotoxique diffusible *inconnu jusqu'à présent, qui a été découvert dans une variété de liquides tumoraux. Il apparaît chez les Souris et les Rats porteurs de tumeurs transplantées, comme chez les Hommes souffrant de tumeurs spontanées malignes de différentes sortes. Dans tous les cas, le polypeptide a présenté une configuration en acides aminés unique, dans ce sens que l'activité était associée à la séquence Tyrosine-Cystéine-Tyrosine. Des tests biologiques suggèrent qu'il exerce une activité sélective dans les cellules normales, en bloquant un processus de synthèse indispensable. On cite brièvement les implications cliniques possibles concernant l'anémie et la cachexie dans les cas de cancer terminal.*

SUMMARY

A progress report is presented on the isolation and composition of a previously unknown diffusible cytotoxic polypeptide *from a variety of tumor fluids. It occurs in mice and rats carrying transplanted tumors as well as in human patients with highly malignant spontaneous tumors of various kinds. In all cases the polypeptide presented a unique amino acid configuration in the sense that the activity was associated with the intact sequence of Tyrosine — Cysteine — Tyrosine. Biological tests suggest that it exerts a* selective activity *inside the normal cells by blocking some vital synthetic process. The possible clinical implications regarding the anemia and cachexia in terminal cancer cases are briefly mentioned.*

ZUSAMMENFASSUNG

In vorliegendem Artikel wird über die bisherigen Forschungsergebnisse mit der Isolierung eines bisher unbekannten dialysierbaren zytotoxischen Polypeptids *und dessen Zusammensetzung berichtet, das in unterschiedlichen Tumorflüssigkeiten gefunden wurde. Es ist ebenso vorhanden in durch Transplantation überführten Tumoren bei Mäusen und Ratten, wie in hochgradig bösartigen Spontantumoren verschiedener Art bei menschlichen Kranken. Das Polypeptid zeigte immer das Vorkommen einer besonderen Aminosäurenzusammensetzung in dem Sinne, dass die Aktivität abhängig ist von der unzerstörten Ordnungsfolge von "Tyrosine — Cysteine — Tyrosine." Biologische Teste deuten auf eine* besondere Art der Aktivität *innerhalb der Normalzellen durch Blockierung eines vitalen syntetischen Prozesses hin. Ueber die evtl. klinische Auswertung in Bezug auf die Anämie und Kachexie im Endstadium der Krebserkrankung wurde berichtet.*

REFERENCES

1. B. Holmberg, Inhibition of cellular adhesion and pseudopodia formation by a dialysable factor from tumour fluids. *Nature, Lond.* **195,** 45–47 (1962).
2. B. Holmberg, A dialysable polypeptide from tumor fluids presenting cytotoxic effects. *Rep. Third Scandinavian Conf. Cell Research.* (1962).
3. B. Holmberg, The isolation and composition of a cytotoxic polypeptide from tumor fluids. *Z. Krebsforsch.* **66,** 65–72 (1964).
4. B. Sylvén and B. Holmberg, Om tumörers toxiska verkan pa värdorganismens cellfunktioner. *Nord. Med.* **70,** 765 (1963). (with English summary).
5. B. Sylvén, Some biochemical factors possibly concerned in the destructiveness of malignant growth. *Elfde Jaarboek van Kankeronderzoek en Kankerbestrijding in Nederland* 127–133. de Bussy, Amsterdam (1961).
6. B. Sylvén, The host-tumor interzone and tumor invasion. Henry Ford Hospital Int. Symp. 635–655. Little, Brown & Co., Boston (1962).

Europ. J. Cancer Vol. 1, pp. 203–209. Pergamon Press 1965. Printed in Great Britain

Lactate Dehydrogenase Isoenzyme Patterns in Human Normal and Malignant Gastric Mucosa

ROSE YASIN and F. BERGEL

Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W.3

INTRODUCTION

THE DISTRIBUTION OF lactic acid dehydrogenase (LDH) in mammalian tissues as five enzymatically active forms (isoenzymes) is now well documented [1, 2, 3, 4, 5]. These isoenzymes can be separated electrophoretically and have been shown to possess distinct physical, chemical and immunological properties [6, 7, 8, 9, 10, 11]. Several workers have now demonstrated that LDH molecules can be composed of two types of sub-units [12, 13]. These will be referred to in this communication as the M-subunit (predominantly occurring in muscle and liver LDH), and the H-subunit (predominantly occurring in heart LDH) [13]. These subunits combine into tetramers yielding two extreme forms (M_4 and H_4) and three hybrids (M_3H_1, M_2H_2 and M_1H_3), which are commonly separated electrophoretically. The presence of different proportions of M and H subunits in LDH would therefore impose different catalytic properties to the enzyme extracted from various tissues. It is thus conceivable that the varying kinetic properties of these isoenzymes could influence the nature of pyruvate metabolism in a cell.

The role of LDH in human neoplasia has been actively investigated for many years. Most reports have been primarily concerned with altered LDH levels in the body fluids associated with cancer [14, 15, 16, 17, 18, 19], but only a limited number of studies comparing LDH isoenzyme patterns in normal and neoplastic tissues have been reported [15, 20, 21, 22]. This report deals with a comparative study of the LDH patterns in normal, malignant and other abnormal tissues found in stomach. The techniques utilised to measure the varying portions of M and H subunits present in the tissue, included starch gel electrophoresis, apparent Michaelis constant (*Km*) for pyruvate, relative ratio of enzyme activity with α-keto-butyrate and pyruvate, and total enzyme activity.

METHODS

Tissue samples

16 normal, 8 tumour, a limited number of uninvolved tissue and gastric ulcer specimens were obtained from patients at the Royal Marsden and St. Stephen's Hospitals. Three of the normal samples were obtained from 8–12 hr autopsy specimens. The remainder were taken from surgical specimens of duodenal ulcer cases. The samples were collected between 10 min to 1 hr after removal, cut into small pieces and frozen in liquid nitrogen. To obtain a histological control, several pieces were selected at random and submitted for histological evaluation. The remainder of the samples were maintained in liquid nitrogen until homogenized (for not more than 10 days). The tissue was weighed, suspended in 0·25M Mannitol containing 0·1 mg EDTA/ml, pH 7·4 [34], and

homogenized with an Ultra-Turrax for two 5-sec periods. When necessary the homogenization was completed by brief grinding with a Potter-Elvehjem. The final concentration of the homogenates was 100–250 mg/ml. The extracts were centrifuged at 144,000 × g for 30 min. All procedures were carried out at 0–5°C. Starch gel electrophoresis was carried on the fresh supernatant; the remainder was frozen at −33°C until assayed. We have determined that the overall stability of the LDH isoenzyme was not affected by the method of storage of tissue and supernatant.

LDH assays

The rate ratio for butyrate to pyruvate was obtained by measuring the LDH activity at 3·3 mM α-ketobutyrate and 0·7 mM pyruvate[23]. The Michaelis constant for pyruvate was determined by the Lineweaver-Burk plot [24], the range of pyruvate concentration being between 5–0·075 mM. Total activity was expressed as the maximum velocity (Vm) per mg nitrogen, i.e. specific activity. The Vm was calculated from the Lineweaver-Burk plot and the nitrogen content of the high speed supernatant was determined by means of the micro-Kjeldahl method. All assays were carried out using a Gilford-optical density converter at 340 mμ in 5×10^{-2}M Tris-HCl buffer, pH 7·4 [25]. The temperature was controlled at 25°C.

Starch gel electrophoresis

Starch gel electrophoresis was performed according to the method of Fine *et al.* [26]. The isoenzymes were visualized on the gel using a modified method of these workers. The staining technique was as follows: 1% agar in 0·1M Tris-HCl buffer, pH 8·0, 10 ml, containing 0·1M lithium lactate, 6 mg nicotinamide adenine dinucleotide (NAD), 0·4 mg N-methyl phenozonium methosulphate, and 2 mg MTT (3-(4,5-dimethyl thiozolyl-2)2,5 diphenyl tetrazolium bromide) was poured over a slice of starch gel and incubated for 15–20 min at 37°C in the dark.

RESULTS

In order to discuss the results it is necessary to consider the histological structure of the stomach. Since the cellular composition of the pylorus and body regions are considerably different, the isoenzymes pattern of each of these areas was ascertained. Specimens taken from cancer patients, but which were not involved in the actual tumour, were often found to be atrophic and/or intestinalized. The term 'atrophic mucosa' denotes glandular degeneration. 'Intestinalization' refers to metaplasia of the gastric gland to intestinal mucosal cells. These conditions were often found to coexist with gastric ulcers.

Figure 1 illustrates some typical LDH starch gel electrophoretic patterns that were obtained for the different gastric specimens studied. The tissues were classified according to their histological appearance. When the tumours are compared with normal body and pylorus mucosa an increase in the amounts of M_4 and hybrid M_3H_1 is discernible. Although the body has a higher H_4 concentration than pylorus, when compared to the tumour, both these tissues contain a relatively small amount of M_4 enzyme. The uninvolved tissue surrounding the tumour also shows some deviation from the normal. It is interesting to note that during body atrophy the relative proportion of H_4 enzyme decreases and the LDH pattern of the tissue begins to resemble that of pylorus. With intestinalization, a striking increase in the M_4 band occurs. A rise in the relative proportion of M_4 enzyme also occurs with intestinalization of the gastric body glands. The patterns obtained here are similar to that illustrated in Fig. 1 for adenocarcinoma plus body glands. It appears therefore that the H subunits are contributed by the body glands, while tumour and goblet cells contribute most of the M subunits. This view is consistent with the work of Leese [27], who demonstrated that most of the H_4-LDH is located in the middle and lower glandular layers of the body, the hybrid LDH's in the upper body glands and the pylorus glands, and muscle, while the M_4 LDH is localized primarily in normal intestinal glands, intestinalized gastric glands and stomach carcinoma cells. The starch gel pattern of jejunal mucosa homogenate was also determined (though not illustrated) and a strong M_4 band was obtained with this tissue.

Although the starch gel pattern shown in Fig. 1 represents the isoenzyme distribution for adenocarcinoma we also observed other stomach tumours. The total maligant samples studied here consisted of five adenocarcinomas, two anaplastic carcinomas and one colloid (signet cell) carcinoma. The LDH gel pattern obtained for the adenocarcinoma was also observed with anaplastic tumours. In general, however, a wide variability within the tumours was found. Although an increase in the relative concentration of the M_4 band was always present, some tumours exhibited a more intermediate pattern, with the hybrid M_3H_1 and

M_3H_2 enzymes contributing the strongest bands. These variations may be due to the relative amounts of muscle, stromal and cancerous elements which may be present.

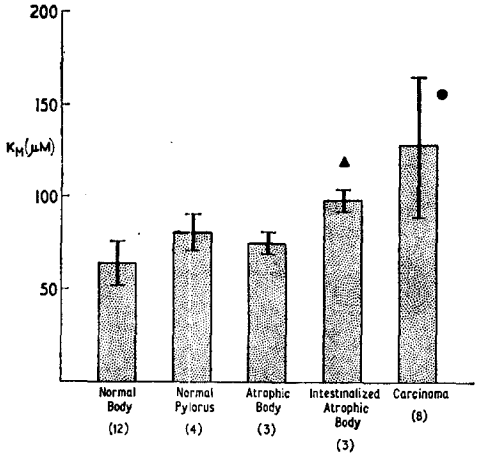


Fig. 2. *Michaelis constants for pyruvate observed in normal and diseased stomach.*
▲ *Single value for intestinalized gastric mucosa.*
● *Single value for jejenum.*

As may be seen in Fig. 2 the *Km* values for the normal and maligant tissue differ by approximately a factor of two. Several workers have reported a two to four fold variation in the Michaelis constant for pyruvate between purified human M_4 and H_4 LDH [23, 28]. A mixture of the isoenzymes therefore would be expected to yield an intermediate *Km* value which will depend on the proportion of M and H subunits present. The data represented in Fig. 2 illustrate the *Km* variation with tissue histology. An increase in the *Km* was found to occur with a corresponding increase in the number of M subunits. The values are in agreement with the *Km's* reported by these other workers and show a direct correlation with the LDH starch gel patterns obtained. The rising Michaelis constant observed in the intestinalized tissue prompted an evaluation of the *Km* of normal intestinal mucosa. This is shown as a closed circle in Fig. 2 and is seen to be similar to that of tumour tissue.

The variation of the LDH activity with α-ketobutyrate has been shown to be dependent on the proportion of M and H subunits present in the enzyme. As the proportion of the M subunits in the molecules increases, the activity ratio of butyrate/pyruvate decreases. For tissue extracts containing mostly M_4 and H_4 enzyme the ratios previously reported by other workers were 0·3 and 1·0 respectively [23]. The results illustrated in Fig. 3 are consistent with the above work, the ratio in tumour being

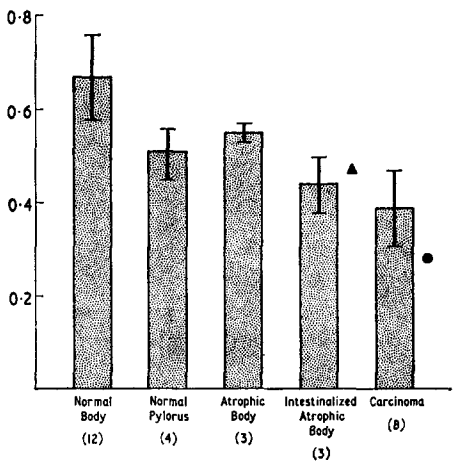


Fig. 3. *LDH butyrate/pyruvate rate ratios obtained for normal and diseased stomach.*
▲ *Single value for intestinalized gastric mucosa.*
● *Single value for jejenum.*

about half that found in normal stomach. The activity ratios for the other abnormal gastric mucosa fall in a continuum between the normal and malignant, showing a correlation with the variation in their content of M and H subunits. These data are in good agreement with that obtained for *Km* and starch gel electrophoretic patterns.

Figure 4 summarizes the values obtained for the LDH specific activity in the grouped tissues. While the mean value for the specific activity is higher for the tumours, the variability of these in each group is so great that this difference cannot be considered as conclusive.

Specific activity represents the combined property of enzyme concentration and isoenzyme composition. To obtain a truer measure of enzyme levels, it is necessary to correct for the

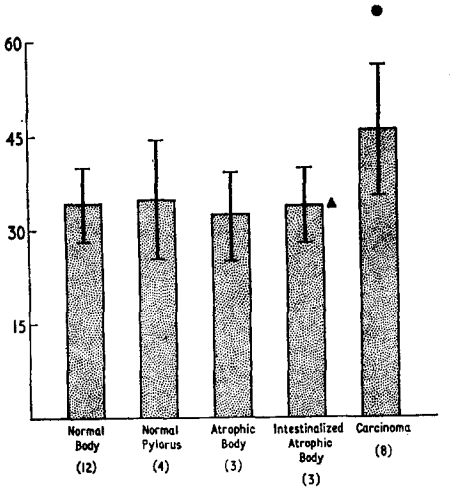


Fig. 4. *LDH specific activities observed in normal and diseased stomach.*
▲ *Single value for intestinalized gastric mucosa.*
● *Single value for jejenum.*

isoenzymes. Since *Km* primarily reflects the isoenzyme distribution of the tissue, we attempted to correct our present enzyme level values by calculating the *Vm/Km* ratios from their respective mean values. Table 1 lists the ratios obtained by this method. The results indicate a lower enzyme concentration in carcinoma than in corresponding normal tissues and show similar trends to that obtained with the other biochemical parameters. Further work on this method must be done before the real implication of these results can be determined.

Table 1.

	$\frac{Vm}{Km}$
Normal body	0·53
Normal pylorus	0·44
Atrophic body	0·43
Intestinalized body	0·34
Carcinoma	0·36
Intestinalized pylorus	0·30
Jejenum	0·41

DISCUSSION

A consistent increase towards the predominance of M-type LDH was found to occur in stomach carcinoma. All biochemical parameters studied corroborated this observation. The finding is in agreement with the study carried out by Goldman *et al.* [20] on lactate dehydrogenases in human normal and neoplastic tissues. Two other findings we obtained were first, the increase of M-type LDH with intestinal metaplasia of the gastric glands. Second, a more limited change in enzyme pattern occurred with body glandular degeneration. Here the enzyme distribution was seen to be approaching that of normal pylorus.

The high glycolytic capacity of most neoplasms [29] compared to corresponding normal tissues is presumably due to several factors such as relative changes in the enzyme and coenzyme concentration and/or changes in the isoenzyme patterns. Overall glycolytic ratios have not been measured here but the observed changes in LDH patterns are consistent with what one would expect for neoplasms, on the basis of Warburg's concept [29]. As the M type LDH increases a higher rate of lactate formation would be favoured. An imbalance in pyruvate metabolism, e.g. pyruvate oxidation and reduction, mediated by other isoenzymes present in the glycolytic and respiratory pathways [30, 31] could result in an increase in the pyruvate concentration. Under these substrate conditions, a tissue having a predominance in the M-type LDH (least inhibited by high pyruvate concentrations) would facilitate lactate production.

The question arises whether the LDH isoenzyme shift is a causal factor in tumour development or a secondary associated factor. It has been proposed that the synthesis of the M and H subunits is controlled by separate genes [13]. A recent publication which has provided some evidence for this hypothesis has also demonstrated the effect of hormone administration on the type LDH synthesized [32]. The possible factors controlling this include (1) intracellular environmental changes (substrate concentration), (2) intercellular environmental changes (e.g. hormones or other endogenous substances), (3) effect of exogenous substances.

Since the predominant LDH variant present in tumours also exists in normal intestinal and intestinalized gastric mucosa, it is probable that the enzyme shift observed is merely a reflection of the changing proportion and nature of the cells. Assuming that the above explanations are valid two questions remained unsolved. Is malignancy preceded by an alteration of the LDH pattern in the cell or does it result from the appearance of a histologically (not necessarily functional) normal cell, which contains its own characteristic LDH pattern, in an abnormal environment? In this latter respect, it might be appropriate to mention Morson's work [33]. He has demonstrated that the extent of intestinal metaplasia of the gastric mucosa increases with age and show a high prevalence in cases with gastric ulcer and more particularly with gastric carcinoma. He has also suggested that intestinalization is a precancerous condition.

Further work on the environmental control of cellular differentiation in the stomach mucosa will have to be carried out before some insight into this problem can be obtained.

It is generally accepted that the early diagnosis of stomach cancer is difficult. An important question is, can one utilize this LDH pattern shift as an aid to early diagnosis. No conclusive results have as yet been obtained with investigations on either LDH patterns in blood [34] or total LDH content present in gastric juice [17]. We are at present commencing a study on the LDH pattern in gastric juice of patients with and without stomach dis-

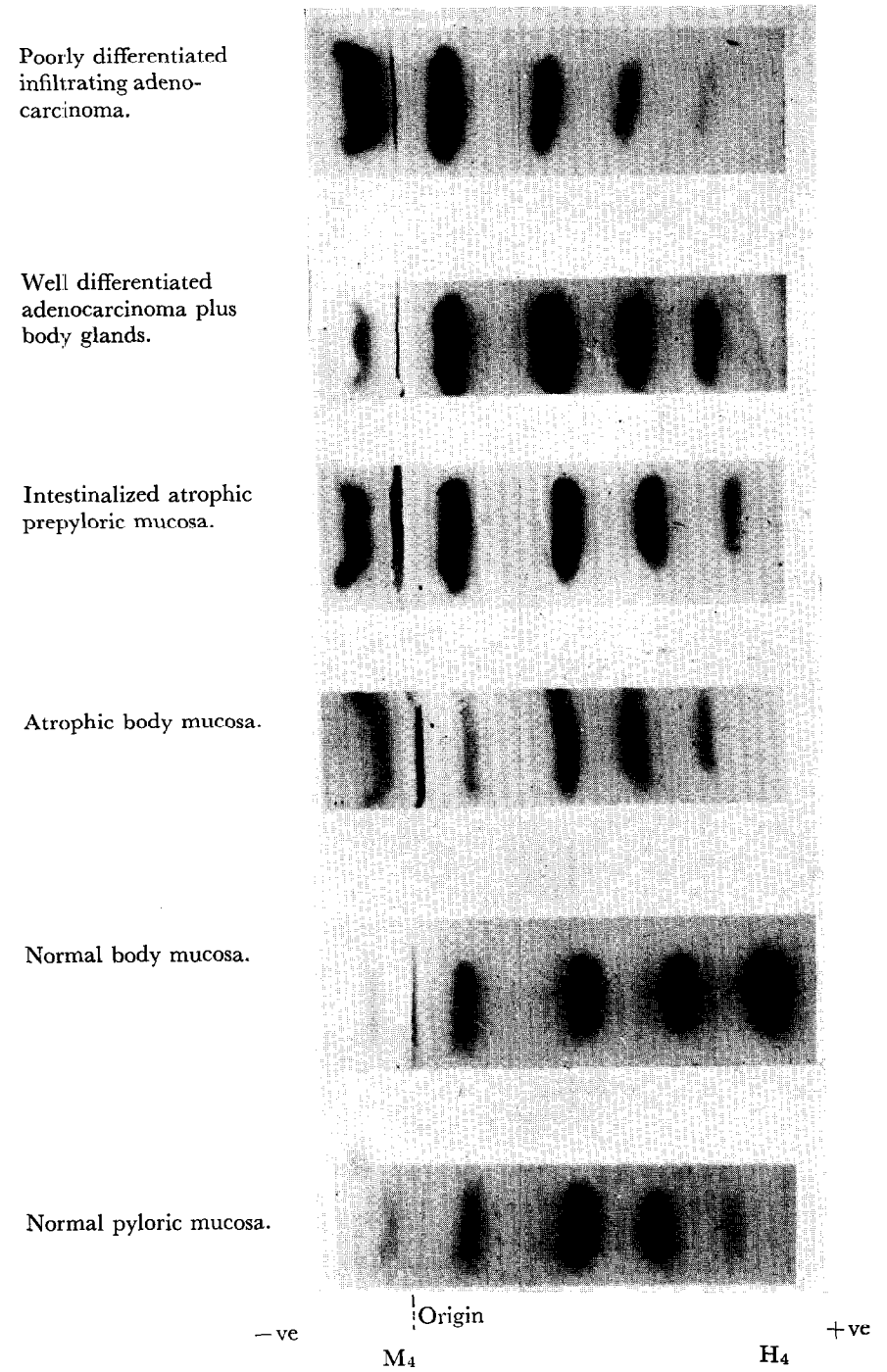


Fig. 1. *Some typical LDH isoenzyme electrophoretic patterns of the various gastric specimens studied.*

(to face p. 206)
Refer to p. 204

orders. A possibility exists that the LDH present there will reflect the changing enzyme pattern observed in this study. This work is still in its initial stages and therefore we cannot comment on the usefulness of this approach in diagnosis.

Acknowledgements — The authors are particularly indebted to Mr. C. I. Cooling and his surgical team, Royal Marsden Hospital for their kind co-operation in providing specimens and to Dr. C. E. Dukes, Chester Beatty Research Institute for his painstaking histological analysis. We also wish to thank Mr. W. H. W. Jayne, St. Stephen's Hospital, Dr. N. F. C. Gowing and co-workers, Pathology Department, Royal Marsden Hospital and Miss P. Giffon, who was responsible for tissue collection and storage. We are also grateful to Dr. D. A. Gilbert for his advice and Mr. J. Biffen for his untiring technical assistance.

This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and the British Empire Cancer Campaign for Research, and by the Public Health Service Research Grant No. CA-03188-08 from the National Cancer Institute, U.S. Public Health Service.

RESUME

1. *Le contenu en isoenzymes de la déshydrognéase de l'acide lactique dans l'estomac normal et pathologique fut étudié à l'aide de plusieurs paramètres biochimiques.*
2. *Une augmentation constante des proportions relatives des sub-unités 'M' fut constatée en présence d'un carcinome gastrique.*
3. *Un changement similaire des déshydrogénases de l'acide lactique fut observé dans les muqueuses gastriques 'intestinalisées.'*
4. *L'importance possible de ces observations est discutée.*

SUMMARY

1. *The LDH isoenzyme content of normal and diseased stomach was studied by means of several biochemical parameters.*
2. *A consistent increase in the relative proportions of 'M' subunits was obtained with stomach carcinoma.*
3. *A similar change in the LDH was observed with "intestinalised" gastric mucosa.*
4. *The possible significance of these results is discussed.*

ZUSAMMENFASSUNG

1. *Der Gehalt an Lactatdehydrogenase in ihren isoenzymatischen Formen wurde in normalem und pathologischem Magengewebe mit Hilfe von einigen biochemischen Parametern studiert.*
2. *Eine anhaltende Zunahme in den relativen Verhaeltnissen der 'M' Einheit wurde im Krebsmagen gefunden.*
3. *Ein aehnlicher Wechsel der LDH wurde auch mit Magenschleimhaut, die eine Verwandlung in eine darm-artige Form erlitten hatte, beobachtet.*
4. *Die moegliche Bedeutung dieser Resultate wird diskutiert.*

REFERENCES

1. N. O. Kalpan and M. Ciotti, Heterogeneity of the lactic dehydrogenases of newborn adult rat as determined with coenzyme analogs. *Biochim. Biophys. Acta* **49,** 425–26 (1961).
2. C. L. Markert and F. Moller, Multiple forms of enzymes: tissue, ontogenetic and species patterns. *Proc. Nat. Acad. Sci.* **45,** 753–63 (1959).
3. P. G. W. Plagemann, F. W. Gregory and F. Wróblenski, The electrophoretically distinct forms of mammalian lactic dehydrogenase. I. Distribution of lactic dehydrogenases in rabbit and human tissues. *J. Biol. Chem.* **235,** 2282–87 (1960).

4. R. Richterich, P. Schafroth and H. Albi, A study of lactic dehydrogenase isoenzyme patterns of human tissues by adsorption-elution on sephadex-DEAE. *Clin. Chim. Acta* **8,** 179–92 (1963).
5. E. S. Vessell and A. G. Bearn, Isozymes of lactic dehydrogenase in human tissues. *J. Clin. Invest.* **40,** 586–591 (1961).
6. J. S. Nisselbaum and O. Bodansky, Purification, kinetic and immunological studies of the major variants of lactic dehydrogenase from human liver, hepatoma and erythrocytes; comparison with the major variant of human heart lactic dehydrogenase. *J. Biol. Chem.* **238,** 969–73 (1963).
7. P. G. W. Plagemann, F. W. Gregory and F. Wróblenski, The electrophoretically distinct forms of mammalian lactic dehydrogenase. II. Properties and inter-relationships of rabbit and human lactic dehydrogenase isozymes. *J. Biol. Chem.* **235,** 2288–93 (1960).
8. D. T. Plummer and F. H. Wilkinson, Organ specificity and lactate-dehydrogenase activity. 2. Some properties of human-heart and liver preparations. *Biochem. J.* **87,** 423–29 (1963).
9. E. O. Wachsmuth and G. Pfleiderer, Biochemische Untersuchungen an Kristallinen Isozymen der Lactatdehydrogenase aus Menschlichen Organen. *Biochem. Z.* **336,** 545–556 (1963).
10. Th. Wieland and G. Pfleiderer, Chemical differences between multiple forms of lactic acid dehydrogenase. *Ann. N.Y. Acad. Sci.* **94,** 691–700 (1961).
11. R. J. Wieme, Application diagnostique de l'enzymoelectrophorèse des deshydrogénase de l'acide lactique. *Clin. Chim. Acta* **4,** 46–50 (1959).
12. E. Appella and C. L. Markert, Dissociation of lactate dehydrogenase into subunits with guanidine hydrochloride. *Biochim. Biophys. Res. Comm.* **6,** 171–176 (1961).
13. R. D. Cahn, N. O. Kaplan, L. Levine and E. Zwilling, Nature and development of lactic dehydrogenases. *Science* **136,** 962–69 (1962).
14. H. R. Bierman, B. R. Hill, L. Reinhardt and E. Emory, Correlation of serum lactate dehydrogenase activity with the clinical status of patients with cancer, lymphomas and leukemias. *Cancer Res.* **17,** 660–67 (1957).
15. A. H. Gelderman, H. V. Gilboin and A. Peacock, Lactate dehydrogenase isozymes in urine from patients with malignancy in the urinary bladder. *J. Lab. Clin. Chem.* **65,** 132–42 (1965).
16. B. R. Hill, Further studies of the fractionation of lactic dehydrogenase of blood. *Ann. N.Y. Acad. Sci.* **75,** 304–10 (1958).
17. D. W. Piper, M. B. Macoun, F. L. Broderick, B. H. Fenton and J. E. Builder, The diagnosis of gastric carcinoma by the estimation of enzyme activity in gastric juice. *Gasteroenterology* **45,** 614–20 (1963).
18. W. E. C. Wacker and L. E. Dorfman, Urinary lactic dehydrogenase activity. I. Screening method for detection of cancer of kidneys and bladder. *J. Amer. Med. Ass.* **181,** 972–78 (1962).
19. F. Wróblewski and K. F. Gregory, Lactic dehydrogenase and their distribution in normal tissues and plasma and in disease states. *Ann. N.Y. Acad. Sci.* **94,** 912–32 (1961).
20. R. D. Goldman, N. O. Kaplan and T. C. Hall, Lactic dehydrogenase in human neoplastic tissues. *Cancer Res.* **24,** 389–99 (1964).
21. G. Pfleiderer, G. and E. D. Wachsmuth, Alters- und funkhonsabhängige Differenzurung der Lactate dehydrogenase Menschilicher organe. *Biochem. Z.* **334,** 185–198 (1961).
22. W. H. Starkweather and H. K. Schoch, Some observations on lactate dehydrogenase of human neoplastic tissue, *Biochim. Biophys. Acta* **62,** 440–42 (1962).
23. D. T. Plummer, B. A. Elliott, K. B. Cooke and F. H. Wilkinson, Organ specificity and lactate-dehydrogenase activity. I. The relative activities with pyruvate and 2-oxo butryrate of electrophoretically separated fractions. *Biochem. J.* **87,** 416–22 (1963).
24. H. Lineweaver and J. D. Burk, The determination of enzyme dissociation constants. *J. Amer. Chem. Soc.* **56,** 658–666 (1934).
25. R. Wu and E. Racker, Regulatory mechanisms in carbohydrate metabolism. III. Limiting factors in glycolysis of ascites tumour cells. *J. Biol. Chem.* **234,** 1029–1035 (1959).
26. I. H. Fine, N. O. Kaplan and D. Kuftinic, Developmental changes of mammalian lactic dehydrogenases. *Biochemistry* **2,** 116–21 (1963).

27. C. L. LEESE, Histological distribution of lactic acid dehydrogenase in human normal and malignant gastric mucosa. *Europ. J. Cancer.* This journal.
28. J. S. NISSELBAUM, D. E. PACKER and O. BODANSKY, Comparison of the actions of human brain, liver and heart lactic dehydrogenase variants on nucleotide analogues and on substrate analogues in the absence and in the presence of oxalate and oxamate. *J. Biol. Chem.* **239,** 2830–34 (1964).
29. O. WARBURG. *The Metabolism of Tumour* (translation F. DICKENS). London Constable (1930).
30. N. O. KAPLAN, Symposium on the multiple forms of enzymes and control mechanisms. 1.–Multiple Forms of Enzymes. *Bact. Rev.* **27,** 155–69 (1963).
31. T. VON WIELAND and G. PFLEIDERER, Multiple formen von enzymen, *Advanc. Enzymol.* **25,** 329–368 (1963).
32. T. L. GOODFRIEND and N. O. KAPLAN, Effects of hormone administration on lactic dehydrogenase. *J. Biol. Chem.* **239,** 130–35 (1964).
33. B. C. MORSON, Precancerous lesions of the upper gastrointestinal tract. *J. Amer. Med. Ass.* **179,** 316–22 (1962).
34. F. WRÓBLEWSKI, Lactic dehydrogenase activity in cancer diagnosis. *Med. Clin. N. Amer.* **45,** 513–20 (1961).

Europ. J. Cancer Vol. 1, pp. 211–216. Pergamon Press 1965. Printed in Great Britain

The Histological Distribution of Lactic Acid Dehydrogenase Isoenzymes in Human Normal and Malignant Gastric Mucosa

C. L. LEESE

Chester Beatty Research Institute
Institute of Cancer Research, London, S.W.3

INTRODUCTION

DIFFERENCES in molecular architecture and amino acid composition of the five isoenzymic forms of lactic acid dehydrogenase (LDH) commonly found in mammalian tissues account for the gradations observed in their physicochemical and immunological behaviour. Composed of two types of polypeptide subunits (*M* and *H*), the biosynthesis of which is believed to be under separate genetic control, each active isoenzyme is a tetrameric complex, the five variants having the compositions M_4, M_3H, M_2H_2, MH_3 and H_4 [1–8].

Of biochemical significance are their different affinities for substrate and co-enzyme in the interconversion of pyruvate and lactate [5, 7, 9]. The H_4 isoenzyme, characteristic of heart muscle, is inhibited at high pyruvate concentrations and its predominance in tissue is thought to be indicative of an oxidative metabolism. Conversely, the M_4 variant facilitates the reduction of pyruvate at high substrate concentrations reflecting a high glycolytic capacity in tissues in which this form predominates. This is of particular interest in connection with the high glycolytic lactate formation frequently reported in neoplastic tissues including those from human sources [10].

The various tissue specific LDH isoenzyme patterns observed in normal adult animals [4, 7] appear to develop from more uniform foetal patterns in late foetal and early postnatal existence [9, 11]. Recent reports have illustrated that a variety of human malignancies are likewise associated with a reorientation of LDH-isoenzyme distribution when compared to their tissue of origin [11, 12, 13]. With the possible exception of seminoma testis [14] these changes involve a reorientation in the direction of the *M* form with the M_4 or M_3H isoenzymes becoming predominant in tumour tissue. Reorientation of this type in gastric carcinoma has recently been confirmed by Yasin and Bergel [15] in our laboratory.

In conventional biochemical techniques employing extracts prepared from gross tissue samples parallel histological control of the material can involve considerable difficulties. This is especially true of complex structures such as gastric mucosa susceptible to localised change and, more particularly, of carcinomas where variation in tissue composition may be considerable within relatively small sample volumes.

In the preliminary quantitative studies described by Yasin and Bergel [15] patterns intermediate between the "normal" and "carcinomatous" LDH isoenzyme distributions were observed in uninvolved mucosal regions from carcinomatous stomachs. In an attempt to explain these "field changes" and to obtain a closer correlation between gross biochemical properties and detailed histological changes a

method based on the use of freeze-dried tissue sections was devised, which permitted a study of the qualitative distribution of LDH isoenzymes in gastric tissues by micro-electrophoretic techniques.

METHODS

Tissue samples were obtained from patients undergoing partial or total gastrectomy for gastric carcinoma, gastric or duodenal ulcer. Thirty tissue specimens have been examined from twelve patients (four Adenocarcinoma, one Colloid carcinoma, two benign gastric ulcer and five duodenal ulcer). As soon as possible following removal of the tissue at operation, specimens from defined regions of the stomach were orientated by fixing to cork pieces and frozen in liquid nitrogen. Subsequently pieces of these specimens were mounted on the chucks of a cryostat and sections 5–10μ thick cut at −20°C. A selection of the sections cut from individual tissue blocks were mounted on cover slips, fixed briefly in formol saline and stained with haematoxylin and eosine. Other sections from the same blocks were freeze-dried at −20°C in high vacuum following the techniques of Lowry [16].

With the additional guidance of the stained sections tissue fragments of closely defined histological characteristics were dissected from the freeze-dried sections under a microscope. In the case of well orientated glandular structures dissection was generally performed as indicated in Fig. 1 to isolate:

(1) Mucous epithelium
(2) Upper glandular structure
(3) Lower glandular structure
(4) Muscularis mucosa and submucosa
(5) External musculature and
(6) A tissue cross-section cut to include a representative sample of all the tissue elements present. A more arbitrary mode of dissection had to be adopted in disorganised carcinomatous specimens. The relative LDH isoenzyme patterns in these tissue fragments were examined electrophoretically.

Electrophoresis was performed on 8 cm^2 glass plates coated with a thin layer (approx 1 mm deep) of 1% agar gel in sodium barbitone buffer (0·07M; pH 8·6; 8 ml per plate) in a manner similar to that described by Wieme [17]. Small wells (1 mm dia.; 6–10 per plate) were punched with the aid of a blunted trochar through the buffered agar layer on a line drawn one third of the distance across the plate. With the aid of needles or short cotton wicks moistened with buffer the dissected tissue fragments (1–5μg dry wt.) were transferred to the wells each of which was then filled with buffer solution from a micro-pipette. In some cases small samples of the high speed supernatant fraction from homogenates of adjacent tissues prepared for quantitative assay [15] were included for comparison with the freeze-dried samples. Electrophoresis was conducted in a modified Kohn Universal Electrophoresis Tank (Shandon Scientific Co. Ltd.) the proteins eluted from the freeze-dried tissue being separated at 4V/cm for 1 hr.

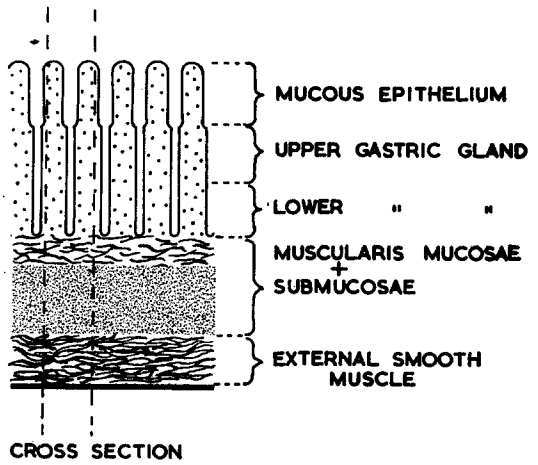


Fig. 1. Diagram illustrating the manner in which freeze-dried sections of glandular mucosa were dissected.

The location of the LDH isoenzymes was detected histochemically by incubating the agar plates at 37°C for 30 min in a solution composed of 6 mg nicotinamide adenine dinucleotide (NAD), 0·5 ml of 2·0 M lithium lactate, 0·5 mg phenazine methosulphate (PMS), 2·0 mg 3(4,5-dimethyl thiazolyl-2)2,5-diphenyl tetrazolium bromide (MTT) all dissolved in 10 ml 0·1 M tris—HCl buffer, pH 8·0 [18]. Following development of the formazan deposits the plates were washed with water, fixed in 4% formol saline for 30 min and transmission photographs were prepared for record purposes.

RESULTS

Preliminary quantitative studies have indicated that the LDH in freeze-dried tissue sections is rapidly eluted into buffer solutions and that butyrate/pyruvate activity ratios are closely comparable to those observed in gross homogenate preparations [15]. This is supported by the identity of electrophoretic patterns from both types of preparation provided the tissues

employed are histologically homogeneous. Freeze-dried sections have previously been used by Lowry *et al.* [19, 20] for LDH assay and a recent immunoelectrophoretic study [21] has indicated their superiority over other tissue preparations in studying soluble tissue proteins.

Typical of the histological detail observable in freeze-dried, unfixed, unstained tissue sections is that seen in a section of body mucosa from a patient with duodenal ulcer (Fig. 2). The relatively unbranched gastric glands extending to the muscularis mucosa beneath which lies the areolar submucosal connective tissue stroma and the external musculature are clearly apparent. The LDH isoenzyme patterns (Fig. 3) in various regions of a serial section, dissected as indicated in Fig. 1, exhibit considerable differences reflecting regional changes in cellular composition. Characteristic of these normal gastric body patterns is the generally weak M_4 component and the very prominent H_4 component located in the mid, and to a lesser extent, in the lower gastric gland regions. The latter is believed to be associated with the oxyntic cells which are particularly abundant in these regions. This band is weakened with increasing degrees of atrophic gastritis. A constant feature of the isoenzyme patterns is that associated with the smooth muscle coat in which the M_2H_2 variant is the major component. This is observed in the stomach, duodenal and jejenal external musculatures. The comparability of the patterns derived from tissue cross-sections and homogenate preparations is indicated in Fig. 3 (bands 6 and 7) both of which qualitatively represent a summation of patterns from individual regions of the specimen (Fig. 3; bands 1–5). Pyloric and cardiac specimens examined in the same manner showed a similar characteristic weak M_4 component. The H_4 band was not as prominent as in the case of the body mucosa due to absent, or considerably reduced, oxyntic cell populations in these regions.

Examination of carcinoma tissue confirmed previous reports [11, 12, 15] of a reorientation of the LDH isoenzyme distribution with the M_4 and M_3H forms predominating irrespective of the degree of anaplasia. An equally significant change was, however, observed in apparently uninvolved mucosa where areas of intestinal metaplasia existed. The patterns observed in these regions closely resembled those from the carcinomatous tissues and offered an explanation for the intermediate isoenzyme distributions observed in the quantitative study of gross homogenate samples [15]. Similar "carcinomatous-like" patterns were observed in intestinalised regions from gastric mucosa of the two gastric ulcer patients in whom there was no pathological evidence to suggest that malignant transformation had occurred.

Through examination of a number of specimens it became apparent that changes in the LDH-isoenzyme distribution paralleled a spectrum of histological changes from normal gastric body mucosa, through pyloric mucosa, atrophic gastritis and intestinal metaplasia to varying degrees of anaplastic carcinoma. Analysis of the quantitative data of Yasin and Bergel [15] revealed a parallel distribution in various biochemical parameters.

This spectrum of histological and enzymic changes is illustrated by various tissue specimens removed from a 47-year-old male having a large adenocarcinoma (5·5×4·5 cm) infiltrating the gastric wall on the inner curvature of the prepyloric region. A section of body mucosa (Fig. 4) remote to the malignancy showed considerable evidence of a trophic disorientation of the gastric glands with a loss in oxyntic cell mass, although no intestinalised areas were present. (This, and subsequent sections were stained with haematoxylin and Mayer's mucicarmine to differentiate gastric and intestinal mucoids and to emphasise the presence of mucous secreting goblet cells.) The isoenzyme patterns in various regions of this mucosal sample (Fig. 5) exhibited a relatively normal body mucosal pattern with some loss in H_4 component relative to the hybrid variants.

A second body mucosal specimen (Fig. 6) closer to, but still separated from the borders of the malignancy, exhibited extensive gastritic changes with regions of intestinalised glands containing typical goblet cells and mucicarmine positive material in the glandular lumen. LDH isoenzyme patterns (Fig. 7) in this region exhibited a distinct shift to the M type whilst the H_4 variant was very weak.

At the borders of the carcinoma (Fig. 8) glandular tissue in varying states of necrosis and atrophy associated with extensive intestinal metaplasia was present. Regions of malignant degeneration of the glandular structures retaining a positive mucicarmine reaction were also apparent. Deeper in the tumour mass varying degrees of differentiation were present (Fig. 9). The LDH isoenzyme patterns derived from areas of heavy intestinalisation at the tumour borders showed (Fig. 10; bands 1 and 2) a close similarity with those from various parts of the tumour (Fig. 10; bands 3–5) each possessing a preponderance of the M_4 and M_3H isoenzymes. For comparison with intestinal tissue a sample of normal jejunal

mucosa from the same patient (Fig. 11) was found to have an isoenzyme pattern (Fig. 12) in which the M_4 band predominated.

A similar investigation of rat tissues has revealed that fore-stomach, gastric glandular tissue and intestinal mucosa possess closely related LDH isoenzyme patterns with the *M* variant predominating. They do not show the sharp demarkation in isoenzyme distribution observed in the human upper gastrointestinal tract.

DISCUSSION

Many authors have emphasized the relationship between gastric carcinoma and the occurrence of intestinal metaplasia in which the normal gastric body and pyloric glands are replaced by a simple tubular epithelium composed of mucous secreting goblet cells, columnar cells with striated borders, paneth and argentaffin cells [22–26]. Unlike the glands in normal gastric mucosa where mitosis occurs predominantly in the mucous neck cell region, intestinalised gastric glands exhibit mitotic activity in the crypts [25, 26]. Histochemical studies have shown that several enzymes e.g. aminopeptidase, whilst normally absent in the gastric mucosa, appear in regions of intestinal metaplasia with histological distributions characteristic of the normal intestinal epithelium [27–29].

The observations reported in the present paper support the conclusion that intestinal metaplastic tissue in the gastric mucosa is morphologically and functionally identical to normal intestinal epithelium. The differences in LDH-isoenzyme patterns between gastric and intestinal epithelium suggests that these tissues are dependent upon different energy metabolism. This is in contrast to the absence of marked differences in LDH-isoenzyme patterns between the gastric and intestinal mucosa of the rat which is of interest in view of the resistance shown by this animal to the development of gastric carcinoma either spontaneous or induced.

The incidence of intestinal metaplasia is known to increase with age and is observed with greatest frequency in the pyloric and inner curvature regions of the stomach, coinciding with those sites in which gastric carcinoma most frequently occurs [23]. The extent and intensity of intestinalisation accompanying gastrointestinal disease increases in the order duodenal ulcer, gastric ulcer and gastric carcinoma. Morson reported an incidence $>90\%$ in the mucosa of carcinomatous stomachs. Gastric carcinoma and intestinal metaplasia have a special association with pernicious anaemia and atrophic gastritis. Both conditions give rise to varying degrees of gastric dysfunction due to loss of functional gastric gland mass. Recent investigations have revealed their frequent association with the presence of circulating auto-antibodies particularly those directed against oxyntic cell constituents [30–32].

Intestinal metaplasia of the gastric mucosa has been considered as a significant preneoplastic condition in man; approximately 30 per cent of gastric carcinomas being shown histologically to arise in intestinalised regions [24]. Järvi and Laurén [29] found that a similar proportion of gastric tumours elaborated mucoid products of an intestinal type and that half of the tumours examined showed cells exhibiting cytological characteristics typical of intestinal epithelial cells. Similar conclusions can be drawn from histochemical studies [27–29, 33] which have shown that a proportion of gastric tumours possess enzyme activities characteristic of intestinal mucosa.

In the present very limited series of carcinomas, an LDH-isoenzyme pattern in which the *M*-subunit predominates has been observed in each instance. Compared with the foregoing observations this would indicate a much more uniform property of gastric tumours. Its significance in relationship to preneoplastic gastric mucosal changes and to the degree of differentiation in gastric carcinomas is not yet clear.

Acknowledgements—The Author wishes to acknowledge the enthusiastic co-operation of Mr. C. I. Cooling and his surgical team at the Royal Marsden Hospital and Mr. W. H. W. Jayne at St. Stephen's Hospital for providing gastrectomy specimens, and to Miss P. Giffon for their collection and storage. A deep debt of gratitude is due to Dr. Cuthbert E. Dukes, Chester Beatty Institute, for his advice and interest in the histopathological aspects of this work and to Professor F. Bergel for his support and encouragement. He is also grateful to Mr. J. Biffen and Mr. G. Munroe for their excellent technical assistance.

This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council, and the British Empire Cancer Campaign for Research, and by the Public Health Research Grant No. CA-03188-08 from the National Cancer Institute, U.S. Public Health Service.

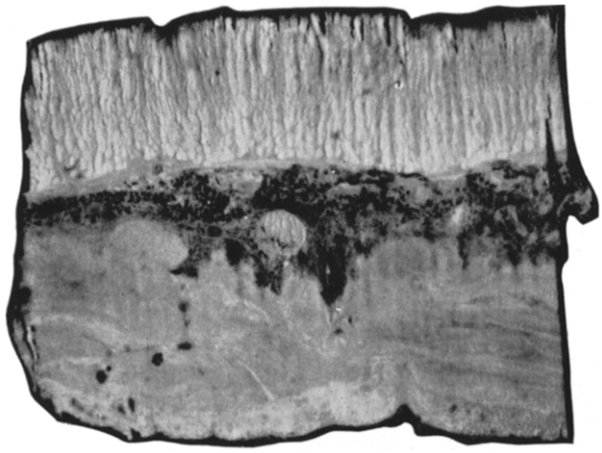


Fig. 2. Unfixed, unstained, freeze-dried section of gastric body mucosa from a duodenal ulcer patient.

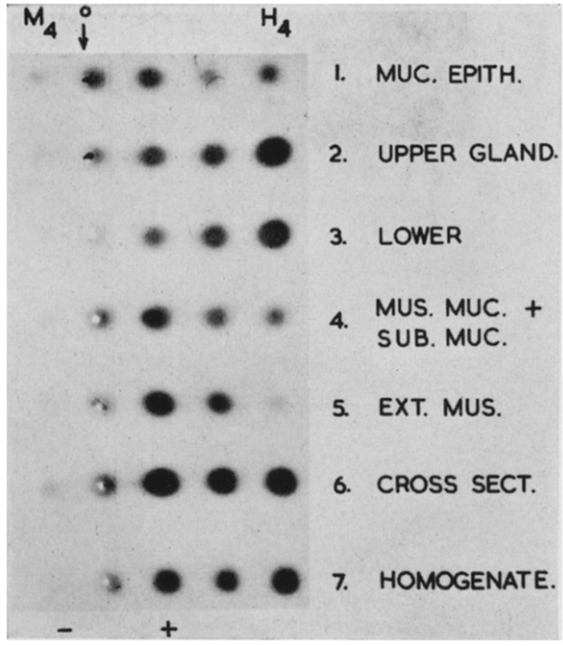


Fig. 3. LDH isoenzyme patterns of normal gastric body mucosa.

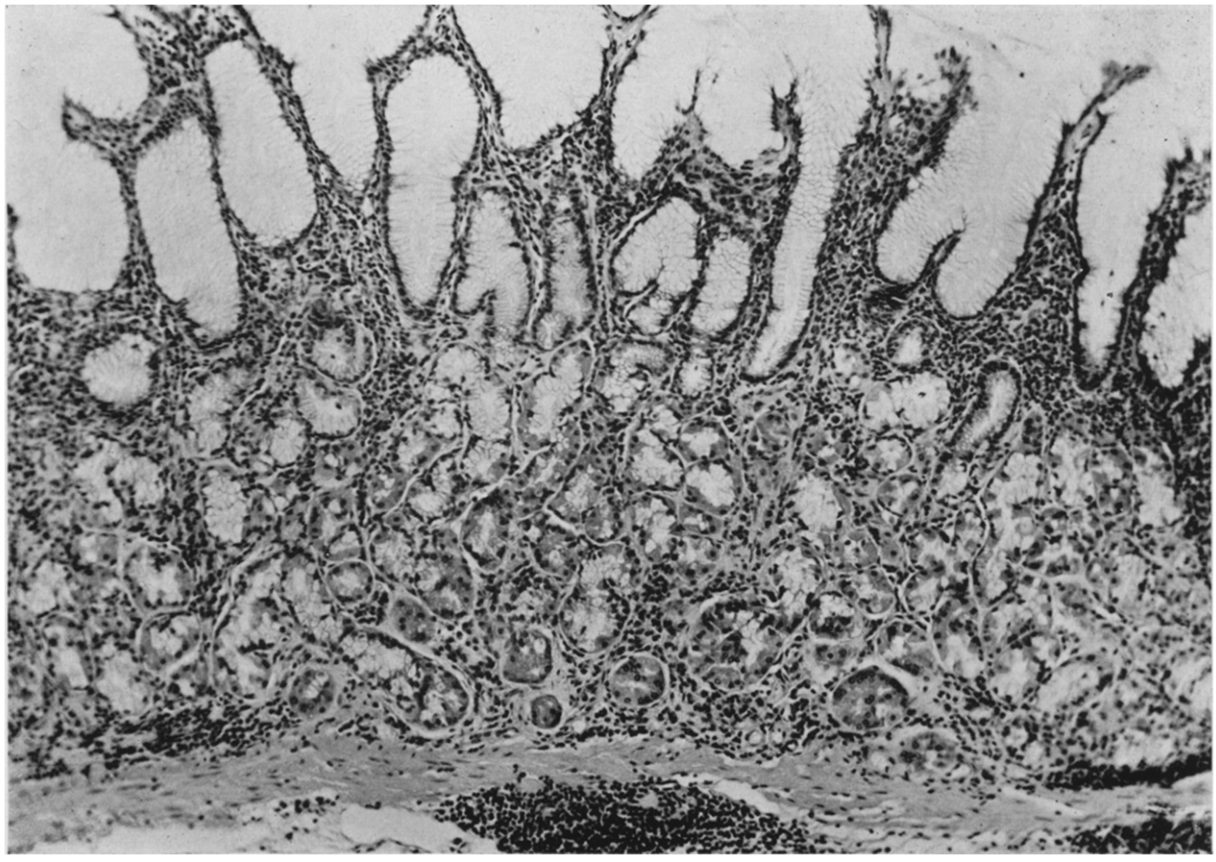


Fig. 4. Section from the body mucosa of carcinomatous stomach showing atrophic changes but no intestinalised glands. Haematoxylin and mucicarmine . ×120.

(to face p. 214)

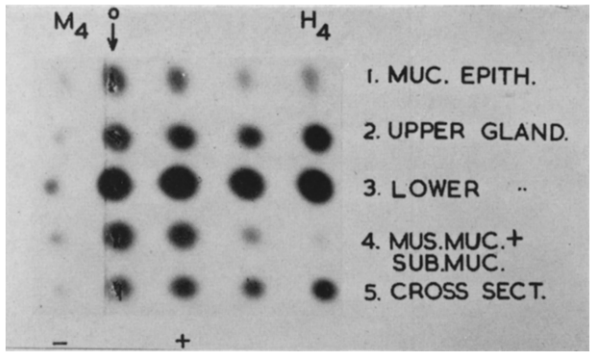


Fig. 5. *LDH isoenzyme patterns present in atrophic body mucosa in the absence of intestinalised glands.*

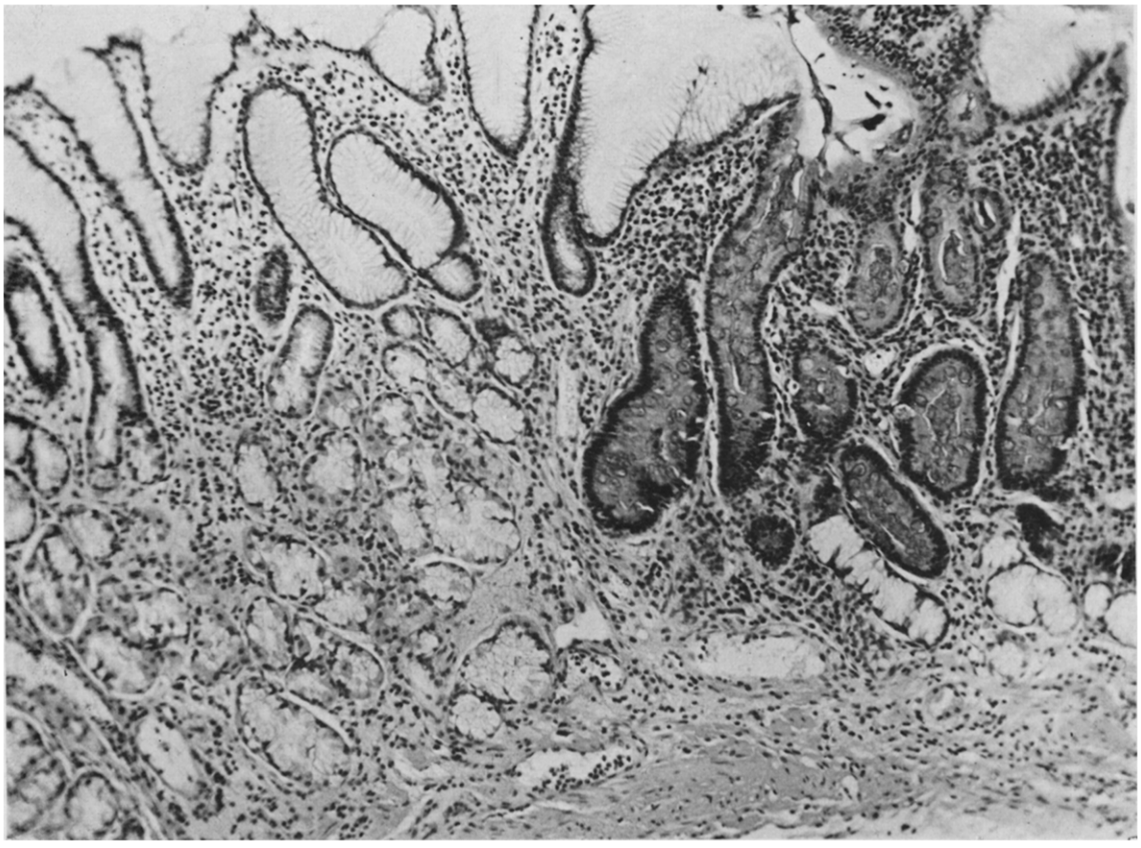


Fig. 6. *Section from the body mucosa of a carcinomatous stomach exhibiting localised areas of intestinal metaplasia. Haematoxylin and mucicarmine.* × 120.

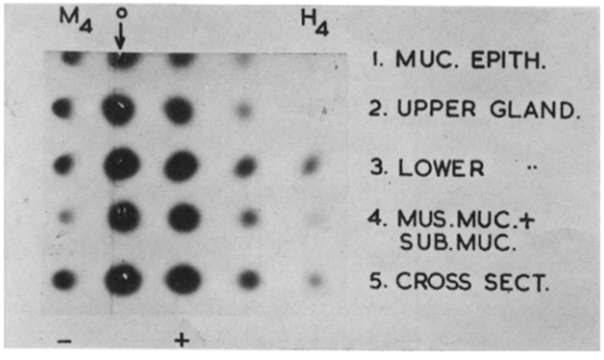


Fig. 7. *LDH isoenzyme patterns present in atrophic body musoca containing localised areas of intestinal metaplasia.*

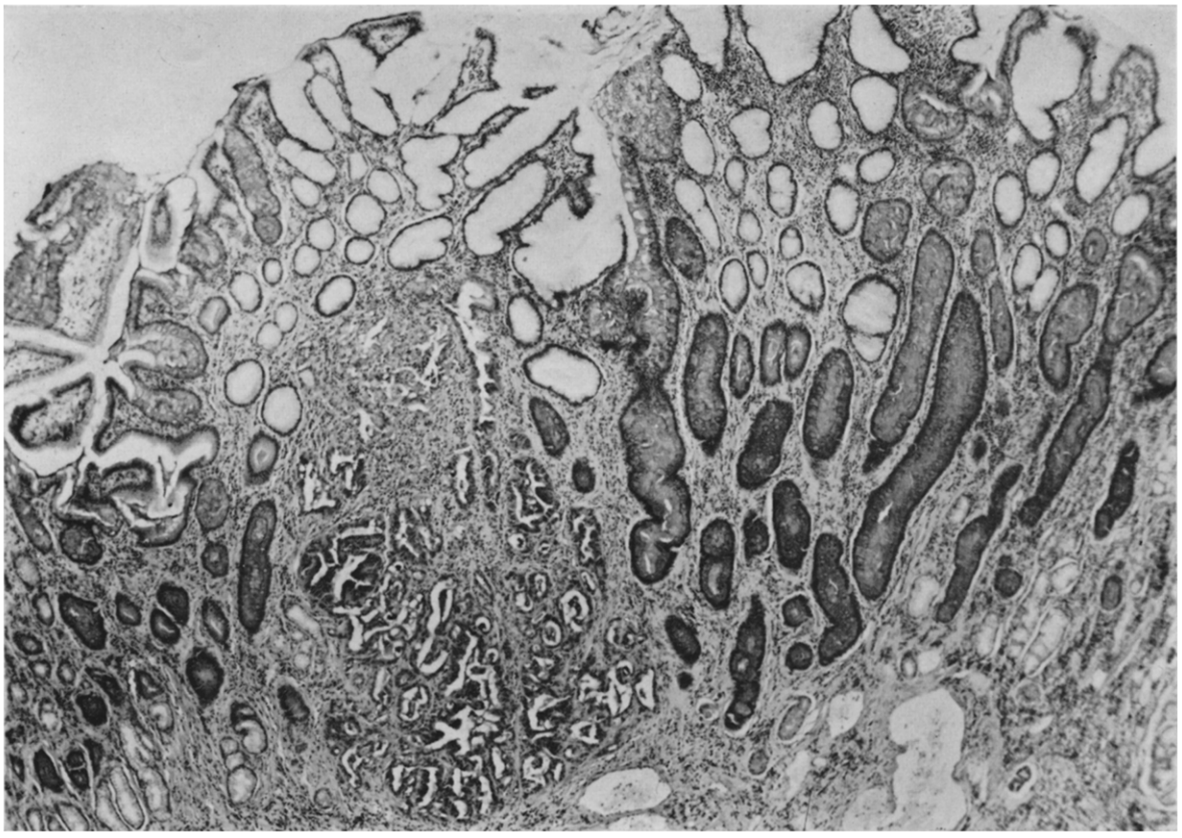


Fig. 8. *Section of glandular tissue at the border of the malignant growth showing intensive intestinal metaplasia and regions of carcinomatous change in the glandular structures. Haematoxylin and mucicarmine.* $\times 50$.

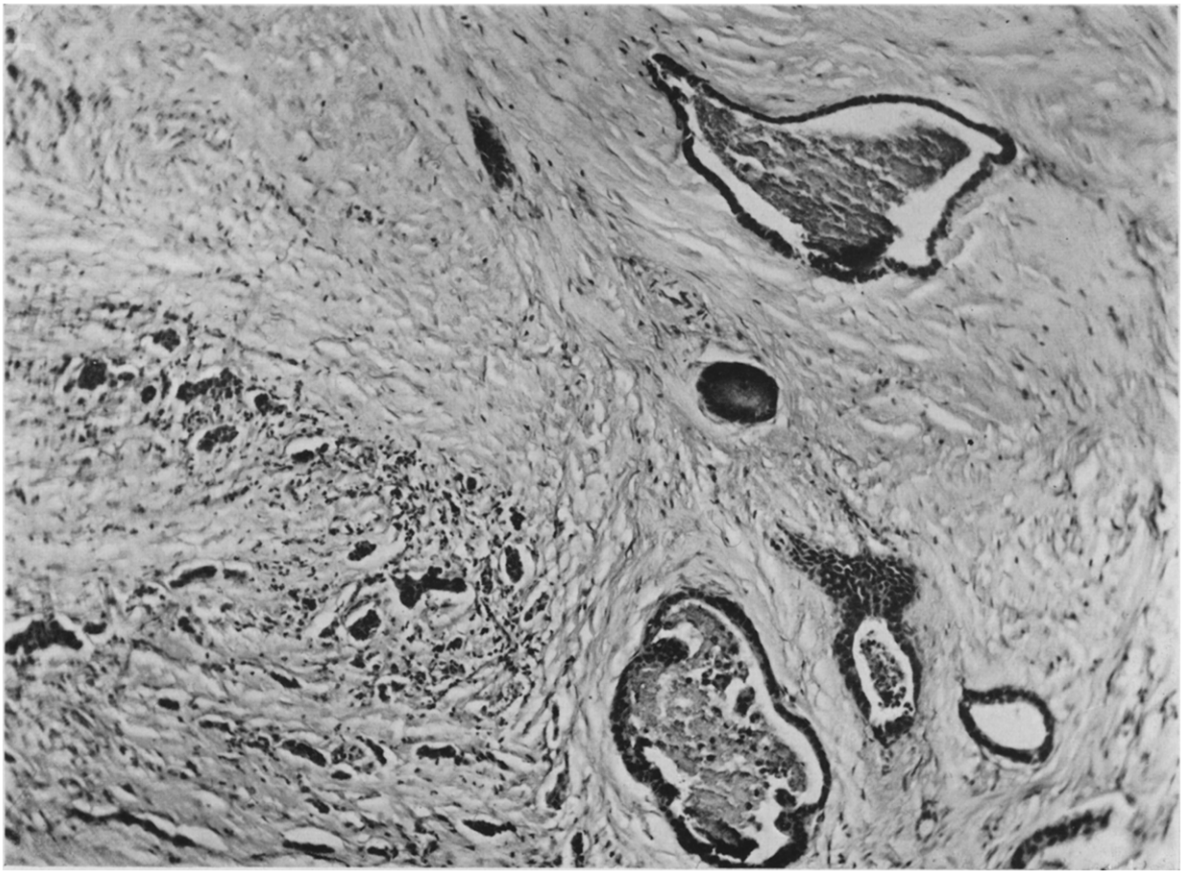


Fig. 9. *Section of the gastric carcinoma exhibiting varying degrees of differentiation. Haematoxylin and mucicarmine.* $\times 120$.

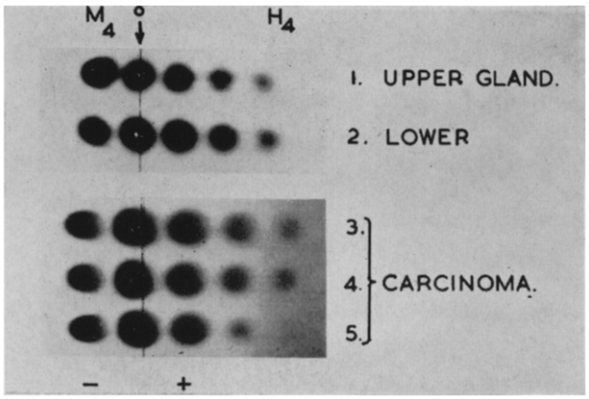


Fig. 10. *LDH isoenzyme patterns present in intestinalised mucosa at the margins of the malignant growth* (*bands* 1 *and* 2) *and in regions of varying degrees of differentiation within the tumour* (*bands* 3–5).

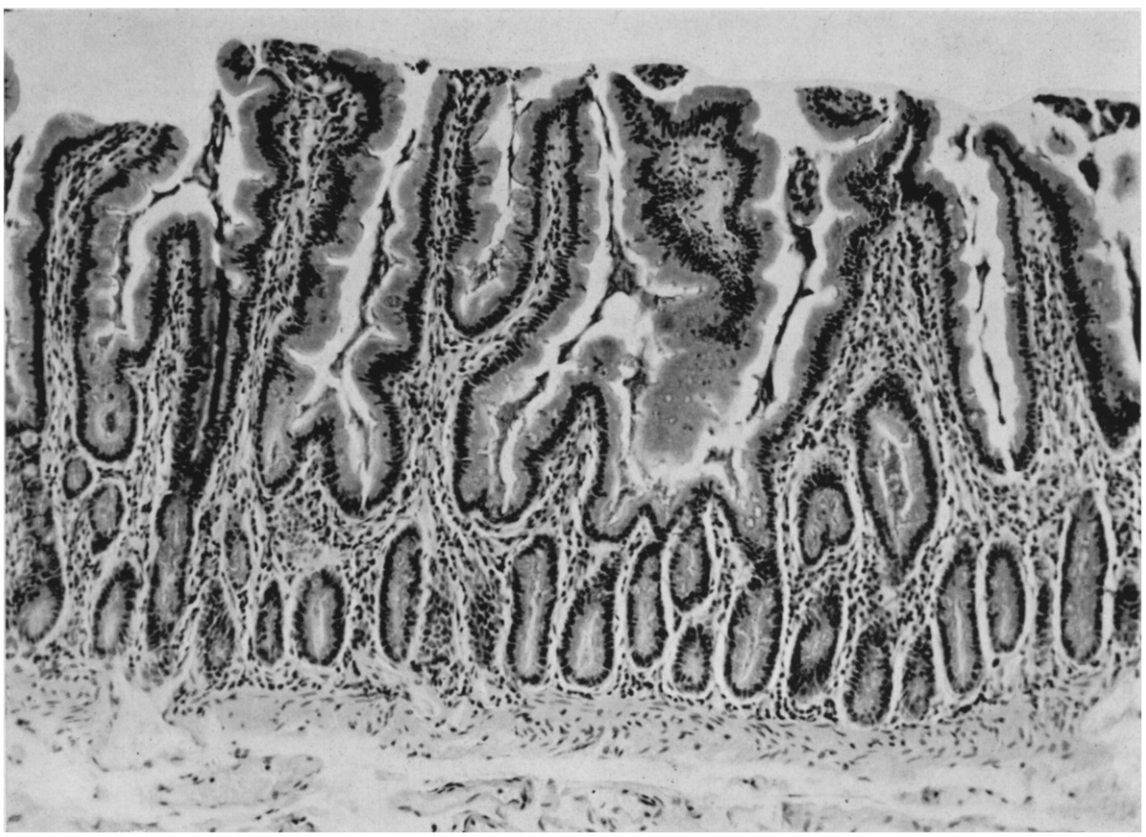


Fig. 11. *Section of normal jejunal mucosa. Haematoxylin and mucicarmine.* × 120.

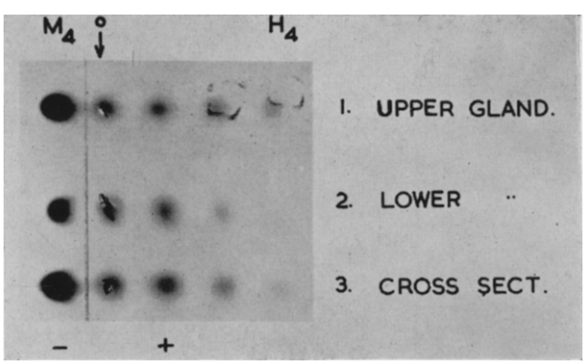


Fig. 12. *LDH isoenzyme patterns present in normal jejunal mucosa.*

RESUME

La distribution histologique d'isoenzymes de la déshydrogénase de l'acide lactique dans la muqueuse gastrique humaine normale et pathologique a été étudiée qualitativement à l'aide d'une technique microélectrophorétique, en employant le système de la congélation des coupes de tissu.

La subunité M *prédomine dans les régions de métaplasie intestinale de l'estomac, tandis que la subunité* H *est le composant principal dans la muqueuse de l'estomac normal. La distribution des isoenzymes observées dans des régions de métaplasie intestinale ressemblent beaucoup à celles observées dans le carcinome gastrique, ce qui renforce l'hypothèse que la métaplasie intestinale est une lésion précancéreuse de l'estomac humain.*

SUMMARY

The histological distribution of lactic acid dehydrogenase isoenzymes in normal and diseased human gastric mucosa has been studied qualitatively by a microelectrophoretic technique employing freeze-dried tissue sections.

Regions of intestinal metaplasia in the stomach exhibit patterns in which the M*-subunit predominates in contrast to the normal stomach where the* H*-subunit was the major component. The isoenzyme patterns observed in regions of intestinal metaplasia closely resembled those observed in gastric carcinoma supporting the contention that intestinal metaplasia is a precancerous lesion in the human stomach.*

ZUSAMMENFASSUNG

Die histologische Verteilung von Lactatdehydrogenaseisoenzymen in normaler und erkrankter menschlicher Magenschleimhaut wurde durch eine mikro-elektrophoretische Technik qualitativ untersucht, wobei lyophilisierte Gewebeschnitte verwendet wurden.

Darmartig-entartete Magenteile zeigen ein Vorherrschen von "M"*-Einheiten im Gegensatz zu dem normalen Magen, wo die* "H"*-Einheiten den Hauptbestandteil darstellen. Die Verteilung der Isoenzyme in den darmartig-entareten Teilen sah derjenigen in dem Magenkrebs sehr ähnlich, woraus der Schluss gezogen werden könnte, dass die darmartige Metaplasie der Magenschleimheut eine präkanzeröse Läsion des menschlichen Magens ist.*

REFERENCES

1. E. Appella and C. L. Markert, Dissociation of lactate dehydrogenase into subunits with guanidine hydrochloride, *Biochem. Biophys. Res. Comm.* **6,** 171–176 (1961).
2. E. Appella and C. L. Markert, Physiochemical properties of purified isoenzymes of lactate dehydrogenase from different sources, *Fed. Proc.* **21,** 253 (1962).
3. T. Wieland and G. Pfleiderer, Chemical differences between multiple forms of lactic acid dehydrogenase, *Ann. N.Y. Acad. Sci.* **94,** 691–700 (1961).
4. C. L. Markert and F. Møller, Multiple forms of enzymes: tissue ontogenic and species specific patterns, *Proc. Nat. Acad. Sci.* **45,** 753–763 (1959).
5. R. D. Cahn, N. O. Kaplan, L. Levine and E. Zwilling, Nature and Development of lactic dehydrogenase, *Science* **136,** 962–969 (1962).
6. K. Rajewsky, S. Avrameas, P. Grabar, G. Pfleiderer and E. Wachsmuth, Immunologische Spezifität von Lactatdehydrogenase. Isozymen dreier Säugetier-Organismen, *Biochim. Biophys. Acta.* **92,** 248–259 (1964).
7. P. G. W. Plageman, K. F. Gregory and F. Wroblewski, The electrophoretically distinct forms of mammalian lactic dehydrogenase I. Distribution of lactic dehydrogenase in rabbit and human tissues, *J. Biol. Chem.* **235,** 2282–2287 (1960).
8. P. G. W. Plageman, K. F. Gregory and F. Wroblewski, The electrophoretically distinct forms of mammalian lactic dehydrogenase II. Properties and interrelationships of rabbit and human lactic dehydrogenase isoenzymes, *J. Biol. Chem.* **235,** 2288–2293 (1963).

9. D. T. Lindsay, Isoenzymic patterns and properties of lactate dehydrogenase from developing tissues of the chicken, *J. Exp. Zool.* **152,** 75–89 (1963).
10. R. A. L. McBeth and J. F. Bekesi, Oxygen consumption and anaerobic glycolysis in human malignant and normal tissue, *Cancer Res.* **22,** 244–48 (1962).
11. G. Pfleiderer and E. D. Wachsmuth, Alters-und funktionsabhängige Differenzierung der Lactatdehydrogenase Menschlicher Organe, *Biochim. Z.* **334,** 185–198 (1961).
12. R. D. Goldman, N. O. Kaplan and T. C. Hall, Lactic dehydrogenase in human neoplastic tissue, *Cancer Res.* **24,** 389–399 (1964).
13. R. Richterich and A. Burger, Lactic dehydrogenase isoenzymes in human cancer cells and malignant effusions, *Enzymol. Biol. Clin.* **3,** 65–72 (1963).
14. H. A. Zondag, Activity of Lactate Dehydrogenase in Patients with Malignant Tumours of the Testis and Ovary, in *Enzymes in Clinical Chemistry*, pp. 120–129. Edited by R. Ruyssen and L. Vandendriessche. Elsevier, Amsterdam (1965).
15. R. Yasin and F. Bergel, Lactic acid dehydrogenase isoenzyme patterns in human normal and malignant gastric mucosa, *Europ. J. Cancer* **1,** 203–209 (1965).
16. O. H. Lowry, The quantitative histochemistry of brain, *J. Histochem. Cytochem.* **1,** 420–428 (1953).
17. R. J. Wieme, An improved technique for agar-gel electrophoresis on microscope slides, *Clin. Chim. Acta.* **4,** 317–321 (1959).
18. I. H. Fine, N. O. Kaplan and D. Kuftinec, Developmental changes in mammalian lactic dehydrogenases, *Biochemistry* **2,** 116–121 (1963).
19. J. L. Strominger and O. H. Lowry, The quantitative histochemistry of brain IV. Lactic, malic and glutamic dehydrogenases, *J. Biol. Chem.* **213,** 635–646 (1955).
20. O. H. Lowry, N. R. Roberts and C. Lewis, The quantitative histochemistry of the retina, *J. Biol. Chem.* **220,** 879–892 (1956).
21. D. E. H. Tee, H. Wang and J. Watkins, A cryostat method for the extraction of soluble tissue components. *Nature, Lond.* **204,** 682–4 (1964).
22. H. A. Magnus, Observations on the presence of intestinal epithelium in the gastric mucosa. *J. Path. Bact.* **44,** 389–398 (1937).
23. B. C. Morson, Intestinal metaplasia of the gastric mucosa. *Brit. J. Cancer* **9,** 365–376 (1955).
24. B. C. Morson, Carcinoma arising from areas of intestinal metaplasia in the gastric mucosa. *Brit. J. Cancer* **9,** 377–385 (1955).
25. H. Teir and T. Räsänen, A study of mitotic rate in renewal zones of non-diseased portions of gastric mucosa in cases of peptic ulcer and gastric cancer with observations on differentiation and so called 'intestinalisation' of gastric mucosa. *J. Nat. Cancer Inst.* **27,** 949–971 (1961).
26. H. Teir, Mitotic rate and cell differentiation in the diseased human gastric mucosa. *Gastroenterology* **44,** 536–539 (1963).
27. L. W. Wattenberg, Histochemical study of aminopeptidase in metaplasia and carcinoma of the stomach. *Arch. Path.* **67,** 281–286 (1959).
28. H. T. Planteydt and R. G. J. Willighagen, Enzyme histochemistry of the human stomach with special reference to intestinal metaplasia. *J. Path. Bact.* **80,** 317–323 (1960).
29. O. Järvi and P. Laurén, On the role of heterotopias of the intestinal epithelium in the pathogenesis of gastric cancer. *Acta. Path. Microbiol. Scand.* **29,** 26–44 (1951).
30. K. B. Taylor, I. M. Roitt, D. Doniach, K. G. Couchman and C. Shapland, Autoimmune phenomena in pernicious anaemia: gastric antibodies. *Brit. Med. J.* **2,** 1347–1352 (1962).
31. N. F. Cogill, D. Doniach, I. M. Roitt, D. L. Mollin and A. Wynn Williams, Autoantibodies in simple atrophic gastritis, *Gut* **6,** 48–56 (1965).
32. G. B. J. Glass, The natural history of gastric atrophy. *Amer. J. Dig. Dis.* **10,** 376–398 (1965).

Europ. J. Cancer Vol. 1, pp. 217–219. Pergamon Press 1965. Printed in Great Britain

Patterns of Lactic Acid Dehydrogenase Isoenzymes in Normal and Malignant Human Tissues

NIS I. NISSEN and L. BOHN

Fibiger Laboratory, Copenhagen and the Central Laboratory, Sundby Hospital, Copenhagen, Denmark

SINCE the demonstration by Warburg in 1923 [1] of a high glycolytic capacity of tumor cells and the demonstration by Cori and Cori in 1925 [2] and Warburg in 1926 [3] of a high rate of glycolysis in malignant tumors *in vivo*, much work has been devoted to this abnormality, which still represents the major biochemical difference between normal and malignant tissues. A focus of interest in that work has been one of the key-enzymes in glycolysis, the lactic acid dehydrogenase (LDH), which reversibly catalyses the conversion of pyruvate to lactic acid. Hill and Levi [4] as the first found this enzyme increased in serum of patients with advanced malignant tumors, and high levels have since been demonstrated in effusions around tumor cells, in animals with transplanted tumors and in some cases in the tumor tissue itself.

The demonstration of heterogeneity of the LDH [5–7] has now made it possible not only to characterize differences between cells in a quantitative way as to the total LDH activity, but also qualitatively as to the LDH isoenzyme pattern.

In the present work an attempt has been made to compare LDH isoenzyme patterns in surgical biopsies of human normal and malignant tissues.

METHODS

Biopsies of human tissues were obtained during operations, usually from a tumor and from the corresponding non-involved normal tissue.

The specimens were kept at 4°C and after a delay of 2–4 hr — in a few cases 24 hr — minced, washed with barbital buffer and homogenized in an Ultra-Turrax homogenizer for 2 min. After centrifugation the supernatant was used for agar-electrophoresis in a way slightly modified from that of Wieme *et al.* [8]. 10 µl samples were placed in the slits in Difco Noble agar (buffered to pH 8·6 with diemal-buffer), covering microscopic slides. The slides were placed on a special watercooled tank (AB Analysteknik, Sweden) and the electrophoresis was done at 1 200 V for 25 min. Using a simple rubber frame the two substrate mixtures containing cyanide, sodiumlactate, DPN, phenazine methosulphate and nitroblue tetrazolium in agar were poured out on each slide. Following incubation at 37°C for 90 min the slides (now showing blue enzyme bands) were fixed in acetic acid-ethanol, dried and scanned in a Densicord (Photovolt) at 530 nm. The bands were numbered from the anode and their enzymatic activities were expressed as per cent of the total activity. All samples were run as duplicates. The standard deviation of the duplicates was less than 4 per cent.

RESULTS

Table 1 summarizes the LDH isoenzyme patterns determined in forty tissue biopsies

from twenty patients. The figures indicate mean values of the relative activity in each enzyme fraction in each group. The two fractions with highest activity in the group have been underlined.

It is rather obvious that for gastric carcinoma there is a displacement in the main activity from band I and II to band II and III. The same difference is found between non-invaded colon-mucosa and colon-carcinoma and between normal and malignant prostatic tissue.

Normal mammary tissue is difficult to obtain due to the heavy infiltration with fatty cells. The normal values therefore rather represent a mixture of adipose and mammary tissues. Compared with this the malignant tissue clearly has a more cathodic pattern.

The normal kidney tissue is from the cortex. It can be discussed if this is the tissue of origin for the hypernephroma's which form the malignant group. Medullary kidney tissue has been shown to have a more cathodic LDH pattern [9].

In three lung carcinomas the isoenzyme pattern was not different from that of the corresponding normal tissue. However the number of cases in this group as in the other groups are still too small for final evaluation. Statistical analysis will be presented in a later, enlarged material.

Preliminary results from studies (with P. Briand) on primary tissue cultures of human normal cells, indicate a similar displacement of the peak activity in the LDH isoenzyme pattern against the cathodic end during cultivation. Studies are now in progress in which these findings are correlated to growth characteristics and metabolic rates.

Table 1. LDH isoenzyme pattern in normal and malignant human tissues. Mean values from forty biopsies in twenty patients. The two fractions with highest activity in each group have been underlined.

Tissue		LDH Isoenzymes per cent activity in fraction				
		I	II	III	IV	V
Stomach	Normal	48	24	20	8	0
	Carcinoma	21	35	30	7	7
Colon	Normal	22	62	17	0	0
	Carcinoma	19	40	41	2	0
Mamma	Normal	30	71	0	0	0
	Carcinoma	15	41	44	0	0
Kidney	Normal (cortex)	35	35	23	7	1
	Carcinoma	13	22	32	33	0
Lung	Normal	39	57	4	0	0
	Carcinoma	32	59	10	0	0
Prostata	Normal	28	45	26	0	0
	Carcinoma	14	42	43	0	0

DISCUSSION

In recent years evidence has been presented that two types of LDH, different with respect to amino acid composition, catalytic characteristics, physical and immunological properties, exist in most animals [9, 10]. The synthesis of both types, which seems regulated by separate genes, can be influenced rather easily by external factors such as hormones and oxygen tension [11]. As well the H-type (H for heart) as the M-type (M for muscle) consist of 4 subunits and hybrids contain both M and H units. The five possible combinations H_4, H_3M, H_2M_2, HM_3, M_4 correspond to the five demonstrable bands in the enzyme-electrophoresis.

Goldman, Kaplan and Hall [12] examined with a direct assay H and M type LDH in human tumors and corresponding normal tissues and could show a consistent shift in the tumors against the M-forms. This corresponds well with our findings of a shift in the isoenzyme bands demonstrable in agar-electrophoresis towards the cathodic end in tumors.

The significance of this finding is not yet clear. As pointed out by Kaplan and his group [9–12] the functional differences between the M and H forms of LDH, especially with respect to pyruvate inhibition, makes the M-form an enzyme more suitable for glycolytic metabolism and the H-form more suitable for respiratory metabolism. Thus the shift towards the M-form in the malignant tissues could be related to their higher rate of glycolysis.

Another possible explanation of the changes in the tumors could be the low oxygen tension, that often has been recorded here, in line with the finding that the synthesis of H and M-forms of LDH are dependent on oxygen tension [11].

RESUME

Les isoenzymes de la déshydrogénase lactique furent étudiées, par une méthode d'électrophorèse sur agar, dans des homogénates de tissus humains, normaux et cancéreux, obtenus par biopsies chirurgicales. A une exception près, le sommet de l'activité, dans la distribution des isoenzymes, se trouvait plus près de la cathode pour les tumeurs que pour les tissus normaux correspondants. La signification de cette observation est discutée.

SUMMARY

Lactic acid dehydrogenase isoenzymes determined with an agar-electrophoretic method were studied in homogenates of human normal and malignant tissues obtained by surgical biopsies. With a single exception the peak activity in the isoenzyme pattern was more cathodic in the tumors than in the corresponding normal tissues. The significance of these findings is discussed.

ZUSAMMENFASSUNG

Die Isoenzyme der Milchsäuredehydrogenase wurden unter Anwendung einer agarelektrophoretischen Methode in Homogenaten von normalen und bösartigen menschlichne Geweben, welche durch Biopsieen erhalten worden waren, bestimmt. Mit nur einer Ausnahme war die Spitzenaktivität in der Verteilung der Isoenzyme mehr in der Richtung der Kathode bei den Tumoren, verglichen mit den normalen Geweben, gelegen. Die Bedeutung dieser Beobachtung wird besprochen.

REFERENCES

1. O. Warburg and S. Minami, Versuche an überlebendem Carcinomgewebe. *Klin. Wchnschr.* **1,** 776–777 (1923).
2. C. F. Cori and G. T. Cori, The carbohydrate metabolism of tumors. II. Changes in the sugar, lactic acid and CO_2-combining-power of blood passing through a tumor. *J. Biol. Chem.* **64,** 11; **65,** 397–405 (1925).
3. O. Warburg, F. Wind and E. Negelein, Uber den Stoffwechsel von Tumoren im Körper. *Klin. Wchnschr.* **5,** 829–832 (1926).
4. B. R. Hill and C. Levi, Elevation of a serum component in neoplastic disease. *Cancer Res.* **14,** 513–515 (1954).
5. J. B. Neilands, Purity of crystalline lactic dehydrogenase. *Science* **115,** 143 (1952).
6. E. S. Vesell and A. G. Bearn, Heterogeneity of lactic and malic dehydrogenase. *Ann. N.Y. Acad. Sci.* **75,** 286–291 (1958).
7. T. Wieland and G. Pfleiderer, Nachweis der Heterogenität von Milchsäuredehydrogenasen verschiedenen Ursprungs durch Trägerelektrophorese. *Biochem. Z.* **329,** 112–116 (1957).
8. R. J. Wieme, M. van Sande, D. Karcher, A. Lowenthal and H. J. van der Helm, A modified technique for direct staining with nitro-blue tetrazolium of lactate dehydrogenase iso-enzymes upon agargel electrophoresis. *Clin. Chim. Acta* **7,** 750–754 (1962).
9. I. H. Fine, N. O. Kaplan, and D. Kuftinec, Developmental changes of mammalian lactic dehydrogenases. *Biochemistry* **2,** 116–121 (1963).
10. R. D. Cahn, N. O. Kaplan, L. Levine and E. Zwilling, Nature and development of lactic dehydrogenases. *Science* **136,** 962–969 (1962).
11. D. M. Dawson, T. L. Goodfriend and N. O. Kaplan, Lactic dehydrogenases. Functions of the two types. *Science* **143,** 929–933 (1964).
12. R. D. Goldman, N. O. Kaplan and T. C. Hall, Lactic dehydrogenase in human neoplastic tissues. *Cancer Res.* **24,** 389–399 (1964).

Europ. J. Cancer Vol. 1, pp. 221–224. Pergamon Press 1965. Printed in Great Britain

Distribution of LDH Isozymes in Mastitis, Fibroadenoma and Carcinoma of the Human Mammary Gland

M. STANISLAWSKI-BIRENCWAJG and F. LOISILLIER

Laboratoire de Chimie des Protéines et Laboratoire d'Immunochimie, Institut de Recherches Scientifiques sur le Cancer, Villejuif (Seine) France

IT HAS been shown [1–7] that the onset of a pathological process may be very sensitively reflected by characteristic alterations of the LDH isozyme pattern. In view of this very basic observation, the determination of the LDH isozyme pattern in disease can have definite diagnostic implications.

In tissues of human neoplastic lesions studied up to date, the LDH 5 ("M type" hybrid) was found to be increased [8]. This aberration has been suggestively associated to impaired respiratory activity of neoplastic tissues [8]. Other workers [9, 10], on the other hand, noted a characteristic predominance of the LDH 3 isozyme in neoplastic tissues, and suggested a reversal to an embryonic [11] LDH pattern.

The present report concerns a study of LDH isozyme patterns of the human mammary gland in cases of mastitis, fibroadenoma and carcinoma.

MATERIALS AND TECHNIQUES

All mammary gland tissue specimens studied here were obtained from surgical interventions. The histological controls were those routinely carried out in the hospitals.

The specimens were frozen (−30°C) within 2 hr after collection from the Paris area hospitals. In some cases fresh unfrozen specimens were assayed immediately upon arrival.

The samples (15 samples were assayed on the same day) were thawed and the affected area was cleared of the apparently unaffected mammary gland tissue adjacent to the lesion. From this adjacent area only gland tissue was selected for analysis after careful dissection of the usually predominating fatty tissue. This gland tissue was called PT, for peritumoral region tissue. PT tissue could, however, only be obtained in cases of epithelioma of the mammary gland (carcinoma of the breast) where the whole breast was available after the surgical intervention.

In all cases, the tissue was finely diced with scissors, admixed to 2 volumes of cold veronal buffer (0·05 M; pH 8·2) and homogenized with an Ultraturrax homogenizer (12,000 rev/min) until complete cell distribution (2 min). The homogenate was then centrifuged at 10 000 rev/min for 30 min, and the supernatant liquid (after removal of the top lipid layer), was immediately subjected to electrophoretic separation in agarose [12] (0·025M veronal buffer; pH 8·2), at 4°C, for 90 min. The electrophoregrams (5 samples/plate) were then developed for enzyme activity [13] using the tetrazolium salt, MTT as final hydrogen ion acceptor. The stained plates were registered densitometrically (with an orange filter) on planimetric paper. The surface of each of the 5 peaks (Fig. 1) representing the LDH isozymes was measured, and the results were

expressed as ratios of activity. One square millimeter on the absorption diagram was arbitrarily chosen to represent one unit of activity.

Total LDH activity of the samples applied at the starting reservoirs (R) of the electrophoretic plates ranged between 900–1600 units. Within this range, the LDH isozyme ratios for a given sample remained constant.

Alternatively, quantitative estimations of total activity were carried out using the spectro-photometric technique of Markert and Appella [14]. These were expressed as units per ml of homogenate supernatant (U/ml).*

RESULTS

The mammary gland lesions studied were mastitis (M), fibroadenoma (Fa), and epithelioma (K) (carcinoma).

Quantitative estimations of total LDH activity done on a few specimens from each of these groups, indicated clearcut differences of enzyme concentration: In 7 epithelioma cases the values range from 35 to 131 U/ml. In 3 cases of this group, the epithelioma tissue was compared to its respective PT region giving the following values: 1. 110–10 U/ml; 2. 131–11 U/ml; 3. 77–31 U/ml (the first value is that of epithelioma, the second of the PT region). In 7 cases of mastitis the values ranged from 6·6 to 23 U/ml; and 2 cases of fibroadenoma gave 2·8 and 17U/ml. These results indicate a significant increase (on a tissue volume basis) in total enzyme activity in cancerous tissue compared to the PT and benign lesion groups (M and Fa.).

The electrophoretic analysis of specimens supplied information regarding qualitative differences among LDH isozymes in these lesions (Table 1). In this table, the specimens were grouped into three categories and their activities were expressed in terms of two ratios; LDH 5/LDH 1 and total activity/LDH 5. It is evident, for instance, that whereas in the K group the activity of LDH 5 is almost 20 times as great as LDH 1, it is only 6 times and 3 times higher in the PT and the M, Fa. groups respectively. Expression of LDH isozyme patterns in terms of ratios of total activity/LDH 5 has also proven to be of statistical significance. It is evident that the activity of the LDH 5 represents practically 1/3 of the total activity in the K group, whereas it is only 1/4 and 1/5 in the PT and M, Fa. groups respectively. Both ratios of activity clearly demonstrate that in epithelioma of the mammary gland the LDH 5 is relatively increased in detriment to LDH 1. In the only case of normal gland tissue studied (a specimen obtained following aesthetic surgery) the LDH 5/LDH 1 was 4·8 and the total activity/LDH 5 was 4·5.

*1 U/ml is arbitrarily defined as an optical density change of 0·1 at 340 mμ in 90 sec/ml$_2$ of homogenate supernatant.

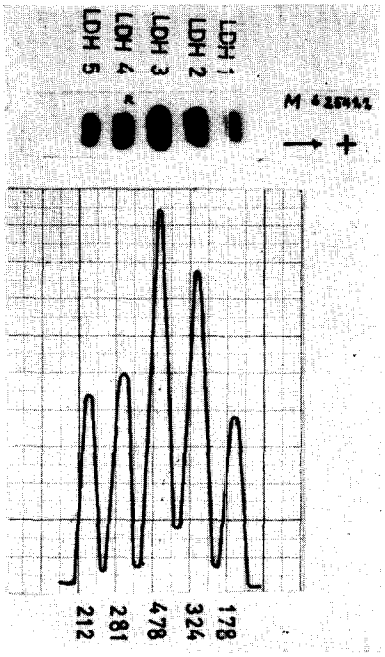
Table 1. *Comparison of iso-*LDH *activity in lesions of the human mammary gland.*

Tissue examined	LDH 5* / LDH 1		Total activity* / LDH 5	
	No. of cases	Mean	No. of cases	Mean
K†	15	19·6 (1·8–63·2)	16	2·8 (1·6–5·8)
PT‡	7	6·33 (2·5–10)	7	3·78 (2·5–6)
M,Fa§	21	3·2 (0·3–5·5)	20	4·6 (3·3–11·2)
	P=0·0001 for group K vs. M,Fa P=0·02 for group K vs. PT		P=0·0001 for group K vs. M,Fa P=0·02 for group K vs. PT	

*Estimated after densitometric reading of electrophoretically separated isozymes revealed by the formazan method (see text).
†Epithelioma of the mammary gland (carcinoma of the breast).
‡Peritumoral region of mammary gland epithelioma.
§Mastitis, and Fibroadenoma of the mammary gland.
Numbers in brackets indicate range values.
The P value was estimated according to Wilcoxon's test of comparison of two paired series.

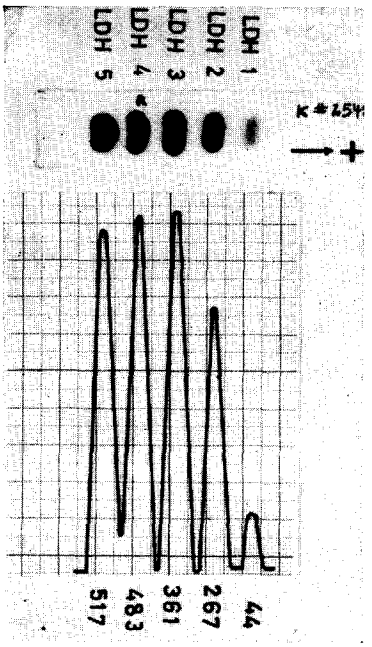
Fig. 1. *Methodology in the study of iso-LDH in lesions of the human mammary gland.*

1. Quantitative estimation of total LDH activity in a sample (see text).



2. Electrophoretic separation in agarose; staining for enzyme activity by the formazan method.

3. Densitometric tracing (orange filter).



4. Analysis of results.

$$\frac{\text{LDH 5}}{\text{LDH 1}} = 1{\cdot}2 \qquad \frac{\text{tot. act.}}{\text{LDH 5}} = 7{\cdot}4$$

$$\frac{\text{LDH 5.}}{\text{LDH 1}} = 11{\cdot}8 \qquad \frac{\text{tot. act}}{\text{LDH 5}} = 3{\cdot}2$$

(to face p. 222)

DISCUSSION

The extreme variations in values in quantitative estimations and particularly in the isozyme ratio values seem to be inherent to human mammary gland. Similar observations were recently reported by Goldman *et al.* [8] in 7 benign and 13 neoplastic mammary gland lesions.

The values exhibited in Table 1 do not seem to be significantly influenced by a possible contamination from serum LDH. Firstly, electrophoretic analysis of several normal human sera and of sera from patients with epithelioma of the mammary gland did not demonstrate the group differences observed when tissues were analysed (the LDH 5 peak could barely be registered densitometrically). In fact, the K sera did not measurably differ from normal sera. Secondly, the K group specimens were macroscopically devoid of blood elements.

In any presentation of results relating to malignant lesions, it is of utmost importance to have available similar estimations done on the normal corresponding tissue, in order to be able to evaluate the significance of such findings. This was one difficulty encountered by Goldman *et al.* [8] in their sampling of epithelioma of the mammary gland specimens. We showed here that comparison of K vs. the benign lesion group (M, Fa.) can be misleading if the latter are considered representative values of the normal corresponding organ. Rather, we consider the PT values as probably more closely resembling normal breast values (the only case studied substantiates this probability).

The presentation of LDH patterns in terms of ratios, strikingly emphasizes the LDH isozyme aberration occuring in neoplastic mammary gland tissue.

Acknowledgements—The authors express their gratitude to Drs. J. Uriel and S. Avrameas for continuous support of the above work, and critical evaluation of this manuscript.

The excellent technical assistance of Mme. M-H. Barsi is acknowledged.

Statistical computations were carried out by M. Lellouch of the Unité de Recherches Statistiques de l'Institut Gustave Roussy, Villejuif, (Seine) France.

RESUME

Dans l'épithélioma de la glande mammaire, l'activité totale de la LDH est augmentée. L'activité de la LDH 5 *est augmentée au détriment de la LDH* 1. *Nos résultats sont conformes à ceux de Goldman* et al. [8] *qui affirment qu'une modification caractéristique de la répartition des isoenzymes de la LDH — avec prédominance de la LDH de type M (LDH* 5) *— se produit dans le tissu cancéreux.*

SUMMARY

In epithelioma of the mammary gland, the total LDH activity is increased. The activity of LDH 5 *is elevated in detriment to LDH* 1. *Our results are in accordance with those of Goldman* et al. [8] *who state that a definite shift in the molecular pattern of LDH with predominance of "M" type LDH (LDH* 5) *occurs in cancerous tissue.*

ZUSAMMENFASSUNG

*In dem Epithelioma der Brustdrüse ist die gesamte LDH-Aktivität gesteigert. Die Aktivität von LDH*5 *ist auf Kosten von LDH*1 *heraufgesetzt. Unsere Resultate stimmen mit denen von Goldman* [8] *überein, der feststellte, dass in Krebsgeweben eine bestimmte Verschiebung in der molekulären Struktur der LDH mit Vorherrschen von LDH des "M-Typs" (LDH*5) *stattfindet.*

REFERENCES

1. C. Manso, K. Sugiura and F. Wroblewski, Glutathione reductase and lactic dehydrogenase activities of tissues of rodents with transplanted tumours. *Cancer Res.* **18,** 682 (1958).
2. A. Meister, Lactic dehydrogenase activity of certain tumours and normal tissues. *J. Nat. Cancer Inst.* **10,** 1263 (1950).

3. G. D. Rees and C. Huggins, Steriod influences on respiration, glycolysis and levels of pyridine nucleotide-linked dehydrogenases of experimental mammary cancers. *Cancer Res.* **20,** 963 (1960).
4. E. D. Wachsmuth and G. Pfleiderer, Alters — und funktionsabhängige Differenzierung der Lactatdehydrogenase menschlicher organe. *Biochem. Z.* **334,** 185 (1961).
5. G. Weber and G. Pfleiderer, Isoenzymes in the human epidermis. *Ann. N.Y. Acad. Sci.* **94,** 933 (1961).
6. R. J. Wieme and Y. van Maercke, The fifth (electrophoretically slowest) serum lactic dehydrogenase as an index of liver injury. *Ann. N.Y. Acad. Sci.* **94,** 898 (1961).
7. F. Wroblewski and K. F. Gregory, Lactic dehydrogenase isozymes and their distribution in normal tissues and plasma and in disease states. *Ann. N.Y. Acad. Sci.* **94,** 912 (1961).
8. R. D. Goldman, N. O. Kaplan and T. C. Hall, Lactic dehydrogenase in human neoplastic tissues. *Cancer Res.* **24,** 389 (1964).
9. N. Dioguardi, A. Agostoni and G. Fiorelli, Lactic dehydrogenase (LDH) molecular forms of myeloblasts, myelocytes and granulocytes. *Prot. Biol. Fluids, 12th Colloquium,* Bruges, p. 278 Elsevier, Amsterdam (1964).
10. W. H. Starkweather and H. K. Schoch, Some observations on the lactate dehydrogenase of human neoplastic tissue. *Biochim. Biophys. Acta* **62,** 440 (1962).
11. W. Gerhardt, B. Ovlisen, J. Clausen and H. Andersen, Differentiation of LDH isozymes during human fetal development. *Prot. Biol. Fluids. 12th Colloquium,* Bruges, p. 203. Elsevier, Amsterdam (1964).
12. J. Uriel, S. Avrameas and P. Grabar, Caractérisation d'enzymes après électrophorèse et analyse immuno-électrophorétique en agarose. *Prot. Biol. Fluids, 11th Col.* Bruges, p. 355 (1963).
13. G. Broun and S. Avrameas, Visualization of different diphospho- and triphosphopyridine nucleotide dehydrogenase isozymes in agar-gel plate. *Nature, Lond.* **197,** 1208 (1963).
14. C. L. Markert and E. Appella, Physiochemical nature of isozymes. *Ann. N.Y. Acad. Sci.* **94,** 678 (1961).

Europ. J. Cancer Vol. 1, pp. 225–231. Pergamon Press 1965. Printed in Great Britain

Activité Mono Amine Oxydase dans les Tumeurs de la Crête Neurale*

A. AMAR-COSTESEC et C. BOHUON
Unité de Biologie Expérimentale, Institut Gustave ROUSSY, Villejuif, France

et O. SCHWEISGUTH
Service de Pédiatrie, Institut Gustave ROUSSY, Villejuif, France

LES TUMEURS de la crête neurale sont remarquables par divers aspects. En particulier, elles sont très souvent "secrétantes." D'importants travaux ont été effectués ces dernières années sur les catécholamines et leurs métabolites retrouvés dans l'urine de malades porteurs de telles tumeurs.

Actuellement, il est nécessaire d'étudier les évènements biochimiques qui se situent dans la tumeur elle-même. Dans un premier travail [1], nous avons discuté la signification possible de la présence de Mono Amine Oxydase (MAO) et de Catéchol O Méthyl Transférase (COMT) dans les Neuroblastomes. Nous n'avions alors étudié qu'un petit nombre de tumeurs. Cette communication rapporte les résultats des dosages de MAO portant sur 33 tumeurs de l'enfant et 3 tumeurs de l'adulte ainsi réparties:

Tableau 1.

Tumeurs de l'enfant:	
Neuroblastomes	24
Ganglioneuroblastomes	4
Ganglioneuromes	5
Tumeurs de l'adulte:	
Pheochromocytomes	3

Le Tableau 2 indique les cas étudiés, l'âge des malades et le diagnostic.

Tableau 2.

		n° de dossier	Age	Sexe
Neuroblastomes				
COU		12/63 7209	2 ans	M
BRA		12/64 3918	8 mois	M
MAR		12/64 1412	1½ mois	F
GRU		12/63 2187	9 mois	F
CAM	1ère tumeur	12/64 2170	8 mois	F
	2ème tumeur		2 ans	
AKR		12/64 2208	4 ans	M
RIT		12/63 6123	3½ ans	F
VIN		12/64 5720	4 ans	F
CAB		12/63 5413	11 mois	F
JES		12/62 2309	2 ans	M
GON		12/64 6562	6 mois	M
FLO		12/63 0270	19 mois	M
SED		12/64 2399	3 ans	M
MART		12/64 3667	4 ans	M
FAU		12/61 2142	3 ans	M
LEC		12/62 3497	2½ ans	M
MAG		12/59 3726	6 ans	M
HOY	1ère tumeur		4 jours	M
	2ème tumeur		21 jours	
HEB		12/65 1040	2 ans	F
FLE		12/58 6519	14 ans	M
MAI		12/63 3765	4 ans	F
OWC			6 mois	F
Ganglioneuromes				
TER			2 ans	F
ROU			13 ans	M
DUV			10 ans	F
SCH			4 ans	M
CAI		12/63 3571	4½ ans	M
Ganglioneuroblastomes				
BAR			2½ ans	F
CAN			4½ ans	F
DOR	1ère tumeur	12/64 0052	3 ans	F
	2ème tumeur		4 ans	
Pheochromocytomes				
NIS			40 ans	F
PEL			34 ans	M
GAS			26 ans	M

*Les Médecins et Chirurgiens des Hôpitaux de Paris, auxquels nous devons une partie du matériel étudié, voudront bien trouver ici l'expression de notre reconnaissance.

MÉTHODES

Prélèvement — Les tumeurs étudiées proviennent surtout d'interventions chirurgicales (33 cas). Les autopsies (3 cas) ont été pratiquées précocement: 3 heures après le décès dans le cas le plus favorable.

Le prélèvement, placé dans un flacon stérile, est porté au laboratoire. Le délai qui s'écoule entre le prélèvement et l'arrivée au laboratoire varie de quelques minutes à deux ou trois heures.

Lorsque cela a été possible, les tumeurs ont été, dès leur prélèvement, plongées dans le mélange carboglace-acétone (−70°C environ).

Diagnostic — Un fragment de la tumeur est réservé au diagnostic. Une partie est destinée à l'histologie, l'autre est mise en culture. La confrontation des deux techniques permet un diagnostic précis [2].

Conservation — Le prélèvement réservé aux déterminations biochimiques est, soit immédiatement exploité, soit conservé à −50°C dans un freezer jusqu'au moment de l'emploi. La décongélation est effectuée à +4°C.

Homogénéisation — Un fragment de tumeur, prélevé dans une zone non nécrotique et d'aspect homogène, est additionné de cinq parties d'eau distillée à +4°C pour une partie de tissus. L'hypotonicité ainsi créée, jointe au broyage, fait éclater les structures cellulaires et libère l'enzyme.

Les opérations suivantes se déroulent en chambre froide. Le tissus, découpé en fragments, est additionné du liquide de dilution. Le tout est placé dans un tube plongeant dans de la glace fondante. On broie (Ultra turax, 20 000 tours-sec) pendant 10 sec. L'opération est répétée, si nécessaire. On passe ensuite à l'homogénéiseur de Potter, sans changer de tube.

Lorsque nous traitons de petites quantités de tissus (volume final avec le liquide de dilution: 1,5 ou 2 ml), nous les découpons en fragments aussi petits que possible avant de les passer au Potter.

L'homogénéisat obtenu est filtré sur gaze. Les déterminations suivantes sont immédiatement pratiquées.

Détermination de l'activité enzymatique — Nous employons la méthode de Weissbach *et al.* [3]. Nous faisons varier les quantités d'enzyme et de substrat pour obtenir des réactions linéaires pendant 30 min. Les déterminations s'effectuent avec et sans semicarbazide (concentration finale 10^{-3}M) afin d'éliminer les effets d'une éventuelle présence de Diamine oxydase. Il n'y a pas de différence notable entre les deux séries de résultats ainsi obtenus.

Dosage des protéines — Les protéines sont dosés par la méthode de Riegler [4] ou par celle de Lowry [5].

Expression des résultats — Les résultats sont exprimés en millimicromôles de substrat (cynuramine) désaminé par minute et par milligramme de protéine.

RÉSULTATS

Les déterminations portent sur 54 fragments de tumeurs dont l'origine est la suivante:

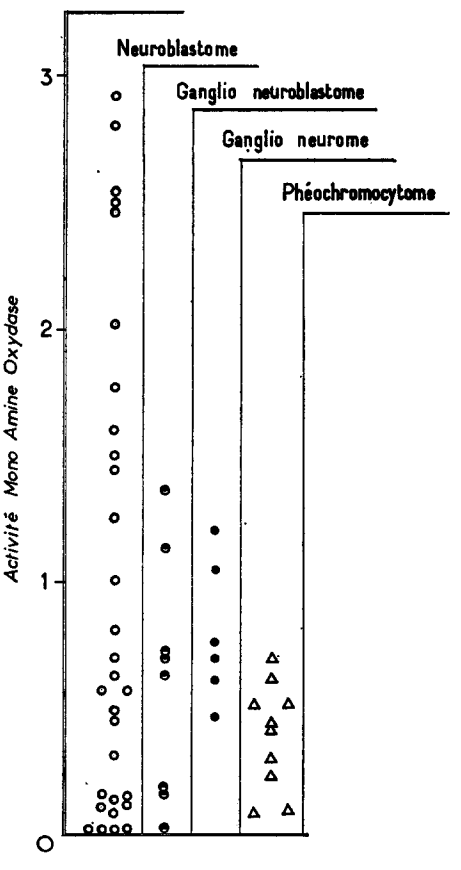
Tableau 3.

Neuroblastomes:	
30 fragments provenant de	24 tumeurs différentes
Ganglioneuroblastomes:	
8 fragments provenant de	4 tumeurs différentes
Ganglioneuromes:	
6 fragments provenant de	5 tumeurs différentes
Pheochromocytomes:	
10 fragments provenant de	3 tumeurs différentes

Les résultats des dosages de MAO sont rapportés dans le Tableau 4.

Afin de pouvoir situer ces valeurs par rapport à celles habituellement trouvées chez l'enfant, nous indiquerons les valeurs suivantes trouvées

Tableau 4.



dans les tissus normaux d'un enfant de deux ans.

Tableau 5.

Foie	3,40
Mitochondries de foie	12,34
Rein	2,75
Mitochondries de rein	8,60
Mitochondries de cerveau	2,20

Les mitochondries étaient préparées par la méthode de Hogeboom *et al.* [6].

Il s'agit là de valeurs moyennes. Nous voyons que certains neuroblastomes possèdent des activités voisines de celles du foie.

DISCUSSION

A. *Critique des dosages d'enzymes dans des fragments de tumeur*

Les dosages d'enzyme effectués sur des fragments de tumeurs ont une signification relative et ne permettent pas une comparaison exacte entre deux tumeurs. Pour faire des comparaisons rigoureuses, il aurait fallu broyer toute la tumeur. L'activité déterminée pourrait alors refléter la quantité de MAO contenue dans la tumeur.

Nous n'avons pu procéder ainsi. D'abord, il faut rappeler que les tumeurs de la crête neurale présentent de larges zones de nécrose. Ensuite, d'autres déterminations devant être faites, un seul fragment en général a pu être réservé au dosage de la MAO.

Pour illustrer cette critique, nous avons découpé un fragment de tumeur en plusieurs morceaux de 0,500 à 1 g (poids de tissus frais). Dans chaque fragment nous avons dosé l'activité MAO. Les résultats sont exposés dans le Tableau 6.

Tableau 6.

BAR	Ganglioneuroblastome					
	0,17	0,69	0,72	0,15		
DUV	Ganglioneurome					
	0,75	1,04				
NIS	Pheochromocytome					
	0,69	0,43	0,51	0,60	0,50	0,40

L'activité MAO varie d'un point à l'autre de la tumeur. Cette donnée n'est pas surprenante et indirectement ces résultats ont été confirmés par histochimie.

Dans un même ordre d'idée, nous avons pu, chez trois malades, doser la MAO dans deux localisations de la tumeur. Voici les résultats obtenus:

Tableau 7.

HEB	Neuroblastome	
	Tumeur rétropéritonéale	1,25
	Ganglion lymphatique satellite envahi	2,80
DOR	Récidive de ganglioneuroblastome thoracique	
	Tumeur pariétale	0,62
	Ganglion envahi	1,13
HOY	Neuroblastome congénital	
	Tumeur intra-rachidienne prélevée à l'âge de 4 jours	1,60
	Tumeur rétropéritonéale préleveé à l'âge de 21 jours	1,44

Chez un malade, nous avons eu la possibilité de doser la MAO dans une première tumeur, puis dans une récidive. Les résultats sont rapportés dans le Tableau 8.

Tableau 8.

CAM	Neuroblastome	
	Tumeur prélevée à l'âge de 8 mois	0,09*
	Récidive métastatique pulmonaire prélevée à l'âge de 2 ans (autopsie)	0,15**

*Homogénéisat.
**Mitochondries.

Les résultats précédents montrent que les indications fournies par les dosages d'enzyme dans les tissus pathologiques doivent être interprétées avec la plus grande prudence.

B. *Relation entre l'activité MAO dans les tumeurs et la secrétion urinaire d'acides phénoliques*

Nous avons recherché une éventuelle relation entre l'activité MAO dans la tumeur et la secrétion d'acide vanillyl mandélique (VMA) et homovanillique (HVA) dans l'urine.

Nous n'avons comparé ici que les excrétions des acides phénoliques sans retenir celle des alcools phénoliques. Bohuon *et al.* ont par ailleurs montré l'étroite corrélation existant entre les excrétions d'acides et d'alcools phénoliques chez les enfants porteurs de tumeurs de la crête neurale [7].

Le schéma suivant rappelle le catabolisme des catécholamines et l'importance de la MAO dans l'apparition des divers catabolites retrouvés dans l'urine.

Pour ce travail, nous avons classé les tumeurs de la crête neurale en quatre catégories (Tableau 9).

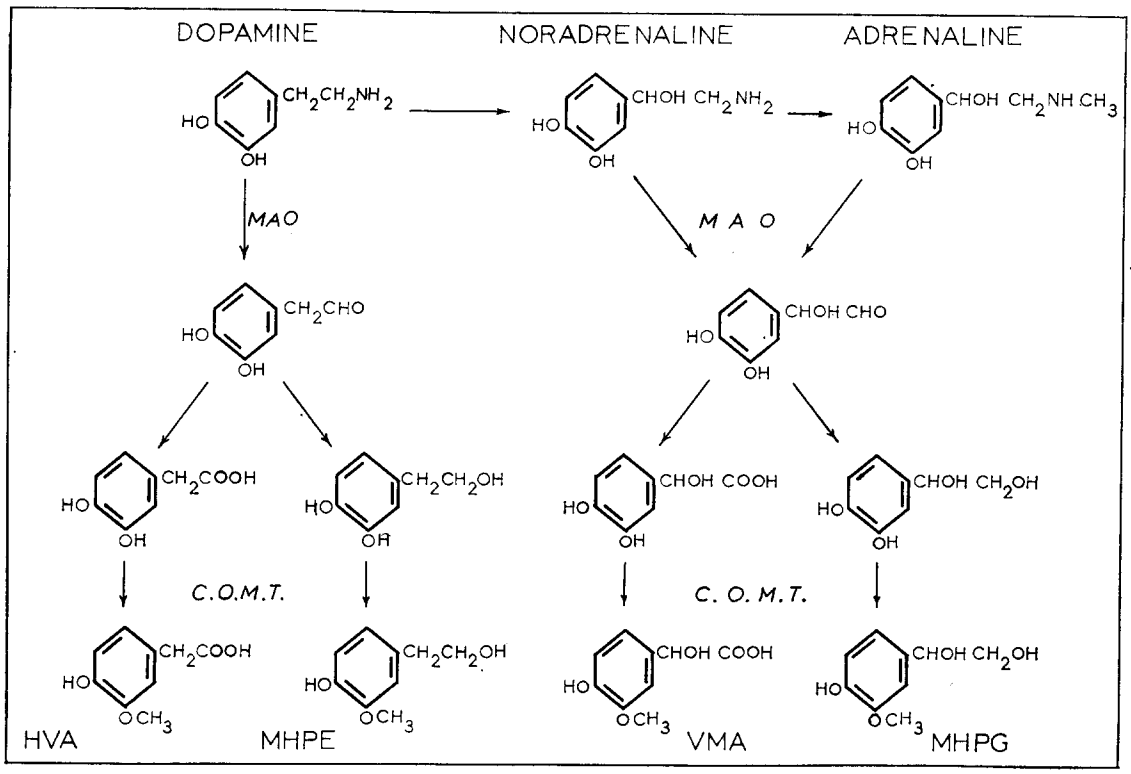


Fig. 1. *Une autre voie existe simultanément où la COMT agit avant la MAO.*
Abréviations: HVA Acide homovanillique
VMA Acide vanillyl mandélique
MHPG Méthoxy hydroxy phényléthylène glycol
MHPE Méthoxy hydroxy phényl éthanol
MAO Mono amine oxydase
COMT Catéchol O méthyl transférase

Tableau 9.

I	Tumeurs non secrétantes	Taux de VMA ou d'HVA dans l'urine inférieurs à 5 µg/mg de créatinine
II	Tumeurs peu secrétantes	Taux n'ayant pas dépassé 20 µg/mg de créatinine
III	Tumeurs moyennement secrétantes	Taux n'ayant pas dépassé 70 µg/mg de créatinine
IV	Tumeurs très secrétantes	Taux atteignant plusieurs centaines de µg/mg de créatinine.

La Fig. 2 montre qu'il n'existe pas de relation nette entre les taux de MAO dans la tumeur et la secrétion des acides phénoliques. Ainsi, chez des malades excrétant peu de VMA ou d'HVA on peut trouver des taux d'enzyme élevés (Colonne I). A l'opposé, des taux d'enzyme faibles peuvent correspondre à une excrétion élevée. (Colonne IV).

Une constatation analogue avait été faite à la suite d'un travail sur l'activité COMT dans les Neuroblastomes [1]. On peut donc penser que l'élimination des acides et des alcools phénoliques dans l'urine de malades porteurs de tumeurs de la crête neurale est influencée par d'autres facteurs que les taux d'enzymes catabolisant présents dans ces tumeurs.

C. *Comparaison entre les taux de MAO dans les neuroblastomes et les phéochromocytomes*

Les phéochromocytomes ont une activité MAO faible par rapport aux Neuroblastomes. C'est ce qui ressort du Tableau 4. Nous n'avons pu étudier que trois phéochromocytomes pour 24 neuroblastomes. Sur les trois tumeurs étudiées, deux avaient une activité MAO pratiquement nulle. Une seule tumeur avait une activité appréciable comprise entre 0,40 et 0,69.

Comment expliquer cette différence d'activité entre phéochromocytomes et neuroblastomes?

Le prélèvement des tumeurs a été fait dans d'excellentes conditions: congélation dans le mélange carboglace-acétone (environ −70°C).

Il est possible que les phéochromocytomes aient une faible activité MAO. On sait, d'autre

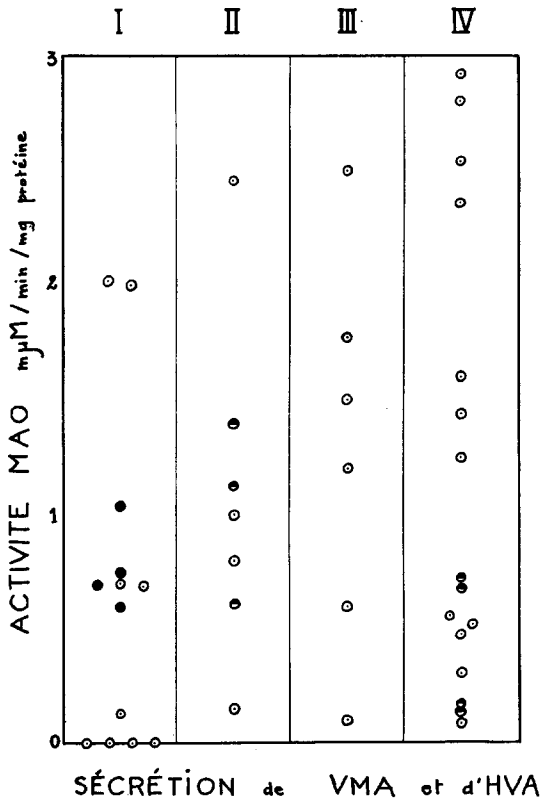


Fig. 2.

part, qu'ils contiennent des taux élevés d'adrénaline et de Noradrénaline [8].

Ces éléments suffisent peut-être à expliquer les accès hypertensifs trouvés chez les malades porteurs de phéochromocytomes. Cependant, la faible activité MAO dans les phéochromocytomes peut s'expliquer autrement. Nous avons montré que les catécholamines sont des inhibiteurs compétitifs de la cynuramine vis-à-vis de la MAO. Il est possible que l'homogénéisation libère des taux d'amine inhibant la réaction de dosage.

D. *Neuroblastomes sans activité MAO*

Enfin, nous évoquerons un dernier point: existe-t-il ou non des neuroblastomes sans MAO?

D'après le Tableau 4, nous avons quatre tumeurs sans activité enzymatique. Comment peut-on expliquer l'absence de MAO dans ces tumeurs?

Les conditions de prélèvement étaient celles que nous utilisons habituellement et qui nous donnent satisfaction. Nous ne retenons donc pas la destruction de l'enzyme.

Le traitement ne semble pas non plus en cause, car aucun des quatre malades n'avait subi de rayons ou de chimiothérapie au moment du prélèvement.

La MAO peut être inhibée par des amines entrant en compétition avec la cynuramine vis-à-vis de l'enzyme. Les neuroblastomes contiennent au moins deux de ces amines en quantité notable: dopamine et tyramine. Nous ferons donc les mêmes réserves que dans le cas des Phéochromocytomes.

L'enzyme peut être masqué ou inhibé par d'autres dérivés que les amines citées ci-dessus. C'est ce que nous essaierons de déterminer par des recherches ultérieures.

Mais il se peut aussi que la MAO ne soit pas présente dans certaines tumeurs.

Dans la mesure où il ne s'agit pas d'une défaillance de méthodologie, le problème de neuroblastomes sans activité MAO nous paraît une question intéressante à approfondir.

RESUME

Les auteurs ont étudié l'activité Mono Amine Oxydase dans 54 *fragments de tumeurs de la crête neurale provenant de* 33 *enfants* (24 *neuroblastomes* 4 *ganglioneuroblastomes*, 5 *ganglioneuromes*) *et* 3 *adultes* (3 *phéochromocytomes*).

A. La répartition de l'enzyme est hétérogène et les valeurs obtenues en différents points de la tumeur varient parfois dans la proportion de 1 à 5. Le dosage des protéines dans les tissus, nécessaire pour calculer l'activité spécifique, peut introduire une cause d'erreur importante.

B. Il ne semble pas y avoir de relation directe entre le taux d'enzyme catabolisant (MAO) dans la tumeur et les taux de catabolites (VMA et HVA) retrouvés dans l'urine.

C. Les phéochromocytomes ont une activité MAO très faible par rapport aux neuroblastomes. La possibilité d'une inhibition compétitive entre le substrat (cynuramine) et les catécholamines libérées par broyage de la tumeur n'est pas exclue.

D. Quatre neuroblastomes ne possédaient pas d'activité MAO. Une inhibition de l'enzyme par la tyramine ou la dopamine libérée par broyage est possible. Il peut aussi s'agir d'un inhibiteur d'une autre nature, d'un masquage de l'enzyme ou d'un type cellulaire différent.

SUMMARY

Mono Amine Oxidase activity in 54 fragments of neural crest tumors from 33 children (24 neuroblastomas, 4 ganglioneuroblastomas, 5 ganglioneuromas) and from 3 adults (3 pheochromocytomas), has been studied by the authors.

A. The repartition of the enzyme is heterogeneous and the values obtained in different parts of the tumors sometimes vary in the proportion of 1 to 5. The estimation of proteins in the tissues, necessary for calculating the specific activity can introduce an important error.

B. It seems there is no direct relationship between the level of catabolic enzyme (MAO) in the tumor and the levels of catabolites (vanillyl mandelic acid and homovanillic acid) found in the urine.

C. Pheochromocytomas have a very low Mono Amine Oxidase activity compared with neuroblastomas. The possibility of a competitive inhibition between the substrate (kynuramine) and catecholamines liberated by disruption of tumor cells is not excluded.

D. No MAO activity was found in four neuroblastomas examined, perhaps for the following reasons:

inhibition of the enzyme by tyramine or dopamine liberated by disruption of cells
an inhibitor of unknown nature
masking of the enzyme
a different cellular type.

ZUSAMMENFASSUNG

Die Autoren haben die Mono Amine Oxydase-Aktivität in 54 Fragmenten von Tumoren der Neuralleiste bei 33 Kindern (24 Neuroblastome, 4 Ganglioneuroblastome, 5 Ganglioneurome) und 3 Erwachsenen (3 Pheochromocytome) studiert.

A. Die Verteilung des Enzyms ist unregelmässig. Die erhaltenen Werte schwanken von 1 : 5 in den verschiedenen Fragmenten desselben Tumors. Die Eiweissbestimmung im Gewebe welche nötig ist zur Berechnung der spezifischen Aktivität, kann eine empfindliche Fehlerquelle sein.

B. Keine direkte Beziehung zwischen der Enzymmenge in Tumor und der Stoffwechselproduktmenge im Urin (Vanillyl mandelic Säure und Homo vanillic Säure) scheint zu bestehen.

C. Pheochromocytome, im Vergleich zu Neuroblastomen, haben eine sehr schwache Aktivität. Es besteht die Möglichkeit einer kompetitiven Inhibition zwischen Substrat (Kynuramin) und, durch Tumorzellsertrümmerung, freigewordene catecholamine.

D. Vier Neuroblastome zeigten keinerlei Mono Amine Oxydase Aktivität. Verschiedene Hypothesen können dazu erwägt werden:

Es ist möglich dass sich, bein Zerreiben des Tumors eine Enzyminhibition einstellt durch freigewordenes Tyramin oder Dopamin.

Es könnte jedoch auch ein anderer, unbekannter Inhibitionsfaktor in Spiele sein.

Das Enzym könnte maskiert sein.

Die Tumorzellen könnten anderswie differenziert sein.

BIBLIOGRAPHIE

1. C. Bohuon, E. H. La Brosse, A. Amar-Costesec and M. Assicot, Réflexions sur le dosage de la COMT et de la MAO dans les Neuroblastomes. Premiers résultats expérimentaux. In: *Neuroblastomas. Biochemical Studies*, Ed. RFA. Springer, Berlin (1965).
2. J. Behleradek, G. Barski and R. Gerard Marchant, La culture de tissu *in vitro*, auxiliaire de l'histodiagnostic des tumeurs humaines. *Arch. Anat. Path.* Sous presse.
3. H. Weissbach, T. E. Smith, J. W. Daly, B. Witkop and S. Udenfriend A rapid spectrophotometric assay of monoamine oxidase based on the rate of disappearance of kynuramine. *J. Biol. Chem.* **235,** 1160 (1960).
4. E. Riegler, Eine kolorimetrische bestimmungs Methode des Eiweisses. *Z. Anal. Chem.* **53,** 242 (1914).
5. O. H. Lowry, N. J. Rosenbrough, A. L. Farrand and R. J. Randall, Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193,** 263 (1951).
6. G. H. Hogeboom, W. C. Schneider and G. E. Palade, Cytochemical studies of mammalian tissues. I. Isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particulate material. *J. Biol. Chem.* **172,** 619 (1948).
7. Bohuon et al. Travaux non publiés.
8. R. Robinson, The Clinical Chemistry of Pheochromocytomas. In: *The Clinical Chemistry of Monoamines* p.63 Elsevier, (1962).

Europ. J. Cancer Vol. 1, pp. 233–243. Pergamon Press 1965. Printed in Great Britain

The Role of Chromosomes in the Characterisation of Human Neoplasms

D. T. HUGHES

Department of Cytogenetics,
Chester Beatty Research Institute,
Institute of Cancer Research: Royal Cancer Hospital,
London

THE IMPORTANCE of chromosomes in the characterisation of neoplasms depends on the role that they play in malignant transformation and progression of tumours. The role of chromosomes in these processes is still not fully understood although many advances have been made since 1914, when Boveri first put forward his chromosome mutation theory for the cause of cancer, thereby stimulating many experimental investigations on tumour mitosis and chromosome patterns [1].

Knowledge of chromosome patterns has advanced with improvements in technique, notably the hypotonic treatment for spreading chromosomes [2] to allow accurate counting and analysis. With the exception of bone-marrow, actively dividing cells are not readily accessible for direct examination of chromosomes. For this reason, short term tissue culture techniques have also made a tremendous contribution to the study of human chromosomes by creating conditions conducive to cell division in tissues from foetuses, biopsies from surgical operations, skin [3, 4] or blood [5].

Since the normal chromosome number of human cells ($2n=46$) was not established before 1956 (Tjio and Levan [6] studied foetal lung cultures), all previous counts in normal and tumour cell populations are regarded as inaccurate, although they clearly showed general trends in ploidy level.

In order to assess the significance of chromosome anomalies in tumour cell populations it is necessary to make a comparison with the extent of chromosome variations in normal tissues of different persons.

1. *Chromosome number*

Variations deviating from the diploid number $2n=46$, have been found in both normal and tumour tissues:

i. *Differentiated tissues* — (Normal and non-malignant pathological conditions).

Levan [7] reviewed the literature for eighteen human tissues and found that the cells were predominantly diploid, ranging from 66 per cent diploid (marrow) to 100 per cent diploid (in lung). Aneuploid cells usually occur in most carefully examined tissues, due to errors in cell division which probably do not proliferate because abnormal clones are not normally found [8].

The incidence of aneuploidy in somatic cells may be related to age: Jacobs *et al.* [9] found a significant increase in aneuploidy of sex chromosomes with increasing age.

A variety of aneuploid individuals have been discovered in human populations. The first one to be found had forty seven chromosomes [10]. Subsequently many aneuploid congenital deviants have been described, involving abnormal numbers of sex chromosomes, such as

XO, XXX, XXXX and (or) other chromosomes [11, 12]. The largest aneuploid number was first found in a boy with forty nine chromosomes [13]. Other cases have been found to be mosaics of diploid and triploid [14] or diploid and aneuploid cells of different aneuploid numbers [15].

Evidence has accumulated to suggest that at least some forms of aneuploid congenital conditions may have a predisposition for the development of neoplasms that may be triggered off by some other factors. Stewart [16] found that acute leukaemia occurred nearly eighteen times more frequently in Down's syndrome (mongolism) than in the general population. In addition, it has been suggested that some genes may affect chromosome non-disjunction, causing the occurrence of several types of aneuploid individuals and leukaemia in the same families [17, 18]. Hauschka *et al.* [19] found familial non-disjunction without leukaemia, possibly because offspring died too soon from other causes.

ii. *Chromosome number in human neoplasms* — In cell populations, chromosomes are an indication of genetic stability or variability. Levan [7] tabulated 40 human cell lines, cultured *in vitro*, half derived from normal tissue and half from cancers. Both had a similar heteroploid chromosome distribution. The highest stemline for a cultured line was 133 whereas no human tumour *in vivo* was known to have a higher stemline than 92. Most human tumours had stemlines in the diploid-triploid range.

Hauschka [20] found that out of 73 malignant human ascites tumours, nine (12%) were diploid or pseudodiploid and 64 (88%) had aneuploid modes; on the other hand 13 benign effusions all had diploid or pseudodiploid modal chromosome numbers. A further analysis of 74 tumours (including 13 types) examined directly (without colchicine or culturing) indicated that 69 (93%) had aneuploid modes, while 5 tumours (7%) had a diploid modal chromosome number. Thirty-four cases of leukaemic bone marrow (63%) out of 56 also had aneuploid or pseudodiploid modes while 22 cases (37%) had apparently normal diploid modes. Hauschka [20] concluded that "Leukoses, on the whole exhibit a near diploid trend whereas the patterns of neoplasms from other tissues extend over the entire range of viable chromosome compliments".

Ishihara *et al.* [21] described twenty cancer effusions which all had abnormal modal chromosome numbers but these may not reflect those of the original tumour.

Table 1. *Ploidy levels.*
(x=numbers 1-11)

	$(2n-x)$	$(2n)$	$(2n+x)$	$(3n-x)$	$(3n)$	$(3n+x)$	$(4n-x)$	$(4n)$	$(4n+x)$	Total cells
	%	%	%	%	%	%	%	%	%	1,282
Gastric carcinoma (30 cases)	4·2	8·7	14·3	41·0	3·1	18·2	5·1	0·6	4·6	
Uterine carcinoma (13 cases)	6·1	1·3	35·8	23·6	2·2	17·9	4·4	0	8·7	229
Rectal carcinoma (4 cases)	20·0	20·0	18·7	11·2	0	3·7	20·0	0	6·2	80
Mammary carcinoma (2 cases)	0	0	8·0	44·0	0	40·0	4·0	0	8·0	25
Ovarian carcinoma (2 cases)	4·1	38·8	5·8	1·6	0	0·8	34·7	2·4	11·6	121
Ascites hepatoma (1 case)	0	0	0	5·0	5·0	85·0	5·0	0	0	20

Calculated from the data of Makino *et al.* [22].

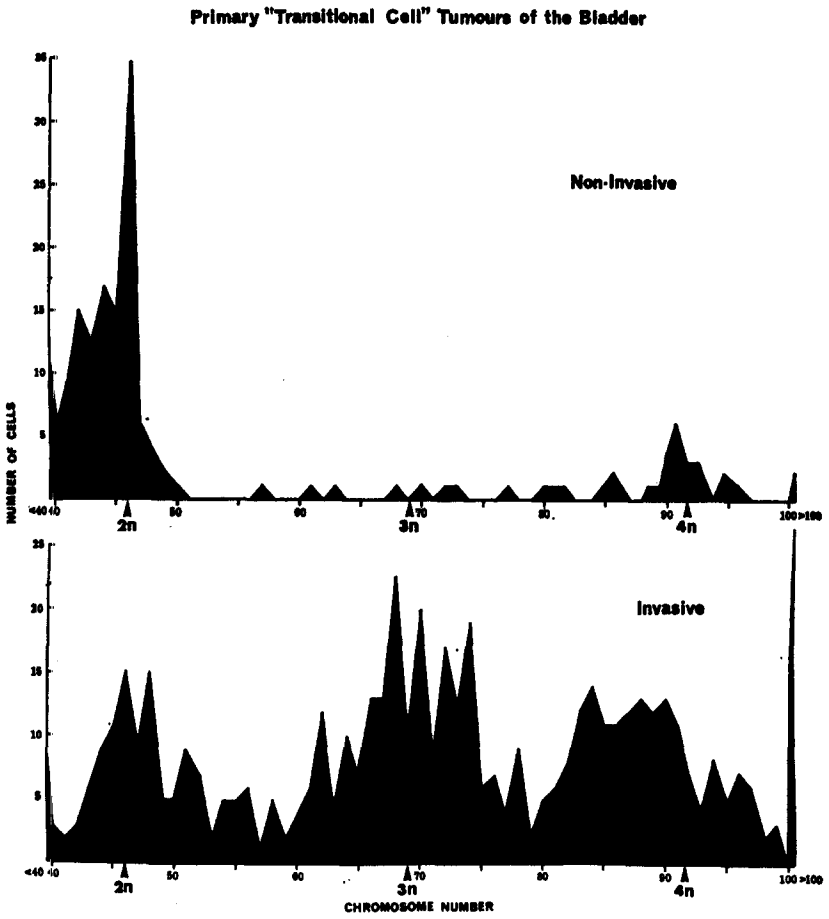


Fig. 1. *Histogram of chromosome number distributions in primary 'transitional cell' tumours of the bladder, including total cells counted in nine non-invasive and twenty one invasive tumours (by courtesy of D. Lamb)*

Makino *et al.* [22] found that many tumours from different sites had characteristic modal chromosome numbers. The majority of these tumours were primary tumours. Table 1 shows the ploidy levels of tumours at six sites, calculated from the data of Makino *et al.* It can be seen that the most frequent chromosome numbers are found at different ploidy levels in the various tumours e.g. the gastric carcinomas are mainly hypotriploid ($3n$-x) whereas rectal carcinomas are mainly distributed in the hypo- to hyper-diploid range and also in the hypo-tetraploid level. There is considerable evidence to suggest that the different ranges of chromosome numbers found in the various neoplasms represent environmental selection of cell variants in the evolution, or progression, of the tumour cell populations towards greater malignancy at the various sites [23]. A general conclusion concerning a characteristic ploidy mode in mammary, ovarian and ascites hepatoma cannot be reached because too few cases were examined.

Very few studies have been made of very early cancer, or premalignant neoplasms, to obtain chromosome data more closely related to the nature of the mechanism of malignant transformation. Information with a bearing on this question has been obtained from studies on cervical lesions [24–27] and on primary tumours of the bladder [28]. Heteroploid chromosome numbers were found in the premalignant lesions of the cervix and premalignant bladder tumours; however, diploid modes were found at these sites. In the cervix the most striking difference in chromosome number was found between dysplasia (with a main range of 40–55) and carcinoma *in situ* (with a range of 40–100) so that considerable aneuploidy was found to occur some time before the onset of malignancy [26] and did not accompany the development of the capacity to invade. It is possible that genetic changes conferring the capacity to invade may remain latent until endocrine changes in the host at menopause enable the transformed cells to invade.

In the bladder the situation seems clearer. Lamb [28] found that extensive changes in chromosome number coincided with the capacity to invade. Nine benign bladder tumours were predominantly in the diploid range

whereas twenty one malignant bladder tumours had chromosome numbers ranging from forty to more than 100 without any common modal ploidy level (see graph Fig. 1). It seems possible to diagnose malignancy in terms of the chromosomes in this situation because over two thirds of the cells in the invasive tumours had chromosome numbers in excess of the diploid range (i.e. from $3n-x$ to $4n+x$). The wide spread in these numbers without a characteristic mode does not rule out the possibility that the primary transformation occurred in the diploid cells, the aneuploid cells being rapidly developing secondary variants in tumour progression. Note that the frequency of the diploid range is as great as the other types (Fig. 1).

The occurrence of primary malignant tumours with diploid modal chromosome numbers shows that aneuploidy is not an universal requirement for malignant transformation. In addition, many diploid stem lines have been reported in primary and even secondary tumours [20, 23]. However, chromosome number is an inadequate criterion of the normality of a chromosome set. Until the chromosomes of cells with a diploid chromosome number have been analysed in detail it cannot be supposed that these cells do not have any chromosome anomaly. The distinction between normal and pseudodiploid karyotypes largely depends on our knowledge of the normal range of variation in chromosome structure

Table 2. Pseudodiploidy.

Material	No. cases examined	Number of Chromosomes per Group in individual cells							References
		A	B	C	D	E	F	G	
Normal female cells		6	4	16	6	6	4	4	
Dysplasia	1 (H.182)	4	4	20	6	5	4	3	Wakonig-Vaartaja and Kirkland [26]
		6	5**	16	6	6	4	3	
		7	3	18	6	6	3	3	
Carcinoma *in situ*	1 (H.210)	6**	4*	16	6	6**	4	4	
		6*	5**	15	6	6	4	4	
		6	5*	13	6	7	4	5	
		7*	5*	15	6	5	5	3	
	1 (Case 1)	7	4	16	6	6	3	4	Boddington *et al.* [25]
	1 (Case 7)	8	4	14	6	6	2	6	
Carcinoma of the cervix	1 (H.70)	6	2	16**	3*	6	4	7 (all*)	Wakonig-Vaartaja [49]
Carcinoma of the corpus uteri	1 (H.96)	6*	4	16	6	6	4	4	
		6	4	16*	6	6*	4	4	
		7*	4	17	5	5	4	4	
Normal male		6	4	15	6	6	4	5	
♂Burkitt tumour (23 cells)	1	5	5*	15*	6	6	4	5	Stewart *et al.* [33]

*grossly abnormal chromosome.

(shape, size, cytochemistry), the size of the anomaly and the accuracy of the technique used:

2. *Karyotype analysis and the recognition of chromosome mutations*

The standard system for the identification of individual chromosomes on the basis of chromosome length and centromere position clearly distinguished seven groups of chromosomes [29, 30]: denoted A, B, C, D, E, F and G. Some or all of the chromosome pairs in groups B, C, D, E, F and G could not be distinguished with certainty by the existing morphological criteria. In 1963, this situation remained largely the same except for identification of the Y chromosome, by its morphology and the X chromosome, by tritiated thymidine [31]. The human karyotype has been found to be a stable pattern consisting of the same number of groups (characterised by their chromosome number and morphology) in all normal cells of normal individuals (with 46 chromosomes). This forms the first level for the recognition of karyotype anomaly:

i. *Chromosome groups* — Male and female cells contain the same number of chromosomes per group in groups A(6) B(4) D(6) E(6) F(6) but differ in groups C and G: Male C(15) G(5), female C(16) G(4), owing to the difference in sex chromosome pairs.

Table 2 shows chromosome group analysis of pseudodiploid cells in primary human neoplasms. The analysed cells in each neoplasm (except the Burkitt Tumour) have different abnormal combinations of chromosomes, indicating that they were not directly derived from the same parent cell. It is not known whether they arose from diploid cells or indirectly by non-disjunction of tetraploid or aneuploid cells. However, cells with forty six chromosomes certainly have a selective advantage in these neoplasms because they form the mode. Structural chromosome abnormalities (especially 'marker' chromosomes) give clues concerning the origin of some of these cells.

ii. *Morphological anomalies* (shape and size) Gross mutations in chromosome size and shape (according to centromere position), due to deletion, inversion or translocation, are readily distinguishable visually as marker chromosomes. Table 2 shows the frequency of marker chromosomes in chromosome groups of pseudodiploid tumour cells. The occurrence of the same marker chromosome in several cells strongly suggests that they originated from the same parent cell (i.e. belong to the same clone), even if these cells have different chromosome group combinations or have different chromosome numbers [32, 25, 26, 33, 27].

In the Burkitt primary tumour, twenty three pseudodiploid cells (modal number) were found with the same chromosome group and marker anomalies indicating that they were derived from the same cell. Furthermore, the chromosome mutation involved, probably occurred in a diploid cell because it can be interpreted as an AC reciprocal translocation, forming two 'markers'.

Figure 2 shows 'double minute' fragment like chromosomes which may be small rings. They were found by Cox [34, 35] in 78 per cent of the

Table 3.

Chr. groups	Normal female cells (46 Chrs)			HeLa cell (51 Chrs)		
	Number of Chrs.	Percentage total karyotype		Number of Chrs.	Percentage total karyotype	
		Chr. Number	Chr. Area		Chr. Number	Chr. Area
A	6	13·0	22·7±1·06	7*	13·0	22·7
B	4	8·7	12·6±0·49	5‡	10·9	13·3
C	16	34·8	38·2±1·40	15	32·6	33·7
D	6	13·0	9·9±0·39	6	13·0	8·5
E	6	13·0	8·5±0·28	11**	23·9	15·7
F	4	8·7	4·1±0·19	3	6·5	2·8
G	4	8·7	2·8±0·34	4	8·7	3·4

*grossly abnormal chromosome.

cells from a neuroblastoma, and 60 per cent of rhabdomyosarcoma cells. These marker chromosomes occurred in both diploid and aneuploid cells. The origin of the minute chromosomes is not known, nor their role in the progression of these tumours. Lubs [36] has also found similar marker chromosomes in a glioma.

Other chromosome anomalies may also occur in primary tumours but require the development of new techniques in order to distinguish them. New methods have been developed for the measurement of chromosome size including automatic planimetry of photographic areas [37]. HeLa cells and chronic myeloid leukaemia cells were used as test systems for measurements on abnormal karyotypes. The chromosomes of the HeLa cells examined were about half normal size, suggesting that they were composed of only half the usual number of strands, i.e. a mutation in the polynemy level of the chromosomes, yet retaining the same relative proportion of substance per chromosome.

Figure 3 illustrates one such HeLa cell (with 51 chromosomes) which is compared with measurements of normal female cells in Table 3. Note the differences in the distribution of chromosomes per group. The comparative differences in percentage total karyotype, in terms of chromosome number, give a distorted representation of the distribution of chromosome material in the abnormal cell, because of individual chromosome size differences between the groups. It was previously shown that chromosome area measurements are related to the distribution of DNA in a karyotype and hence the distribution of genes [37]. The genetic expression of an abnormal karyotype may well depend not only on what genes are present but in what amount and whether there is a balance of chromosomes of different types. Chromosome size measurements can indicate the proportional contributions of individual chromosomes and chromosome groups to the genome.

Large scale human population chromosome studies are needed in order to obtain valuable control data on the normal range of variation and to determine whether any anomalous karyotypes are associated with pre-disposition to cancer ontogeny. For this reason the normal tissues of cancer patients need to be examined in addition to the tumour. Thus Gunz *et al.* [38] found a deletion of the short arm of a chromosome twenty one (designated Ch^1) in two families in which some of the carriers of this anomaly have lymphoid leukaemia.

Other examples are known that suggest the influence of recessive genes (i.e. on a subchromosomal level) on predisposition to neoplasia. Lele *et al.* [39] found a deleted chromosome No. 15 in a retinoblastoma and suggested that there is a recessive gene promoting this type of tumour on the long arm of a normal chromosome No. 15. Another possible example is a rare syndrome characterised by congenital erythema with an apparently recessive mode of inheritance [40]. Cultured cells had a high frequency of chromosome breakage; and three out of nineteen cases developed neoplasms.

Although some chromosome anomalies may not be detectable by light microscopy, it is desirable to detect all possible anomalies to the utmost limitations of microscopy. These limitations have not yet been reached. Attempts have been made to obtain more accurate measurements of chromosome substance by determining their DNA content.

iii. *DNA content per chromosome* — Direct methods of ultramicrospectophotometry and ultramicrointerferometry of individual chinese hamster chromosomes have formed a basis for further studies on human chromosomes, when some preliminary problems have been overcome [41].

Rudkin *et al.* [42], using a photographic method, found that about 40 per cent of the DNA of a chromosome No. 21, would have been lost in the formation of the Ph^1 chromosome of chronic myeloid leukaemia cells. More accurate methods are needed in order to determine whether the deleted portion of a chromosome twenty one has been translocated to another part of the same karyotype.

In order to recognise the origin of translocations it is necessary not only to be able to distinguish the abnormal chromosome but also to determine the origin of its component parts, by developing techniques for linear differentiation of chromosome structure.

DISCUSSION AND CONCLUSIONS

1. *Chromosomes and metabolism*

Since DNA is mainly responsible for the chemical basis of heredity and forms a major component of chromosome structure, chromosome anomalies are capable of modifying cell metabolism. Since some genes only express themselves in differentiated tissues, not all chromosomal anomalies may express themselves physiologically in undifferentiated cell proliferation. It is therefore important to determine which chromosome anomalies affect cell growth

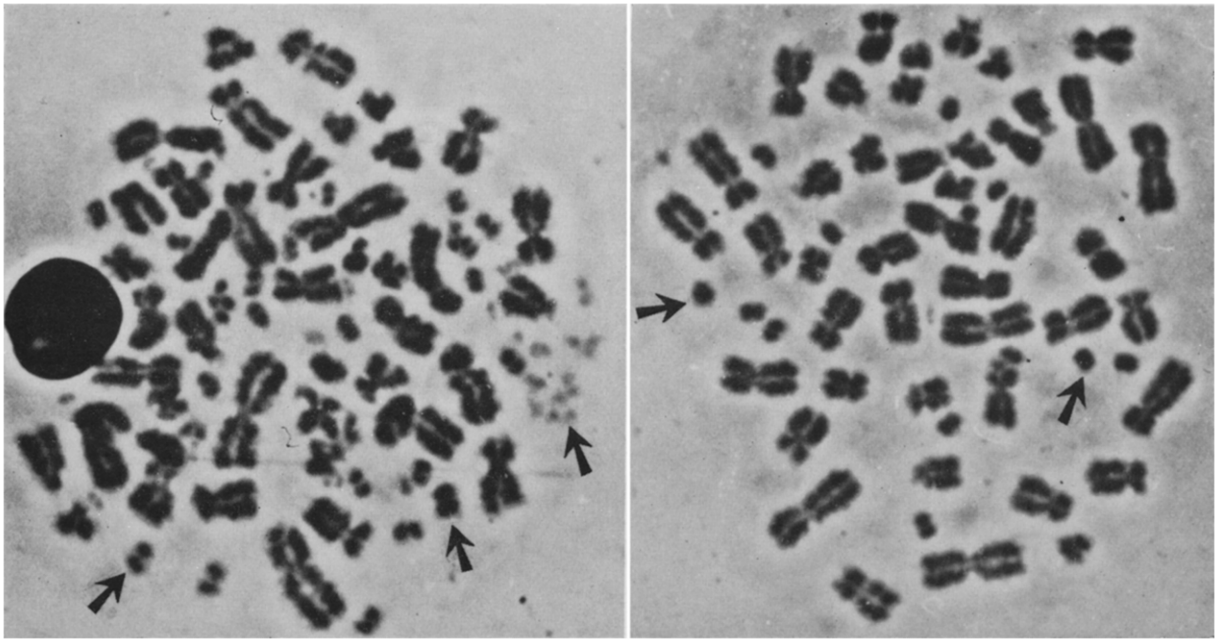


Fig. 2. Two cells from a neuroblastoma showing a range of 'double-minute' fragments (by courtsey of D Cox).



Fig. 3. Aneuploid karyotype of a HeLa cell with 51 chromosomes, showing an abnormal distribution of chromosomes per group and structurally altered 'marker' chromosomes (M^1 and B^1).

(to face p. 238)

and reproduction. Some evidence on this question has been obtained by correlated studies on cytological, cytochemical and biochemical levels for the same population of cells. Cloned cell lines from Chang liver [43] and HeLa [44, 45] have demonstrated the metabolic effect of different karyotypes on the activity of several enzymes. De Carli *et al.* [44] showed that short acrocentric chromosomes (Group G) had the greatest effect on alkaline phosphatase activity and probably carry the genes controlling this enzyme.

More studies of this kind are needed to determine the role of chromosome groups and individual chromosomes in cell metabolism.

2. *Chromosome anomalies and malignant transformation*

An anomalous chromosome pattern, common to all malignant tumours, has not been found; so that widely different patterns may result in malignant transformation (or are secondary products of the change.) Anomalous chromosome numbers of chromosomes per cell are not restricted to malignant cells. However, chromosome heteroploidy of cell populations has been found to be associated with malignancy in some primary tumours and in tissue culture, in some cases, and forms a basis for diagnosing malignancy [26]. More studies are needed on premalignant conditions and early neoplasms. The change from diploid chronic-leukaemia to acute leukaemia may be equivalent to malignant transformation and could contribute to this question.

Hayflick and Moorhead [46] showed that diploid cell strains have only a limited life in tissue culture conditions, malignant transformation being necessary in order to attain the capacity for indefinite growth *in vitro*. Further studies on chromosomal and biochemical changes involved in cell transformation *in vitro* can also make a valuable contribution. The malignancy of cultured hamster cells has been assessed by cultivation in chinese hamster cheek pouches, chorioallantoic membrane of chick embryos and by injection into last stage cancer patients.

Evidence from late stages of primary tumours and in secondary tumours and heteroploid cell cultures with the characteristic stem lines, may not be related to the primary malignant transformation: only showing the selected secondary cell varients that arose during tumour progression towards greater malignancy.

Cells with a chromosome number of forty six are always found in primary tumours. However, since only a small number of tumours containing diploid cells have been karyotyped in detail, it is not yet known whether pseudodiploidy is a general phenomenon in malignant transformation. Studies on nucleolar organising chromosomes [47] suggest that non-random changes in chromosome groups may be expected.

Further studies on the distribution of chromosomes per group and with new techniques for detecting structural anomalies of individual chromosomes are needed. Boveri's contention that abnormal chromosome constitution causes malignancy has not yet been disproved. It may yet be possible to diagnose further types of malignant cells by their karyotype.

3. *Genetic predisposition*

It is well known that the incidence of cancer is high in certain strains of experimental animals and certain human families [48]. Earlier, examples were described in which families seem to be predisposed to chromosome nondisjunction and chromosome structural changes. They may inherit genes that affect the stability of the chromosome mechanism. Genetically unbalanced individuals with abnormal karyotypes also have a greater tendency for cancer ontogeny, probably in response to environmental factors.

Karyotype analysis of families with a high cancer incidence may reveal structurally unbalanced karyotypes causing their predisposition to cancer

Acknowledgements — I wish to thank Mr. D. Cox (The Hospital for Sick Children, Great Ormond Street, London) for the loan of his preparations of neuroblastoma to enable Fig. 2 to be made; Dr. D. Lamb (Brompton Hospital, Fulham Road, London) for Fig. 1; Dr. Resa Wakonig-Vaartaja for making data (in the press) available for analysis and for reading this paper and giving me her criticisms.

This investigation has been carried out during tenure of the Shepherd Research Fellowship of the Institute of Cancer Research, London; it has also been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and the British Empire Cancer Campaign for Research, and by the Public Health Service Research Grant No. CA-03188-08 from the National Cancer Institute, U.S. Public Health Services.

RESUME

L'auteur fait une revue critique et analytique de la littérature, à partir de 1959, *concernant le nombre de chromosomes par cellule (degré de ploïdie) et le caryotype (nombre de chromosomes par catégories, structure et contenu en ADN des chromosomes).*

Des mensurations des chromosomes sont présentées, afin de montrer comment l'équilibre des types chromosomiques, dans le caryotype, peut être déterminé, pour les cellules pseudodiploïdes et aneuploïdes.

Ces données sont discutées en fonction du rôle des anomalies chromosomiques dans le métabolisme cellulaire, la transformation maligne et la théorie de Boveri, selon laquelle ces anomalies sont la cause du cancer. Des objections à la théorie de Boveri proviennent des observations suivantes:

a. l'existence d'aneuploïdie dans certaines conditions pathologiques et dans des lignées de cellules non cancéreuses;

b. l'existence de tumeurs malignes, primitives et secondaires, avec un caryotype diploïde normal. Néanmoins, des études de certaines familles ont montré que des syndromes liés à des anomalies chromosomiques peuvent prédisposer les cellules à la transformation maligne, à cause de leur instabilité métabolique; la possibilité persiste que des cancers apparamment diploïdes pourraient être pseudodiploïdes (de nouvelles techniques seraient nécessaires pour leur détection).

Les anomalies chromosomiques ont, de toute évidence, un effet métabolique dans les néoplasies et participent, de façon considérable, à la progression de celles-ci vers une plus grande malignité. L'avantage sélectif de ces mutations résulte en l'évolution des niveaux caractéristiques de ploïdie des tumeurs, en différentes localisations.

Les études cytogénétiques peuvent apporter une aide pour le diagnostic de malignité dans certaines tumeurs où des modifications marquées de la constitution chromosomique sont directement associées à leurs capacités invasives.

SUMMARY

Observations reported in the literature, since 1959, *have been assessed and analysed for chromosome number per cell (ploidy levels) and karyotype (number of chromosomes per classification group, structure and DNA content of individual chromosomes).*

Measurements of chromosome size are presented, to show how the genetical balance of chromosomal types within a karyotype can be determined in pseudodiploid and aneuploid cells.

These findings are discussed in relation to the role of chromosome anomalies in cell metabolism, malignant transformation and Boveri's theory that chromosome anomalies cause cancer. Objections to Boveri's Theory arise from the occurrence of (a) certain aneuploid pathological conditions and cell lines that are non-malignant, and (b) diploid primary and secondary malignant neoplasms with apparently normal karyotype. However, studies on families have indicated that syndromes due to chromosome anomalies may predispose cells for malignant transformation because of their metabolic instability; and the possibility remains that apparently diploid neoplasms may be pseudodiploid (and await further techniques for their detection).

Evidently, chromosome anomalies have a metabolic effect in neoplasms and are of considerable importance in aiding the progression of neoplasms towards greater malignancy. The selective advantage of cell variants results in the evolution of characteristic ploidy levels of tumours at different sites.

Chromosome studies may aid the diagnosis of malignancy in some neoplasms, where striking changes in the chromosome constitution is directly associated with their capacity for invasion.

ZUSAMMENFASSUNG

Der Verfasser hat die seit 1959 *in der Litteratur mitgeteilten Beobachtungen kritisch ausgewertet in Bezug auf die Chromosomenzahl pro Zelle (Ploïde-Grad) und auf den Karyotyp (Zahl der Chromosomen pro Klassifikationsgruppe, Struktur derselben, ADN-Gehalt der einzelnen Chromosomen).*

Die Chromosomengrösse wurde bestimmt, um zu zeigen, wie das genetische Gleichgewicht der Chromosomentypen innerhalb des Karyotyp erhalten wird und zwar sowohl in pseudodiploiden als auch in aneuploiden Zellen.

Diese Ergebnisse werden in Bezug auf die Rolle der chromosomalen Anomalieen bei Zellstoffwechselstörungen, bei bösartiger Entartung und in Bezug auf Boveri's Theorie über den Einfluss von chromosomalen Abweichungen auf die Krebsentstehung besprochen. Wiedersprüche gegen Boveri's Theorie ergeben sich aus der Existenz von (a) gewissen pathologischen aneuploiden Syndromen und Zellinien, welche nicht bösartig sind und (b) aus der Existenz von diploiden primären und sekundären bösartigen Tumoren mit anscheinend normalem Karyotyp. Trotzdem scheinen Untersuchungen von Familienstammbäumen zu zeigen, dass chromosomale Abweichungen Zellen zu bösartiger Entartung prädisponieren, wahrscheinlich auf Grund der Instabilität ihres Stoffwechsels; die Möglichkeit bleibt aber offen, dass scheinbar diploide Neoplasmen in Wirklichkeit pseudodiploid sind und dass in Zukunft feinere Untersuchungsmethoden angewandt werden müssten.

Es erscheint wahrscheinlich, dass chromosomale Abweichungen den Stoffwechsel der Tumoren beeinflussen und von grosser Wichtigkeit für die progressive Malignität der Geschwulste sind. Selektive Vorzüge von gewissen Zellvarianten führen zu der Entwicklung von charakteristischen Ploidiegraden in verschiedenen abschnitten desselben Tumors.

Die Untersuchung der Chromosomen kann der Diagnose der Bösartigkeit gewisser Tumoren zu Gute kommen und zwar insbesondere dort, wo auffallende Veränderungen der chromosomalen Zusammensetzung direkt mit der Invasionsfähigkeit in Zusammenhang zu stehen scheinen.

REFERENCES

1. P. C. Koller, Chromosomes in neoplasia. In: *Cellular Control Mechanisms and Cancer.* Elsevier, Amsterdam (1964).
2. T. C. Hsu, Mammalian chromosomes *in vitro* I. The karyotype of man. *J. Hered.* **43,** 167–172 (1952).
3. T. T. Puck, S. J. Ciecura and A. Robinson, Genetics of somatic mammalian cells. III. Long term cultivation of euploid cells from human and animal subjects. *J. exp. Med.* **108,** 945 (1958).
4. D. G. Harnden, A human skin culture technique used for cytological examinations. *Brit. J. exp. Pathol.* **41,** 31 (1960).
5. P. S. Moorhead, P. C. Nowell, W. J. Mellman, D. M. Battips and D. A. Hungerford, Chromosome preparations of leucocytes cultured from human peripheral blood. *Exper. Cell Res.* **20,** 613–616 (1960).
6. J. H. Tjio and A. Levan, The chromosome number of man. *Hereditas* **42,** 1 (1956).
7. A. Levan, Relation of chromosome status to the origin and progression of tumours. In: *Genetics and Cancer.* (1961) Peter Owen Ltd., London (1959).
8. A. A. Sandberg, T. Ishihara, T. Miwa and T. S. Hauschka, The in vivo chromosome constitution of marrow from 34 human leukemias and 60 non-leukemic controls. *Cancer Res.* **21,** 678–689 (1961).
9. P. A. Jacobs, M. Brunton, W. M. Court Brown, R. Doll and H. Goldstein, Change of human chromosome count distributions with age: evidence for a sex difference. *Nature* **197,** 1080–1081 (1963).
10. P. A. Jacobs, A. G. Baikie, W. M. Court Brown, H. Forrest, J. R. Roy, J. S. S. Stewart and B. Lenox, Chromosomal sex in the syndrome of testicular feminisation. *Lancet* **2,** 591 (1959).
11. W. M. Court Brown, D. G. Harnden, P. A. Jacobs, N. Maclean and D. J. Mantle, Abnormalities of the sex chromosome complement in man. M.R.C. Special Report Series, No. 305, H.M. Stationery Office, London (1964).
12. R. Turpin and J. Lejeune, *Les Chromosomes Humains.* Gauthier-Villars, Paris (1965).
13. M. Fragarro, K. Kaijser and J. Lindsten, A child with 49 chromosomes. *Lancet* **2,** 899–902 (1960).
14. J. A. Böök, Further studies in clinical cytogenetics. Lunds Univ. *Arsskrift, N.R. Avd.* **56,** 19 (1960).

15. M. Fracarro, A. I. Taylor, M. Bodian and G. H. Newns, A human intersex (true hermaphrodite) with XX/XXY/XXYY sex chromosomes. *Cytogenetics* **1,** 104–112 (1962).
16. A. Stewart, J. Webb and D. Hewitt, Aetiology of childhood malignancies. *Brit. Med. J.* **1,** 452–460 (1961).
17. O. J. Miller, W. R. Breg, R. D. Schmickel and W. Tretter, a family with an xxxxy male, a leakaemie male, and two 21-trisomic mongoloid females, *Lancet* **2,** 78 (1961).
18. A. G. Baikie, K. E. Buckton, W. M. Court Brown and D. G. Harnden, Two cases of leukaemia and a case of sex chromosome abnormality in the same sibship. *Lancet* **2,** 1003–1004 (1961).
19. T. S. Hauschka, J. E. Hasson, M. N. Goldstein, G. F. Koepf and A. A. Sandberg, An XYY man with progeny indicating familial tendency to non-disjunction. *Am. J. Hum. Genetics* **14,** 22–29 (1962).
20. T. S. Hauschka, Chromosome patterns in primary neoplasms. *Exper. Cell Res., Suppl.* **9,** 86–98 (1963).
21. T. Ishihara, Y. Kikuchi and A. A. Sandberg, Chromosomes of twenty cancer effusions. *J. Nat. Cancer Inst.* **30,** 1301–1361 (1963).
22. S. Makino, M. S. Sasaki and A. Tonomura, Cytological studies of tumours. XL. Chromosome studies of 52 human tumors. *J. Nat. Cancer Inst.* **32,** 741–777 (1964).
23. T. C. Hsu, Evolution of cell populations. *Int. Rev. Cytol.* **12,** 69–161 (1961).
24. A. I. Spriggs, M. M. Boddington and C. M. Clarke, Carcinoma of the cervix uteri. *Lancet* **1,** 1383–1384 (1962).
25. M. M. Boddington, A. I. Spriggs and M. R. Wolfendale, Cytogenetic abnormalities in carcinoma-*in-situ* and dysplasia of the uterine cervix. *Brit. Med. J.* **1,** 137–202 (1965).
26. R. Wakonig-Vaartaja and J. A. Kirkland, A correlated chromosomal and histopathologic study of pre-invasive lesions of the cervix. *Cancer,* In press (1965).
27. N. B. Atkin and M. C. Baker, Chromosomes in carcinoma of the cervix. *Brit. Med. J.* **1,** 522 (1965).
28. D. Lamb, Personal communication (1965).
29. T. T. Puck, A proposed standard system of nomenclature of human mitotic chromosomes. *Lancet* **1,** 1063 (1960).
30. K. Patau, The identification of individual chromosomes, especially in man. *Amer. J. Hum. Genet.* **12,** 250–276 (1960).
31. London Conference, The normal human karyotype. *Ann Hum. Genet.* **27,** 295 (1964).
32. V. R. Wakonig, A human tumour with identifiable cells as evidence for the mutation theory. *Brit. J. Cancer* **16,** 616–618 (1962).
33. S. E. Stewart, E. Lovelace, J. J. Whang and V. A. Ngu, Burkitt tumour: tissue culture, cytogenetic and virus studies. *J. Nat. Cancer Inst.* **34,** 319–327 (1965).
34. D. Cox, Chromosome studies on solid childhood tumours. *Ann. Report Brit. Emp. Cancer Campgn.* **42,** In press (1964).
35. D. Cox, C. Yuncken and A. I. Spriggs, Minute chromatin bodies in malignant tumours of childhood. *Lancet,* In press (1965).
36. H. A. Lubs and J. H. Salmon, Chromosome complement of human solid tumours. II. Karyotype of glial tumours. *J. Neurosurgery* **22,** 160–168 (1965).
37. D. T. Hughes, Quantitative studies in karyotype analysis. Oxford Chromosome Conference, 1964. *Heredity,* In press (1965).
38. F. W. Gunz, P. H. Fitzgerald and A. Adams, An abnormal chromosome in chronic lymphocytic Leukaemia. *Brit. Med. J.* **2,** 1097–1099 (1962).
39. K. P. Lele, L. S. Penrose and H. B. Stallard, Chromosome deletion in a case of retinoblastoma. *Ann. Hum. Genet.* **27,** 171 (1963).
40. J. German, R. Archibald and D. Bloom, Chromosome breakage in a rare and probably genetically determined syndrome of man. *Science* **148,** 506–507 (1965).
41. L. Carlson, T. Caspersson, G. E. Foley, J. Kudynowski, G. Lomakka, E. Simonsson and L. Sörén, The application of quantitative cytochemical techniques to the study of individual mammalian chromosomes. *Exp. Cell Res.* **31,** 589–594 (1963).

42. G. T. RUDKIN, D. A. HUNGERFORD and P. C. NOWELL, DNA contents of chromosome Ph¹ and chromosome 21 in human chronic granulocytic leukaemia. *Science* **144,** 1229–1232 (1964).
43. U. R. KONIGSBERG and H. M. NITOWSKY, Studies of the karyotype of clonal strains of Chang liver differing in alkaline phosphatase activity. *J. Nat. Cancer Inst.* **29,** 699–705 (1962).
44. L. DE CARLI, J. J. MAIO and F. NUZZO, Alkaline phosphatase activity and chromosome variation in human cells in culture. *J. Nat. Cancer Inst.* **31,** 1501–1509 (1963).
45. J. L. GERMAN, III, V. J. EVANS, J. A. CORTNER and B. B. WESTFALL, Characterisation of three human cell lines by chromosomal complement and by certain biochemical parameters. Reversible alteration of isozyme patterns by different media. *J. Nat. Cancer Inst.* **32,** 681–707 (1964).
46. L. HAYFLICK and P. S. MOORHEAD, The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25,** 585–621 (1961).
47. S. OHNO, J. M. TRUJILLO, W. D. KAPLAN and R. KINOSITA, Nucleolus-organisers in the causation of chromosomal anomalies in man. *Lancet* **2,** 123–126 (1961).
48. P. C. KOLLER, The genetic component of Cancer. In *Cancer* **1.** (Edited by R. W. RAVEN) Butterworth, London (1958).
49. R. WAKONIG-VAARTAJA, Chromosomes in gynaecological malignant tumours. *Austral. & N.Z. J. Obst. & Gynaecol.* **3,** 170-177 (1963).

Europ. J. Cancer Vol. 1, pp. 245–251. Pergamon Press 1965 Printed in Great Britain

Effect of Alkylating Agents on the Life Cycle of Ehrlich Tumour Cells

Autoradiographic Studies

G. PALME and E. LISS

I. Medical Clinic, Freie Universität Berlin

Department of Nuclear Medicine

A NUMBER of cytostatic agents, the prototype of which is nitrogen mustard, have the common property of producing an alkylating effect in a physiological medium. It is obvious that the great number of theoretically possible alkylations *in vivo* allows numerous points of attack of these compounds in cell metabolism [1]. The aim of morphological and biochemical investigations is to detect these individual points of attack in order to establish the primary and perhaps critical effects of the cytostatic agents on cell metabolism. The findings could serve as a starting point towards an effective chemotherapy.

In previous experiments we were able to demonstrate by parallel determination of various metabolic indices such as DPN content, glycolysis rate, nucleic acid synthesis, and others that the *de novo* DNA synthesis is especially sensitive to alkylating agents [2, 3]. It was of interest to study in detail the effect of these carcinostatic agents on the DNA synthesis at the cellular level by means of the autoradiographic technique. With this method, the effect of the respective substances on other phases of the life cycle of dividing cells can also be examined.

In this report, a few results of our investigations will be discussed. For an example, the effect of Cyclophosphamide(N,N-bis-(2-chlorethyl)-N′-O-propylenephosphoric acid esterdiamide) and Trenimon (2,3,5-tris-ethylenimino-benzoquinone-1,4) on Ehrlich ascites tumour cells *in vivo* will be employed.

MATERIAL AND METHODS

The tumour used in our studies was an ELD-Ehrlich ascites tumour line carried in CF-1 mice. The cells were harvested 6 days after inoculation of 16×10^6 tumour cells per animal. The experiment groups of six animals each were given a single dose i.p. of Trenimon and Cyclophosphamide respectively, with the size of the doses varying. All the pre-treated groups and a control group of untreated animals were then given an injection of 1·5/μC/g ^{3}H-Thymidine (^{3}H-TdR) with a specific radioactivity of 1·9 C/mM (Schwarz BioResearch Laboratories, Mt. Vernon, N.Y.) i.p. The labeled precursor was applied 3 hr after the administration of Trenimon and 6 hr after administration of Cyclophosphamide. Ninety minutes after injection of ^{3}H-TdR, all the animals were killed by cervical dislocation. In a further experiment, animals pre-treated with Cyclophosphamide and an untreated control group were tapped at different intervals after the ^{3}H-TdR injection. The tumour cells were removed with a heparinized syringe.

In all cases, the tumour cells were carefully cleared from the serum by washing them twice with Ringer's solution enriched by inactive Thymidine in excess. Thin smears of ascites cells were prepared. The smears were dried, then fixed in absolute methanol for 5 min, and immersed in tap water for 30 min to remove the unbound radioactivity. Radioautographs were prepared according to the dip-coating method [4] by using K2 and G5 emulsions (Ilford, London). The time of exposure varied from 4 to 12 days. After photographic processing, the radioautographs were stained with haematoxylin and eosin.

The labeling index, that is the percentage of tumour cells labeled by a single injection of ^{3}H-TdR, was determined on 3,000 cells taken at random on each slide. The mean grain count per labeled cell was conducted on 300 interphase nuclei taken at random on each smear. The percentage of the labeled mitoses was determined on every 100 mitoses.

RESULTS AND DISCUSSION

After administration of Cyclophosphamide or Trenimon to mice and subsequent injection of ^{3}H-TdR, in all the normally proliferating cell systems investigated (small intestine, basal epithelium of the tongue, spleen and bone marrow) the incorporation of ^{3}H-TdR into DNA is reduced, as we have shown in previous biochemical and autoradiographic experiments [5, 6]. On account of the short duration of the experiments and the respective doses applied, an alteration of the nucleoside or nucleotide pool can be excluded. Therefore, we have interpreted the decrease of the incorporation rate of the labeled precursor as an inhibition of the *de novo* DNA synthesis.

These investigations were then extended on Ehrlich ascites tumour cells in the phase of exponential growth. Table 1 shows the effect of Cyclophosphamide and Trenimon on the incorporation of ^{3}H-TdR into the interphase nuclei of ascites tumour cells in dependency of the dose of cytostatic agents administered. As Table 1 indicates, the mean grain count per interphase nucleus 6 hr after application of Cyclophosphamide in a dose of 60 mg/kg body weight and more is significantly reduced in comparison with the untreated controls, whereas with a dose of 30 mg/kg body weight no inhibition of the uptake of ^{3}H-TdR is existent. However, the labeling index, that is the percentage of DNA synthetizing nuclei in relation to the total number of interphase nuclei, does not show any difference between treated cells and controls, independent of the doses administered. An analogous observation can be made after application of Trenimon, except that the incorporation of ^{3}H-TdR is significantly inhibited already after the lowest dose applied. With the prolongation of the duration of the influence of cytostatic agents the inhibitory effect on the uptake of ^{3}H-TdR is still more pronounced. These results were confirmed, by *in vitro* investigations with comparable cell concentrations which were incubated with a defined ^{3}H-TdR concentration. These additional investigations were necessary to exclude influences which could result from differences in the size of the proliferating cell pools in individual animals.

It is now necessary to demonstrate if the above-described inhibition of ^{3}H-TdR uptake affects the entire cell population at the time of the DNA synthesis phase (S-phase) while the labeled precursor is available or if the DNA synthesis is suppressed only in some cells of the population, while synthesis continues at the normal rate in the remainder cells of the population. The results of such an experiment are shown in Fig. 1. In the figure, grain count distributions of ascites tumour cells labeled with

Table 1. *Effect of Cyclophosphamide and Ttrenimonon ^{3}H-thymidine uptake and labeling index of Ehrlich ascites cells* in vivo *(expressed in percent of the normal value).*

Cyclophosphamide*				Trenimon†			
Body weight (mg/kg)	Labeling index (±S)	Mean grain count/ interphase nucleus (±S)	(P)	Body weight (mg/kg)	Labeling index (±S)	Mean grain count/ interphase nucleus (±S)	(P)
Controls	100·0 ±4·1	100·0 ±16·5	—	Controls	100·0 ±4·9	100·0 ±7·8	—
30 mg	102·0 ±1·2	101·8 ±2·5	—	0·1 mg	98·2 ±3·9	48·7 ±5·4	<0·001
60 mg	96·7 ±5·9	45·3 ±15·1	<0·001	0·2 mg	94·0 ±1·8	34·2 ±5·8	<0·001
120 mg	104·9 ±5·8	34·9 ±9·6	<0·001	0·4 mg	101·0 ±5·3	11·0 ±3·4	<0·001

*^{3}H-TdR was applied 6 hr after Cyclophosphamide.
†^{3}H-TdR was applied 3 hr after Trenimon.

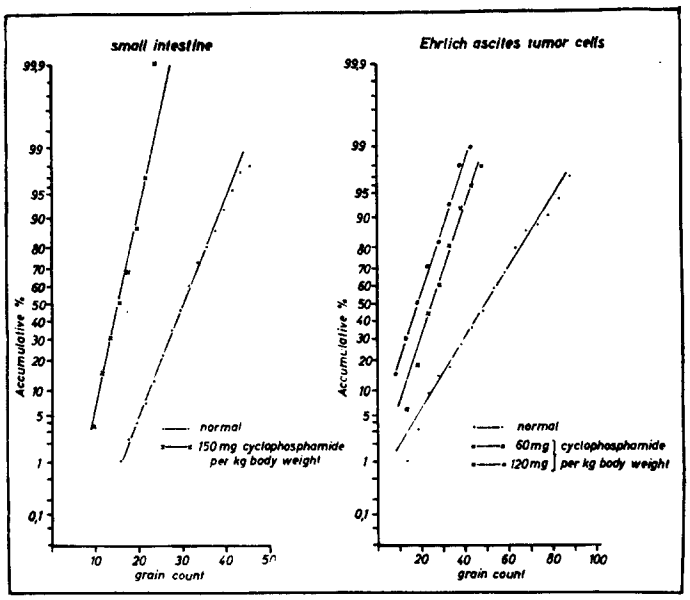


Figure 1. *Grain count distribution in the form of probability plots of labeled cells of untreated and with Cyclophosphamide pre-treated animals* 90 *min after injection of* ^{3}*H-TdR. On the left: small intestine of mice; on the right: Ehrlich ascites tumour cells.*

^{3}H-TdR 6 hr after application of Cyclophosphamide in doses of 60 and 120 mg/kg body weight are presented in comparison with the distributions of the untreated control cells. These distributions are presented in form of cumulative distributions on a probability plot. A straight line on such a plot is characteristic of a normal distribution. The slope of a line expresses the standard deviation of the distribution. Figure 1 indicates that the pre-treated cell populations as well as the controls show a normal distribution of grain counts. The mean of the distribution of the treated cells is shifted towards a lower grain count value, which indicates that, after application of Cyclophosphamide, the entire cell population in DNA synthesis shows a reduced uptake rate of ^{3}H-TdR. As we are dealing with an asynchronously growing cell population, it can be concluded that, in presence of the doses applied, the entire DNA synthesis phase in the cell cycle must be affected. In all cases, the inhibition of ^{3}H-TdR incorporation precedes morphological at least light-optically recognizable alterations of cells, as an expression of a general toxical damage.

We were then concerned with the question, if the inhibition of the DNA synthesis, that is the effect on the S phase, constitutes the primary point of attack of the alkylating agents in the

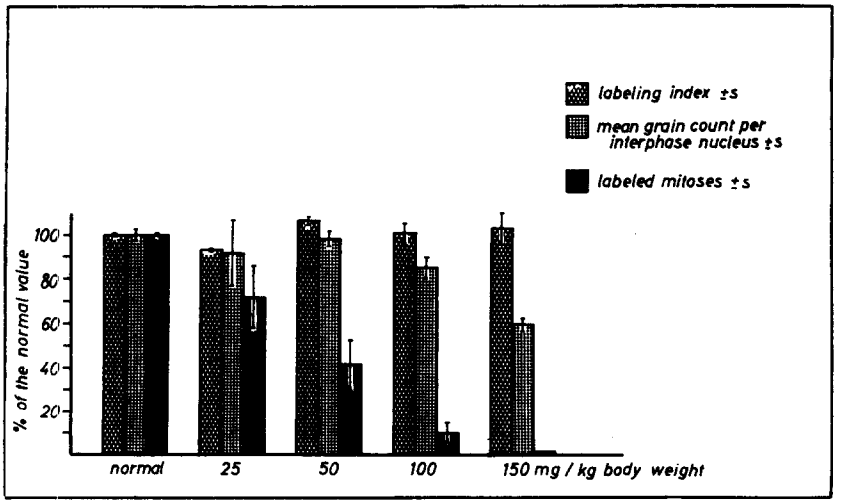


Fig. 2. *Effect of Cyclophosphamide on labeled mitoses, labeling index, and mean grain count per interphase nucleus in the small intestine of mice.*

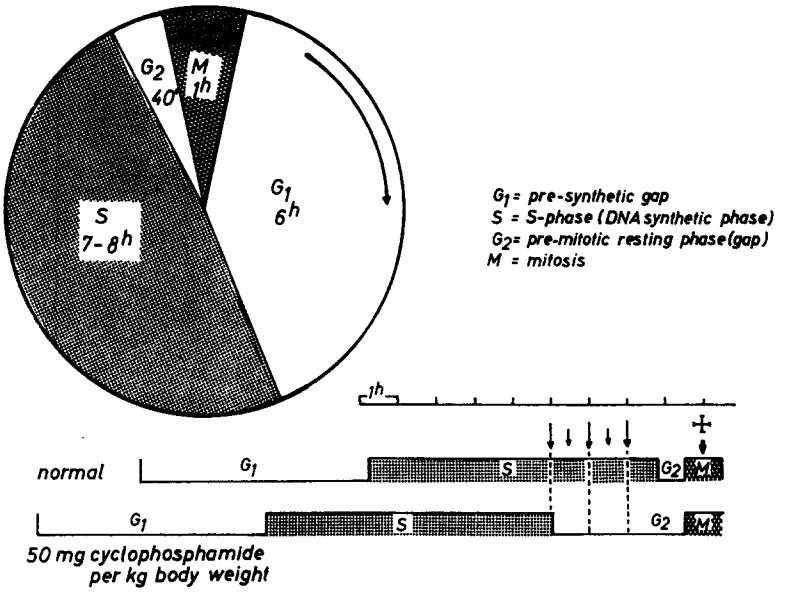


Fig. 3. Life cycle of the small intestine epithelium.

cell cycle, or if another phase of the life cycle could be still more sensitive towards these cytostatic agents. As before, the small intestine epithelium of mice was used for the experiments, because in these cells the individual phases of the life cycle are comparatively constant [7].

Figure 2 shows that, with different doses of Cyclophosphamide administered, a decrease of the percentage of labeled mitoses can be observed, and this already after the smallest dose applied while the overall mitotic index only gradually decreases [8]. In accordance with the findings of Quastler and Sherman [7], Koburg and Maurer [9] always 100 per cent of the mitoses are labeled 2 hr after injection of ^{3}H-TdR in the controls. However, with the same duration of experiment, the percentage of labeled mitoses decreases rapidly with the increase of the dose of Clycophosphamide. The ^{3}H-TdR incorporation is inhibited only in the presence of higher doses. The labeling index, however, remains constant with all the doses applied. It only decreases in the presence of sublethal doses, as was shown in previous investigations [6]. These results will now be interpreted with the help of a model showing the life cycle of a cell of the small intestine epithelium (Fig. 3). The life cycle of a cell is usually divided into four phases [10, 11]: (I) a pre-synthetic phase, called G-1; (II) an S phase, during which new DNA is synthetized; (III) a pre-mitotic phase, called G-2; and (IV) mitosis.

The first change to be observed regularly after administration of different doses of Cyclophosphamide is a rapid decrease of the percentage of labeled mitoses, as we have seen. How can the finding be explained, and what is its significance?

The linear model of the life cycle of a cell will clarify the point. If the interval between the administration of ^{3}H-TdR and dissection is longer than the G-2 phase — 2 hr in this case — all of the mitoses present at the time of dissection must be labeled, because these cells were in the S phase (DNA synthesis) when ^{3}H-TdR was applied (indicated by the arrows). Cells which were in the G-2 phase have already undergone mitosis, cells then in the G-1 phase have not yet entered the mitotic phase. If one gradually extends the usually very constant G-2 phase of the small intestine epithelium by means of the effect of Cyclophosphamide, more and more cells will not be in the S phase anymore but already in the extended G-2 phase, when ^{3}H-TdR is injected, and thus will not appear as labeled mitoses. When finally the G-2 phase becomes longer than the interval between the ^{3}H-TdR injection and dissection, all of the present mitoses will be unlabeled. If one procedes in the opposite direction and gradually extends the interval between ^{3}H-TdR injection and dissection further than to the original duration of experiment of 2 hr, an increase of the labeled mitoses results until finally all of them are labeled. The time reached at this point corresponds to the duration of the extended G-2 phase. In a corresponding experiment, a prolongation of the premitotic resting phase of normally about 40 min up to 3,5 to 4 hr was measured, after an injection of Cyclophosphamide of 50 mg/kg body weight.

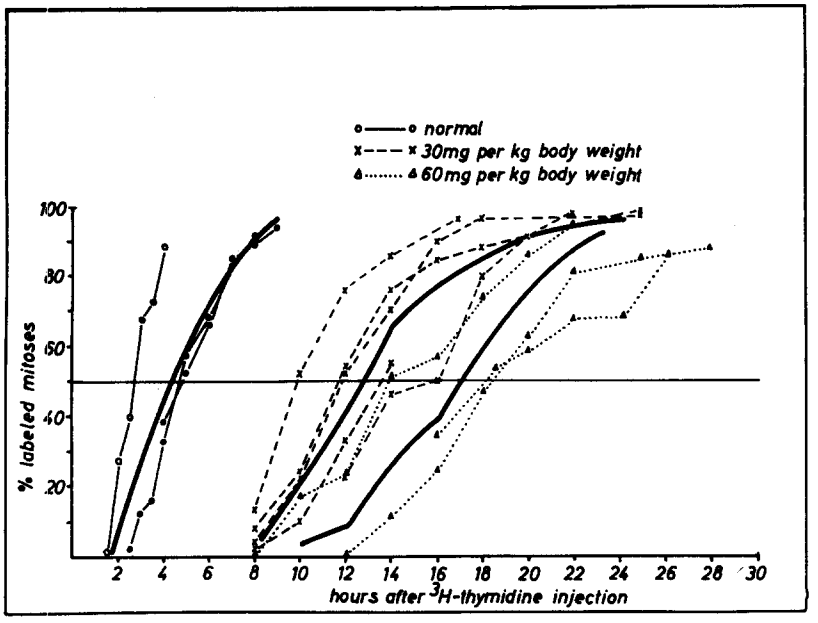


Fig. 4. Occurrence of labeled mitoses per unit time in untreated and with Cyclophosphamide pre-treated Ehrlich ascites tumour cells.

These findings of a distinct sensitivity of the G-2 phase to alkylating agents, first demonstrated in normally proliferating cells, gains significance when compared to the results of identically conducted experiments on ELD-Ehrlich ascites tumour cells. As Fig. 4 shows, the G-2 phase of ascites tumour cells was extended to a medium duration of 12 hr after administration of a single dose of Cyclophosphamide of 30 mg/kg, up against 4 to 6 hr [12, 13] in the untreated ascites cells. After a dose of 60 mg/kg the occurrence of labeled mitoses is being delayed up to even 16 hr. Thus, by simply extending the pre-mitotic phase of the life cycle, a considerable cytostatic effect has been produced, resulting in the doubling of the generation time of the tumour cells from normally 18 hr to about 34 hr, in other words, the proliferation process was delayed by about 50 per cent. Further investigations will have to explain in how far the inhibition of the DNA synthesis, as demonstrated in the presence of a dose of Cyclo. of 60 mg/kg, could bear an additional effect on the delayed occurrence of the labeled mitoses.

In summary, the above-described results indicate that the individual phases of the life cycle of proliferating cells differ in their sensitivity to the various doses applied. In the presence of low to medium size doses, the duration of the pre-mitotic resting phase is being increasingly extended which delays the progression of the cells into mitosis and thus slows down the proliferation rate. Higher doses additionally affect the DNA synthesis by inhibiting the incorporation of thymidine. Still larger doses finally injure the entire cell cycle.

Acknowledgements — This work was supported by funds of the German Ministry of Scientific Research and by grants from the Deutsche Klassenlotterie Berlin. The authors gratefully acknowledge the invaluable technical assistance of Miss S. Schiller and Miss U. Asheuer.

RESUME

1. *Après application des agents alkylants Trénimon et Cyclophosphamide à des cellules de tumeur ELD d'Ehrlich, une inhibition de l'incorporation du ^{3}H-TdR fut démontrée par la méthode autoradiographique, au moyen du compte des grains. L'importance de l'inhibition dépendait des doses employées, aussi bien que de la durée d'action des agents.*

2. *Une analyse de la distribution du compte des grains dans les noyaux d'interphase a démontré que toutes les cellules, au moment de la phase S, sont affectées de façon plus ou moins uniforme par l'inhibition de la synthèse de l'ADN.*

3. *Des études sur l'action de doses différentes de Cyclophosphamide sur le cycle de vie de la cellule ont révélé que la phase de repos prémitotique (G-2) est encore plus sensible au Cyclophosphamide que la phase S. Dans le cas des cellules tumorales d'Ehrlich, l'effet inhibiteur du Cyclophosphamide sur la phase G-2 est spécialement intense et nettement plus prononcé que dans les divers types de cellules normales étudiées chez la souris.*

SUMMARY

1. *After application of the alkylating agents Trenimon and Cyclophosphamide to ELD-Ehrlich ascites tumours cells, an inhibition of the incorporation of ^{3}H-TdR was determined autoradiographically by means of determining the grain count. This inhibition was found to be dependent on the size of the doses applied as well as on the duration of the influence of the agents.*

2. *An analysis of the grain count distribution among the interphase nuclei showed that the entire cell population, at the time of the S-phase, is relatively uniformly affected by the inhibition of the DNA synthesis.*

3. *Investigations concerning the effect of different size doses of Cyclophosphamide on the life cycle of the cell indicated that another phase of the cell cycle, the pre-mitotic resting phase (G-2) is even more sensitive to Cyclophosphamide than the S-phase. In Ehrlich ascites tumour cells, the inhibiting effect of Cylcophosphamide on the G-2 phase is especially distinct and is definitely more pronounced than in the normal cell systems of mice investigated.*

ZUSAMMENFASSUNG

1. *Nach Verabreichung der alkylierenden Substanzen Trenimon und Cyclophosphamid wurde an ELD-Ehrlich-Ascitestumorzellen au oradiographisch durch Silberkornauszählung eine Hemmung der Tritium-TdR-Inkorporation festgestellt. Diese Hemmung war von der angewendeten Dosis und von der Dauer der Einwirkung der Substanzen abhängig.*

2. *Die Analyse der Häufigkeitsverteilungen der Silberkornzahlen der Interphasekerne zeigte, dass die gesamte in der S-Phase befindliche Zellpopulation ziemlich einheitlich von der Hemmung der DNS-Synthese betroffen wird.*

3. *Untersuchungen über den Effekt von verschieden hohen Dosen von Cyclophosphamid auf den Generationscyclus ergaben, dass eine andere Phase des Zellcyclus, nämlich die prämitotische Ruhephase (G-2), noch empfindlicher als die S-Phase gegen Cyclophosphamid ist. In den Ehrlich-Ascitestumorzellen war der Hemmeffekt des Cyclophosphamid auf die G-2-Phase desonders ausgeprägt und viel intensiver als in den untersuchten normalen Zellsystemen der Maus.*

REFERENCES

1. G. P. Wheeler, Studies related to the mechanisms of action of cytotoxic alkylating agents: A review. *Cancer Res.* **22,** 651 (1962).
2. E. Liss und G. Palme, DPN-Gehalt, anaerobe Glykolyse und DNS-Synthese von Ascitestumorzellen unter dem Einfluß von Trenimon. *Naturwissenschaften* **50,** 672 (1963).
3. E. Liss und G. Palme, Der Einfluß von alkylierenden Cytostatica auf den Nucleinsäurestoffwechsel von Ehrlich-Ascites-Tumorzellen. *Z. Krebsforsch.* **66,** 196 (1964).
4. B. Messier and C. P. Leblond, Preparation of coated radioautographs by dipping sections in fluid emulsion. *Proc. Soc. Exptl. Biol. N.Y.* **96,** 7 (1957).
5. G. Palme, Die Beeinflussung der DNS-Synthese durch Endoxan. Autoradiographische Untersuchungen mit tritium-markiertem Thymidin. In: *Chemotherapie maligner Tumoren,* S. 125–131. F. K. Schattauer Verlag, Stuttgart, (1964).

6. G. PALME, E. LISS, K. OEFF und A. PLATIS, Der Einfluß von Cyclophosphamid auf die Desoxyribonucleinsäure-Synthese von normalen proliferierenden Zellen sowie Ascites-Tumorzellen. Autoradiographische und biochemische Untersuchungen. *Arzneimittel-Forsch.* **13,** 1034 (1963).
7. H. QUASTLER and F. G. SHERMAN, Cell population kinetics in the intestinal epithelium of the mouse. *Exptl. Cell Res.* **17,** 420 (1959).
8. G. PALME und E. LISS, Autoradiographische Untersuchungen über das Verhalten einzelner Interphaseabschnitte des Dünndarmepithels der Maus unter Einwirkung von Cyclophosphamid (Endoxan). *Naturwissenschaften* **51,** 197 (1964).
9. E. KOBURG und W. MAURER, Autoradiographische Untersuchung mit ^{3}H-Thymidin über die Dauer der DNS-Synthese und ihren zeitlichen Verlauf bei den Darmepithelien und anderen Zelltypen der Maus. *Biochim. Biophys. Acta.* **61,** 229 (1962).
10. A. HOWARD and S. R. PELC, Synthesis of desoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. *Heredity (Suppl.)* **6,** 261 (1952).
11. L. G. LAJTHA, Bone marrow cell metabolism. *Physiol. Rev.* **37,** 50 (1957).
12. R. BASERGA, Mitotic cycle of ascites tumor cells. *Arch. Pathol.* **75,** 156 (1963).
13. W. OEHLERT, N. SEEMAYER und P. LAUF, Autoradiographische Untersuchungen über den Generationszyklus der Zellen des Ehrlich-Ascitescarcinomas der weißen Maus. *Beitr. Pathol. Anat.* **127,** 63 (1962).

Europ. J. Cancer Vol. 1, pp. 253–258. Pergamon Press 1965. Printed in Great Britain

Sampling of Cells From Human Tumours by Aspiration Biopsy for Diagnosis and Research

J. ZAJICEK

From the Cytology Department of Radiumhemmet
Institute of Radiopathology and the General Department of Radiumhemmet
Karolinska Sjukhuset, Stockholm, Sweden

ASPIRATION biopsy can be defined as a procedure in which negative pressure is created in a syringe and, as a result of pressure differences, cellular material is drawn from the tissue into the needle. It has long been an accepted method of obtaining material for diagnostic purposes. Aspiration biopsy is performed mainly on blood-forming tissues, but is increasingly utilized also in the diagnosis of human tumours in various organs—prostate, thyroid and salivary glands, lung, breast, etc. [1].

The value of aspiration biopsy for cytologic diagnosis of lesions of the female breast was recently analyzed at Radiumhemmet in Stockholm [2] by comparing cytologic and histologic reports in 1,220 cases. This study covered the years from 1955 (when mammary aspiration biopsy was introduced at Radiumhemmet) through 1962. The present paper reports a detailed analysis of cases in which aspiration biopsy of the female breast was performed at Radiumhemmet in 1962, when the staff had had 7 years' experience of the biopsy technique and of reading the slides prepared from aspirate. The total number of women who underwent aspiration biopsy of the breast in 1962 was 925. Surgical treatment was subsequently carried out in 417 cases and thus comparison could be made between cytologic and histologic observations. In the 508 cases in which surgery was not performed (54·9% of the total group), the clinical and cytologic diagnoses were inoperable cancer or benign conditions such as cysts, mastitis and fibroadenoma.

Table 1 permits comparisons between the cytologic and the histologic findings. (Table compiled in collaboration with S. Franzén and T. Ångström).

In 211 cases the histologic report stated benign mammary lesion. The cytologic examination of biopsy aspirate likewise was negative for malignancy in 81 per cent of these cases. In a further 17·1 per cent, cellular atypia was observed on the cytologic slides, and in 1·9 per cent there was cytologic suspicion of cancer. No false positive diagnosis of cancer was made.

The histologic examination showed precancerous or possible precancerous lesion in sixteen cases. In thirteen of these cases no signs of malignancy were found in biopsy aspirate. The cytologic diagnosis was suspected malignancy in two cases and cancer in one case. It seems noteworthy that no case in this group was cytologically classified as showing cellular atypia.

The histologic diagnosis was cancer in 190 cases (in one case, however, malignancy was only suspected). In 5·8 per cent of these histologically proven malignant tumours, the cytologic report was negative for cancer. As a rule the discrepancy was attributable to an unsatisfactory aspirated specimen. In 2·1 per cent of the histologically demonstrated malignancies, the cytologic report stated cellular atypia. In 12·6 per cent malignancy was cytologically suspected, and in 79·5 per cent a diagnosis of cancer was made from study of biopsy aspirate. It may seem remarkable that obvious clinical signs of mammary carcinoma such as fixation of the skin or the muscle and

Table 1. Cytological findings in 417 cases with histologically diagnosed mammary gland lesion.

	Histologic findings			
Cytologic findings	Benign 211	Precancerous lesion 16	Cancer 190*	Total 417
Benign or unsatisfactory specimen	171 (81·0%)	13 (81·3%)	11 (5·8%)	195
Cellular atypia	36 (17·1%)	0	4 (2·1%)	40
Suspectedly malignant	4 (1·9%)	2 (12·5%)	24 (12·6)%	30
Cancer	0	1 (6·2%)	151 (79·5%)	152

*In 1 case malignancy was only suspected.

retraction of the nipple were lacking in 40·6 per cent of the cases in which a diagnosis of cancer was indicated by aspiration biopsy.

These figures show that the possibility of a false positive diagnosis of mammary carcinoma from aspiration biopsy can be disregarded if the cytologist is fully familiar with the morphology of mammary gland cells in various pathologic conditions. The cytologic diagnosis of mammary cancer is considered at Radiumhemmet to be fully reliable, and operable cases are now referred for radical mastectomy without prior surgical biopsy. In our series of cases, however, 7·9 per cent of the cytologic reports were false negatives. This implies that a negative aspiration biopsy report does not unconditionally declare the lesion to be benign. If the clinical suspicion of malignancy persists, a second aspiration or a surgical biopsy should be performed.

The results in Table 1 may be summarized by saying that the cytologic diagnosis agreed with the histologic diagnosis in 98·1 per cent of the benign mammary conditions, in 12·5 per cent (two of sixteen cases) of the group histologically classified as precancerous, and in 92·1 per cent of the histologically proven malignancies. The overall accuracy of the aspiration biopsy method as compared with conventional histologic examination was 92 per cent in this series.

Aspirate obtained from human epithelial tumours through a fine (22 gauge) needle consists largely of plugs of epithelial cells mixed with interstitial fluid and variable amounts of blood (Figs 1 and 2). In earlier investigations [3] the epithelial component was isolated by means of centrifugation for quantitative enzymic analysis. Dipeptidase and acid and alkaline phosphatase activities were determined in cells aspirated from mammary and prostatic carcinomas. The readings were compared with results from analyses of epithelial plugs aspir-

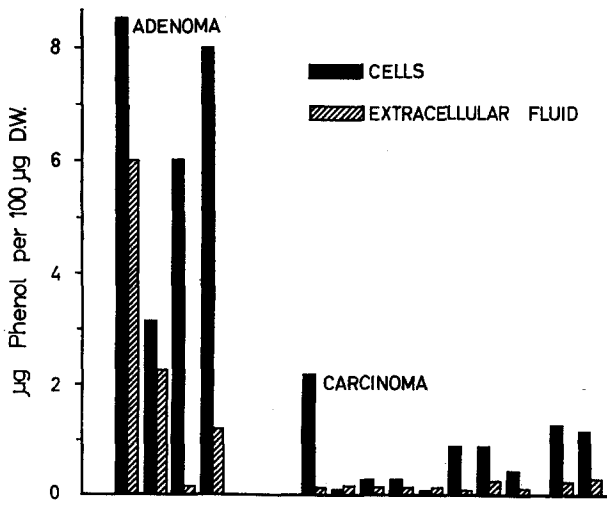


Fig. 3. Alkaline phosphatase activity in material aspirated from mammary fibroadenoma (4 cases), primary mammary carcinoma (first 8 cases) and axillary metastasis of mammary carcinoma (last 2 cases). The activity is expressed as µg phenol liberated during 1 hr per 100 µg air-dried material. Disodium phenylphosphate was used as substrate. pH 9·8. Incubation time 1 hr at 37°C. The filled columns denote activity in the washed cells and the shaded columns represent activity in the washing medium, containing also the interstitial fluid.

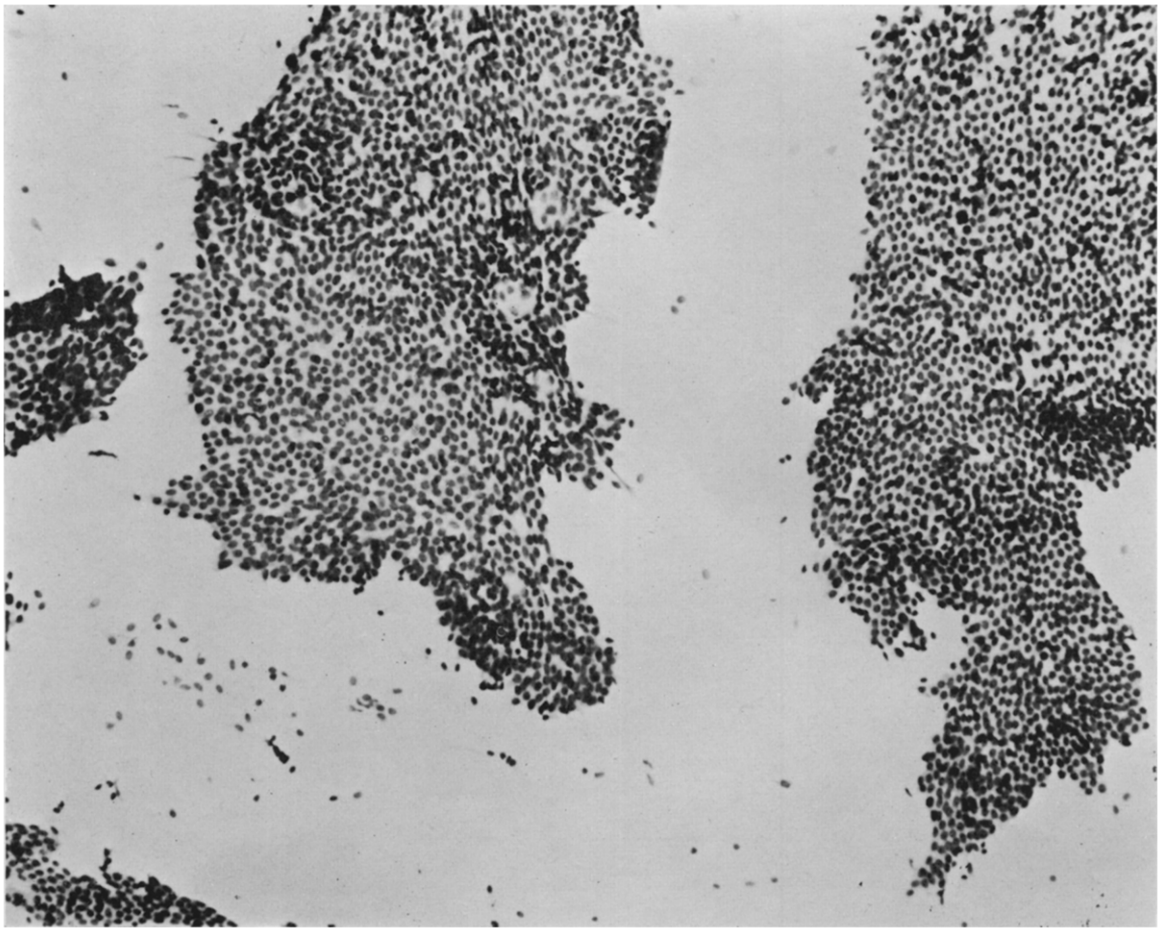


Fig. 1. *Plugs of epithelial cells aspirated from mammary fibroadenoma and spread on a glass slide. Papanicolaou stain.* × *136.*

(to face p. 254)

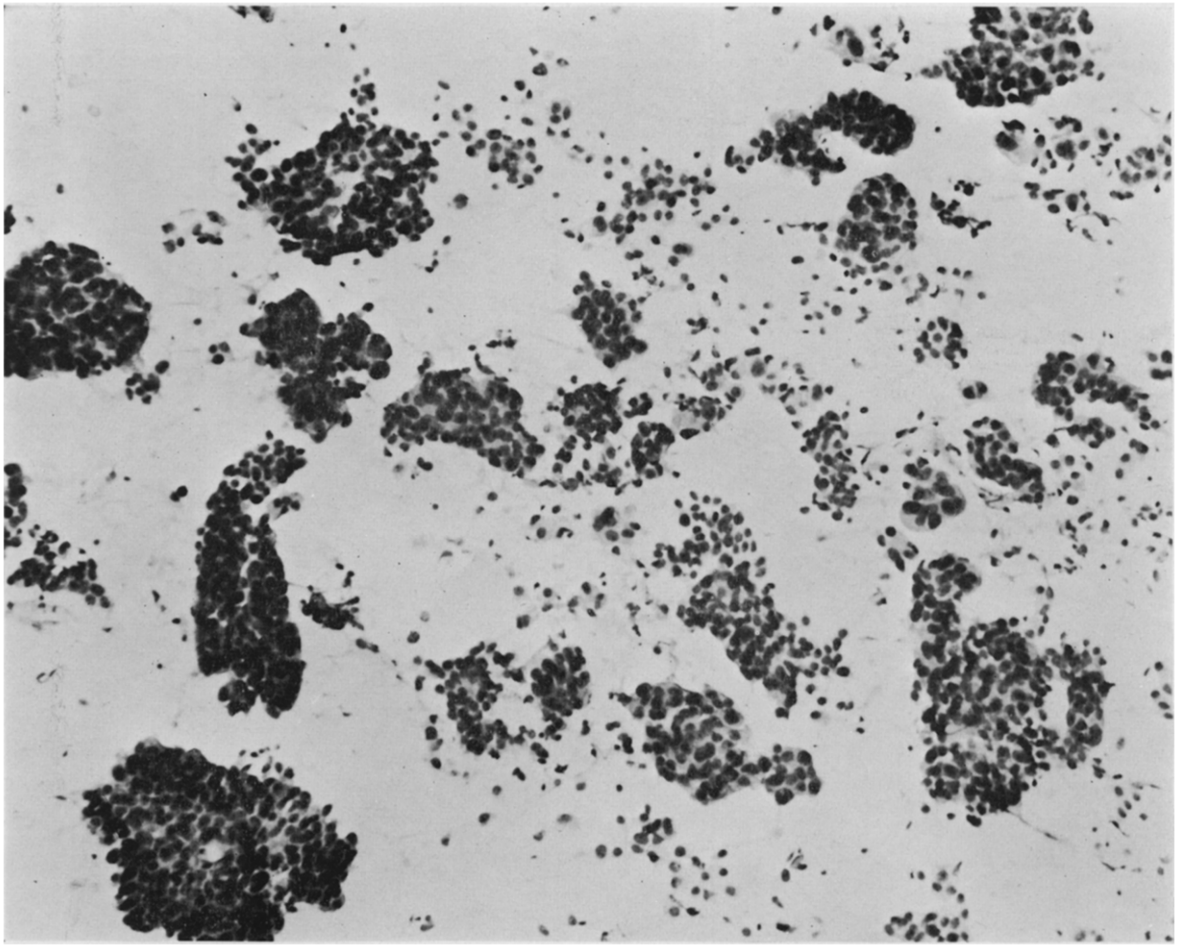


Fig. 2. Plugs of carcinoma cells aspirated from primary mammary carcinoma and spread on a glass slide. Papanicolaou stain × 136.

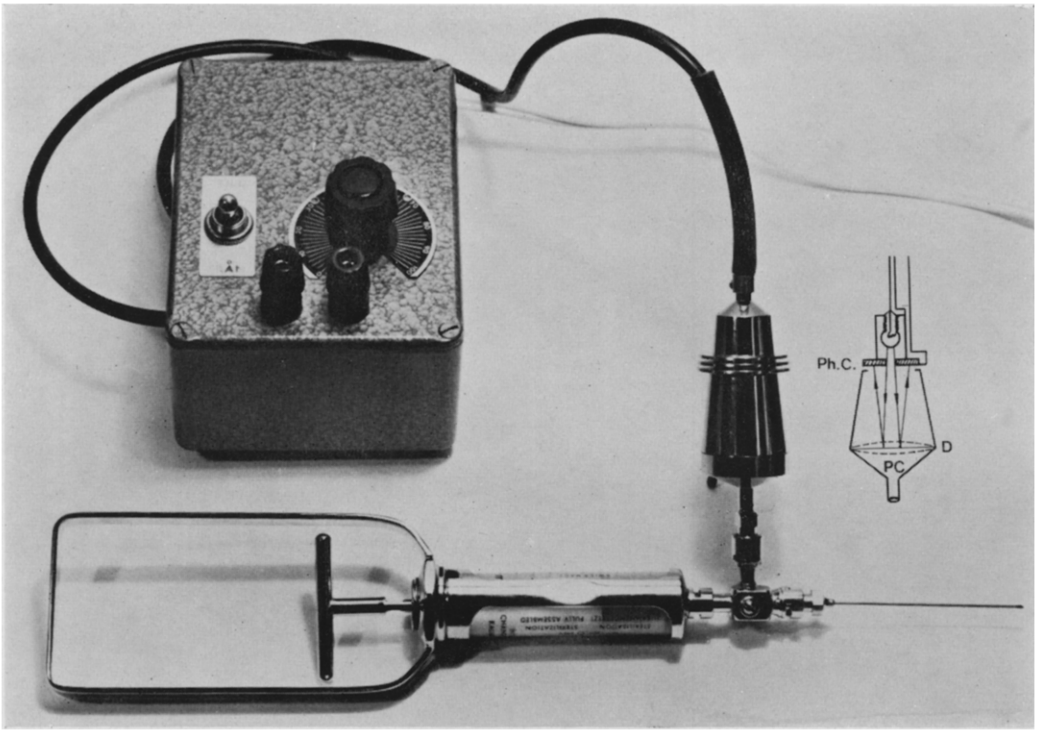


Fig. 4. Apparatus for measuring pressures during aspiration biopsy. The pressure gauge is connected with the syringe and the needle by a three-way stopcock.
The diagram shows the pressure chamber (PC), which is in communication with the syringe. The chamber is bounded above by a flexible diaphragm (D) which reflects light from a filament bulb on to a selenium photocell (PhC). The cell transmits pressure readings to the recorder (shown above the syringe).

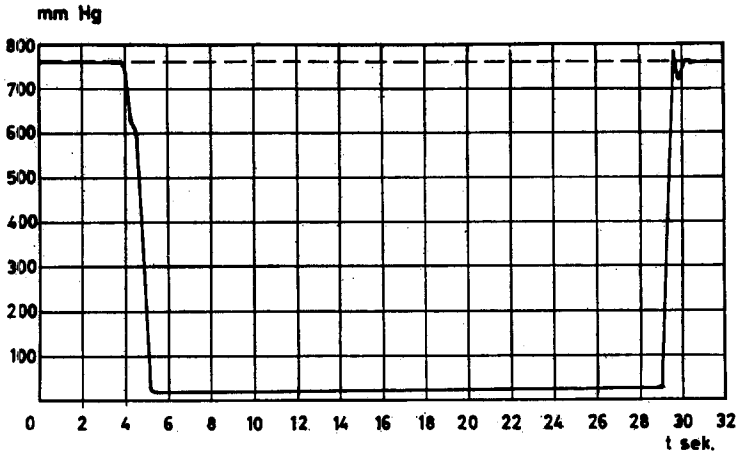


Fig. 5. *Pressure differences in the syringe during aspiration biopsy of a lymph node.*

ated from benign mammary and prostatic lesions. Figure 3 expresses graphically the alkaline phosphatase activity in aspirates from mammary fibroadenomas and from mammary carcinomas.

Since patients experience relatively little discomfort from aspiration biopsy with a fine needle, repeated biopsies are well tolerated. The method therefore offers possibilities for studying the biologic behaviour of tumour-cell populations in different stages of tumour development. Such use of aspiration biopsy, however, raises the question of whether or not the procedure as such may affect the viability of the sampled cells.

In order to answer this question, we attempted to analyze the physical factors that are involved in aspiration biopsy and their possible influence on cell viability. Particular attention was devoted to the effects of negative pressure.

An apparatus for registering pressure differences during aspiration biopsy was evolved [4]. It is shown in Fig. 4. The apparatus is connected by means of a three-way stopcock to a Franzén-Kifa syringe [5], which has a special handle permitting single-handed management. The pressure-recording device consists of a conical tube having in its lower part a pressure chamber which connects with the syringe but is separated from the rest of the apparatus by a flexible, light-reflecting diaphragm of stainless steel. This diaphragm is about 0·5 mm thick. In the upper part of the tube a filament lamp is inserted in such a way that the diaphragm is illuminated from a circular opening in the centre of a photocell, which is shielded from the direct light of the lamp. When a subatmospheric pressure is produced in the pressure chamber, the diaphragm bends and as a result the light from the circular opening is increasingly focused on the photocell (in this case a selenium cell). From there the voltage is transmitted to a recorder. When the apparatus has been calibrated against a manometer, pressure differences can readliy be calculated.

Figure 5 shows the pressure changes in the syringe during aspiration biopsy of a lymph node. The time required for the biopsy was 29 sec. During this time the pressure in the syringe was maintained at about 16 mm Hg. In order to obtain sufficient material, the needle was moved back and forth in the node four or five times during aspiration. When the sample had been taken, the pressure in the syringe was allowed to return to atmospheric equilibrium by releasing the piston to its original position before the needle was withdrawn from the lymph node. The graph illustrates how the pressure in the syringe normalized during the manipulation.

From the experiment illustrated in Fig. 5 it would seem that the pressure differences arising in the needle during aspiration biopsy amount to about 740 mm Hg.

Subsequent investigations concerned the extent to which such pressure differences may influence the viability and proliferation rates of the aspirated cells. It was found that pressure differences similar to those created during aspiration biopsy had no apparent effect on the viability of He-La and bone marrow cells [6]. After subjection to the negative pressures, the cells remained impermeable to the eosin and lissamine-green dyes which are commonly used in testing the viability of human tumour cells. The proliferation rates of both He-La cells and bone-marrow cells were likewise unchanged by the negative pressures. Proliferation of He-La cells was studied in tissue cultures and proliferation of marrow cells in transplantation experiments on lethally irradiated mice [6].

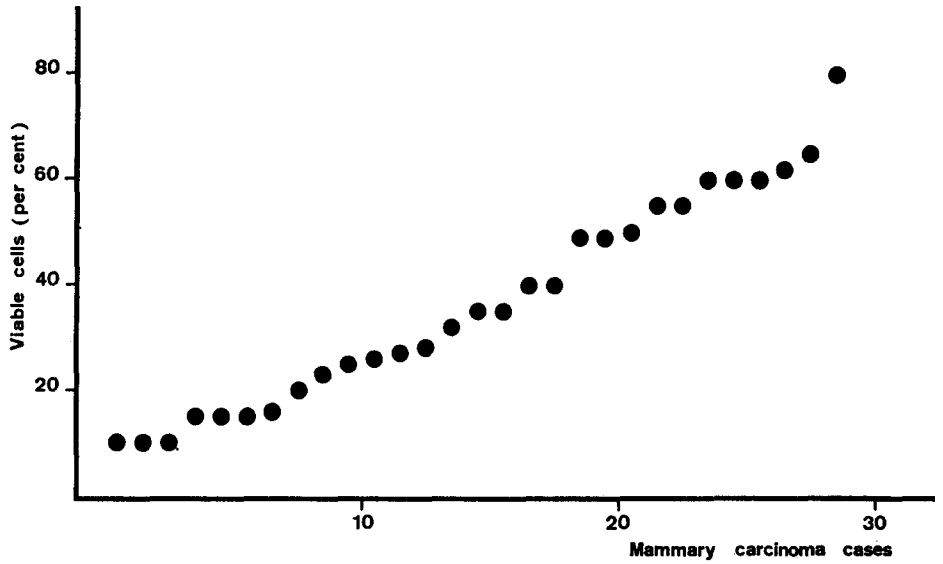


Fig. 6. Percentages of viable cells in aspirates from 29 primary carcinomas of the breast. The cases are arranged in order of frequency of viable cells. (Eosin dye permeability test).

This absence of effect on the viability and the proliferation rates of aspirated cells indicates that aspiration biopsy is suitable for sampling cell material from human tumours for biologic work.

Figure 6 shows cell viability rates in aspirates from twenty-nine primary carcinomas of the breast. In sampling the cell material, the needle was moved back and forth in the tumour four or five times and in some cases it was inserted into different areas of the tumour. The cases are arranged in order of frequency of viable cells. It is seen that aspirates from some of these mammary carcinomas contained only about 10 per cent of viable cells and that the highest values in the case series were in the range 60–80 per cent.

Study was also made of the viability rates of cell populations sampled from human tumours by aspiration biopsy on consecutive days during radiotherapy. Figure 7 shows the percentages of permeable cells in populations aspirated from a glandular metastasis (dia ca. 1 cm) of a squamous cell carcinoma and from a tonsillar lymphoblastic lymphoma (dia ca. 3 cm). A conventional X-ray apparatus (200 kV) was used and the skin doses were recorded. The metastatic squamous carcinoma was clinically resistant to irradiation and persisted after treatment, whereas the lymphoma was radio-

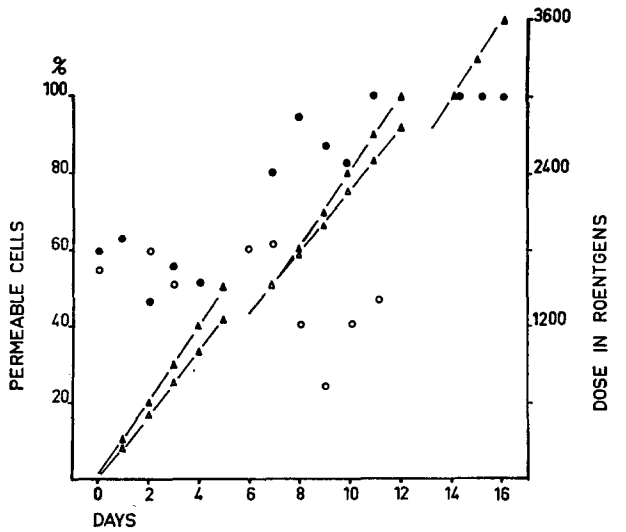


Fig. 7. Percentages of permeable cells in tumour populations aspirated on consecutive days during radiotherapy.

●=lymph node metastasis from squamous cell carcinoma
●=lymphoblastic lymphoma
▲△=radiation dosage.

sensitive and disappeared after about 11 days.

An apparent paradox is that cell death during radiotherapy progressively increased in the squamous carcinoma aspirates, reaching a maximum in the final days of treatment, but showed no measurable increase in aspirates from the radiosensitive lymphoma, even on the eleventh day, when the tumour had almost completely disappeared (Fig. 7). It is conceivable that this discrepancy in results was attributable to selective removal of damaged cells from the tonsillar lymphoma during irradiation, before cell death could be registered by means of the dye permeability test in biopsy aspirate. An observation which favours this hypothesis is that cells aspirated from tumours which clinically are regarded as radiosensitive (e.g., lymphoblastic lymphomas, seminomas and some poorly differentiated carcinomas or sarcomas) display in smear preparations marked decrease of mutual adhesiveness and behave as individual free cells. Contrary behaviour is shown by highly differentiated carcinomas and sarcomas [7].

Figure 7 further shows that aspiration biopsy was performed thirteen times on the patient with squamous carcinoma and nine times on the patient with lymphoblastic lymphoma. On all occasions the patients willingly accepted the biopsy.

Because of its simplicity, its acceptance by patients and its apparent innocuousness as regards the viability and proliferation rates of the sampled cells, aspiration biopsy is recommended as a method for collecting material from human tumours for morphologic and biologic studies.

RESUME

Une analyse critique des protocoles cytologiques de biopsies par aspiration des lésions mammaires a montré qu'un diagnostic de cancer a été posé, par la cytologie, dans 92,1 *pour cent des* 190 *cas de carcinomes vérifiés histologiquement. Les* 7,9 *pour cent restant consistaient en faux négatifs. La possibilité de faux positifs peut être écartée, si le cytologiste est pleinement familiarisé avec la morphologie des cellules mammaires, dans les différentes conditions pathologiques.*

Le matériel recueilli par aspiration des tumeurs mammaires fut aussi utilisé pour des études cytochimiques et pour des cultures de tissu . Le but de ces investigations comportait, notamment, la détermination de la viabilité des cellules cancéreuses pendant le traitement.

Afin de déterminer si la technique de la biopsie par aspiration peut, en elle-même, influencer la viabilité des cellules prélevées, l'action de pressions négatives fut étudiée dans des expériences séparées. La viabilité et la vitesse de croissance des cellules n'étaient pas modifiées par ces pressions négatives.

SUMMARY

A critical analysis of cytologic reports from aspiration biopsy of mammary gland lesions showed that a positive cancer diagnosis was cytologically made in 92·1 *per cent of* 190 *histologically verified carcinomas. In the remaining* 7·9 *per cent, false negative cytologic reports were given. The possibility of false positive diagnosis can be disregarded if the examining cytologist is fully familiar with the morphology of mammary gland cells in various pathologic conditions.*

Cell material aspirated from mammary tumours was used also for cytochemical studies and for tissue cultures. The aims of these investigations included determination of the viability of cancer cell populations during treatment.

In order to determine whether or not the aspiration biopsy procedure as such can influence the viability of the sampled cells, the effect of the negative pressures was studied in special experiments. The viability and proliferation rates of the cells were not impaired by these pressures.

ZUSAMMENFASSUNG

Die kritische Auswertung der zytologischen Untersuchungsprotokolle betreffend Aspirationsbiopsieen von Brustdrüsengeschwulsten ergab, dass eine positive Krebsdiagnose zytologisch in 92,1% *von histologisch verifizierten Karzinomen gestellt wurde. In den verbleibenden* 7,9% *wurde eine falsche negative zytologische Diagnose gestellt. Die Möglichkeit von falschen positiven Diagnosen kann praktisch ausser Acht gelassen werden, sofern der untersuchende Zytologe wohl vertraut mit der Morphologie der Brustdrüse unter den verschiedenen pathologischen Bedingungen ist.*

Aspiriertes Zellmaterial aus Brustdrüsengeschwulsten wurde ausserdem für zytochemische Untersuchungen und für Gewebskulturen verwendet. Das Ziel dieser Untersuchungen war festzustellen, wie sich die Wachstumsfähigkeit der Krebszellen während der Behandlung Verhält.

Um festzustellen, ob die Aspirationsprozedur als solche einen etwaigen Einfluss auf die Lebensfähigkeit der Zellen haben könnte, wurde der Einfluss von negativen Druckverhältnissen auf solche Zellen in besonderen Experimenten untersucht. Die Lebensfähigkeit und die Teilungsgeschwindigkeit wurden unter den angewendeten Bedingungen nicht beeinflusst.

REFERENCES

1. P. L. Cardozo, *Clinical Cytology*, L. Stafleu, Leyden (1954).
2. J. Zajicek, S. Franzén, P. Jakobsson and C. Rubio, Aspiration biopsy from mammary tumours in cytologic diagnosis and research. *Proc. 2nd. Int. Congr. Exfoliative Cytology.* In press.
3. J. Zajicek, P. L. Esposti and M. Vozelj, Quantitative determination of enzymic activities in cells obtained from human carcinomas by aspiration biopsy. *Proc. 1st Int. Congr. Exfoliative Cytology* **166,** (1962).
4. C. O. Criborn, S. Franzén, B. Unsgaard and J. Zajicek, Studies on the effect of aspiration biopsy on the viability of aspirated cells. *Scand. J. Haemat.* **1,** 272 (1964).
5. S. Franzén, G. Giertz and J. Zajicek, Cytological diagnosis of prostatic tumours by transrectal aspiration biopsy: A preliminary report. *Brit. J. Urol.* **32,** 193 (1960).
6. B. Unsgaard and J. Zajicek (data to be published).
7. B. Johansson and J. Zajicek, Sampling of cell material from human tumours by aspiration biopsy. *Nature, Lond.* **200,** 1333 (1963).

Europ. J. Cancer Vol. 1, pp. 259–264. Pergamon Press 1965. Printed in Great Britain

Lymphocytes from Leukaemic Blood Cultured with Phytohemagglutinin

G. ASTALDI, G. COSTA, R. AIRO and N. DUARTE
The Blood Research Foundation Center †, *Hospital of Tortona, Italy*

INTRODUCTION

IT HAS been shown that a good percentage of human blood lymphocytes in tissue culture with phytohemagglutinin (P.H.A.) [1–3] undergoes morphological, cytochemical, ultrastructural, as well as biological changes [4–14]. In particular, lymphocytes synthetise DNA and RNA and become first prolymphocytes, secondly lymphoblasts, and finally proliferating blast-like cells. The proliferating capacity of these cells is very high, as after 12 hr of colchicine (0·25 gammas/ml) they show a statmokinetic index of 315·6±26·4 [15]. It has been also observed that the development of lymphocytes to proliferating blast-like cells may be arrested by ionizing irradiation [16] as well as by a radiomimetic compound such as DAUNOMYCIN [17].

In the last few years P.H.A. has been tested also on peripheral blood lymphocytes cultivated from different leukaemias. The results of these researches show that lymphocytes from chronic lymphocytic leukaemia (*CLL*) give a deficient response when stimulated with P.H.A. [18–25], whereas lymphocytes from chronic granulocitic leukaemia (*CGL*) show a normal development. In our experiments, we obtained a percentage of 59·0±10·2 blast-like cells when cultivating for 3 days peripheral blood lymphocytes from normal beings, a percentage of 61·6±13·0 blasts when cultivating lymphocytes from *CGL*, and finally a percentage of 7·8±5·8 blasts when cultivating lymphocytes from *CLL* [26–28]. This behaviour seems to support the hypothesis, that a deficient development after P.H.A.-stimulus is one of the biological properties that lymphocytes acquire when becoming leukaemic. In other words, the mentioned behaviour seems to suggest that lymphocytes are "leukaemic" in *CLL*, and "normal" in CGL were only the granulocytic series should be leukaemic.

Another theory which seems to get support from the above-mentioned observations, is the one assuming that lymphocytic population is composed of two different cellular clones, characterized by different kinetics [29–31], different immunological functions [31–32], different sensitivity to glycocorticoid hormones [33] and to Roentgen irradiation [34], different site of their formation [31], and now a different response to the P.H.A.-stimulus. It is admitted that one clone of lymphocytes is formed in the thymus cortex, bone marrow and germinal centres of lymphoid follicles, while the other one is located around the lymphoid germinal centres, in the medullary cords and in the pulp of lymph nodes and spleen.

It is also possible that the type of response to P.H.A. — positive or negative — deals with the immunological stage of the single lymphocytes, as P.H.A. acts through an immunological mechanism. Perhaps, the immunological-competent-lymphocytes are P.H.A. responsive, and the immunological-non-competent-lymphocytes are those which do not respond to P.H.A. Possibly also, the defect of response to P.H.A. shown by *CLL*-lymphocytes does not depend directly on the cells, but on their environmental factors, i.e., on the *CLL*-plasma. In order to clarify this point, we cultivated blood lymphocytes from normal subjects in a tissue culture medium containing P.H.A., and added with

*These researches have been performed with the support of "Istituto Sieroterapico Milanese," Milano, Italy.
†Supported by "The Blood Research Foundation," Washington D.C., USA.

Table 1.

No. of Cases		Percentages of blast-like cells after 72 hr of tissue culture			
		Lymphocytes from Chr. Lymph. Leukaemia	Lymphocytes from normal subjects		
Chr. Lymph. Leukaemia	Normal	Own plasma	Own plasma	Heterologous plasma	
				Normal	Leukaemic
1	1/5	14·0	69·0±3·2	67·0±6·9	12·6±3·2
2	6/10	9·0	60·2±9·3	66·0±6·0	7·8±5·3
3	11/15	7·0	69·8±3·0	66·8±10·2	9·0±1·9
4	16/20	8·0	65·2±4·1	71·4±4·3	13·6±3·2
5	21/25	5·0	78·2±7·1	73·6±8·2	8·2±5·2
6	26/30	2·0	63·8±8·8	65·4±5·8	8·8±4·6
Mean±S.D.		9·0±4·4	67·7±6·1	68·3±3·5	10·0±2·7

CLL-plasma. In particular, the following experiment was undertaken:

METHODS

Peripheral blood lymphocytes from normal subjects were cultivated in NCTC 109 (Microb. Ass.) added with P.H.A. and divided in 3 lots. The first lot was added with autologous plasma (20%), the second lot with heterologous normal plasma (20%), the third one with plasma from *CLL*-patients (20%). Contemporarily, the peripheral blood lymphocytes from those *CLL*-patients were cultivated in the same NCTC 109, added with 20% of their own plasma. In conclusion, plasmas from 6 *CLL*-patients have been tested on blood lymphocytes from 30 normal subjects, each *CLL*-plasma being tested on the lymphocytes from 5 different normal beings.

RESULTS

The results are shown in Fig. 1 and summarized in Table 1. It appears that blood lymphocytes from *CLL*-patients after 3 days of tissue culture with their own plasma, developed a percentage of blast like cells of 9·0±4·4, while the blood lymphocytes from normal subjects developed the following percentages of blast-like cells: a. 67·7±6·1, when cultivated with their own plasma; b. 68·3±3·5, when cultivated with an heterologous normal plasma; c. 10·0±2·7, when cultivated with plasma from *CLL*. Results similar to these reported in c., have been obtained testing concentrations of 10% and 5% of the same *CLL*-plasmas.

From the reported data, it appears that both normal and *CLL*-lymphocytes show a defect in development when cultivated in *CLL*-plasma, whereas normal lymphocytes show a normal development when cultivated in normal plasma, either autologous or heterologous. This seems to prove that in *CLL*-plasma a condition exists which causes the defect of response of peripheral blood lymphocytes to the P.H.A.-stimulus.

Another observation, that we should like to submit for your criticism, deals with the relationships between ionizing irradiation of the spleen and the *CLL*-lymphocyte development after P.H.A.-stimulus. In particular, we have irradiated the spleen of 2 *CLL*-patients with 400 and 480 r, respectively, administered in different doses (5–6). Before irradiation (Table 2), the blood lymphocytes in tissue culture with P.H.A. (3 days) developed a percentage of 5·5 and of 13 blast-like cells, respectively; i.e., they showed a typical *CLL*-behaviour. After irradiation, the blood lymphocytes from the same patients developed a percentage of 67 and of 76 blast-like cells, when cultivated with P.H.A. for 3 days.

In order to explain the reported observation it may be assumed that X rays have destroyed the P.H.A.-non-responsive-lymphocytes (the leukaemic ones), and preserved the P.H.A.-responsive (the normal ones). Nevertheless, this does not seem to be an adequate explanation. In fact, after irradiation (Fig. 2) we do not observe a definite relation between the decrease in the lymphocyte counts and the increase of the blast-like cells in tissue cultures with P.H.A. Moreover, it appeared that 4–8

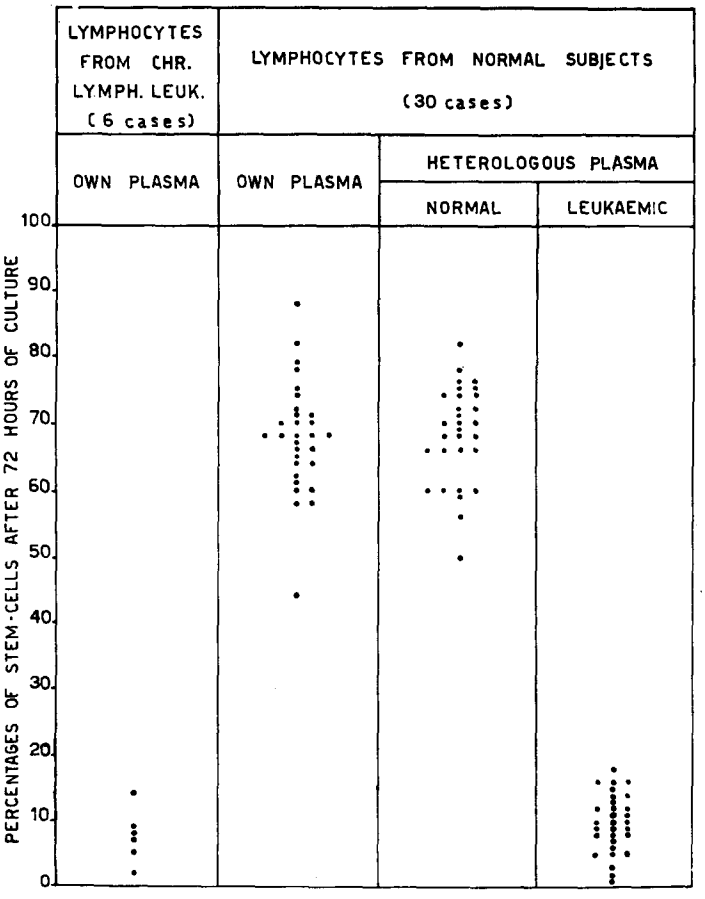

LYMPHOCYTES FROM CHR. LYMPH. LEUK. (6 cases)
LYMPHOCYTES FROM NORMAL SUBJECTS (30 cases)
OWN PLASMA
OWN PLASMA
HETEROLOGOUS PLASMA
NORMAL
LEUKAEMIC
PERCENTAGES OF STEM-CELLS AFTER 72 HOURS OF CULTURE
100
90
80
70
60
50
40
30
20
10
0

Fig. 1.

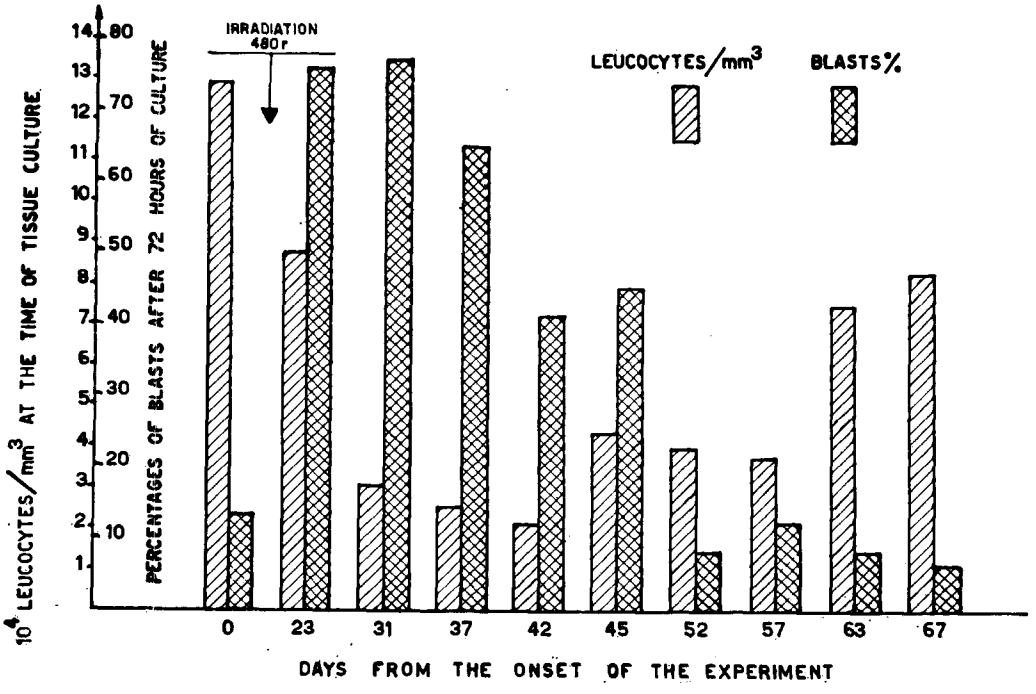

IRRADIATION 480 r
LEUCOCYTES/mm3
BLASTS%
10⁴ LEUCOCYTES/mm3 AT THE TIME OF TISSUE CULTURE
PERCENTAGES OF BLASTS AFTER 72 HOURS OF CULTURE
0
23
31
37
42
45
52
57
63
67
DAYS FROM THE ONSET OF THE EXPERIMENT

Fig. 2.

Table 2.

	Days from the onset of the experiment	Spleen irradiation (r)	Leucocytes/mm³	Percentages of blasts after 72 hours of culture
Case N.1	0	—	110,000	5·5
	2/12	400	—	—
	28	—	8,500	67
	72	—	45,000	7
Case N.2	0	—	129,000	13
	1/13	480	—	—
	23	—	86,400	76
	31	—	30,200	77
	37	—	25,000	65
	42	—	21,000	41
	45	—	43,000	45
	52	—	39,400	8
	57	—	37,000	12
	63	—	74,000	8
	67	—	82,000	6

weeks after the spleen irradiation, lymphocytes were P.H.A.-non-responsive again, independently from the degree of increase in their count. Because of this, we believe that the spleen irradiation may interfere with the *CLL*-plasma in such a way that its inhibiting factor(s) on lymphocytic development is (are) removed.

CONCLUSION

The results taken as a whole seem to prove that the defect of the response to P.H.A. presented by *CLL*-lymphocytes does not depend on lymphocytes themselves but in some plasmatic condition, which may be temporarily removed by the spleen irradiation.

RESUME

Les lymphocytes du sang périphérique de la leucémie lymphoïde chronique (LLC) réagissent mal à la phytohémagglutinine en culture de tissu, tandis que les lymphocytes de la leucémie myéloïde chronique réagissent comme les lymphocytes du sang normal. Il apparaît que cette absence de réponse au stimulus P.H.A. des lymphocytes de la LLC ne dépend pas directement des cellules mêmes, mais du plasma. En fait, le plasma de LLC ajouté à des lymphocytes de sang normal, en culture de tissu avec P.H.A., empêche leur transformation en cellules proliférantes semblables à des cellules blastiques. L'irradiation de la rate par rayons X peut supprimer cette action inhibitrice du plasma de la LLC.

SUMMARY

Peripheral blood lymphocytes from chronic lymphocytic leukaemia (CLL) *react very poorly to PHA in tissue culture, whereas lymphocytes from chronic granulocitic leukaemia* (CGL) *react as lymphocytes from normal blood do. It seems that this defect of response to PHA-stimulus of lymphocytes from* CLL *does not directly depend on the cells themselves, but on the plasma. In fact, the plasma from* CLL *added to normal blood lymphocytes in tissue culture with PHA inhibits their development to proliferating blast-like cells. X-ray irradiation of the spleen may remove that inhibiting condition of the* CLL*-plasma.*

ZUSAMMENFASSUNG

Peripherische Blutlymphozyten von Fällen von chronischer lymphatischer Leukämie (CLL) reagieren nur schlecht auf PHA in Gewebskulturen und zwar im Gegensatz zu Lymphozyten von chronischer granulozytischer Leukämie (CGL), die wie Lymphozyten aus normalem Blut ansprechen. Es scheint, dass diese mangelnde Reaktion der Lymphozyten von CLL auf den PHA-Stimulus nicht von den Zellen selbst, sondern von dem Plasma abhängt. Tatsächlich ist es so, dass die Zugabe von Plasma von CLL zu normalen Blutlymphozyten in Gewebskulturen mit PHA die Entwicklung der letzteren zu blastähnlichen Zellen verhindert. Röntgenbestrahlung der Milz ist fähig die hemmende Wirkung des CLL-Plasmas aufzuheben.

REFERENCES

1. I. G. Li and E. E. Osgood, A method for rapid separation of leukocytes and nucleated erythrocytes from blood or marrow with phytohemagglutinin from red beans. (*Phaseoulus Vulgaris*). *Blood* **4,** 670 (1949).
2. D. A. Rigas and E. E. Osgood, Purification and properties of the phytohemagglutinin of *Phaseoulus Vulgaris*. *J. Biol. Chem.* **212,** 607 (1955).
3. D. A. Rigas and E. A. Johnson, Studies on the phytohemagglutinin of "Phaseoulus Vulgaris" and mitogenecity. In: Leukopoiesis in health and disease. *Ann. N.Y. Acad. Sci.* **113,** 800 (1964).
4. D. A. Hungerford, A. J. Donnelly, P. C. Nowell and S. Beck, The chromosome constitution of a human phenotipic intersex. *Amer. J. Hum. Genet.* **11,** 215 (1959).
5. P. C. Nowell, Phytohaemagglutinin an initiator mitosis in cultures of normal human leukocytes. *Cancer Res.* **20,** 462 (1960).
6. K. Carstairs, The human small lymphocyte: its possible pluripotential quality. *Lancet* **1,** 829 (1962).
7. A. A. MacKinney, Jr. F. Stohlman, Jr. and G. Brecher, The kinetics of cell proliferation in culture of human peripheral blood. *Blood* **19,** 349 (1962).
8. L. Bermann and G. S. Stulberg, Primary cultures of macrophages from normal human peripheral blood. *Lab. Invest.* **11,** 1332 (1962).
9. G. Astaldi, E. Strosselli and S. E Sauli, Le cellule emiche nella ricerca citogenetica. *Haematological* **47,** 1 (1962).
10. W. H. Marshall and K. B. Roberts, The growth and mitosis of human small lymphocytes after incubation with phytohemagglutinin. *Quart. J. Exp. Physiol.* **48,** 146 (1963).
11. E. H. Cooper, P. Barkhan and A. J. Hale, Observation on the proliferation of human leukocytes cultured with phytohemagglutinin. *Brit. J. Haemat.* **9,** 101 (1963).
12. W. M. Elves and J. F. Wilkinson, The effect of phytohemagglutinin on normal and leukemic leucocytes when cultured *in vitro* *Exp. Cell Res.* **30,** 200 (1963).
13. Y. Tanaka, L. B. Epstein, G. Brecher and F. Stohlman, Jr. Transformation of lymphocytes in cultures of human peripheral blood. *Blood* **22,** 614 (1963).
14. G. Astaldi, Das Phytohemagglutinin und des Problem des Lymphocytes. *Med. Klin.* **59,** 368 (1964).
15. G. Astaldi, e G. Costa, Sull'attività proliferativa delle cellule staminali ottenute dal sangue normale con la fitoemagglutinina. *Bioch. e Biol. Sper.* **3,** 364 (1964).
16. G. Astaldi et G. Costa, Influence des rayons X sur l'évolution des cellules souches derivées des lymphocytes cultivés en présence de phytohémagglutinine. Congr. Swiss. Soc. Haematol., Luzern, May 1965. *Schweiz. med. Wochr.* In press.
17. G. Costa et e G. Astaldi, Effetto inibente della daunomicina sull'evoluzione staminale dei linfociti. *Gazz. Int. Med. Chir.* **70,** 597 (1965).
18. P. C. Nowell, Differentiation of human leukemic leukocytes in tissue culture. *Exp. Cell. Res.* **19,** 267 (1960).

19. R. Schrek and Y. Rabinowitz, Effects of phytohemagglutinin on rat and normal and leukaemic human blood cells. *Proc. Soc. Exp. Biol. N.Y.* **113,** 191 (1963).
20. D. Quaglino and D. C. Cowling, Cytochemical studies on cells from chronic lymphocytic leukaemia and lymphosarcoma cultures with phytohaemagglutinin. *Brit. J. Haemat.* **10,** 358 (1964).
21. D. Quaglino, Cytochemistry in relation to short-term tissue cultures. *Proc. 9th Congr. Europ. Soc. Haemat.* **1,** 112, Lisbon 1963. Karger, Basel.
22. C. Bernard, A. Geraldes and N. M. Boiron, Action de la phytohemagglutinine *in vitro* sur les lymphocytes de leucémies lymphoïdes chroniques. *Nouv. Rev. Franc. Hémat.* **4,** 69 (1964).
23. J. H. Robbins, Human peripheral blood in tissue culture and the action of phytohemagglutinin. *Experientia* **20,** 164 (1964).
24. J. H. Robbins, Tissue culture studies of the human lymphocytes. *Science* **146,** 1648 (1965).
25. M. W. Elves, S. Roath and M. C. Israels, Failure of lymphocytes from hypogammaglobulinaemic subjects to transform in culture. *Brit. Med. J.* **2,** 1051 (1965).
26. G. Astaldi, R. Airò and S. Sauli, *In vitro* studies on leukaemic cells. In *Symposium on Current Research in Leukaemia.* Cambridge (1964).
27. G. Astaldi, R. Airò e S. Sauli, Problemi di biologia delle cellule leucemiche studiati con la cultura *in vitro*. *Min. Med.* **56,** 1099, (1965).
28. G. Astaldi, S. Sauli, R. Airò, L. Ratto and G. Costa, With reference to the effect of phytohemagglutinin on lymphocytes from different leukemias. Texas Rep. (1965). In press.
29. E. E. Osgood, H. Tivey, K. B. Davidson, A. J. Seam and J. G. Li, The relative rates of formation of new leukocytes in patients with acute and chronic leukemias. *Cancer* **51,** 331 (1952).
30. N. B. Everett, R. W. Caffrey and W. C. Riecke, Recirculation of lymphocytes. *Ann. N.Y. Acad. Sci.* **113,** 887 (1964).
31. G. C. Craddock, G. S. Nakai, H. Fakuta and L. M. Vaslager, Proliferative activity of the lymphatic tissues of rats as studied with tritium labelled tymidine. *J. Exp. Med.* **120,** 389 (1964).
32. J. G. Gorman and J. Chandler, Is there an immunologically incompetent lymphocyte? *Blood* **23,** 117 (1964).
33. R. Schrek, Prednisolone sensitivity and cytology of viable lymphocytes as tests for chronic lymphocytic leukemia. *J. Nat. Cancer Inst.* **33,** 837 (1964).
34. C. A. Trowell, Lymphocyte, In *The Biology of Cells and Tissues in vitro.* **2,** p. 95. Academic, New York. (In press).

Europ. J. Cancer Vol. 1, pp. 265–273. Pergamon Press 1965. Printed in Great Britain

Observations on Primary Short-term Cultures of Human Tumors

A second 5-year Study*†

DOROTHY G. WALKER, MICHAEL M. LYONS and JANE C. WRIGHT‡

Departments of Surgery and Pathology, New York University Medical Center, Bellevue Hospital and University Hospital.

THIS report deals primarily with the *in vitro* behavior of five major human tumor groups in explant and dispersed cell cultures: lymphomas, sarcomas, melanomas, carcinomas, and tumors of the nervous system.

The growth of human tumors *in vitro* dates back to 1911 [1] when Carrell and Burrows reported on the growth of "human sarcomata and carcinomata." Since that time a number of investigators have grown human tumors for a variety of purposes. Notable among the works which provided guidelines, allowed for correlations or served as reference sources for the present study, are the investigations of Cobb *et al.* [2–4], Murray and Stout [5], Papanicolaou and Maddi [6], Orr and McSwain [7], DiPaolo [8], Lasfargues and Ozzello [9], Hu *et al.* [10] and Dobrynin [11].

Our study was initiated in an effort to develop an *in vitro* drug sensitivity test system capable of predicting the clinical response in an individual patient with cancer. Evaluation of drug sensitivity was based on morphological evidence of cytotoxicity. Some of these results have been published [12].

The growth phase of the study was, therefore, secondary and was limited to observations of cultures up to the time they were considered ready for testing, usually 4–10 days.

SUMMARY OF THE FIRST 5-YEAR STUDY

The growth phase of the first 5-year study, conducted by Dr. Jewel Cobb, was concerned with the growth characteristics and cellular morphology of a variety of human tumors and the determination of an optimal culture medium for the majority of these tumors. A small number of "normal"§ tissues were also studied. Some of the distinguishing features of the various tumor types were recorded [12]. 1. Lymphoma cultures were characterized by the presence of a large number of immature lymphocytic forms. 2. Sarcoma cultures, though identified with some difficulty, exhibited cells with rounder nuclei as compared with other fibroblastic cells, marked fibrinolysis and a swirling growth pattern. 3. Melanoma cultures generally exhibited radial outgrowths. The melanocyte was observed in various stages of differention ranging from the undifferentiated uni-polar or bipolar form, in cultures of amelanotic melanomas, to the differentiated dendritic form in cultures of more heavily pig-

*Supported in part by grants from the American Cancer Society, Nos. T-244 and T-244a, and United States Public Health Service (1) CA-0735401 and (2) CA-05760-03.

†Presented in part at the International Symposium on the Biological Characterisation of Human Tumours, Abbaye de Royaumont, France, May, 1965.

‡The authors are deeply indebted to the surgeons, Drs. Stephen Gumport, Frederick Golomb and Matthew Harris, for their co-operation in supplying the operative specimens; to Mrs. Barbara Merriday for her invaluable assistance in compiling the tabular data, to Dr. Carlton Nadolney for his help in the interpretation of these data, and to Mrs. Bertha Tyler, Miss Corinne Deihl, Mrs. Carole Brodie, Mrs. Adele Colon and Miss Celia Hindin for their technical and secretarial assistance.

§"Normal" tissues were obtained at surgery performed for (a) excision of a questionable enlarged lymph node, (b) herniorrhaphy, or (c) removal of adjacent non-pathological tissue from patients with a diagnosed malignancy.

mented melanomas [4]. 4. Carcinomas in culture were distinguished by the following criteria: cells with polygonal to round shape, round vesicular nuclei, prominent large nucleoli, marked basophilic cytoplasm, and variation in nuclear and total cell diameters within groups of cells.

A variety of media were used in an attempt to improve the rate and extent of growth. These included Eagle's basal medium, NCTC 109, and Medium 199 supplemented with varying proportions (10, 20 and 40 per cent) of human, horse, and calf sera. A comparative study evaluating the effects of heterologous (horse), homologous (pooled human) and autologous sera, each combined with Eagle's basal medium was reported [13]. Forty-one culture series of "normal" and malignant tissues were studied. Of 21 "normal" and malignant culture series, 19 exhibited better outgrowths in Eagle's medium with autologous serum.

Results of the *in vitro* growth were recorded for a four-year period. A total of 549 tissue and pleural and peritoneal effusion specimens from 366 patients were prepared *in vitro*. Three hundred and sixty-four, or 66·4 per cent, grew in culture. The per cent growth of the major groups studied was: lymphomas, 84 per cent; sarcomas, 70·3 per cent; melanomas, 72·1 per cent; carcinomas — skin, 43·7 per cent; breast, 54·6 per cent; respiratory system, 73·3 per cent; alimentary system, 61·2 per cent; primary site undetermined, 70·5 per cent; male and female urogenital system, 54·1 per cent; malignant neoplasms of the nervous system, 78·5 per cent; benign tumors, 72·7 per cent; "normal" tissues, 69·5 per cent.

The experiences gained from the first 5-year study were applied to the design of the present study, particularly with regard to the cytomorphological criteria of growth and the optimal culture medium. Attention could then be directed to the semiquantitative characterization of critical growth parameters. This is of special importance in our evaluation of the cytostatic and cytotoxic effects of the cancer chemotherapeutic agents under study.

The following parameters will be presented for a representative sample of each tumor type: the per cent growth; relative growth rates and survival times; mitotic activity; and preliminary findings on the relative levels of incorporation of tritiated nucleosides. Examples of structural or organotypic growth patterns and morphologically distinct cell types will be cited.

MATERIALS AND METHODS

The specimens cultured included fresh biopsies of primary or metastatic solid tumor masses and pleural and peritoneal effusions from patients with far advanced neoplastic disease.

The solid tumor specimens were collected aseptically in 1 oz jars containing 5 cml^3 of Medium 199 (without bicarb) plus 150 mcg/ml of neomycin. The specimens were then cut into 1–2 mm^3 fragments (explants). Three explants were placed on a 9×22 mm coverslip in 1–1 chicken plasma-embryo extract clot. Twenty to 30 such cultures were prepared from each specimen. The coverslip cultures were inserted into Leighton tubes and fed initially with 1–1·5 ml of Medium 199, with 150 mcg/ml of neomycin sulfate added, plus 20 per cent autologous or pooled human serum. A maintenance solution of Medium 199 plus 10 per cent pooled human serum was used for all subsequent feeding.

Medium 199, without (w/o) and with $NaHCO_3$ (bicarb), was used in proportions which, after serum was added, would result in a physiological level of bicarb, thought to be better suited for cells of low proliferative activity. Thus, the initial growth medium consisted of 80% Medium 199 without bicarb +20% autologous or pooled human serum. The maintenance medium consisted of 70% Medium 199 w/o bicarb, 20% Medium 199 with bicarb and 10% pooled human serum. This gave 45·2 and 47·6 mg/100 ml of $NaHCO_3$ in the growth and maintenance media, respectively. Complete Medium 199 contains 125 mg/100 ml.

The fluid specimens were centrifuged and the sedimented cells redistributed in Medium 199 plus 40% of the supernatant fluid. One ml aliquots containing approximately 50,000 cells were placed in Leighton tubes with coverslips.

Autoradiography

Cultures were exposed to 1 μc/ml of tritiated thymidine (H^3-TDN) or tritiated uridine (H^3-UR) for 1 hr and then fixed in 1:3 parts glacial acetic acid and absolute ethanol. The fixed cultures were processed by the film-stripping technique outlined by Schwartz Bioresearch Laboratories.*

RESULTS AND DISCUSSION

Our present series comprised a total of 607 tumor specimens isolated from 580 patients.

*Schwartz Bioresearch, Inc., Orangeburg, New Jersey.

Table 1. *Summary of growth of human tumors in tissue culture.*

	Tumor type	Positive growth	Total number (%)
I.	*Lymphomas*		
	Lymphosarcoma	14	15
	Hodgkin's disease	10	14
	Mycosis fungoides	18	23
	Reticulum cell sarcoma	5	6
	Malignant lymphoma:		
	Undetermined type	15	16
	Total:	62	74 (83·6%)
II.	*Sarcomas*	21	32 (65·6%)
	Fibrosarcoma		
	Rhabdomyosarcoma		
	Osteogenic sarcoma		
	Liposarcoma		
	Hemangiosarcoma		
III.	*Melanomas*	57	81 (70·3%)
IV.	*Carcinomas*		
	Skin: basal cell (BCE)	31	34
	squamous cell	7	9
	Lung and bronchus	10	18
	Mouth and esophagus	5	9
	Stomach	6	9
	Colon and rectum	18	26
	Liver	5	5
	Breast	37	57
	Ovary	23	28
	Testis	3	3
	Prostate	0	3
	Kidney	9	13
	Urinary bladder	7	9
	Metastatic carcinoma 1°?	10	15
	Total:	171	236 (72·4%)
V.	*Tumors of the nervous system*		
	Malignant		
	Astrocytoma	12	16
	Glioblastoma	12	17
	Medulloblastoma	3	3
	Reticulum cell sarcoma in brain	1	2
	Metastatic carcinoma in brain	6	9
	Miscellaneous	5	8
	Benign		
	Meningioma	7	10
	Acoustic neuroma	1	1
	Total:	47	64 (73·4%)
VI.	*Miscellaneous neoplasms*	51	54 (94·4%)
	Carcinoma of thyroid		
	Carcinoma of pancreas		
	Carcinoma of vulva		
	Mesothelioma		
	Dysgerminoma		
	Multiple myeloma		
	Neuroblastoma		
	Chronic lymphatic leukemia		
VII.	*Tumor type undetermined*	7	11
VIII.	*Benign tumors*	15	52 (28·8%)
	Fibroma		
	Lymphadenopathy		
	Prostate hyperplasia		
IX.	*Normal*		
	Brain	1	2
	Kidney	1	1
	Total:	2	3
Totals:		433	607

Approximately ten per cent of the specimens received were classified as lost-to-study because of contamination, necrosis, absence of cells in pleural and peritoneal effusion specimens, and mishaps in the culture procedures.

Growth of the various tumor types

Collectively, 75 per cent of the specimens cultured exhibited growth, i.e. migration of cells, with or without mitotic activity. Specimens exhibiting growth in any or all of the explants are included. The per cent growth for a given 1-year period ranged from 63·8 to 85·2 per cent. The highest per cent growth was recently achieved after the $NaHCO_3$ concentration was adjusted to a physiological level better suited for cells of low proliferative activity. The data for the entire series is given in Table 1.

Lymphomas exhibited the highest per cent growth (83·6 per cent) and the benign tumors the lowest (28·8 per cent). The relative per cent growth in decreasing order is: lymphomas, tumors of the nervous system, carcinomas, melanomas, sarcomas, benign tumors. The high per cent growth (94·1 per cent) of a group of miscellaneous neoplasms (Table I, Group VI), because of the variety, cannot be explained in the context of this report. The low per cent growth of the benign tumors, in this series, was attributed to the exceedingly poor growth (17·7 per cent) of 46 benign prostate hyperplasias from males over 60 years of age. By comparison, there was 72·7 per cent growth of benign tumors in the first five year series and this was attributed to the inclusion of several benign tumors from children (neurofibroma, craniopharyngioma, and benign giant cell tumor). The age factor cannot be applied to the group of "normal" tissues which exhibited 69·5 per cent growth, but rather, as the sources indicate, that these tissues were in a hyperreactive state.

The relation of age of patient and growth in culture has not been fully examined, but the general impression has been that tumors of children exhibit a very high per cent and rate of growth in culture. A representative sample of the age range for each group was recorded: Lymphomas, 30–64 years; sarcomas, 13 years and 31–50 years; melanomas, 38 years and 62–80 years; carcinomas, 40–70 years; tumors of the nervous system, 2, 3, 8 years and 46–58 years.

Variable rates of growth were observed in the tumor groups. These were defined in an earlier publication and are as follows: *slow*, migration, 1 to 100 μ and/or density 10 to 100 cells in 150× fields per 72 hr; *moderate*, migration 100 to 200 μ and/or density 100 to 200 cells in 150× fields per 72 hr; *fast*, migration beyond 200 μ and/or density over 200 cells in 150× fields per 72 hr.

Table 2 shows the distribution of growth rates of the various tumors.

Table 2. Comparative growth rates of various tumor groups.

Tumor type	Total number	Fast	Moderate	Slow
Lymphomas	73	16·4%	49·3%	34·2%
Sarcomas	31	19·4%	51·6%	29·0%
Melanomas	59	15·3%	47·5%	37·2%
Carcinomas	197	22·8%	35·1%	42·1%
Tumors of the nervous system	47	31·9%	29·8%	38·3%

Moderate growth was observed in the majority of lymphomas, sarcomas, and melanomas, while the majority of carcinomas grew slowly. The tumors of the nervous system were almost evenly divided with regard to the number exhibiting fast and slow growth. All the nervous system tumors from children were in the fast-growing group.

The relation of initial growth rate to survival in culture was noted. In this context, the criterion of survival is viability (cultures exhibiting good cellular definition, cellular migration and/or mitotic activity) up to or beyond day 21. The number of survivor cultures was determined from those found to be suitable for testing within three weeks after explantation (Table 3).

From the data presented in Table 3, it can be concluded that the higher number of survivors in the fast growing group may be explained by their physiological autonomy. Examination of the data concerning the slow-growing group further supports this premise. In particular, the

Table 3. Relation of initial growth rates to survival in culture.

Tumor type	Growth rates		
	Fast	Moderate	Slow
Lymphomas	12/12	33/36	13/25
Sarcomas	6/6	12/16	6/9
Melanomas	9/9	28/28	15/22
Carcinomas	31/45	65/69	39/83
Tumors of the nervous system	15/15	12/14	18/18

The expression is a ratio of the number surviving the three week period over the total number which grew in culture.

carcinomas had the highest per cent of slow growth (Table 2) and the smallest number of survivors (Table 3). A low number of survivors was also noted in the slow-growing lymphomas and a relatively low number in the melanomas. It appears that tumors having biochemically differentiated cell types, e.g., melanin producing melanocytes, lymphoid elements, and mucin producing cells of adenocarcinomas, usually had the smallest number of survivors. These findings are consistent with current concepts of the balance of cellular autonomy and differentiation.

Growth patterns and cell types

Several patterns of growth were observed but these were not specific for given tumor groups.

First, there is the simple spreading out of the explant and a drifting out of cells. This was best demonstrated in a metastatic adenocarcinoma to brain (Fig. 11). There appeared to be no cellular adhesiveness either to the glass surface, plasma, substrate or other cells. The cells were gradually lost in the supernatant media during refeeding.

Second, is the random migration of single cells. This is observed to some degree in all cultures and is the dominant pattern of growth in a majority of tumor and tissue specimens in culture (Figs. 9, 10, 15, 17).

The factors responsible for cell mobility have not been examined critically as such. However, the orientation of cells has been seen to be modified by physical surface phenomena: plasma clot thickness, microetchings in the glass surface, amorphous fibers, wire mesh cross-hatchings, perforations in cellophane strips and cellulose sponge matrices.

Biochemical reactions, such as those resulting in clot liquefaction would seem to play a role. Liquefaction of the plasma clot, in several cases, appeared to be associated with the migration of a 'front' of spindle cells followed by the emergence of a confluent sheet of polygonal cells. In some cases, the spindle cells were just ahead of the advancing sheet and formed an attached fringe. In other cases, the spindle cells preceded the sheet of polygonal cells leaving a clear zone several micra wide. In either case, the spindle cells usually became oriented both circumferentially at the edge of the liquefaction pool and radially into the plasma clot at the periphery.

Third, is the migration of cells in confluent sheets, with or without a fringe of spindle cells (Figs. 1, 2, 6, 10). This was generally observed in carcinoma and other tissue specimens of epithelial origin. It was never observed in the lymphomas, except in one case of mycocis fungoides, and this was believed to be an outgrowth of skin.

Fourth, is the organotypic migration in which the cells appear to be structurally oriented in a three dimensional matrix. This was best demonstrated in a thyroid hyperplasia culture (Figs. 3, 4). Initially, growth was fast, but the extent of cellular migration was restricted by a palisading of cells visualized as an alternating pattern of ridges in the sheet of cells.

The cell types observed, like the growth patterns were not specific for a given tumor type. In general, cells were round (Fig. 9), polygonal (Fig. 6), bipolar and multipolar spindle shaped (Figs. 7 and 8). One or all of these cell types have been found in a single culture. Whether they represent one or multiple origins in a given culture is not known.

In our laboratory, several cells having some of the characteristics observed in tissue sections were seen. Notably, the signet ring cells of adenocarcinoma (Fig. 9), the immature lymphoid cells (Fig. 11) and reticulum cells (Fig. 12) of the polymorphic infiltrate in mycosis fungoides, and the melanin containing or producing cells in melanotic (Fig. 10) and amelanotic melanomas, respectively.

Mitotic activity in primary cultures

Seventy-four cultures exhibiting good growth, i.e., fast growth within 12 hours or extensive migration, or, in the case of effusion specimens, a dense cell population, were examined for evidence of mitotic activity. A range of 0·1 per cent to 7·8 per cent was found, based on cell counts of 500–1000 cells.

It was thought that fluctuations in the mitotic index of a given culture series could be used to determine drug cytotoxicity. This was not possible, however, since upon examination, no evidence of mitosis was found in many of the control cultures.

Mitotic indices were calculated at varying times during the culture period. These findings are summarized in Table 4.

Examination of the mitotic indices in several individual culture series of particular tumor types revealed definite peaks of mitotic activity (underlined in Table IV). Furthermore, there is a notable coincidence between the time in culture in which there is mitotic activity and survival for three weeks in culture, as defined above.

Carcinomas and lymphomas attain their peak mitotic activity early in culture and have a correspondingly small number of survivor cultures, 13/25 and 39/83, respectively. These

Table 4. Mitotic indices of human tumors in primary culture: Comparisons of tumor types and aging cultures.

Name/TC	Diagnosis	Mitotic index					
		3–7 days	8–12 days	13–17 days	18–22 days	23–27 days	28 days and over
	Lymphomas						
MD/286D	Lymphosarcoma	0·3*		0·2			0·1
MY/897	Reticulum cell sarcoma	2·0			0·1		
GL/991	Malignant lymphoma: Undetermined type	0·9				1·0	
	Sarcomas						
AC/1001	Fibrosarcoma (P.F.)†	0		0·3	0·1		0
CK/962	Fibrosarcoma	3·5	0				
RM/1003	Osteogenic sarcoma	1·3					0·9
	Melanomas						
HS/532A	Recurrent melanoma			1·7		2·3	
	Carcinomas						
WM/904	Stomach		0				0
BG/987A	Breast (P.F.)†			0·3	0·1	0·1	0
MK/995	Breast		0	0			
WJ/1000	Kidney	0·6	1·4	0·6	0·6		0
CP/999	Kidney		0·3	0·1	0·3	0	
MM/811	Testis				0·1	0	
MS/970A	Thyroid (metastatic)	0·9		0·3			0
D/895	Undifferentiated carcinoma 1° Unknown	0		1·0	0·4		
AP/1002	Metastatic carcinoma 1° Unknown	0·6	0·1	0			
	Tumours of the nervous system						
SM/960	Cystic glioblastoma		2·4			0·1	0
MG/957	Glioblastoma	0					0
SV/979	Brain tumor, type?		2·0				0·9
NK/998	Ganglioneuroma			0	0		

*The underscored mitotic index number is the calculated peak of mitotic activity.
†P.F. — Pleural and peritoneal fluids.

data are compatible with the previously discussed relationship between cellular autonomy and differentiation.

Preliminary study of tritiated nucleoside incorporation

Twenty culture series were studied for the relative incorporation of the tritiated nucleosides, H^3-thymidine and H^3-uridine. Cultures were exposed to 1 μc/ml of H^3-thymidine or H^3-uridine for one hour at 37°C and then fixed. Each labelled nucleoside presented a distinct pattern as determined from cytological and cytochemical examination (Figs. 13–16). H^3-thymidine labelling was confined to the nucleus in an even, and usually dense, distribution. H^3-uridine labelling was also confined to the nucleus, but there was increased density in the area of nucleolar material. This was best demonstrated in a few preparations with slightly displaced films (Fig. 17).

Evaluation of the levels of incorporation was based on the per cent of labelled nuclei. An attempt was made to relate nucleoside incorporation, indicative of DNA and RNA synthesis or the phases of the mitotic cycle with the mitotic index. (Table 5).

It was expected that high levels of H^3-uridine incorporation, e.g., would coincide with a low mitotic index. Alternatively, high levels of thymidine incorporation would parallel high mitotic indices: such relationships were found in only a few cases. Agreement of H^3-thymidine incorporation and mitotic activity were found in the lymphomas, but in only one case each of sarcomas, carcinomas, and tumors of the nervous system. With H^3-uridine, this relationship was found in 1/4 sarcomas, 3/6 carcinomas,

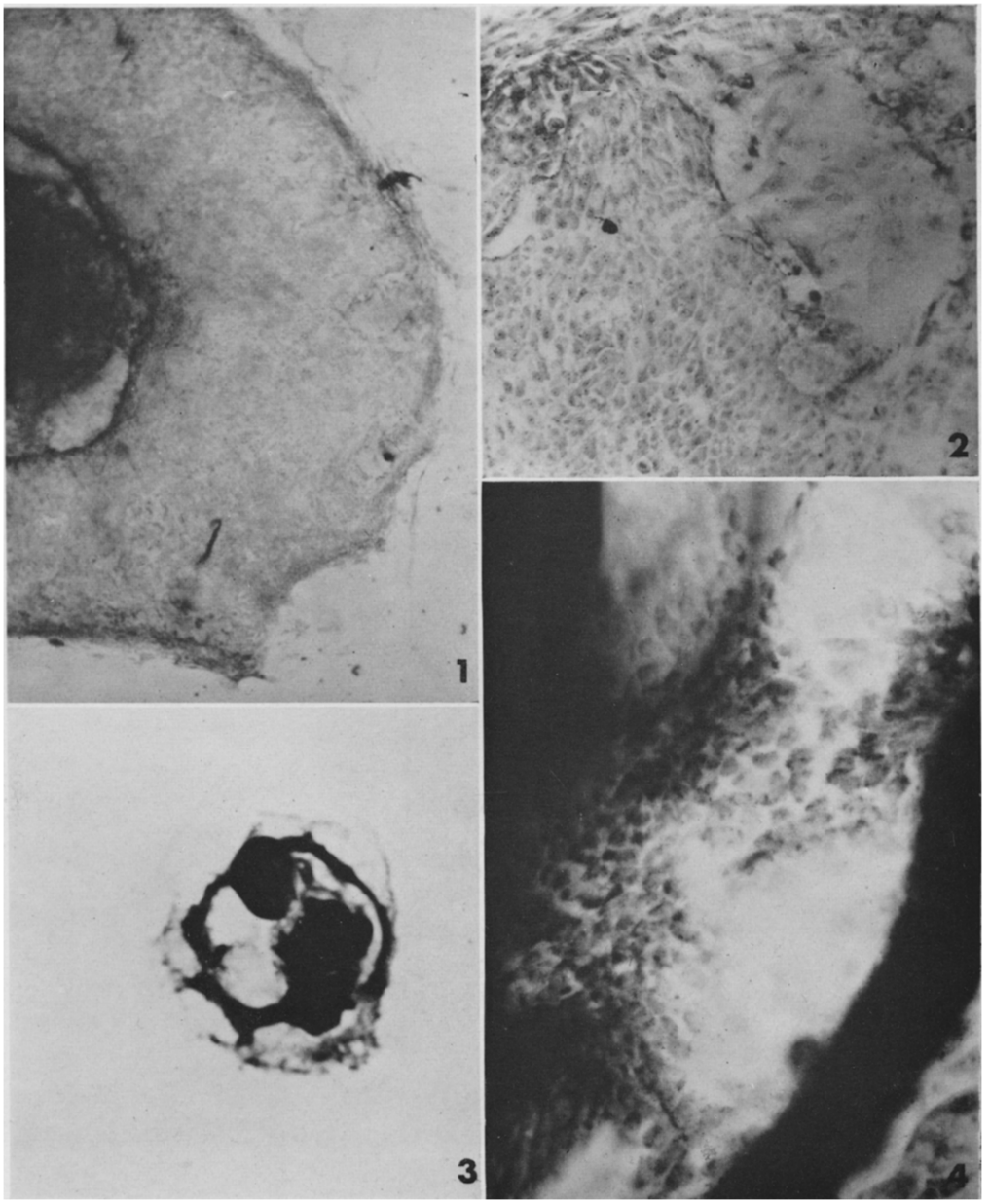


Fig. 1. *Sheet of polygonal cells surrounding explant, circumvented by narrow "border" of spindle cells. From a mycosis fungoides skin biopsy.* ×25.

Fig. 2. *Higher magnification of Fig.* 1, *showing cyst-like formation in epithelial sheet. Independent cells appear within the area bound by cells arranged circumferentially.* ×100.

Fig. 3. *Organotypic growth of explant from a thyroid hyperplasia.* ×25.

Fig. 4. *Higher magnification of Fig.* 3, *showing the structure-like reorganization of the cells. There is a palisading of cells visualized as an alternating pattern of ridges in the sheet of polygonal cells.* ×150.

(to face p. 270)

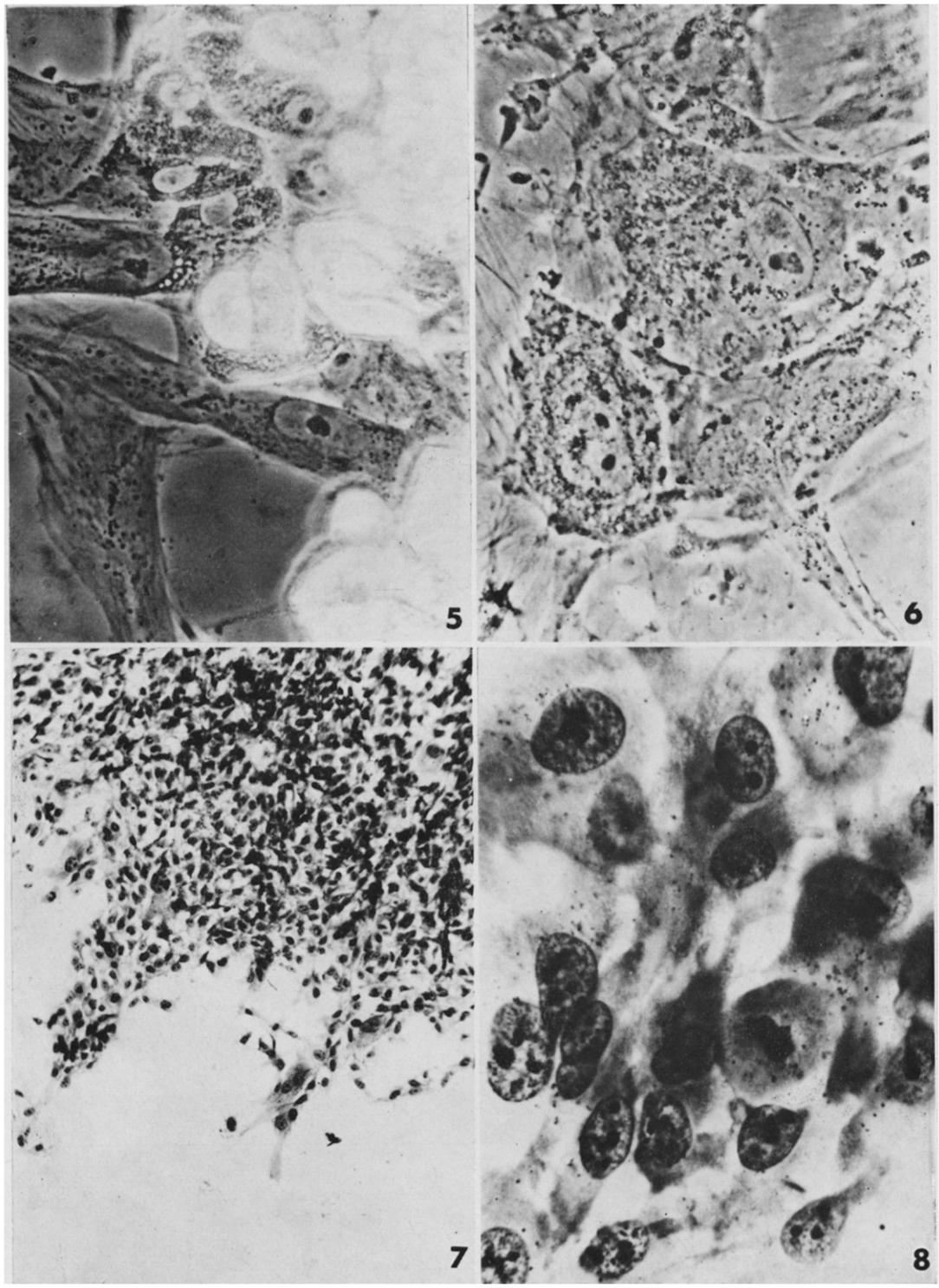


Fig. 5. *Cells from a melanoma biopsy in the living culture. Spindle cells in random orientation are on the left. The dense cluster of small refractile cells are macrophages with coarse melanin granules.* ×630.

Fig. 6. *Cells from a carcinoma of breast biopsy. Polygonal cells growing confluently. A large number of microfibrils can be seen. These may account for the increased adhesiveness of cells apparent in this culture.* ×630.

Fig. 7. *Low power view of the undifferentiated multipolar cells seen in the majority of human tumor explant cultures. From a meningioma.* ×100.

Fig. 8. *Higher magnification of Fig.* 9. ×400.

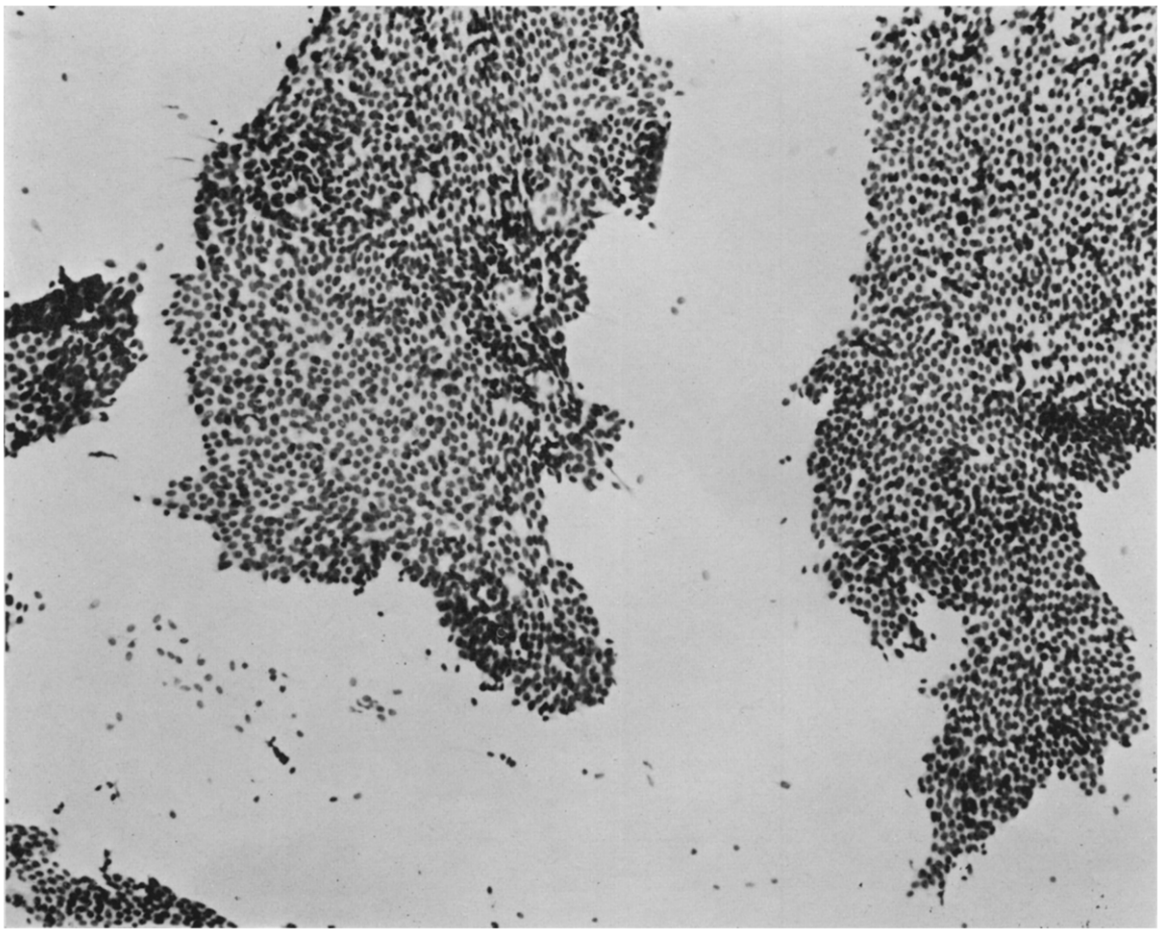


Fig. 1. *Plugs of epithelial cells aspirated from mammary fibroadenoma and spread on a glass slide. Papanicolaou stain.* × *136.*

(to face p. 254)

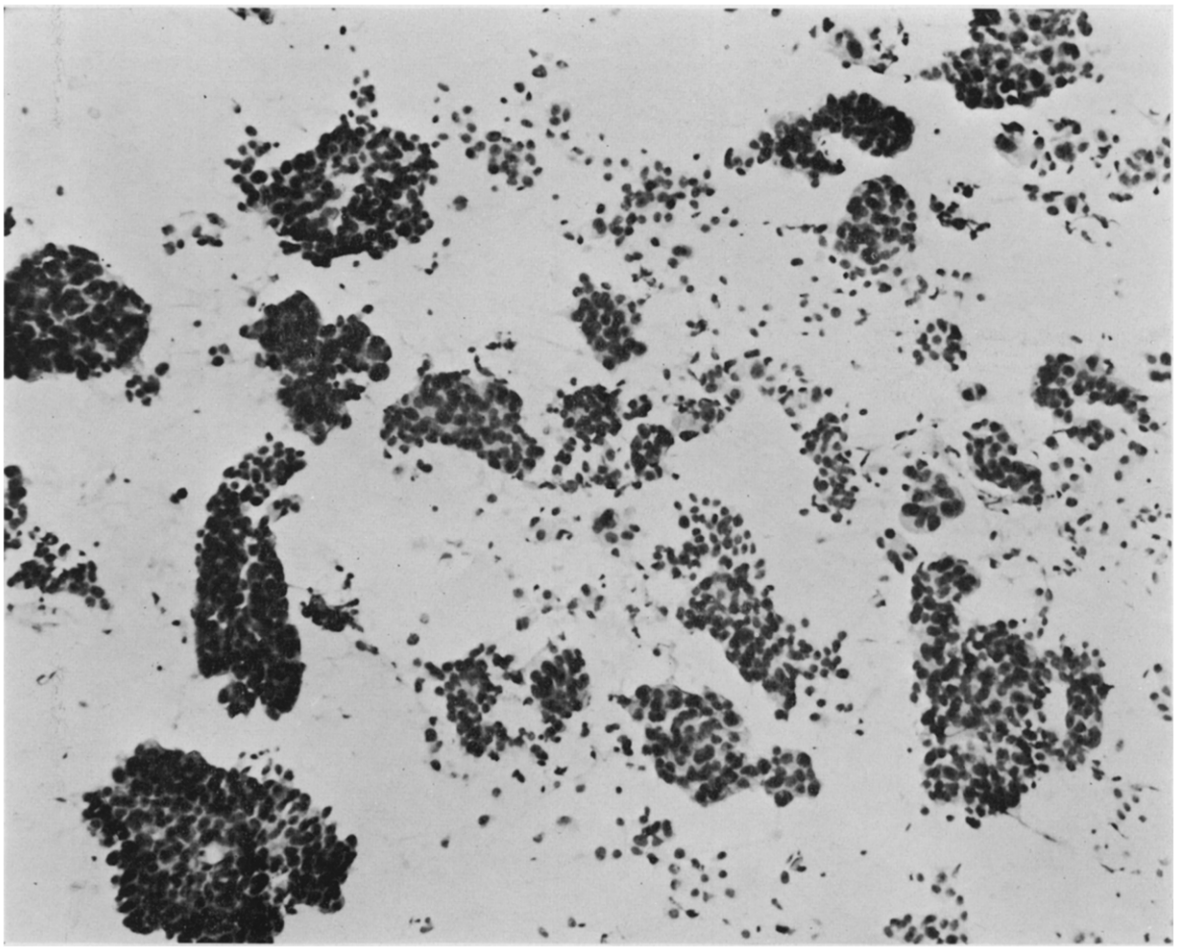


Fig. 2. Plugs of carcinoma cells aspirated from primary mammary carcinoma and spread on a glass slide. Papanicolaou stain × 136.

Table 5. *Comparison of the incorporation of the tritiated nucleosides and the Mitotic index in a variety of tumors.*

Name/TC	Diagnosis	Age of culture (days)	Mitotic index	% Labelled cells	
				H^3–TDN	H^3–UR
MY/897	Reticulum cell sarcoma	12	2·0	9·2%	0
ED/912	Mycosis fungoides	9	·4	21·5%	54%
SG/940	Malignant lymphoma, type undetermined	9	1·5	24·6%	0
PC/918	Osteosarcoma	12	0	4·5%	100%
KL/947	Osteosarcoma	14	0	0?	0?
FS/985	Rhabdomyosarcoma	7	·4	25%	14%
CK/962	Fibrosarcoma	18	3·5 (5 dys) 0 (12 dys)	0	19%
MC/968	Recurrent melanoma	21	0·2	0	0
LD/952	Carcinoma of breast	18	0	0	0
WM/904	Carcinoma of stomach	11	0	24·5%	0
HS/942	Carcinoma of kidney	28	0	0	100%
PB/944	Carcinoma of kidney	16	0	0?	0?
MM/961	Carcinoma of testis	18	0·1	5%	100%
TF/850A	Adenocarcinoma, 1° unknown	11	0	4%	100%
MG/957	Glioblastoma	28	0	27·5%	69%
GV/984	Glioblastoma	8	0	31·5%	63%
SM/960	Cystic glioblastoma	18	2·4	4·5%	90%
CL/951	Brain tumor, type undetermined	20	0	8·8%	100%
JA/975	Fossa tumor	12	0	0	0
HS/942A	Normal kidney	28	0	0	0

and 1/4 tumors of the nervous system.

These data show that there is minimal correlation between nucleoside labelling and mitotic activity under the present experimental conditions. Future studies using the nucleosides in different concentrations and exposure times on a larger series of tumors should allow for a more significant interpretation.

RESUME

Les auteurs ont étudié le comportement in vitro *de* 607 *échantillons de tumeurs humaines, réparties en* 5 *grands groupes histologiques. Ils ont établi, pour ces tumeurs, le pourcentage relatif de croissance (pourcentage de cultures positives), le temps de survie, l'activité mitotique et le degré d'incorporation de nucléosides tritiés.*

Des modalités de croissance et des types cellulaires morphologiquement définis ont été observés.

Le pourcentage relatif de croissance, en ordre décroissant, était le suivant: 83,6% *pour les lymphomes;* 73,4% *pour les tumeurs du système nerveux;* 72,4% *pour les carcinomes;* 70,3% *pour les mélanomes et* 65,6% *pour les sarcomes.*

Une croissance modérée fut observée dans la majorité des lymphomes, tandis que la croissance était lente dans la majorité des carcinomes. Les carcinomes qui avaient le plus grand pourcentage de croissance lente avaient aussi le plus petit nombre de survies des cultures après 21 *jours. Un petit nombre de survies était également noté dans les cas de lymphomes à croissance lente. Les lymphomes à croissance rapide avaient, par contre, un grand nombre de survies.*

L'activité mitotique était très variable parmi les divers spécimens d'un même groupe de tumeurs. L'examen des index mitotiques dans plusieurs séries de cultures mit en évidence des pointes d'activité mitotique. Il y avait une coïncidence remarquable entre le moment où apparaissait ce maximum d'activité mitotique dans la culture et le temps de survie des cultures, pour les lymphomes et les carcinomes.

On tenta d'établir une relation entre l'incorporation de nucléosides marqués (thymidine et uridine traitées) — témoin de la synthèse d'ADN et d'ARN — et l'index mitotique. Une telle corrélation ne fut observée que dans un petit nombre de cas.

SUMMARY

The in vitro *behavior of representative samples of five major human tumor groups comprising a total of* 607 *tumor specimens was studied with regard to the relative per cent growth, growth rates and survival time, mitotic activity, and levels of incorporation of tritiated nucleosides.*

Growth patterns and morphologically distinct cell types were cited.

The relative per cent growth in decreasing order was: lymphomas, 83·6%. *tumors of the nervous system,* 73·4%, *carcinomas,* 72·4%, *melanomas,* 70·3%, *and sarcomas,* 65·6%.

Moderate growth was observed in the majority of lymphomas, sarcomas and melanomas, while the majority of carcinomas grew slowly. The carcinomas with the highest per cent of slow growth also had the smallest number of survivors. A low number of survivors was also noted in the slow-growing lymphomas. The fast-growing lymphomas had a correspondingly high number of survivors.

The mitotic activity was highly variable among the individual specimens within a tumor group. Examination of the mitotic indices in several individual culture series revealed definite peaks of mitotic activity. There was a notable coincidence between the time in culture in which there is mitotic activity and the survival time of lymphomas and carcinomas.

An attempt was made to relate labelled nucleoside (H^3-thymidine and H^3-uridine) incorporation, indicative of DNA and RNA synthesis, with the mitotic index. Agreement was found in only a few cases under the experimental conditions applied.

ZUSAMMENFASSUNG

Die Verfasser untersuchten das Verhalten in vitro *von* 607 *Tumoren menschlichen Ursprungs, welche in* 5 *grössere Untergruppen aufgeteilt wurden, und zwar in Bezug auf den relativen Prozentsatz der Fälle, bei denen es zu einem Wachstum kam, auf die Wachstumsgeschwindigkeit, auf die Ueberlebenszeit, auf die Zellteilungsaktivität und auf die Grössenordnung der Inkorporation von tritiumhaltigen Nukleosiden.*

Der relative Prozentsatz der Fälle, bei welchen es zu einem Wachstum kam, war in absteigender Grössenordnung der folgende: Lymphome; 83,6%; *Tumoren des Nervensystems:* 73,4%; *Karzinome:* 72,4%; *Melanome:* 70,3%; *Sarkome:* 65,6%.

Beschränktes Wachstum wurde bei der Mehrzahl der Lymphome, Sarkome une Melanome beobachtet, während die Mehrzahl der Karzinome langsam wuchs. Die Karzinome mit dem grössten Prozentsatz von langsamem Wachstum hatten auch die kleinste Anzahl von überlebenden Kulturen. Eine kleine Anzahl von überlebenden Kulturen gab es auch bei den langsam-wachsenden Lymphomen. Die schnell-wachsenden Lymphome zeigten eine entsprechend grosse Zahl von überlebenden Kulturen.

Die Zellteilungsaktivität war höchst unterschiedlich innerhalb der einzelnen Tumorgruppen. Untersuchungen über die Zellteilungsindexe in verschiedenen individuellen Kulturserien zeigten umschriebene Höhepunkte der Zellteilungsaktivität. Ein nennenswertes Uebereinstimmen zwischen der Dauer von Zellteilungsaktivität und Ueberlebenszeit der Kueturen von Lymphomen und Karzinomen wurde festgestellt.

Es wurde versucht, eine Beziehung zwischen der Einverleibung von tritiumhaltigen Nukleosiden (H^3-Thymidin und H^3-Uridin), welche ein Anzeichen für DNA-und RNA-Synthese darstellten, und dem Zellteilungsindex zu finden. Ein derartiges Uebereinstimmen konnte nur in wenigen Fällen unter den angewendeten Versuchsbedingungen festgestellt werden.

REFERENCES

1. A. Carrel and M. Burrows, Cultivation *in vitro* of malignant tumors. *J. Exp. Med.* **13,** 571–575 (1911).
2. J. P. Cobb, J. H. Kiefer and F. H. Wood, Human bladder neoplastic cells in tissue culture. *J. Urol. (Baltimore)* **73,** 1039–1044 (1955).
3. J. P. Cobb and J. C. Wright, Studies on a craniopharyngioma in tissue culture. I. Growth characteristics and alterations produced following exposure to two radiomimetic agents. *J. Neuropath. Exp. Neurol.* **18,** 563–568 (1959).
4. J. P. Cobb and D. G. Walker, Studies on human melanoma cells in tissue culture. I. Growth characteristics and cytology. *Cancer Res.* **20,** 858–867 (1960).
5. M. R. Murray and A. P. Stout, The classification and diagnosis of human tumors by tissue culture methods. *Tex. Rep. Biol. Med.* **12,** 898–915 (1954).
6. G. N. Papanicolaou and F. V. Maddi, Diagnostic value of cells of endometrial and ovarian origin in human tissue culture. *Acta. Cytol. (Philad.)* **5,** 1–16 (1961).
7. M. F. Orr and B. McSwain, Tissue culture of human breast carcinoma. *Amer. J. Path.* **31,** 125–141 (1955).
8. J. A. DiPaolo, The use of human tumor effusions in primary culture for testing chemotherapeutic compounds. *Cancer* **17,** 391–394 (1964).
9. E. Y. Lasfargues and L. Ozzello, Cultivation of human breast carcinoma. *J. Nat. Cancer Inst.* **21,** 1131–1147 (1958).
10. F. Hu, R. J. Staricco, H. Pinkus and R. P. Fosnaugh, Human melanocytes in tissue culture. *J. Invest. Derm.* **28,** 15–32 (1957).
11. Y. V. Dobrynin, Establishment and characteristics of cell strains from some epithelial tumors of human origin. *J. Nat. Cancer Inst.* **31,** Vol. 5, 1173–1194 (1963).
12. J. P. Cobb, D. G. Walker and J. C. Wright, Comparative chemotherapy studies on primary short-term cultures of human normal, benign and malignant tumor tissues — A five-year study. *Cancer Res.* **21,** 583–590 (1961).
13. J. P. Cobb and D. G. Walker, Effect of heterologous, homologous, and autologous serums on human normal and malignant cells *in vitro*. *J. Nat. Cancer Inst.* **27,** 1–14 (1961).

Europ. J. Cancer Vol. 1, pp. 275–279. Pergamon Press 1965. Printed in Great Britain

In Vitro Studies of Cell Proliferation In Tumours—I

Critical Appraisal of Methods and Theoretical Considerations

G. GORDON STEEL and J. P. M. BENSTED,
Biophysics Department, Institute of Cancer Research, (Surrey Branch), Belmont, Surrey

THE AMOUNT of data available on the rate of growth of human tumours is small. The data which has been obtained has come from serial radiographic examination, mainly of lung metastases and it is clear that the cases in which this is possible form a highly-selected group. Only in certain anatomical sites can an accurate tumour diameter be obtained from radiographs and even more serious selection occurs in the choice of cases which can remain untreated during the period of serial radiography.

The present work is part of a broad study of cell proliferation in tumours, which has raised the possibility of predicting tumour growth rate from studies of cell proliferation in human specimens removed at operation. The only useful parameter which can at present be determined in such material is a thymidine labelling index. Knowledge of a labelling index does not allow the cell cycle time or growth fraction of the tumour to be determined, but it does enable a potential volume doubling time to be predicted. Tritium labelled thymidine (see the review by Kiesileski *et al.* [1]) is a tracer which is widely used for labelling cells which are in course of DNA synthesis. Such cells normally proceed to division within a few hours of completing DNA synthesis in mammalian tissues, and hence a measurement of the proportion of cells labelled by H^3-Thymidine gives information on the rate of cell production. The labelling index gives information which is analogous to the mitotic index but it is preferable for two main reasons: firstly that the duration of DNA synthesis is more easily measured than the duration of mitosis (and these times must be known in order to predict cell production rate); secondly, since the mitotic time is only about one tenth of the DNA synthetic period the mitotic index is more likely to be affected by a period of devascularisation during operation.

It should be emphasised that in the present work the aim is to obtain an *in vivo* labelling index. Although the labelling is accomplished *in vitro*, the conditions are designed to ensure that only those cells which were in synthesis in the patient do in fact incorporate thymidine.

MATERIAL AND METHODS

Procurement of human tumour specimens

Specimens have so far almost entirely been obtained from tumours removed at operation. These are preferred to biopsy specimens, because it is essential for any prediction of tumour growth rate to have a group of specimens which are representative of the tumour as a whole. Where possible, the tumour was bisected and specimens taken from the cut face. Tumours have been made available to us through the Characterisation of Human Tumours Programme of the Royal Marsden Hospital and also from the St. Helier Hospital. Specimens of a wide variety of types of tumour have been accepted in this preliminary work; some of the recorded labelling indices are given in Table 1. Figs. 1 and 2 show the typical autoradiographic appearance.

Table 1.

Specimen	Diagnosis	Labelling index (%)
HI 1	Ca. lower 1/3 oesophagus (epithelium)	14·8
HI 2	Malignant melanoma	10
HI 3	Parotid gland tumour	0·04
HI 4	Ca. stomach (epithelium)	50
	(stroma)	3
HI 5	rhabdomyosarcoma of testis	7·3
HI 8	phaeochromocytoma	0·2

Specimens are obtained in the fresh state without sterile precautions immediately after removal from the patient, cooled and transported in ice-cold saline. No change has been found in the ability of specimens to incorporate thymidine after at least 6 hr at 4°C.

Technique of Incubation with Thymidine

Tumour specimens can be labelled simply by incubation for half an hour in agitated medium containing 1 μc/ml or more of H^3-thymidine. The choice of medium is not critical. However, it has been shown [2, 3] that under such conditions labelled cells are not usually found deeper than about 150μ from the surface of a solid tissue specimen. We have found, and Rajewsky has confirmed [4] that this is due to anoxia within the specimen inhibiting the uptake of thymidine: thymidine itself appears to penetrate well, but only those cells which can obtain oxygen by diffusion from the medium actually utilise it. The evidence for this is that the depth of the zone of incorporation depends as Warburg's theory [5] would predict on the oxygen tension and the Q_{O_2} of the tissue being studied [4]. In order, therefore, to increase the depth of the zone of incorporation and thus obtain reliable labelling indices, incubations have been carried out under two atmospheres excess oxygen pressure in medium which was first supersaturated with oxygen at two atmospheres. Parker 199 medium has been used, although Earle's solution seemed just as satisfactory; the medium was continuously agitated and 5%CO_2 in air was added up to one atmosphere in order to keep the pH within normal limits. Tumour specimens were normally taken as slices up to 1 mm thick and 5 mm square.

Incubations have been carried out for up to 1 hr, the object being to label only those cells which were in DNA-synthesis in the patient. The rationale of the technique is that those cells which in the patient possessed the enzymes for thymidine localisation are put in contact with thymidine just long enough for an easily detectable amount of bound tritium to be incorporated. Complete DNA synthesis or cell viability beyond this limited sense is not assumed.

More recently, an improved technique has been developed in which high pressure oxygen is produced by the decomposition of hydrogen peroxide in disposable polystyrene specimen tubes (2½ in. × ⅜ in., Luckhams Ltd., London). It has been found that when the well-fitting polythene caps of these tubes are held by a screw clamp, pressures of 10–20 atmospheres can easily be generated and withstood by the tube. Preliminary results have confirmed that thymidine labelling is not prevented under these conditions, that tissue damage is not serious and that the depth of the zone of penetration is good.

Perfusion experiments

As an alternative to *in vitro* labelling of tissue slices, some preliminary work has been done using a technique of total perfusion of isolated human tumours. This technique is being developed by Griffiths [14] for the study of tumour vascularity, but it is also an attractive method of labelling whole tumour specimens. Labelling of tumour cells has been observed in a large hypernephroma which was perfused following surgical removal of a kidney and subsequent perfusion with perfusate containing blood of the same group as that of the patient. Two mc of thymidine was added to the 4½ l. of perfusate, 3 hr after the start of perfusion. It has been demonstrated that thymidine is not degraded by the perfusing plasma during the period of the experiment, but it is yet to be established that all viable regions of the tumour are reached and labelled by thymidine in such a system.

Interpretation of the labelling index data

In any proliferating tissue, and particularly in tumours, not all proliferating cells have the same duration of intermitotic cycle. There is a

distribution of cell cycle times, and in tumours there is reason to believe that this distribution may be very broad. In addition to cells which can be regarded as proliferative, there may also be many which, probably as a result of metabolite deficiency, are prevented from dividing though still able to do so when given a suitable environment. Mendelsohn has used the term Growth Fraction [6] to specify the size of the proliferative compartment and this is a useful concept provided it is realised that an arbitrary boundary between 'proliferating' and 'non-proliferating' has to be set.

In the interpretation of labelling index data it is assumed that any cell which takes up thymidine would have divided a few hours later. There is some support for this assumption in the fact that both in normal tissues and in tumours the fraction of mitoses which become labelled after a single injection of H^3-thymidine invariably reaches a maximum of about 100 per cent, generally within 6 hr of injection. Consider first a volume of tumour tissue containing N cells, Np of which are proliferative and spend on average a time t_s in DNA synthesis. Assume first that the population is in linear growth. Then, provided the distribution of cell cycle times is invariant, the size of the proliferative compartment must be constant and the probability of finding a cell at any point in the cell cycle is everywhere the same (rectangular phase distribution diagram). If the fraction of proliferating cells entering mitosis per hr (the mitotic rate) is m, then

$$\text{Time to Double } (T) = \frac{N}{m.Np}$$

and the fraction of cells in DNA synthesis is given by

$$L.I. = \frac{t_s \cdot m \cdot Np}{N}$$

thus

$$T = \frac{t_s}{L.I.} \qquad \text{(i)}$$

For cell populations in other than linear growth one must take into account the fact that the probability of finding a cell in different parts of the cell cycle is not constant. In the extreme case of exponential growth, for instance, the phase distribution diagram is an exponential function [7]. The effect of this on equation (*i*) is to introduce a constant of proportionality.

$$T = \lambda \frac{t_s}{L.I.} \qquad \text{(ii)}$$

where λ must be found from the shape of the phase distribution diagram. In the case of tumours, accurate values for λ are in general not known, but for a wide range of tumour doubling times an assumed value of 0·80 is accurate within $\pm 10\%$.

Equation (ii) therefore allows tumour doubling times to be predicted for measured values of labelling index provided that some assumption can be made for the duration of DNA synthesis. The duration of this phase has been measured in a wide variety of mammalian cell systems and it has been claimed that its length is 'constant' [8–10]. There are certainly exceptions to this claim [11–13] but until measurements of the length of t_s in human tumours become available there is some justification for assuming a period of 10 hr.

On the basis of the theory outlined above, Table 2 lists corresponding values of labelling index and predicted doubling time.

Table 2.

Potential doubling time (days)	Labelling index %
1	34
5	6·9
10	3·4
50	0·69
100	0·34
500	0·069

Some comment must be made on the effect of necrosis on tumour growth rate. In many tumours there are large regions of necrosis, fluid-filled cysts and other forms of acellularity. In general cell proliferation does not take place in such regions but they nevertheless contribute to the overall growth curve. Provided that resorption of necrotic regions does not take place rapidly, their effect on overall tumour growth will not be different from regions containing only non-proliferating cells (regions of zero growth fraction). Cysts on the other hand may arise or swell suddenly, perhaps as a result of a vascular catastrophe, and have an important and unpredictable effect on overall growth.

The importance of cell loss

An implicit assumption in the above theory is that cell loss from the tumour does not occur to an appreciable extent and it is for this reason that the predicted doubling times are regarded as being 'potential'. The rate of cell loss from tumours is an important parameter and yet one which seems to have been neglected in studies of tumour growth. So far as the authors are aware no quantitative data on cell loss from tumours appear in the literature. Cell

loss is here thought of as being due to all sources, by exfoliation, by cell loss into the circulation, by cell death when this does not merely result in the accumulation of necrotic material. In some tumours cell loss may well be negligible, and in such cases the potential doubling times predicted from equation (ii) should correspond to actual volume doubling times. Early metastatic growth in lymph nodes may well be an example of this situation. In other cases it is difficult to believe that cell loss could be small, for instance in carcinomata of the gastro-intestinal tract. The normal epithelium is in a state of continuous cell replacement [15], with turnover times as short as 2 days in the small intestine, and it is difficult to imagine that in carcinomata this property has been completely lost. In such cases the rate of growth of the tumour may largely be determined by rate of cell loss and the importance of obtaining estimates of this parameter is clear.

The value of estimates of the cell production rate which can be obtained by measurement of labelling index thus depends to a large extent on the question of cell loss. What is now required is a basis of experience on how closely potential doubling times correspond to actual overall growth rate in a range of tumour types. This may have to be obtained largely by work on a variety of experimental animal tumours but some human data may also be available. The acquisition of such experience should in some measure help to provide information both on the rate of growth of human tumours and the extent to which cell loss is a controlling factor.

Acknowledgements — Tumour specimens were obtained through the Characterisation of Human Tumours Programme of the Royal Marsden Hospital and from the St. Helier Hospital. In particular we are grateful to Mr. C. I. Cooling, Mr. J. D. Griffiths and Dr. T. E. W. Goodier.

We wish to thank Margaret Lord and Lilian Baker for their technical assistance and Professor L. F. Lamerton for his continual support and advice.

RESUME

La prolifération cellulaire dans les tumeurs humaines est étudiée par un marquage in vitro *à la thymidine-^3H, réalisé sous une forte pression d'oxygène, de façon à préserver la synthèse de l'ADN dans les cellules situées dans la profondeur des explants. Les 'indices de marquage' par la thymidine-^3H servent à prédire la vitesse de la prolifération cellulaire dans la tumeur et de là, un temps potentiel de duplication de la tumeur, Les postulats utilisés dans la méthode sont discutés, en particulier le rôle de la perte cellulaire.*

SUMMARY

Cell proliferation in human tumours is being investigated by in vitro *labelling with H^3-Thymidine. This is carried out under high-pressure oxygen in order to preserve DNA synthesis of cells deep within the excised specimen. Thymidine labelling indices determined in this way are used to predict the cell production rate within the tumour and thus a potential tumour doubling time. The assumptions involved in this procedure are discussed, in particular the rôle of cell loss.*

ZUSAMMENFASSUNG

Die Zellproliferation in einer Reihe menschlicher Tumoren wurde mit Hilfe der in vitro *Markierung mit H^3-Thymidin untersucht. Dazu wurden hohe Sauerstoff-Partialdrucke verwendet, um die DNA-Synthese in den Zellen im Innern der inkubierten Tumorproben aufrechtzuerhalten.*

Durch die so gewonnenen Thymidin-Markierungsindices wird die Berechnung der Zellneubildungsrate und damit einer potentiellen Tumorverdoppelungszeit ermöglicht. Die zu diesem Verfahren nötigen Voraussetzungen, insbesondere die Bedeutung des Verlustes von Zellen aus der Tumorzellpopulation, werden diskutiert.

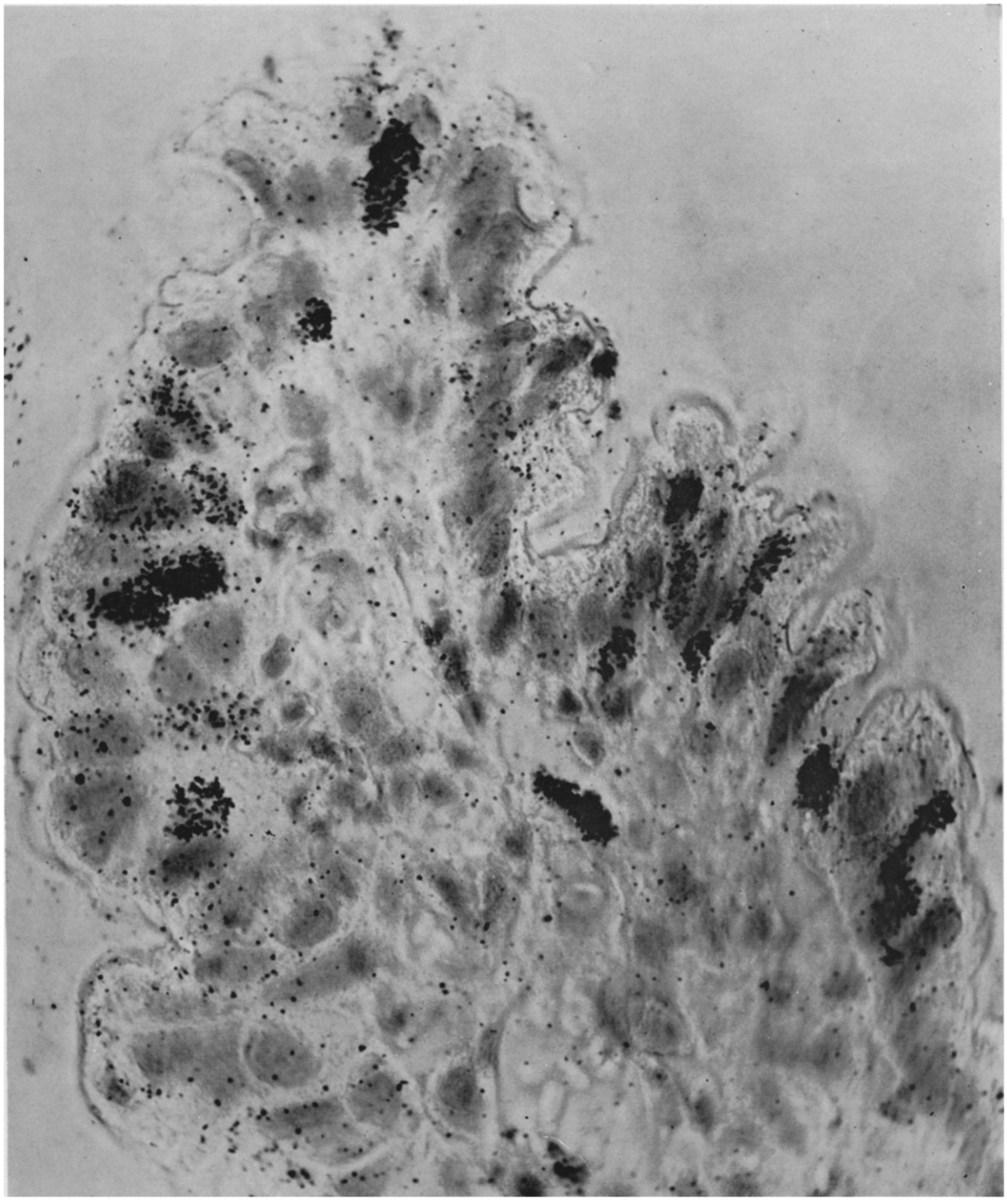


Fig. 1. *The autoradiographic appearance of an oesophageal carcinoma resected from an* 80 *year old male.*

(to face p. 278)
Refer to p. 276

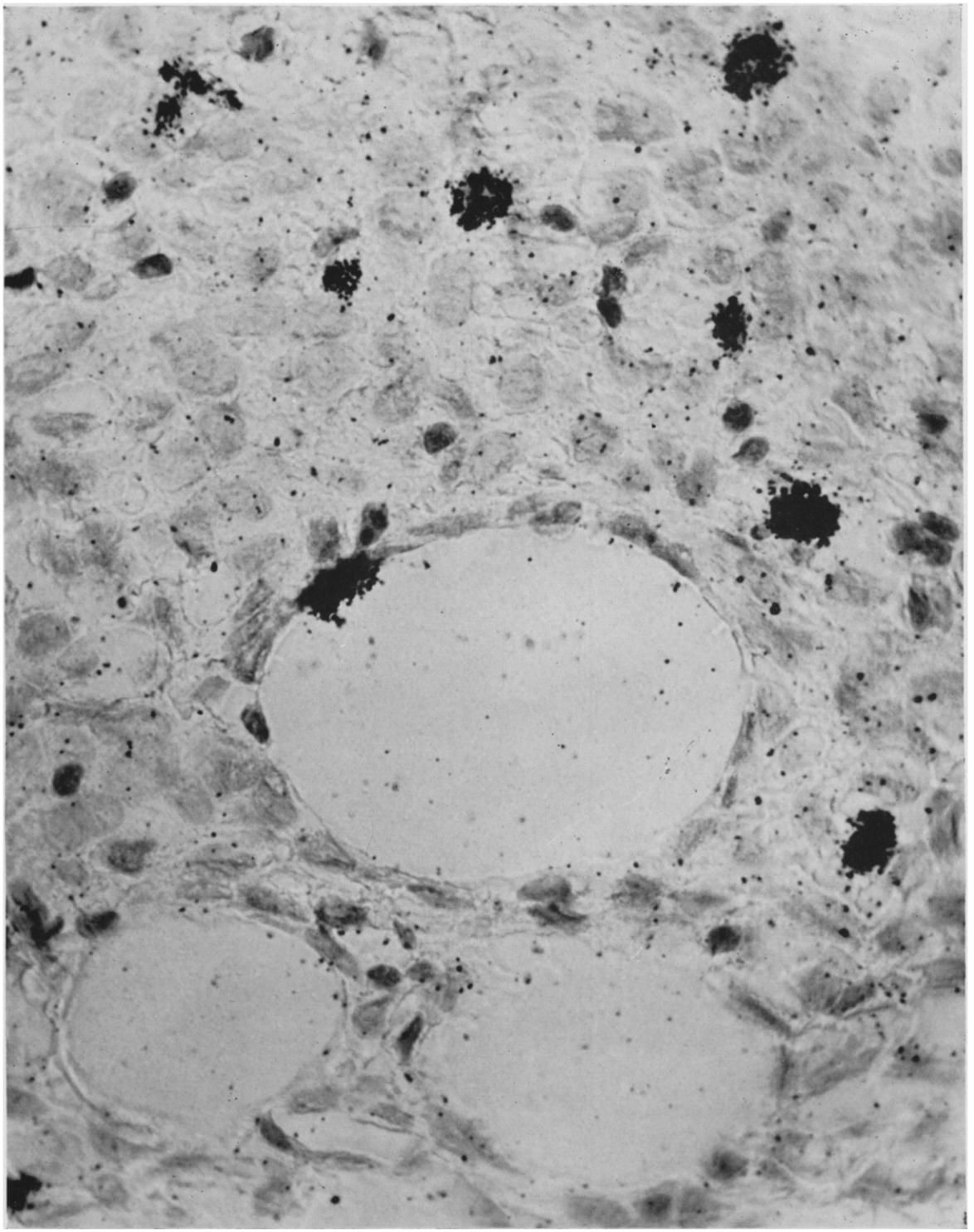


Fig. 2. The autoradiographic appearance of a recurrent malignant melanoma from a 68 year old female.

REFERENCES

1. W. E. KISIELESKI, R. BASERGA and H. LISCO, Tritiated thymidine and the study of turnours. *Atompraxis* **7,** 81 (1961).
2. J. R. RUBINI, E. P. CRONKITE, V. P. BOND and S. KELLER, *In vitro* labelling of proliferating tissues and tumours with triatiated thymidine. *J. Nucl. Med.* **2,** 223 (1961).
3. C. GROBSTEIN, Cytodifferentiation and its controls. *Science* **143,** 1 (1964).
4. M. F. RAJEWSKY, *In vitro* studies of cell proliferation in tumours II.—Characteristics of a standardized *in vitro* system for the measurement of 3H-thymidine incorporation into tissue explants. *Europ. J. Cancer* **1,** 281 (1965).
5. O. WARBURG, *The Metabolism of Tumours.* London, Constable (1930) 77.
6. M. L. MENDELSOHN, Autoradiographic analysis of cell proliferation in spontaneous breast cancer of C3H mouse. III. The growth fraction. *J. Natl. Cancer Inst.* **28,** 1015 (1962).
7. H. A. JOHNSON, Some problems associated with the histological study of cell proliferation kinetics. *Cytologia* **26,** 32 (1961).
8. I. L. CAMERON and R. C. GREULICH, Evidence for an essentially constant duration of DNA synthesis in the renewing epithelia of the adult mouse. *J. Cell Biol.* **18,** 31 (1963).
9. W. MAURER, CH. PILGRIM, K. WEGENER, S. HOLLWEG and J. LENNARTZ, Messung der Dauer der DNS — Verdopplung und der Generationszeit bei Versehiedenen Zellarten von Maus und Ratte durch Doppel — Markierung mit H^3 und C^{14} Thymidin. *6th Int. Bad Gastein Symp. Austria* (1964).
10. DEFENDI, V. and L. A. MANSON, Analysis of the life-cyle of mammalian cells. *Nature, Lond.* **198,** 359 (1963).
11. V. MONESI, An autoradiographic study of DNA synthesis and the cell cycle in spermatogonia and spermatocytes of mouse testis using tritiated thymidine. *J. Cell Biol.* **14,** 1 (1962).
12. M. LIPKIN, P. SHERLOCK and B. M. BELL, Generation time of epithelial cells in the human colon. *Nature, Lond.* **195,** 175 (1962).
13. F. BRESCIANI, A comparison of the cell generative cycle in normal, hyperplastic and neoplastic mammary gland of the C3H mouse, in *Symp. Fundamental Cancer Research and Cellular Radiation Biology,* Houston, Texas (1964).
14. J. D. GRIFFITHS and A. J. SALSBURY, *Circulating Cancer.* Thomas, Illinois (1965).
15. F. D. BERTALANFFY, Cell renewal in the gastrointestinal tract of man. *Gastroenterology* **43,** 472 (1962).

Europ. J. Cancer Vol. 1, pp. 281–287. Pergamon Press 1965. Printed in Great Britain

In Vitro Studies of Cell Proliferation in Tumours—II

Characteristics of a Standardised *In Vitro* System for the Measurement of ^{3}H-thymidine Incorporation into Tissue Explants

MANFRED F. RAJEWSKY

Max Planck Institut für Biophysik, Frankfurt am Main, Germany

THE PERCENTAGE of labelled nuclei (thymidine labelling index, L.I.) [1–3] is usually measured autoradiographically after an *in vivo* pulse exposure to the specific [4] DNA precursor thymidine, labelled with ^{3}H or ^{14}C. The L.I. is an indicator for the rate of cell proliferation and may also be used to predict the doubling time of growing cell populations (such as tumours), provided the duration (t_S) of the S-period of the cell cycle [5] is known and the rate of cell loss from the population can be estimated [6]. Whereas at the present time hardly any information is available concerning cell loss, there is good evidence that with a few exceptions [7, 8] t_S is fairly constant (6–10 hr) for most mammalian cells [9]. Considerably fewer data have been accumulated on t_S of tumour cells but dramatic differences when compared with t_S of normal cells seem to be unlikely. These limitations have to be borne in mind when the L.I. is used for the characterisation of the proliferative activity.

The normal procedure for the measurement of an overall L.I. in a given tissue consists of microscopical determination of the ratio of the number of labelled nuclei to the total number of nuclei in autoradiograms. The accuracy of the measurements is therefore not only limited by the inherent errors of the technique but also by the 'counting capacity' of the investigator. This last point is of special significance in tissues with a low L.I. or with a highly heterogeneous distribution of labelled nuclei (such as tumours, especially in more advanced stages), where counts would be necessary from many different regions of the specimen to obtain a satisfactory overall L.I.

The aim of the studies presented in this paper was to establish and analyse an *in vitro* system for the measurement of ^{3}H-thymidine (^{3}H-Tdr) incorporation into tissue explants shortly after removal of the tissue from the animal (or from patients at operations or biopsies). It was hoped that with a properly standardized technique and the use of liquid scintillation counting instead of the more time consuming histoautoradiographic method, a correlation could be demonstrated between tissue-specific ^{3}H-activity values and the respective L.I.s. If successful, such a method might not only provide results more quickly than autoradiography, but it might also be a better approach to true average values for the tissues in question, provided a sufficient number of explants cut randomly from different regions of the tissue were included in the measurements.

MATERIAL AND METHOD

In order to test the system, tissues from rats and mice of various inbred strains have been used. As a publication on the technique is in preparation [10], only a short description will be given in this context.

Cubic explants of 1 mg wet wt from freshly removed tissues were cut under sterile conditions with two cataract knives at about 4°C. For each experiment four of five culture tubes were prepared, each containing 3 ml of cold synthetic medium 199 and 20 explants (=20 mg±3% S.D.). Ninety min after removing the tissue from the animal, 2 ml of a standard solution of ^{3}H-Tdr in Hanks' balanced salt solution (1 μc/ml) were added per tube, the final ^{3}H-activity in the medium thus being 0·4 μc/ml. Taking into account the specific activity of the ^{3}H-Tdr (~3 mc/mM), the thymidine concentration in the medium was about 1–10 per cent of the concentration of free thymidine in the tissues [11, 12]. The tubes were then immediately incubated in a roller incubator [13] at 37·5°C and 23·5 rev/min, the gas phase being air. After a standard incubation time of 400 min, thymidine incorporation was stopped by cooling to 4°C. The explants were placed into stainless steel baskets and washed in three changes of Hanks' solution containing non-radioactive thymidine in 300-fold concentration for 15, 30 and 75 min respectively, followed by an extraction with cold 5% TCA for 10 min. After vacuum drying to constant weight at 40°C, the dry weights of the explants were determined. All explants of one experiment (4–5 tubes) were then pooled and combusted using a modified dry oxidation technique [14] and methanol as the absorbent for the resulting HTO. The ^{3}H-activity/mg dry wt was measured in a three-channel liquid scintillation counter using the channel ratio method for quench correction.

CHARACTERISTICS OF THE *IN VITRO* SYSTEM

1. *Distribution of labelled nuclei in the explants*

This problem was studied autoradiographically in 5 μ sections from incubated explants [15]. A ^{3}H-Tdr concentration of 4 μc/ml was chosen for the autoradiographic experiments. Confirming observations by other authors [6, 16, 17], it was found that the occurrence of labelled nuclei is restricted to a peripheral zone of the explant, independent of the time of incubation (see Fig. 1). The depth of the labelled zone depends on the tissue and increases if higher O_2 partial pressures are applied. The latter point has already been observed after *in vitro* labelling of tumour material [16] and use has recently been made of this observation in the incubation of human tumour specimens for determination of L.I.s [6].

In tissue slices constant Q_{O_2} values (Q_{O_2} = O_2 consumption at 38°C in mm^3/mg dry wt/hr) can only be measured as long as a 'limiting section thickness' (d') is not exceeded, or, in other words, as long as the O_2 concentration in the central cell layer of the section does not fall to zero [18]. The 'limiting section thickness' d' (mm) is obtained from the equation

$$\frac{d'}{2} = 109{\cdot}55 \sqrt{\left(\frac{C_0 \cdot D \cdot G}{Q_{O_2}}\right)} \quad (1),$$

where C_0 = O_2 partial pressure (atm); D = diffusion constant for O_2 in tissues (= 1·4 · 10^{-5} cm^3/min/cm^2 at a pressure gradient corresponding to 1 atm/cm and at 38°C) [19]; G = wet wt/dry wt.

Measurements in explants of liver, kidney, spleen and the transplantable 'Marshall' rat carcinoma have shown that the depth (ζ) of the labelled zone corresponds very well to the values for $d'/2$, calculated from equation (1) [15]. Therefore, ζ is essentially a function of Q_{O_2} and C_0 and can be predicted from equation (1). In the *in vitro* system C_0 has been kept constant as all incubations have been performed in air, and hence only G and Q_{O_2} remain as tissue-specific variables for the calculation of ζ. Once ζ is known, the labelled volume (V) of the explant can be calculated. For the cubic 1 mg explants used in the *in vitro* system, V (mm^3) is $5{\cdot}6\,\zeta - 11{\cdot}6\,\zeta^2 + 8{\cdot}0\,\zeta^3$.

A correction of the ^{3}H-activity/mg dry wt values, so far only representing thymidine incorporation into the labelled volume V of the explants, to ^{3}H-activity/explant or ^{3}H-activity/mg wet wt is now possible by application of a correction factor $K = G^{-1} \cdot V^{-1}$ (see Table 1).

2. *Kinetics of ^{3}H-thymidine incorporation* in vitro

Thymidine incorporation into DNA synthesizing nuclei occurs for less than 1 hr after parenteral injection, because of rapid catabolism and elimination from the body [20] (time for 50 per cent incorporation (t_{50}) of the order of a few minutes). On the other hand, t_{50} in the *in vitro* system is about 70 min, the final incorporation being reached after between 400 and 500 min. Fig. 2 shows the kinetics of

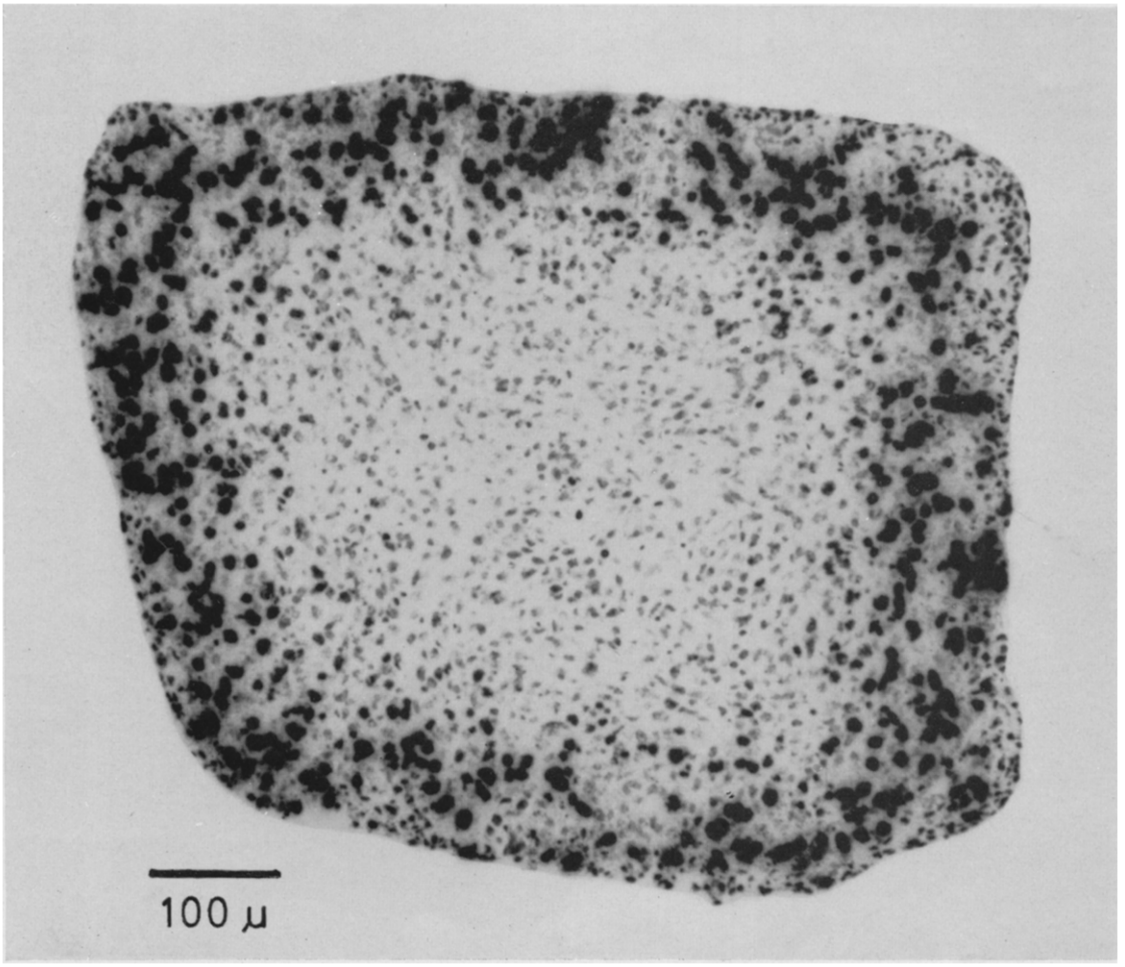


Fig. 1. *Peripheral zone of* ^{3}H-*labelled nuclei in a 'Marshall' tumour explant after incubation in* ^{3}H-Tdr *containing medium (Feulgen stain; autoradiogram).*

(to face p. 282)

Table 1. *Tabulated data on the* in vitro *uptake of* ^{3}H-Tdr *by various tissues under standard conditions, including the correction factor* $K(=G^{-1} \cdot V^{-1})$ *for the transformation of the original dpm/mg dry wt value to relative* ^{3}H-*activity/mg wet wt.*

Tissue	G	Q_{O_2}[1]	Z (mm)	V (mm^3)	K ($=G^{-1} \cdot V^{-1}$)	*dpm*‡ mg *dw*	Relative 3*H*-activity mg *ww*	Percentage of ^{3}*H*-labelled nuclei*	Percentage of ^{3}*H*-labelled nuclei†
skeletal muscle, adult rat	4·1	2·7	0·225	0·770	$31{\cdot}7\times10^{-2}$	798	253	<0·1[1]	0·06
kidney, adult rat	5·4	23	0·089	0·413	$44{\cdot}8\times10^{-2}$	2401	1077	0·4	0·3
liver, adult rat	3·2	9	0·109	0·487	$64{\cdot}2\times10^{-2}$	3363	2158	0·5	0·5
liver, adult mouse	3·2	9	0·109	0·487	$64{\cdot}2\times10^{-2}$	3440	2209	0·5	0·5
liver, 1 day old mouse	3·2	9	0·109	0·487	$64{\cdot}2\times10^{-2}$	146 093	93 780	17·9	23·0
spleen, adult rat	4·0	13	0·102	0·461	$54{\cdot}2\times10^{-2}$	7862	4263	2·2	1·1
Yoshida sarcoma, rat	6·6	7	0·178	0·674	$22{\cdot}6\times10^{-2}$	309 008	69 681		18·4
DS carcinosarcoma, rat	8·3	7	0·199	0·723	$16{\cdot}7\times10^{-2}$	116 108	19 350		4·8
induced hepatoma, rat	5·3	6·7	0·162	0·642	$29{\cdot}4\times10^{-2}$	547 543	160 900		39·5

*autoradiography.
†calculated after relative ^{3}*H*-activity/mg *ww*.
‡standard deviations ≦ 27%.
[1]from the literature.

incorporation for rat liver explants. Similar curves have been obtained for the other tissues studied.

If the kinetics of ^{3}H-Tdr incorporation is a function of the number of synthesizing cells at any given time during the period of incubation the first derivative of the kinetics curve should represent the number of synthesizing nuclei as a function of the incubation time. This was studied by adding ^{3}H-Tdr to the culture tubes at various intervals after starting the incubation. The number of labelled nuclei in the explants was determined autoradiographically. As can be seen from Fig. 3 the curve for the number of labelled cells per explant does indeed decrease, but somewhat

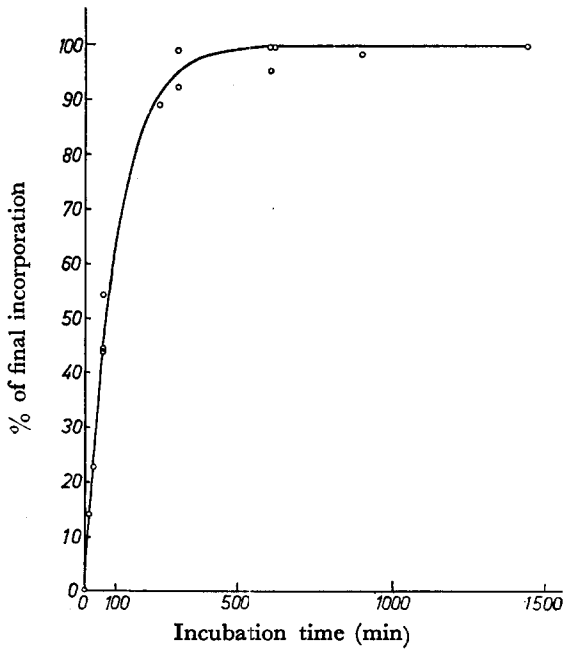


Fig. 2. *Kinetics of* ^{3}H-Tdr *incorporation into rat liver explants in the standardized 'in vitro' system.*

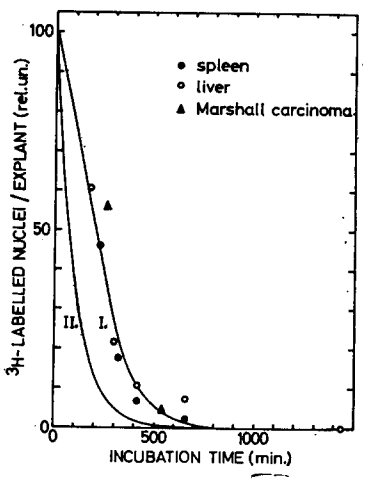


Fig. 3.
Curve I: Number of ^{3}H-*labelled nuclei per explant as a function of the incubation time before the addition of the label. Curve II: First derivative of the* ^{3}H-Tdr *incorporation kinetics curve for comparison.*

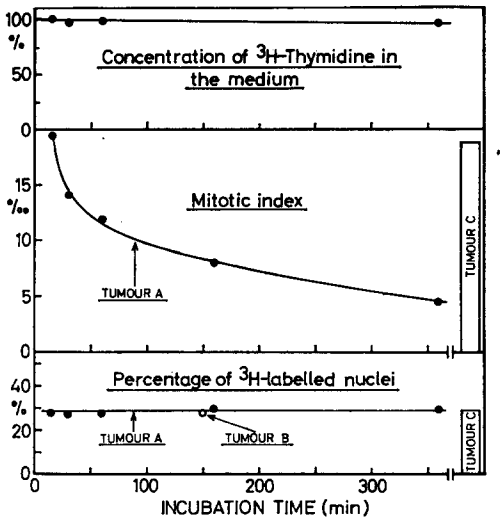


Fig. 4. ^{3}H-Tdr *labelling of three rat 'Marshall' tumours of similar size (A:* 440 *mg, B:* 330 *mg, C:* 360 *mg).*
Tumour A: Explants incubated under standard conditions.
Tumour B: As A, but using medium preincubated for 160 *min with explants from tumour A.*
Tumour C: Intraperitoneal injection of the tumour bearing animal.

less steeply (t_{50} about 180 min) than the first derivative of the incorporation kinetics curve. Consequently, the rate of thymidine incorporation is not constant over the period of incubation, but decreasing, possibly being impaired by unfavourable conditions *in vitro*. Extrapolating from the straight part of the curve one again arrives at a time between 400 and 500 min, which obviously lies within the range of the duration of the S-period [9]. It can, therefore, be assumed that under the conditions of the *in vitro* system those cells which had been in their S-period at the moment of explantation proceed with DNA synthesis, and that very few new cells enter the S-period from G_1.

Further experiments with the rat 'Marshall' carcinoma have extended these observations. ^{3}H-Tdr was added to the samples at the start of incubation and the L.I. in the explants then measured autoradiographically as a function of the incubation time. Fig. 4 shows the result of an experiment with three 'Marshall' tumours of almost identical size. Tumour C was labelled by intraperitoneal injection of the tumour-bearing animal with ^{3}H-Tdr. Explants of tumour A were labelled *in vitro* and explants of tumour B were incubated in medium preincubated with explants from tumour A for 150 min. In an additional chromatographic study* no significant decrease in thymidine concentration in the medium and very little degradation were found during this experiment and after incubation of various other tissues. The main implications of the 'Marshall' tumour experiments are, however, that (a) the L.I. in the explants remained constant up to 24 hr of incubation and corresponds well to the values obtained after *in vivo* labelling of similar material, (b) the mitotic index decreased to about 25 per cent within 6 hr, and (c) no labelled mitotic figures could be detected during the incubation, indicating that cells labelled during their S-period did not proceed to mitosis.

Clearly, although the conditions of the *in vitro* system do not prevent the cells from being progressively damaged, the process of thymidine incoporation seems to be relatively well preserved. Furthermore, the L.I.s measured *in vivo* and *in vitro* are in good agreement. It therefore appeared justifiable to investigate in a number of tissues the correlation between autoradiographic *in vivo* L.I. and relative ^{3}H-activity/mg wet wt as measured by the standardized *in vitro* technique.

3. *Correlation between autoradiographic* in vivo *L.I. and relative* ^{3}H-*activity/mg wet wt measured* in vitro

Extensive measurements in a variety of tissues were carried out to obtain information on this problem. In Fig. 5, mean values for the relative ^{3}H-activity/mg wet wt of six representative tissues are plotted against the

*The author is indebted to Dr. E. H. Cooper, St. Mary's Hospital, London, for the chromatographic analysis of the media.

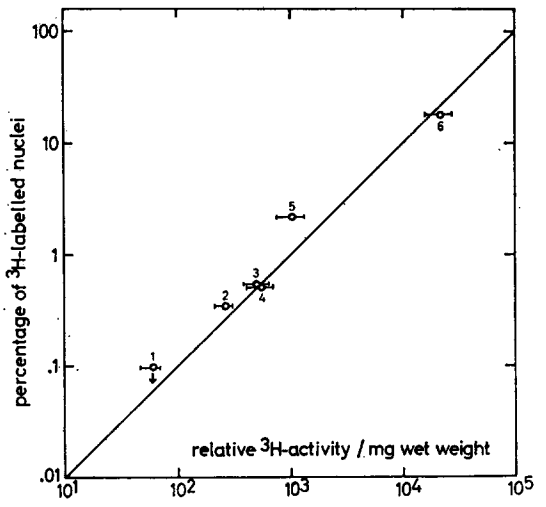


Fig. 5. *Correlation of autoradiographic 'in vivo' L.I. and relative* ³H-*activity/mg wet wt after incubation under standard conditions.*

1. *skeletal muscle, rat.*
2. *kidney, rat.*
3. *liver, adult rat.*
4. *liver, adult mouse.*
5. *spleen, rat.*
6. *liver, newborn mouse.*

corresponding autoradiographic *in vivo* L.I.s. The good proportionality indicates that a prediction of L.I.s is possible from the *in vitro* data. In addition, these findings seem to be evidence for a relatively constant pool of free thymidine in the various rat and mouse tissues studied.

The *in vitro* measurements have also been extended to several malignant tissues; mean values for the L.I.s of three rat tumours as calculated from the respective *in vitro* data, are presented in Table 1 together with data on several normal tissues. The factors necessary for the correction of the original dpm/mg dry wt values to relative ³H-activity/mg wet wt are also included in the Table.

The good correlation between overall L.I. and relative ³H-activity/mg wet wt has led to the use of the *in vitro* system in studies where the overall L.I. of a given tissue has to be monitored in a large number of samples. For instance, measurements of cell proliferation in rat liver in the course of chemical carcinogenesis, or of cell proliferation as a function of age, are in progress. It is hoped that the technique will also prove to be applicable for proliferation studies in human tumour material.

Acknowledgments — These studies were in part carried out under a NATO Research Fellowship at the Biophysics Department of the Institute of Cancer Research: Royal Cancer Hospital, London.

The author is greatly indebted to Prof. L. F. Lamerton and Dr. G. G. Steel for valuable discussions and helpful criticism.

Appreciation is also expressed to Mrs. G. Dose-Augstein, Mrs. M. Lord and Miss L. Baker for their able technical assistance.

RESUME

Dans cet article est présenté un système étalon, in vitro, *servant à mesurer l'incorporation de la thymidine-³H dans les explants tissulaires; les résultats révèlent une bonne corrélation entre l'activité relative du tritium par mg de poids frais et indices de marquage globaux, spécialement dans les tissus ayant des indices bas ou une distribution très hétérogène des noyaux synthétisant l'ADN, ce qui est souvent le cas des tumeurs.*

L'importance de la pression partielle d'oxygène dans les explants, pour la synthèse d'ADN et de là, pour la mise en évidence des noyaux marqués par la thymidine tritiée, est discutée à la fois sur le plan théorique et à partir de données expérimentales. Des mesures de la cinétique de l'incorporation de la thymidine-³H dans la synthèse in vitro *sont présentées.*

SUMMARY

A standardized in vitro *system for the measurement of ^{3}H-thymidine incorporation into tissue explants is described, and by its use a good correlation is demonstrated between relative tritium activity per mg wet wt and autoradiographic thymidine labelling index (L.I.). The value of this technique as a method of determining overall L.I.s is discussed, especially as regards tissues with a low L.I. or with a highly inhomogeneous distribution of DNA synthesizing nuclei, as often occurs in tumours.*

The importance of the oxygen partial pressure in the incubated explants for the process of DNA synthesis and hence for the demonstration of ^{3}H-labelled nuclei is considered both theoretically and on the basis of experimental data. Results of measurements on the kinetics of ^{3}H-thymidine incorporation in the in vitro *system are presented.*

ZUSAMMENFASSUNG

Ein standardisiertes in vitro *System zur Messung des Einbaus von ^{3}H-Thymidin in Gewebeexplantate wird beschrieben. Es wird gezeigt, daß eine gute Korrelation besteht zwischen der relativen ^{3}H-Aktivität pro mg Frischgewicht und dem nach* in vivo *Applikation von ^{3}H-Thymidin autoradiographisch ermittelten Thymidin-Markierungsindex. Die Möglichkeit, Thymidin-Markierungsindices über diese Methode zu bestimmen, wird diskutiert, insbesondere im Hinblick auf Gewebe mit niedrigem Thymidin-Markierungsindex oder mit einer inhomogenen Verteilung DNA-synthetisierender Zellen im Gewebe, wie es oft in Tumoren der Fall ist.*

Experimentelle Befunde und theoretische Überlegungen weisen auf die Bedeutung des Sauerstoff-Partialdruckes in den inkubierten Explantaten für die DNA-Synthese und damit für die ^{3}H-Markierung der Zellkerne hin.

Mit Hilfe verschiedener Versuchsansätze wird die Kinetik des ^{3}H-Thymidineinbaus in vitro *analysiert.*

REFERENCES

1. H. A. Johnson, W. E. Haymaker, J. R. Rubini, T. M. Fliedner, V. P. Bond, E. P. Cronkite and W. L. Hughes, A radioautographic study of a human brain and glioblastoma multiforme after the *in vivo* uptake of tritiated thymidine. *Cancer Res.* **13,** 636 (1960).
2. E. Koburg, Autoradiographische Untersuchungen zum Nucleinsäurestoffwechsel einzelner Zellarten der Lunge. *Verh. Dt. Ges. Path.* **44,** 160 (1960).
3. B. Messier and C. P. Leblond, Cell proliferation and migration as revealed by radioautography after injection of thymidine-H^3 into male rats and mice. *Amer. J. Anat.* **106,** 247 (1960).
4. P. Reichard and B. Estborn, Utilization of desoxyribosides in the synthesis of polynucleotides. *J. Biol. Chem.* **188,** 839 (1951).
5. A. Howard and S. R. Pelc, Nuclear incorporation of P^{32} as demonstrated by autoradiographs. *Exp. Cell Res.* **2,** 178 (1951).
6. G. G. Steel and J. P. M. Bensted, *In vitro* studies of cell proliferation—I. *Europ. J. Cancer,* **1,** 275 (1965).
7. F. G. Sherman, H. Quastler and D. R. Wimber, Cell population kinetics in the ear epidermis of mice. *Exp. Cell Res.* **24,** 114 (1961).
8. F. Bresciani, A comparison of the cell generative cycle in normal, hyperplastic and neoplastic mammary gland of the C_3H mouse. In *Cellular Radiation Biology, 18th Ann. Symp. Fundamental Cancer Research,* University of Texas, M.D. Anderson Hospital and Tumor Institute (1964).
9. Ch. Pilgrim and W. Maurier, Autoradiographische Untersuchung über die Konstanz der DNS-Verdoppelungs-Dauer bei Zellarten von Maus und Ratte durch Doppelmarkierung mit ^{3}H und ^{14}C-Thymidin. *Exp. Cell Res.* **37,** 183 (1965).
10. M. F. Rajewsky, Biophysik (1965). In preparation.
11. W. C. Schneider, Deoxyribosides in animal tissues, *J. Biol. Chem.* **216,** 287 (1955).

12. R. L. POTTER, Cited in Ref. 3.
13. H. WRBA and H. RABES, Zur Methode der quantitativen Bestimmung des Umsatzes an markierten Stoffen durch Organkulturen. *Exp. Cell Res.* **26,** 62 (1962).
14. F. KALBERER and J. RUTSCHMANN, Eine Schnellmethode zur Bestimmung von Tritium, Radiokohlenstoff und Radioschwefel in beliebigem organischem Probenmaterial mittels des Flüssigkeits-Scintillations-Zählers. *Helv. Chim. Acta.* **242,** 1957 (1961).
15. M. F. RAJEWSKY, Thymidin-Inkorporation und O_2-Konzentration in Explantaten normaler und maligner Gewebe *in vitro*. *Nat. Wiss.* **52,** 341 (1965).
16. H. A. JOHNSON and V. P. BOND, A method of labeling tissues with tritiated thymidine *in vitro* and its use in comparing rates of cell proliferation in duct epithelium, fibroadenoma, and carcinoma of human breast, *Cancer* **14,** 639 (1961).
17. W. OEHLERT, P. DÖRMER and R. LESCH, Autoradiographische Untersuchungen über die DNS-Synthese im überlebenden Tumorgewebe des Menschen. *Beitr. Path. Anat.* **128,** 3, 468 (1963).
18. O. WARBURG, *The Metabolism of Tumours*. Constable, London, (1930).
19. A. KROGH, The rate of diffusion of gases through animal tissues, with some remarks on the coefficient of invasion. *J. Physiol.* **52,** 391 (1919).
20. V. R. POTTER, Metabolic products formed from thymidine. In *The Kinetics of Cellular Proliferation,* Ed. by F. STOHLMAN, Grune & Stratton, New York (1959).

Europ. J. Cancer Vol. 1, pp. 289–293. Pergamon Press 1965. Printed in Great Britain

The Interaction of Steroid Hormones with Methylcholanthrene in the Rat Prostate Gland Grown in Organ Culture

ILSE LASNITZKI

Strangeways Research Laboratory
Cambridge, England

INTRODUCTION

IT IS well known that the development and growth of certain human tumours such as the carcinoma of the cervix, of the mammary gland and of the prostatic gland are connected with changes in the hormonal status of the host, but the exact nature of the hormonal action is not known.

The role of hormones in the experimental induction of animal tumours by chemical carcinogens has been widely investigated but has not produced clear cut results. Dunning *et al.* [1] found that methylcholanthrene-induced tumours of the rat prostate gland grew equally well in female and male hosts, Mirand and Staubitz [2], on the other hand, reported that such tumours could be transplanted more successfully into female rats and Allen [3] obtained a higher incidence of prostatic tumours in castrates than in intact rats or in castrates treated with testosterone.

The influence of corticosteroids on the induction of tumours has also been studied. Asboe-Hansen [4] and Baserga and Shubik [5] showed that cortisone delayed tumour production in mouse epidermis painted with DMBA or methylcholanthrene while Green and Savigear [6] claimed that cortisone did not reduce mitosis in mouse epidermis made hyperplastic with DMBA.

These contradictory results may have been due to differences in the timing of the hormonal treatment relative to the exposure to the carcinogens, to variations in general systemic factors between different groups of animals or, most important of all, to the interference of other hormones present in the organism.

In culture these complications are largely eliminated, the variables can be isolated and the effect of individual hormones and their interaction with carcinogens studied under controlled experimental conditions. In earlier work [7, 8] the effect of methylcholanthrene alone on the rat prostate gland in organ culture has been studied. The carcinogen induced a rapid and extensive hyperplasia of the prostatic epithelium which persisted after transfer to control medium.

Recently the influence of oestrone, oestradiol, testosterone and hydrocortisone on the effect of methylcholanthrene has been examined in organ cultures of the ventral prostate gland of the adolescent rat. These hormones play an important role in the maintenance of this organ, its growth and function depend on the presence of androgenic hormones and are impaired by exposure to oestrogens.

MATERIALS AND METHODS

Ventral prostate glands obtained from 6 to 10 week old rats were grown in natural and semi-defined medium by techniques described in detail elsewhere [7]. One group of explants was treated with oestrone, testosterone or

Table 1. The influence of oestrogens, testosterone and hydrocortisone on the effect of methylcholanthrene (MC) *in organ cultures of the rat prostate grown in natural medium.*

Treatment	Total number of cultures	Number of treated cultures showing various degrees of hyperplasia*			
		0	I	II	III
MC only for 11 days	37	4	13	17	3
MC and oestrone for 11 days	16	2	0	6	8
MC and testosterone for 11 days	11	7	3	1	0
MC and hydrocortisone for 11 days	18	16	2	0	0
MC only for 11 days followed by control medium for 6 days	21	1	2	9	9
MC only for 11 days followed by oestrone or oestradiol for 6 days	15	0	0	7	8
MC for 11 days followed by testosterone for 6 days	12	0	12	0	0
MC for 11 days followed by hydrocortisone for 6 days	10	0	4	3	3

* 0—no hyperplasia
I—less than half the number of alveoli show mild hyperplasia
II—half to two-thirds of the alveoli show medium to marked hyperplasia
III—most of the alveoli show marked hyperplasia.

hydrocortisone alone and fixed after 11 days' cultivation; another group was exposed to methylcholanthrene alone. This latter group was subdivided into two sets, one of which was fixed after 11 days' growth in the presence of the carcinogen, while the other was treated with methylcholanthrene (MC) for 11 days and transferred to natural control medium for an additional 6 days.

Another series was exposed to three different carcinogen-hormone combinations.
MC (4 μg/ml) and oestrone (4 μg/ml) or oestradiol (1·5 μg/ml).
MC (4 μg/ml) and testosterone (30 μg/ml).
MC (4 μg/ml) and hydrocortisone (7 μg/ml).

One set of this series was treated with the carcinogen and hormone simultaneously, fixed after 11 days' cultivation and compared with explants grown with the carcinogen alone. In another set the cultures were first treated with the carcinogen for 11 days and then transferred to medium containing hormone for an additional 6 days. These were compared with explants pretreated with MC and maintained in control medium for 6 days. (also see Tables 1 and 2).

The explants were fixed with 3% acetic Zenker's solution, embedded in paraffin, serially sectioned at 6 μ and stained by the periodic acid Schiff reagent after diastase digestion.

RESULTS

Controls

The prostate gland consists of alveoli lined with one row of slightly folded cuboidal or columnar secretory epithelium (Fig. 1). After explantation into medium without additional hormones the epithelium undergoes regressive

Table 2. The influence of oestrone, testosterone and hydrocortisone on the effect of methylcholanthrene (MC) *in organ cultures of the rat prostate gland grown in semi-defined medium.*

Treatment	Total number of cultures	Number of treated cultures showing various degrees of hyperplasia*			
		0	I	II	III
MC only for 11 days	14	5	9	0	0
MC and oestrone for 11 days	10	0	4	5	1
MC and testosterone for 11 days	12	12	0	0	0
MC and hydrocortisone for 11 days	10	10	0	0	0

* 0—no hyperplasia
I—less than half the number of alveoli show mild hyperplasia
II—half to two-thirds of the alveoli show medium to marked hyperplasia
III—most of the alveoli show marked hyperplasia.

All sections have been stained by the periodic acid Schiff reagent after diastase disgestion (PAS)

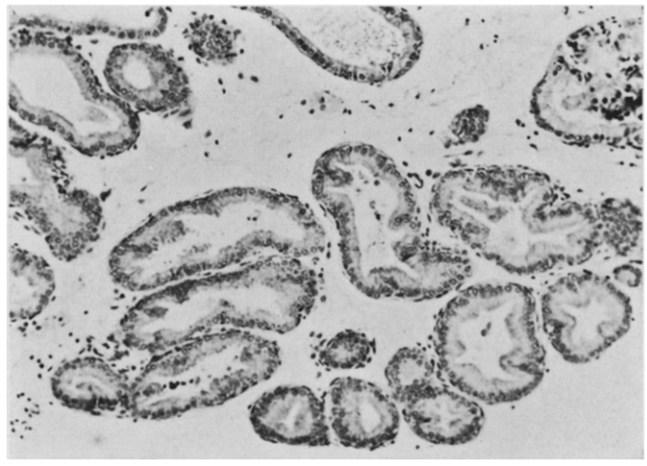


Fig. 1. *Section through a ventral prostate gland of an* 8 *week rat before explantation. Note high, slightly folded epithelium.* ×130.

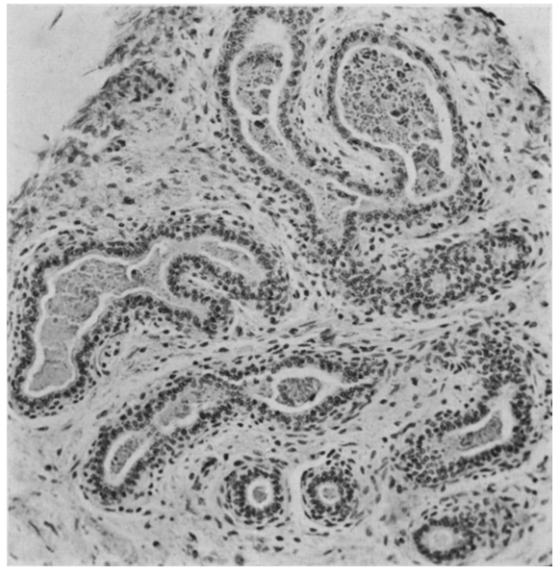


Fig. 2. *Section through a similar gland grown for* 11 *days in natural control medium. Note low epithelium and increase of connective tissue.* ×130.

(to face p. 290)

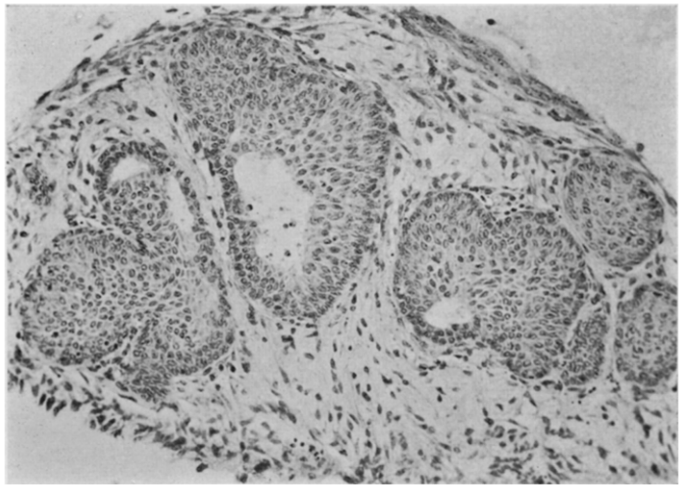


Fig. 3. *Section through a gland grown for* 11 *days in natural medium in the presence of methylcholanthrene, showing marked hyperplasia of the alveolar epithelium.* ×130.

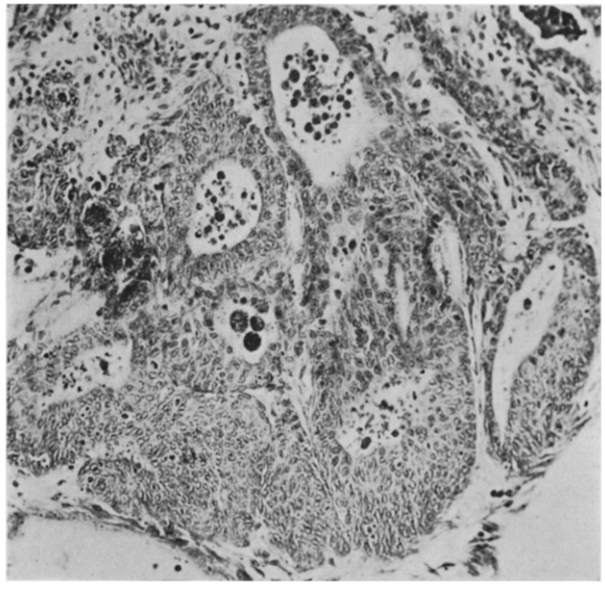


Fig. 4. *Section through a gland grown for* 11 *days in natural medium in the presence of methylcholanthren and transferred to natural control medium for* 6 *days. Note persistence of hyperplasia.* ×130.



Fig. 5. *Section through a gland grown in natural medium for* 11 *days with methylcholanthrene and oestrone showing extensive hyperplasia.* ×130.



Fig. 6. *Section through a gland grown for* 11 *days in natural medium with methylcholanthrene and transferred to medium containing oestradiol. Note hyperplasia.* ×130.

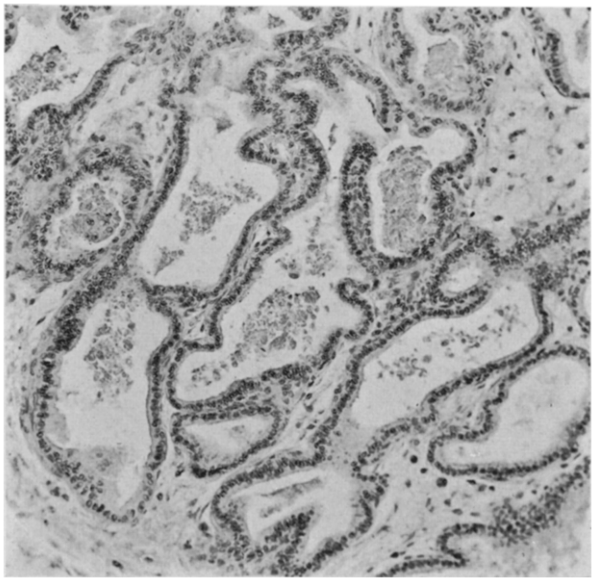


Fig. 7. Section through a gland treated in natural medium with methylcholanthrene and testosterone for 11 days. Note absence of hyperplasia. ×130.

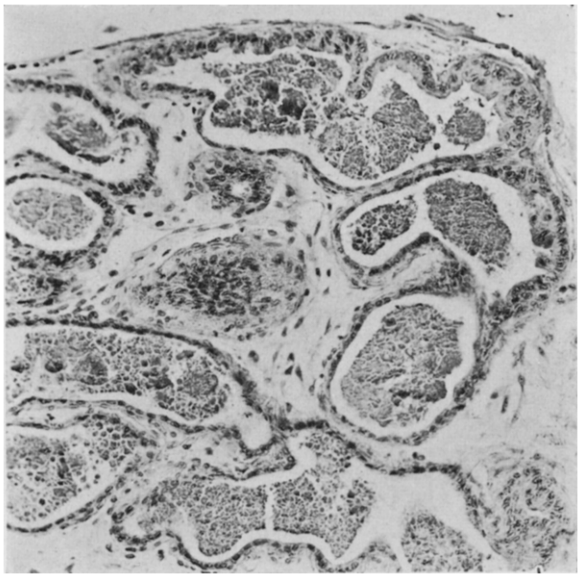


Fig. 8. Section through a gland grown first with methylcholanthrene in natural medium and transferred to medium containing testosterone. Only two alveoli at the periphery show very mild hyperplasia. ×130.

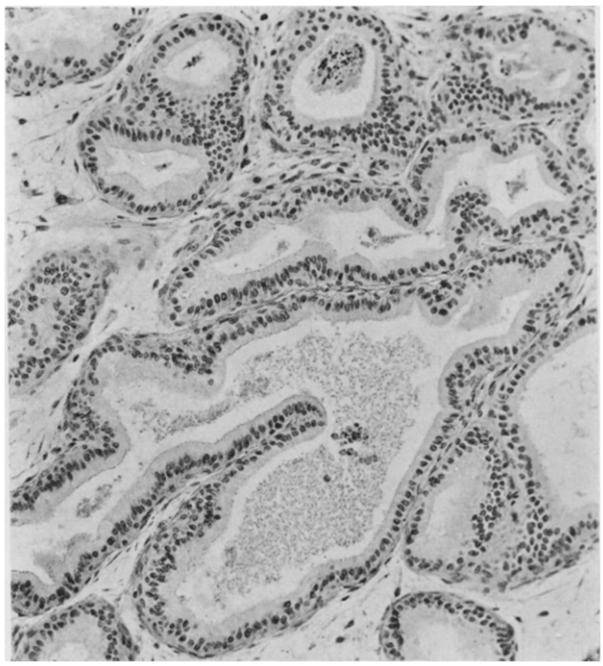


Fig. 9. *Section through a gland treated in natural medium with methylcholanthrene and hydrocortisone for* 11 *days. Note absence of hyperplasia and high epithelium.* ×130.



Fig. 10. *Section through a gland treated first with methylcholanthrene and transferred to medium containing hydrocortisone. Note both normal alveoli and hyperplastic foci.* ×130.

changes. The alveolar lumen becomes narrow and straight and the cells become lower and cease to secrete. In the natural medium this regression is accompanied by an increase in the connective tissue (Fig. 2).

Effect of Hormones

Addition of oestrone or oestradiol to the medium accelerated the atrophy of the epithelium in some alveoli, in others it produced a slight epithelial hyperplasia. In contrast, treatment with either testosterone or hydrocortisone prevented the regression of the epithelium observed in the control medium, and the alveoli were lined with tall actively secreting elements.

Effect of methylcholanthrene

These experiments confirmed the results of earlier work [8]. In the natural medium the carcinogen induced a rapid multiplication of the alveolar cells in most treated cultures (Table 1); layers of crowded cells were formed which partially or completely filled the alveolar lumen (Fig. 3). The superficial cells continued to secrete but at a later stage they were shed and replaced by flat non-secretory elements.

After the transfer of the tissue to control medium the hyperplasia became more marked (Fig. 4). Thus, all except one explant showed hyperplastic foci and in a greater number the hyperplasia was more extensive than in explants exposed to methylcholanthrene for 11 days only (Table 1).

In the semi-defined medium the carcinogen was less effective, the epithelial cells in a few alveoli had multiplied to form not more than 2–3 layers and a smaller proportion of explants were affected (Table 2).

Effect of methylcholanthrene and oestrogens

During simultaneous treatment in the natural medium, the hormone increased the effect of the carcinogen to a moderate degree. A similar proportion of treated cultures as those grown with MC alone showed hyperplasia but in most of these the changes were more extensive (Table 1, Fig. 5).

In the semi-defined medium, however, the action of the hormone was much more pronounced; more treated cultures showed hyperplasia and in most of them cell multiplication was active (Table 2). In both media the secretory cells at the lumen were shed and replaced by flat cells at an earlier stage than in the MC controls.

In explants pretreated with the carcinogen and transferred to medium containing oestrone or oestradiol for 6 days, the hormones also enhanced the effect of methylcholanthrene; a greater number of cultures showed hyperplasia which was more extensive than in controls grown without hormones (Table 1, Fig. 6).

Effect of methylcholanthrene and testosterone

Simultaneous application of the two compounds drastically reduced the incidence and the extent of hyperplasia induced by the carcinogen alone. In the natural medium hyperplasia was present in only four out of eleven explants; in three of these it was very mild and increased cell proliferation was seen in only one explant. In the remaining seven cultures all alveoli were lined with one row of secretory cells (Fig. 7, Table 1).

In the semi-defined medium hyperplasia was completely absent in all explants (Table 2).

Transfer of carcinogen-treated tissue to medium containing testosterone for a further 6 days also diminished the hyperplastic changes. Thus, in most cultures only a few peripheral alveoli showed mild hyperplasia (Fig. 8, Table 1).

Effect of methycholanthrene and hydrocortisone

Simultaneous treatment was followed by an almost complete suppression of epithelial hyperplasia (Table 1). In the natural medium hyperplasia was absent in sixteen out of eighteen treated explants and in the remaining two only a very few alveoli at the periphery showed an increase to 2–3 rows of cells; elsewhere the alveoli were lined with a single layer of tall columnar epithelium (Fig. 9).

In the semi-defined medium hyperplasia was absent in all explants (Table 2).

Transfer of pretreated cultures to medium containing hydrocortisone for 6 days was also followed by a reduction of cell proliferation, but the inhibition of growth was less complete than after simultaneous application (Table 1). In three out of ten explants hyperplasia was still extensive, three showed medium and four mild hyperplasia (Fig. 10).

DISCUSSION

The results show that methylcholanthrene induces a rapid and extensive hyperplasia of the prostatic epithelium in the natural medium and a mild hyperplasia in the semi-defined medium. The lower activity of the carcinogen is probably due to its reduced solubility and hence, its lower concentration in the tissue in the protein poor semi-defined medium.

The oestrogens enhance the hyperplasia to a moderate degree in the natural medium and considerably so in the semi-defined medium where the MC induced hyperplasia is mild. The hormones also accelerate the dedifferentiation of the epithelium. Testosterone or hydrocortisone, on the other hand, suppresses or inhibits the hyperplasia in both media and prevents the regression of the epithelium.

Although the promotion or suppression of hyperplasia varies in degree in the natural and the semi-defined medium, the direction of hormonal action is identical under both conditions. This suggests that it is independent of the traces of other hormones or substances present only in the natural medium.

Possible mechanisms for these effects were considered. The promotion or inhibition of epithelial hyperplasia may be related to the degree of differentiation of the cells, in the sense that only cells which have lost their functional activity, i.e. in explants grown in control medium or in medium containing oestrogens are susceptible to the carcinogen, whereas epithelium which is maintained in an actively secreting state by either testosterone or hydrocortisone is unable to respond.

A direct interaction of the carcinogen with the hormones cannot be ruled out however. Heidelberger and his group [9, 10] showed that carcinogenic hydrocarbons are taken up by cytoplasmic key proteins and testosterone or hydrocortisone may either prevent the binding by competing for sites on the protein molecule during simultaneous application, or break the protein-carcinogen bond when given to cultures pretreated with methylcholanthrene.

Another possibility involves the lysosomes. Allison and Mallucci [11] found that in cells treated *in vitro* with carcinogenic hydrocarbons these substances were localised within the lysosomes. The authors contend that the compounds may initiate carcinogenesis by altering the pattern of lysosomal enzymes. Measurements of lysosomal acid phosphatase in the rat prostate in culture [12] indicate that testosterone or hydrocortisone protects the lysosomal membranes while oestrogens increase their fragility. On this basis it could be argued that testosterone and hydrocortisone may impede the entry of the carcinogen into the lysosomes and that the oestrogens facilitate it. Experiments to test this view have begun.

Acknowledgements—I would like to thank Professor Dame Honor Fell, F.R.S. for constructive criticism in the preparation of this manuscript and Mr. G. Lenney, A.I.T.S. for the microphotographs.

RESUME

L'influence des oestrogènes, de la testostérone et de l'hydrocortisone sur l'action du méthylcholanthrène sur la prostate du rat a été étudiée en culture organotypique dans des milieux naturels et semi-synthétiques.

Dans le milieu naturel, le méthylcholanthrène provoque une très forte hyperplasie de l'épithélioma prostatique. Dans le milieu semi-synthétique, l'hyperplasie est plus modérée. L'oestrone et l'oestradiol augmentent l'hyperplasie épithéliale, tandis que la testostérone et l'hydrocortisone la suppriment dans les deux milieux.

SUMMARY

The influence of oestrogens, testosterone and hydrocortisone on the effect of methylcholanthrene on the rat prostate gland grown as organ cultures in natural and semi-defined medium has been examined.

The hormones were either administered simultaneously with methylcholanthrene or applied to cultures pretreated with the carcinogen.

In natural medium methylcholanthrene alone causes a rapid and extensive hyperplasia of the prostatic epithelium. In the semi-defined medium it causes a mild hyperplasia.

In natural medium oestrone and oestradiol increase the hyperplasia to a moderate degree, in the semi-defined medium they enhance it considerably.

Testosterone and hydrocortisone inhibit or suppress the hyperplasia in both media.

ZUSAMMENFASSUNG

Der Einfluss von Oestron, Oestradiol, Testosteron und Hydrocortison auf die Wirkung von Methylcholanthren wurde in Organkulturen der Rattenvorsteherdrüse (Prostata), die in natürlichen und semi-synthetischen Medien gezüchtet wurden, untersucht.

Methylcholanthren allein verursacht eine starke Hyperplasie des Prostataepithels.

Oestron und Oestradiol steigern diese Wirkung, während Testosteron und Hydrocortison sie in beiden Medien hemmen.

REFERENCES

1. W. F. Dunning, R. R. Curtis and A. Segaloff, Methylcholanthrene squamous cell carcinomas of the rat prostate with skeletal metastases and failure of the rat liver to respond to the same carcinogen. *Cancer Res.* **6,** 256–62 (1946).
2. E. A. Mirand and W. J. Staubitz, Prostatic neoplasms of the Wistar rat induced with methylcholanthrene. *J. Natl. Cancer Proc. Soc. Exp. Biol. Med.* **93,** 457–62 (1956).
3. J. M. Allen, Responses of the rat prostate gland to methylcholanthrene, *J. Exp. Zool.* **123,** 289–13 (1953).
4. G. Asboe-Hansen, Hormonal effects on connective tissues. *Connective Tissues Reaction Trans. 5th Conf., New York.* J. Macy Junior Foundation, 123–82 (1954).
5. A. Baserga and P. Shubik, The action of cortisone on transplanted and induced tumours in mice. *Cancer Res.* **14,** 12–16 (1954).
6. H. N. Green and M. Savigear, Shock and Neoplasia. *Brit. Med. J.* **1,** 498–500 (1951).
7. I. Lasnitzki, The action and interaction of hormones and methylcholanthrene on the ventral prostate gland of the rat *in vitro*. *J. Natl. Cancer Inst.* In press (1965).
8. I. Lasnitzki, The effect of methylcholanthrene on rat prostate glands grown in natural and semi-defined medium. *Cancer Res.* **24,** 973–76 (1964).
9. C. W. Abell and C. Heidelberger, Interaction of carcinogenic hydrocarbons with tissue. VIII. Binding of tritium labelled hydrocarbons to the soluble proteins of mouse skin. *Cancer Res.* **22,** 931–46 (1962)
10. B. C. Giovanella, L. E. McKinney and C. Heidelberger, On the reported solubilisation of carcinogenic hydrocarbons in aqueous solutions of DNA. *J. Mol. Biol.* **8,** 20–27 (1964).
11. A. C. Allison and L. Mallucci, Uptake of hydrocarbon carcinogens by lysosomes. *Nature* **203,** 1024–27 (1964).
12. I. Lasnitzki, S. Waite and J. T. Dingle, The effect of hormones on the lysosomal acid phosphatase in the rat prostate in culture. *Brit. Emp. Canc. Camp. Report,* 375 (1964).

Europ. J. Cancer Vol. 1, pp. 295–298. Pergamon Press 1965. Printed in Great Britain

Autoradiographische Untersuchungen des RNS- und DNS-Stoffwechsels in menschlichen Tumoren

W. OEHLERT und R. LESCH

Ludwig-Aschoff-Haus,
Pathologisches Institut der Universität Freiburg i.Br.

In einer Reihe von autoradiographischen, Untersuchungen konnte gezeigt werden, daß in menschlichem Excisionsmaterial aus der Haut dem Bronchialsystem und dem weiblichen Genitaltrakt nach kurzzeitiger Inkubation mit Eiweiß- oder Nukleinsäurevorläufern ein Einbau in Proteine und Nucleinsäuren erfolgt, dessen Ausmaß als Maßstab für die in vivo ablaufenden Stoffwechselprozesse genommen werden kann (Oehlert, Lesch und Dörmer [1]; Lesch, Schiessle und Oehlert [2]; Fettig und Oehlert [3]). Von systematisch durchgeführten Untersuchungen, in denen jeweils neben der Routinehistologie der Einbau von ^{3}H-Thymidin und ^{3}H-Cytidin autoradiographisch bestimmt wurde, liegen nun so umfangreiche Ergebnisse vor, daß Grenzen und Möglichkeiten der Inkubationsmethode zu überblicken sind.

TECHNIK DER INKUBATION UND AUTORADIOGRAPHIE

Bei der Inkubation der Gewebsproben gingen wir folgendermaßen vor. Unmittelbar nach der Entnahme wurden noch im Operationssaal die kleinen Gewebsbröckel oder die durch Katheterbiopsie, bzw. durch die Abrasio gewonnenen Schleim- und Gewebsanteile in sterile Ringerlösung gebracht, der entweder ^{3}H-Thymidin oder ^{3}H-Cytidin in einer Konzentration von 4 µC/ml zugesetzt worden war. Bei Körpertemperatur wurde 30 Minuten lang inkubiert. Anschliessend erfolgte die Fixation in üblicher Weise in 10% igem neutralem Formalin und die Einbettung in Paraffin. Von jeder Gewebsprobe wurden zwei Objektträger mit meist 5–6 Gewebsschnitten der Routinehistologie zugeführt. Fünf weitere Objektträger mit jeweils der gleichen Anzahl einzelner Schnitte wurden mittels der dipping — Technik unter Verwendung der Emulsion Ilford G5 zu Autoradiogrammen verarbeitet. Nach Belichtungszeiten von 5–10 Tagen und anschliessender photographischer Entwicklung und Fixierung erfolgte die Färbung mit Hämatoxylin — Eosin und die Auswertung der Autoradiogramme.

ERGEBNISSE

Etwa 70% der angefertigten Präparate waren für eine qualitative und nur etwa 40% für eine quantitative Auswertung durch Zellzählungen brauchbar. In etwa 30% der Autoradiogramme war eine Markierung, d.h. ein Einbau der markierten Nucleinsäurevorläufer DNS oder RNS der untersuchten Zellen nicht nachweisbar. Hierbei handelte es sich fast ausschließlich um Präparate, in denen Schnitte besonders grosser Gewebsbröckel zur Untersuchung gekommen waren.

Die Eindringtiefe des Inkubationsmediums betrug etwa 200–300 mµ, sie war umso grösser,

je lockerer die untersuchten Gewebe aufgebaut waren. Nur solche Präparate, bei denen grössere Anteile markierte Zellen enthielten, sodaß Zellzählungen durchführbar waren, konnten für eine quantitative Bestimmung des Markierungsindex herangezogen werden.

Das Markierungsmuster der Gewebe und Zellen entsprach bei den Inkubationsversuchen in jeder Hinsicht demjenigen, das nach parenteraler Applikation von ^{3}H-Thymidin oder ^{3}H-Cytidin im Tierversuch gefunden wird. So waren nach Inkubation mit ^{3}H-Thymidin jeweils nur einzelne Zellkerne markiert. Die autoradiographischen Silberkörner lagen dabei ausschließlich über dem Zellkern der markierten Zellen, wobei bei günstiger Schnittebene der Nucleolus unmarkiert innerhalb des mit Silberkörnern bedeckten Caryoplasmas lag (Abb. 1a). Nach Inkubation mit ^{3}H-Cytidin, welches vorwiegend in die RNS eingebaut wird, beobachtet man eine Markierung aller Zellen im Bereiche der Eindringtiefe des Inkubationsmediums, wobei jedoch die Silberkorndichte über verschiedenen Zellarten auf ein und demselben Präparat unterschiedlich war. Bei der von uns gewählten Inkubationszeit von 30 Min war nur der Zellkern mit Silberkörnern bedeckt, wobei jedoch im Gegensatz zur Markierung mit ^{3}H-Thymidin die Silberkorndichte über dem Nucleolus größer als über dem übrigen Kernraum war (Abb. 1b).

Bei der bisher durchgeführten autoradiographischen Untersuchung von insgesamt 196 Biopsiepräparaten von 110 Patienten nach 30 Min Inkubation in ^{3}H-Thymidin oder ^{3}H-Cytidin konnten wir Aufschlüsse über die Proliferationsrate bei verschiedenen Formen der Hyperplasie und Metaplasie des Bronchialepithels und in Bronchuscarcinomen gewinnen.

Im normalen Bronchialepithel (Abb. 2) beobachtet man nur einige wenige markierte Zellkerne der sogenannten Ersatzzellen, die an der Basis des zum Teil mehrreihigen Zylinderepithels liegen. Im sogenannten regeneratorischen Übergangsepithel des Bronchus, das häufig bei chronischen Bronchitiden zur Beobachtung kommt, bestand praktisch stets eine Vermehrung der DNS— synthetisierenden Zellen, die nicht nur in der Basalzellschicht sondern auch in oberen Epithelschichten aufzufinden waren (Abb. 3). Das gleiche galt von metaplastisch umgewandeltem Bronchialepithel. Hier fanden sich stets zahlreiche Zellelemente markiert, die zum Teil im Stratum basale zum Teil aber auch in oberen Zellschichten lokalisiert waren (Abb. 4). Durch die Inkubation mit ^{3}H-Thymidin konnte in einer ganzen Reihe von Bronchuscarcinomen die Zahl DNS — synthetisierender Zellen bestimmt werden. Sowohl im Plattenepithelcarcinom als auch im kleinzelligen Bronchuscarcinom wurde dabei im allgemeinen eine große Zahl DNS — synthetisierender Zellen aufgefunden (Abb. 5 und 6). Bei mehreren Carcinomen konnte durch eine quantitative Auswertung der Autoradiogramme der Markierungsindex (Zahl der markierten Zellen pro Gesamtzellzahl) ermittelt werden. Unter Berücksichtigung dieser Werte und einer DNS — Synthesezeit von 6 Stunden war es möglich, die Generationszeit, d.h. die Wachstumsgeschwindigkeit derartiger Tumoren abzuschätzen.

Der RNS — Umsatz, gemessen am Einbau von ^{3}H-Cytidin, war im Tumorgewebe stets grösser als im normalen Bronchialepithel (Abb. 7).

Ähnliche Untersuchungen wurden am gynäkologischen Abrasionsmaterial und an Portioexcisionen durchgeführt. Auch hier wurde mit zunehmender Entdifferenzierung des mehrschichtigen Plattenepithels der Portio eine Vermehrung DNS — synthetisierender Zellen in allen Epithelschichten nachgewiesen.

BESPRECHUNG DER ERGEBNISSE

Sowohl das an Hand einiger Beispiele besprochene Einbaumuster der untersuchten Gewebe und Zellen nach Inkubation mit ^{3}H-Thymidin und ^{3}H-Cytidin als auch das an anderer Stelle mitgeteilte Ergebnis von Vergleichsuntersuchungen (Oehlert, Lesch und Dörmer 1963) und die Versuchsergebnisse, die von Herrn Rajewski in einem vorhergehenden Vortrag mitgeteilt wurden, dürfen als Beweis dafür gelten, daß der autoradiographischen Schwärzung in unseren Inkubationsversuchen ein Einbau von ^{3}H-Thymidin in DNS bzw. eine Incorporation von ^{3}H-Cytidin in RNS zugrunde liegen. Wir können also mit unseren Versuchen die im überlebenden menschlichen Gewebe auslaufenden Stoffwechselvorgänge fassen, die uns Hinweise auf die Verhältnisse in vivo geben können. Die von uns angewandte Technik mit kurzer Inkubationszeit und schonender Behandlung der inkubierten Gewebe sowie mit verhältnismäßig indifferentem Inkubationsmedium bietet unseres Erachtens nach bessere Aussagemöglichkeiten als die von anderen Autoren angegebenen Verfahren. Bei diesen wurden relativ lange Inkubationszeiten, Kulturmedien, die für Gewebekulturen verwandt werden, bzw. nach Einfrieren dünne Gewebsscheiben verwandt (Wolberg und Brown [4]; Veenema, Fingerhut und Girgis [5]).

Schwierigkeiten in der Auswertung der Ergebnisse bereitet die Tatsache, daß nur in einem Teil der untersuchten Fälle so grosse Gewebsanteile in engen Kontakt mit dem Inkubationsmedium kommen, daß quantitative Aussagen über den Markierungsindex durch Zellzählungen möglich sind. Durch sorgfältiges Einblocken der inkubierten Gewebe in Paraffin und völliges Aufschneiden in Serien wird man jedoch die Anzahl negativer Autoradiogramme gering halten können.

Soweit die bisher vorliegenden Ergebnisse eine Aussage über den Wert unserer Methode erlauben, kann man Folgendes sagen. Die Grenzen der autoradiographischen Untersuchungstechnik an inkubiertem menschlichem Gewebe werden durch die verhältnismäßig geringe Eindringtiefe des Inkubationsmediums in das zu untersuchende Gewebe gesteckt. Kleine Gewebsbröckel eignen sich besser als grössere, zusammenhängende Gewebspartien. Hierdurch werden sowohl die qualitative als auch die quantitative Auswertung der Autoradiogramme erschwert und die Aussagemöglichkeiten eingeschränkt. Trotz der genannten Nachteile sind von systematisch durchgeführten autoradiographischen Stoffwechseluntersuchungen bei entzündlichen Prozessen, Präcancerosen und Carcinomen Aufschlüsse über die Vorstadien des Carcinoms beim Menschen und über die Wachstumsgeschwindigkeit bestimmter Tumoren zu erwarten.

Die autoradiographische Untersuchung am Inkubationsmaterial unter Verwendung von ^{3}H-Thymidin erbringt Aufschlüsse über die Proliferationsaktivität bestimmter menschlicher Gewebe und Tumoren, die weit über diejenigen hinausgehen, die mit Hilfe von Mitosezählungen erhalten werden können.

Die beschriebene Untersuchungsmethode kann vor allem bei der histologischen Beurteilung von Katheterbiopsien und eventuell auch von Ausstrichpräparaten, von Spülflüssigkeiten und Sputum erhebliche Bedeutung erlangen, wenn es gelingt, den Zeitraum zwischen Gewebsentnahme und histologischer Untersuchung der Autoradiogramme entsprechend zu verkürzen.

Die Inkubation von Gewebsproben mit ^{14}C-markiertem Thymidin statt des ^{3}H-markierten Thymidin würde zum Einbau eines Betastrahlers mit grösserer Reichweite führen und damit die Möglichkeit des leichteren Nachweises der eingebauten Aktivität mit Hilfe des Zählrohres geben. Unter diesen Voraussetzungen liesse sich dann eine Methodik entwickeln, welche den vollautomatischen Nachweis DNS — synthetisierender Zellen in einer grossen Zahl von Ausstrich-oder Abstrichpräparaten gestattet. Dies wiederum würde dem Kliniker die schnelle und leichtere Anwendung zur Auswertung einer grossen Zahl von cytologischen Präparaten, wie sie bei routinemässigen Untersuchungen in der Gynäkologie oder bei Sputumuntersuchungen anfallen, ermöglichen.

RESUME

Ayant examiné, au moyen d'auto-radiographies, le métabolisme de l'ADN et de l'ARN d'environ 500 *biopsies gynécologiques et bronchiques, après incubation* in vitro *de celles-ci, les auteurs discutent la valeur de cette méthode.*

Si l'on y apporte certaines modifications, on peut s'attendre à obtenir de précieuses indications quant à la tendance proliférative de différents tissus au cours d'un pré-screening de grandes séries de frottis cytologiques.

SUMMARY

RNA and DNA metabolism of about 500 *human tissue specimens obtained by gynecological and bronchial biopsy has been studied by means of autoradiography after* in vitro *incubation. The value of this method is discussed.*

With suitable modifications, the method may be expected to give valuable information on cell proliferation in different tissues through pre-screening of large series of gynecological smears.

ZUSAMMENFASSUNG

Nach autoradiographischer Untersuchung zum DNS- und RNS-Stoffwechsel von etwa 500 *gynäkologischen und bronchologischen Biopsien menschlichen Gewebes mit Hilfe der in vitro-Inkubation wird über den Wert dieser Methode berichtet.*

Bei entsprechender Modifizierung sind wertvolle Hinweise auf die Proliferationstendenz verschiedenster Gewebe durch ein "praescreening" grosser Serien cytologischer Ausstriche zu erwarten.

LITERATURVERZEICHNIS

1. W. Oehlert, R. Lesch u. P. Dörmer, Autoradiographische Untersuchungen des DNS- und RNS- Stoffwechsels an menschlichem Excisionsmaterial. *Naturwiss.* **50,** 713 (1963).
2. R. Lesch, W. Schiessle u. W. Oehlert, Autoradiographische Untersuchungen zur DNS.— Synthese an menschlichem Excisionsmaterial aus dem Bronchialbaum. *Beitr. path. Anat.* **129,** 295 (1963/64).
3. O. Fettig, u. W. Oehlert, Autoradiographische Untersuchungen der DNS- und Eiweiß-Neubildung im gynäkologischen Untersuchungsmaterial. *Arch. Gynäkol.* **199,** 649 (1964).
4. W. H. Wolberg u. R. R. Brown, Autoradiographic Studies of *In Vitro* Incorporation of Uridine and Thymidine by Human Tumor Tissue. *Cancer Res.* **22,** 1113 (1962)
5. R. J. Veenema, B. Fingerhut u. A. S. Girgis, A Possible Guide to Therapy of Bladder Tumors. *J. Urology* **90,** 736 (1963).



Abb. 1. *Autoradiogramme von Zellen aus einem nicht verhornenden Plattenepithelcarcinom des Bronchus,* 30 *Minuten nach Inkubation* (*a*) *mit* ^{3}H-Thymidin: *Ausschließliche Markierung des Zellkernes mit Aussparung des Nucleolus;* (*b*) *mit* ^{3}H-Cytidin: *Fast ausschließliche Markierung des Zellkernes, größte Silberkorndichte über dem Nucleolus* (*Hämatoxylin-Eosin, Emulsion Ilford G*5, *Belichtung* 10 *Tage,* 1600×)

(to face p. 298)
Refer to p. 296

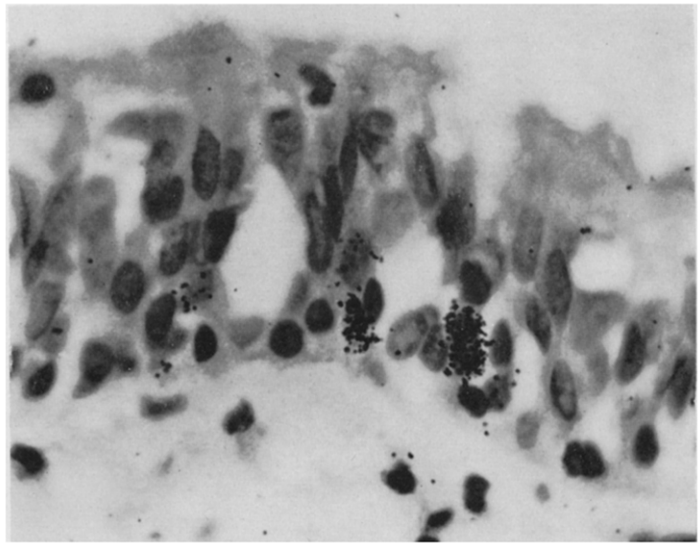


Abb. 2. *Autoradiogramm eines normalen Bronchialepithels mit DNS — synthetisierenden Ersatzzellen.* 30 *Minuten nach Inkubation mit* ^{3}*H-Thymidin* (*Hämatoxylin-Eosin, Emulsion Ilford G*5, *Belichtung* 10 *Tage*, 360×)

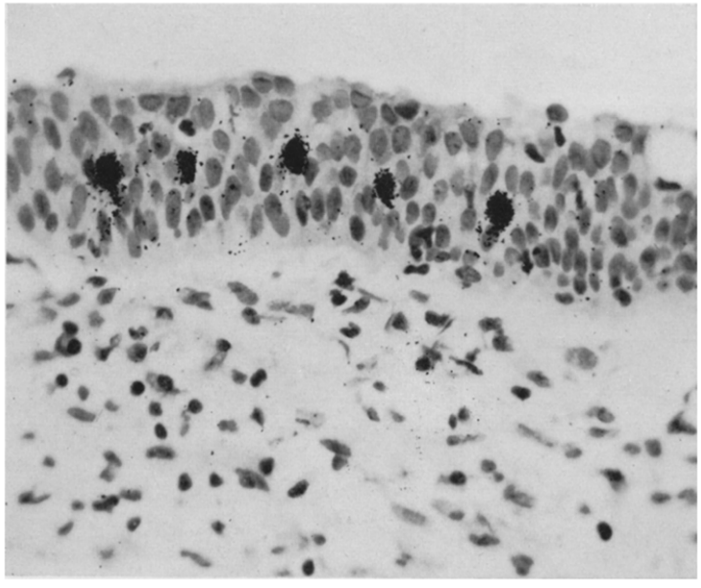


Abb. 3. *Autoradiogramm eines regeneratorischen übergangsepithels des Bronchus mit zahlreichen DNS — synthetisierenden Zellen in mittleren Zellagen, keine Mitosen nachweisbar.* 30 *Minuten nach Inkubation mit* ^{3}*H-Thymidin.* (*Hämatoxylin-Eosin, Emulsion Ilford G*5, *Belichtung* 10 *Tage*, 250×)

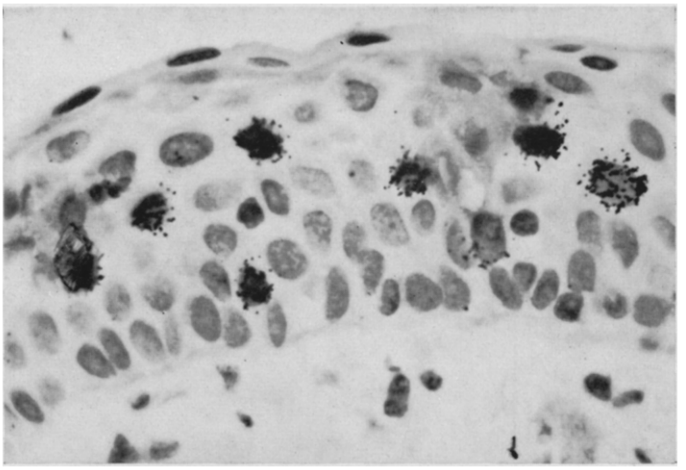


Abb. 4. *Autoradiogramm eines metaplastisch umgewandelten Bronchialepithels; zahlreiche DNS — synthetisierende Zellen im Stratum basale und oberen Zellagen.* 30 *Minuten nach Inkubation mit* ^{3}H-*Thymidin.* (*Hämatoxylin-Eosin, Emulsion Ilford G*5, *Belichtung* 10 *Tage,* 360×)

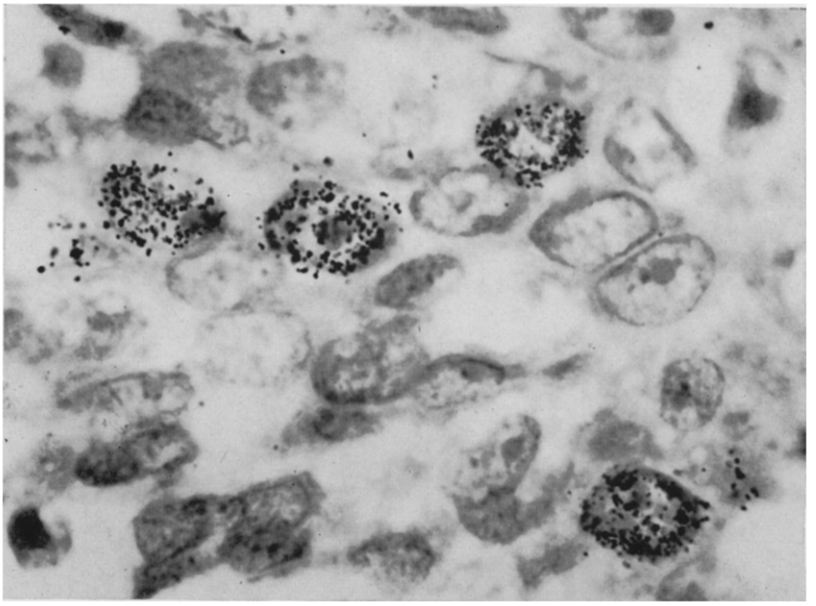


Abb. 5. *Autoradiogramm eines Plattenepithelcarcinoms des Bronchus mit zahlreichen unregelmässig verteilten DNS — synthetisierenden Zellen.* 30 *Minuten nach Inkubation mit* ^{3}H-*Thymidin.* (*Hämatoxylin Eosin, Emulsion Ilford G*5, *Belichtung* 10 *Tage,* 400×)

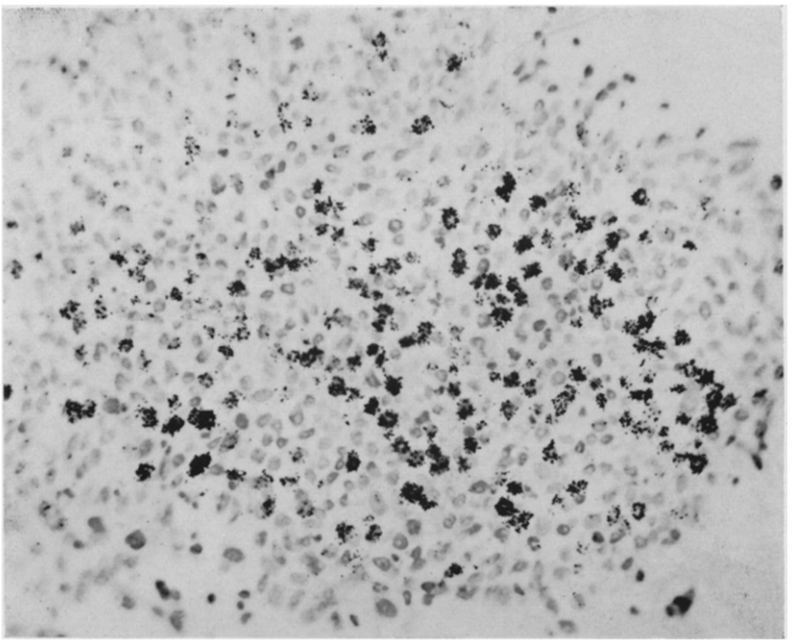


Abb. 6. *Autoradiogramm eines kleinzelligen Bronchuscarcinoms; zahlreiche unregelmäßig verteilte DNS — synthetisierende Zellen.* 30 *Minuten nach Inkubation mit* ^{3}*H-Thymidin.* (*Hämatoxylin-Eosin, Emulsion Ilford G*5, *Belichtung* 10 *Tage,* 150×)

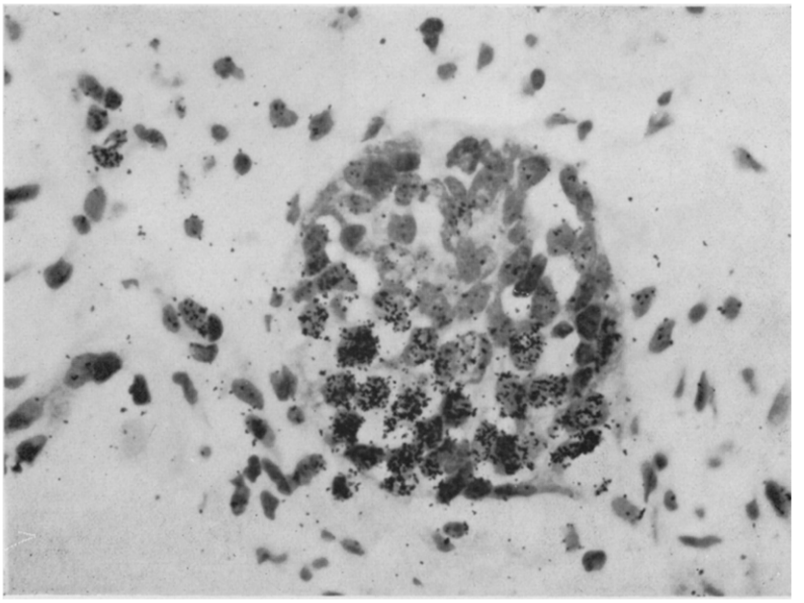


Abb. 7. *Autoradiogramm eines Zellverbandes eines kleinzelligen Bronchuscarinoms; starke RNS — Neubildung in Carcinomzellen, geringere in mesenchymalen Zellen der Umgebung.* 30 *Minuten nach Inkubation mit* ^{3}*H-Cytidin.* (*Hämatoxylin-Eosin, Emulsion Ilford G*5, *Belichtung* 10 *Tage,* 320×)

Europ. J. Cancer Vol. 1, pp. 299–302. Pergamon Press 1965. Printed in Great Britain

A Quantified Periodic Acid-Schiff Reaction in Normal and Leukemic Leukocytes*†

GÖSTA GAHRTON

Institute for Medical Cell Research, Karolinska Institutet, Stockholm, Sweden and the Department of Medicine, University Hospital, Lund, Sweden

THE periodic acid-Schiff reaction is used in histochemistry and cytochemistry to demonstrate 1,2-glycol groups or the equivalent amino- or alkylamino derivates or the oxidation product CHOH-CO, as constituents of polysaccharides, muco- and glycoproteins or some glycolipids, unsaturated lipids or phospholipids [3]. The PAS positive material in most myeloid blood and bone marrow cells is digestible with α-amylase and is therefore considered to consist of the polysaccharide glycogen [2, 4–7]. Mature blood neutrophils contain considerable amounts of α-amylase digestible PAS positive material, distributed as a diffuse or somewhat granular red stain in the cytoplasm, leaving the nucleus unstained. Accurate semi-quantitative microscopic evaluation of the amount of stain in the cells is very difficult, although such estimations have been made in a number of investigations [4–12]. The present work is firstly a study of the quality, stoichiometry and kinetics of the PAS reaction in a model system of glycogen microdroplets and neutrophil leukocytes, and secondly a clinical study of the quantified reaction in neutrophils from patients with chronic myeloproliferative diseases (chronic myelocytic leukemia (CML), myeloid metaplasia and polycythemia vera). The tool for the estimation of the glycogen amount (dry mass) in individual microdroplets was a rapid scanning microinterferometer [13–15]. The amount of stain in glycogen microdroplets and neutrophils was estimated by measuring the absorption of light in a rapid scanning microspectrophotometer [13–15].

METHODS

The technique for the methodological study was the following [2]:

Neutrophils from venous blood were obtained from the buffy coat after sedimentation with a 1 per cent human fibrinogen solution for 20–30 min. The cells were smeared on one half of a hemocytometer cover glass (Bürker), air dried, fixed in absolute methanol for 15 min and air dried again. Glycogen microdroplets were produced on the other half of the hemoctyometer cover glass by spraying an 0·5–1·0 per cent solution of pure glycogen. The droplets were air dried. The whole glass was then covered by a semi-permeable formvar membrane. The glycogen amount (dry mass) of individual localized glycogen microdroplets was measured in a rapid scanning microinterferometer. About 10 individual microdroplets were measured in each preparation. The smears were then submitted to various modifications of the Mc Manus PAS reaction [16] to study the quality, stoichiometry and kinetics of the reaction.

*This work has been supported by grants from the U.S. Public Health Service (Grant No. C-4716), as well as the Swedish Medical Research Council and the Wallenberg Foundation.

†Presented in part at the Xth Congress of the International Society of Hematology, Stockholm 1964 [1] and published in part in Exptl. Cell Research **34**, 488 1964 [2].

RESULTS

Studies of the quality of the PAS reaction

Absorption curves were registered from arbitrary points in glycogen microdroplets and neutrophils at various modifications of the reaction, varying the time of periodic acid oxidation and the Schiff reaction. It was shown that the shape of the absorption curves was approximately the same in glycogen microdroplets and neutrophil leukocytes at various modifications of the reaction with an absorption maximum between 550 and 565 mμ. The formvar coating did not change the absorption curve.

Studies of the relation between glycogen amount and total extinction at 560 mμ in glycogen microdroplets

The relation between the glycogen amount (dry mass) and the total extinction at 560 mμ in localized glycogen microdroplets of different size was linear with various modifications of the PAS reaction (periodic acid oxidation times of 2, 5, 15 and 60 min and Schiff reaction times of 15, 30 and 60 min). That means that the reaction was stoichiometric and the absorption of light followed Beer's law.

Studies of the kinetics of the PAS reaction

The rate of color development in the microdroplets and the neutrophils was studied by comparing the reaction in neutrophils and glycogen droplets in the same preparation 1) at various periodic acid oxidation times with a constant Schiff reaction time of 15 min and 2) at various Schiff reaction times with a constant periodic acid oxidation time of 5 min. It was shown that the rate of color development as a function of the periodic acid oxidation time was considerably more rapid in neutrophils than in glycogen microdroplets. A plateau in the color development curve was reached at about 60-min periodic acid oxidation time. The rate of color development as a function of the Schiff reaction time was somewhat more rapid in neutrophils than in glycogen microdroplets reaching an approximate plateau in both neutrophils and microdroplets after about 60 min Schiff reaction time. It was considered that the plateau in the color development curves at 60 min periodic acid oxidation and 60 min Schiff reaction was the most suitable time for quantitative measurements. If the glycogen microdroplet curve was used as a reference to estimate the true glycogen amount in neutrophils, a mean value of 6×10^{-12} g glycogen per normal neutrophil leukocyte was obtained. This value is somewhat lower but in the same range as the previously biochemically estimated glycogen amount in separated leukocytes [8, 17, 18].

Comparative quantified estimation of the PAS reaction in neutrophils from patients with chronic myelocytic leukemia, myeloid metaplasia and polycythemia vera

Twenty-three cases of chronic myelocytic leukemia (CML) (ten untreated or in relapse, thirteen in remission or partial remission), eighteen cases of myeloid metaplasia and seventeen cases of polycythemia vera were studied. Fifty individual PAS stained mature neutrophils were measured at 546 mμ in each case in a rapid scanning microspectrophotometer. A reference consisting of normal neutrophils from a known control person was stained and measured together with the pathologic cells. The reference was chosen from a control material of twenty normal cases so that the mean value for fifty neutrophils in the control was about the same as the group mean for the twenty normal cases. About fifty reference smears were prepared and fixed at the same time from the control person and could then be used as controls throughout the investigation. A new series of control smears was always compared to the original reference series. A continuous control system was thus obtained and all values were expressed in percentage of the original control, representative for a control material. Neutrophils from CML untreated or in relapse had lower mean total extinction than normal (group mean 59 per cent). The frequency distribution of total extinction per cell did not differ significantly from normal. After treatment with Myleran the group mean for CML was approximately normal (group mean 101 per cent) with a normal frequency distribution of total extinction per cell. Neutrophils from myeloid metaplasia had elevated mean total extinction (group mean 119 per cent) compared to normal. The variance between cells within subjects was usually considerably elevated although a few cases did not differ from normal. Neutrophils from polycythemia vera had the highest mean total extinction (group mean 138 per cent) usually with considerably augmented variance between cells within subjects. The difference in group means and variances in neutrophils from CML and myeloid metaplasia (the group mean was twice as high in myeloid metaplasia as in CML and the variance was 4 times as high in myeloid metaplasia as in CML) is considered to have diagnostic value for the differentiation between these two diseases.

RESUME

La réaction de Mc Manus — P.A.S. a été rendue quantitative par la combinaison de la microinterférométrie et de la microspectophotométrie, appliquées à un modèle consistant en des gouttelettes microscopiques de glycogène et à des polynucléaires neutrophiles. La qualité, la stoïchiométrie et la cinétique de la réaction ont été étudiées La réaction quantitative a été appliquée à l'étude des neutrophiles des malades atteints de leucémie myéloïde chronique, de métaplasie myéloïde et de polycytémie de Vaquez. Les données expérimentales semblent conférer à cette technique une valeur pronostique pour différencier la métaplasie myéloïde de la leucémie myéloïde chronique.

SUMMARY

The periodic acid-Schiff reaction has been quantified by means of combined microinterferometry and microspectrophotometry in a model system of glycogen microdroplets and neutrophil leukocytes. The quality, stoichiometry and kinetics of the reaction have been studied. The quantified reaction has been used in a clinical investigation of neutrophils from patients with chronic myelocytic leukemia, myeloid metaplasia and polycythemia vera. It is considered to have diagnostic importance in the differentiation of chronic myelocytic leukemia and myeloid metaplasia.

ZUSAMMENFASSUNG

Die P.A.S.-Reaktion wurde von den Autoren in eine quantitative Untersuchungsmethode überführt, indem sie die Mikrointerferometrie und die Mikrospectrophotometrie für die Untersuchung eines Modellsystems bestehend aus Glycogen-Mikrotropfen und neutrophilen Leukozyten verwendeten.

Die Qualität, die Stoichiometrie und der Ablauf der Reaktion wurden überprüft.

Die quantiative Reaktion wurde auf die klinische Untersuchung von neutrophilen Leukozyten stammend von Patienten mit chronischer myeloischer Leukämie, mit myeloischer Metaplasie und mit Polycythämia vera angewendet.

Aus diesen Untersuchungen wurde der Schluss gezogen, dass diese Reaktion von differentialdiagnostischer Wichtigkeit bei der Unterscheidung zwischen myeloischer Leukämie und myeloischer Metaplasie ist.

REFERENCES

1. G. GAHRTON, Microspectrophotometric quantitation of the periodic acid-Schiff (PAS) reaction in normal and pathologic neutrophil leukocytes. *Abstract Xth. Congr. Int. Soc. Haematology*, p. 0: 2 (1964).
2. G. GAHRTON, Microspectrophotometric quantitation of the periodic acid-Schiff (PAS) reaction in human neutrophil leukocytes based on a model system of glycogen microdroplets. *Exp. Cell Res.* **34,** 488 (1964).
3. A. G. E. PEARSE, *Histochemistry, Theoretical and Applied.* Churchill, London (1960).
4. G. ASTALDI, E. BERNARDELLI and E. G. RONDANELLI, Ricerche sul contenuto in glicogeno delle cellule del sangue e del midollo osseo. *Haematologica* **36,** 749 (1952).
5. R. P. GIBB and R. E. STOWELL, Glycogen in human blood cells. *Blood* **4,** 569 (1949).
6. M. WACHSTEIN, The distribution of histochemically demonstrable glycogen in human blood and bone marrow cells. *Blood* **4,** 54 (1949).
7. G. B. WISLOCKI, J. J. RHEINGOLD and E. W. DEMPSEY, The occurrence of the periodic acid-Schiff reaction in various normal cells of blood and connective tissue. *Blood* **4,** 562 (1949).

8. A. DAHLQVIST, G. GAHRTON, and A. NORDÉN, The content of total and acid-soluble glycogen in the white blood cells and its relation to the staining with the periodic acid-Schiff reagents. *Acta. Med. Scand.* **172,** 32 (1962).
9. I. GÅRTNER and Å. NORDÉN, Studies on periodic acid-Schiff reactive material in white blood cells from the peripheral blood of patients with diabetes, polycythaemia and chronic lymphocytic leukaemia. *Acta Med. Scand.* **169,** 289 (1961).
10. K. LAMBERS and P. BAUER-SIC, Zur Zytochemie der Blutzellen. *Dtsch. Med. Wschr.* **38,** 1913 (1962).
11. K. LAMBERS and P. BAUER-SIC, Cytochemical and functional findings on leukocytes in chronic myelosis, *Fola. Haemat.* **8,** 436 (1963).
12. H. MERKER and C. YIU HUI, Alkalische Phosphatase und Glykogenge halt der reifen Neutrophilen im Verlauf von idiopathischen myeloproliferativen Erkrankungen. *Verh. Dtsch. Ges. Inn. Med.* **66,** 1050 (1960).
13. T. CASPERSSON and G. LOMAKKA, Scanning microscopy techniques for high resolution quantitative cytochemistry. *Ann. N.Y. Acad. Sci.* **97,** 449 (1962).
14. T. CASPERSSON, G. LOMAKKA and O. CASPERSSON, Quantitative cytochemical methods for the study of tumor cell populations. *Biochem. Pharmacol.* **4,** 113 (1960).
15. T. CASPERSSON, G. LOMAKKA and G. SVENSSON, A co-ordinated set of instruments for optical quantitative high cytochemistry. *Exp. Cell Res.* Suppl **4,** 9 (1957).
16. J. F. A. MCMANUS and R. W. MOWRY, *Staining Methods. Histologic and Histochemical.* Hoeber, Medical Division of Harper and Brothers, New York (1960).
17. V. ESMANN, The glycogen content of leukocytes from diabetic and non-diabetic subjects. *Scand. J. Clin. Lab. Invest.* **13,** 134 (1961).
18. W. N. VALENTINE, J. H. FOLLETTE and J. S. LAWRENCE, The glycogen content of human leukocytes in health and in various disease states. *J. Clin. Invest.* **32,** 251 (1953).

Europ. J. Cancer Vol. 1, pp. 303–307. Pergamon Press 1965 Printed in Great Britain

DNA Content of Tumours

Cytophotometric Measurements

WALTER SANDRITTER

Department of Pathology, Justus Liebig University, Giessen, Germany

CYTOPHOTOMETRIC measurements of normal cells reveal a regular distribution of DNA values which corresponds to the chromosome number [1]. In human tumours, aneuploid DNA distribution patterns were often found that deviated from the normal [2]. Our own measurements [3] demonstrated that the dry weight (interference microscopy) as well as the DNA and histone protein content showed values that deviated from the normal. Measurements on an oat cell carcinoma of the lung serve as an example. The dry weight of the cell nuclei amounted to $20–30 \times 10^{-12}$g (normal cell nuclei of the bronchial tree $40–50 \times 10^{-12}$g).

*Supported by the Deutsche Forschungemeinschaft.

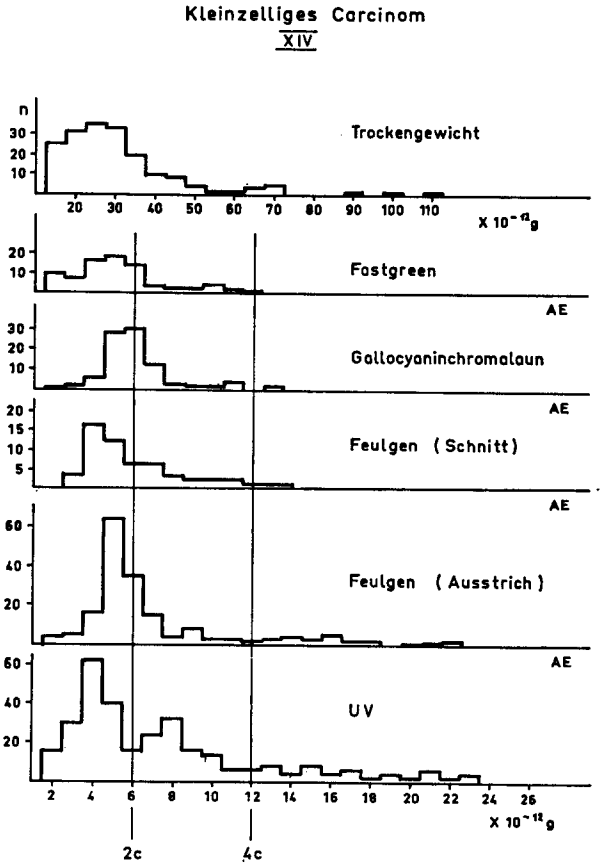


Fig. 1. *Dry weight, histone protein content (Fastgreen pH* 8·2) *and DNA distribution patterns of an oat cell carcinoma of the bronchus.*

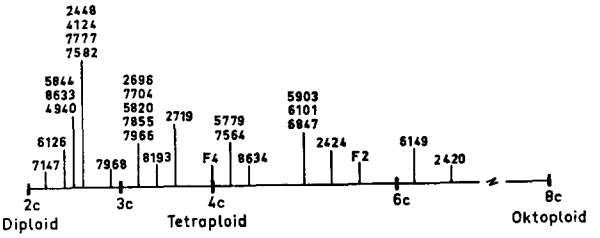


Fig. 2. Appearance of DNA stem lines from thirty human malignant tumours.

The histone protein content (fastgreen staining pH 8·2) as well as the DNA content corresponded to a hypodiploid DNA content (Fig. 1). Investigations on thirty human malignant tumours (Fig. 2) showed that most of the tumours have a DNA "stem line" lying between diploid and tetraploid or octoploid values. However, normal DNA values were also found. These measurements of smears do not indicate whether this atypical DNA distribution occurs only in the peripheral zone or as well in the centre of the carcinoma cell cord. Figure 3 shows measurements made from tissue sections. It is clearly seen that the outer and inner zones of the carcinoma cell cord had cells with an atypical DNA content and that the DNA stem line is expressed in the same way in both zones.

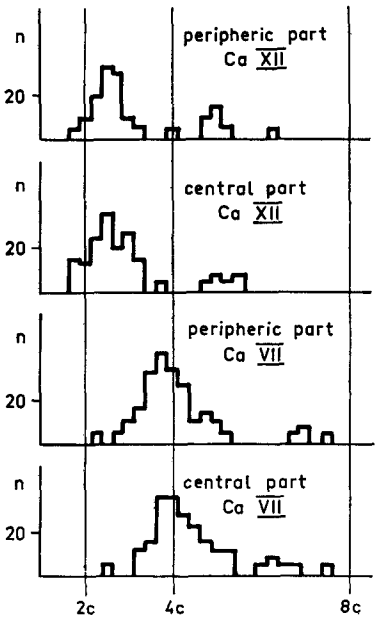


Fig. 3. DNA distribution pattern in the peripheric and central regions from two carcinomatous cell cords (squamous cell carcinoma).

In experimental animal tumours in contrast to the situation in humans, a diploid DNA content is usually found [4].

What do early cancerous lesions show in the human being? Carcinoma *in situ* of the exocerxvix exhibited, contrary to simple atypical epithelium (dysplasia) (Fig. 4), an aneuploid DNA stem line as seen in a total of eight cases. Figure 5 shows that this stem line was found in all layers of the carcinoma *in situ*. The stem line persisted throughout the different steps of the invasion (Fig. 6) up to the microcarcinoma [5]. This could also be found in a case of malignant lung adenomatosis (Fig. 7). The DNA distribution pattern represents a nearly triploid stem line, independent of localisation of tumour cell population (intra-alveolar, lymphangiosis carcinomatosa and lymph node metastasis) [6].

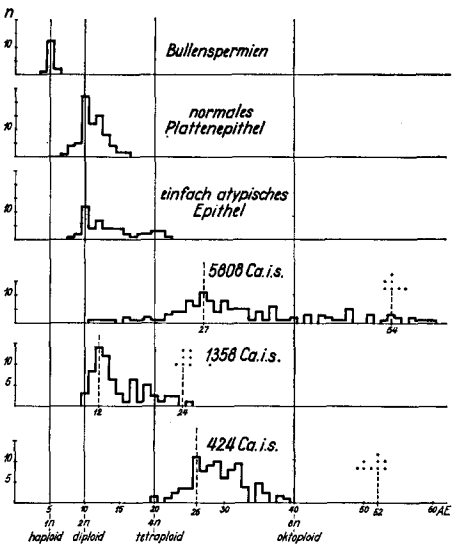


Fig. 4. DNA distribution patterns of normal portio epithelium, atypical epithelium and three cases of carcinoma in situ *of the exocervix.*

During cancerisation *in vitro* [7], usually after one year a new DNA stem line develops that persists unchanged over a long period of time (Fig. 8). After a stem line has developed the rat fibroblasts can be transplanted back into rats. These transplanted tumour cells show another stem line than the original cells. Upon retransplantation of the animal tumours in tissue culture the stem line changes often again.

These investigations show that malignant tumours are often distinguished from normal

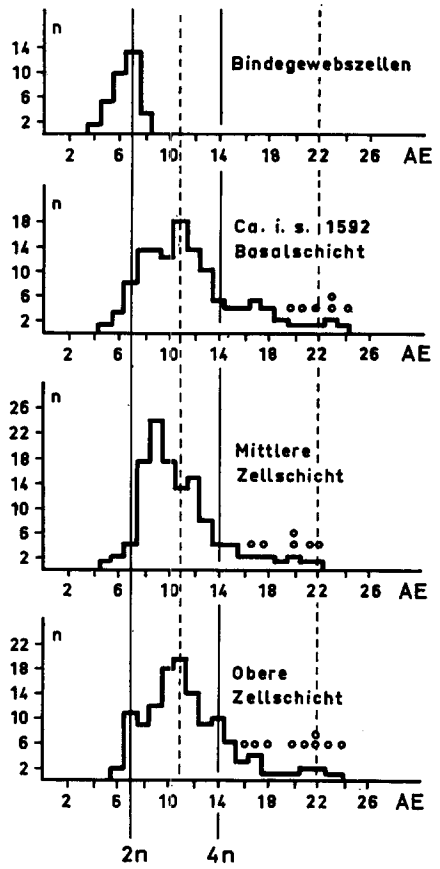


Fig. 5. *DNA distributions in the different layers of a carcinoma* in situ (*basal, middle and surface*).

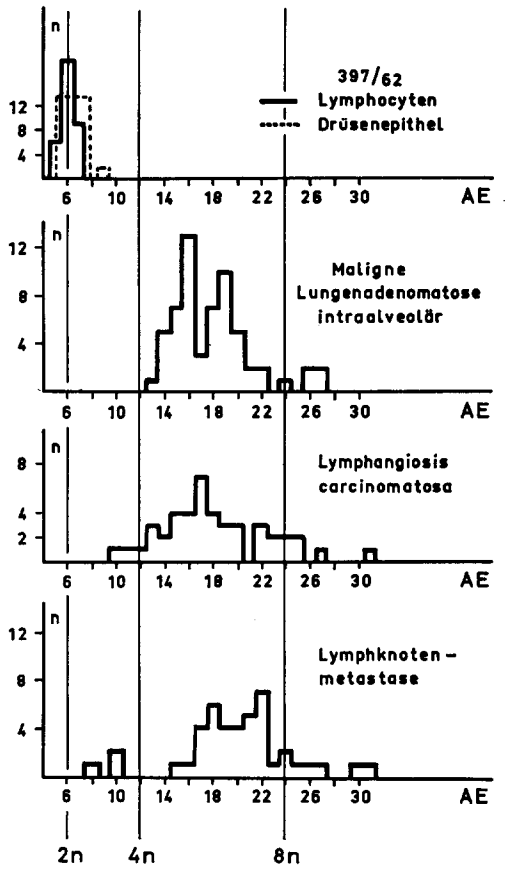


Fig. 7. *Malignant lung adenomatosis (alveolar cell carcinoma) lymphangiosis carcinomatosa, and lymph node metastases, DNA distribution patterns.*

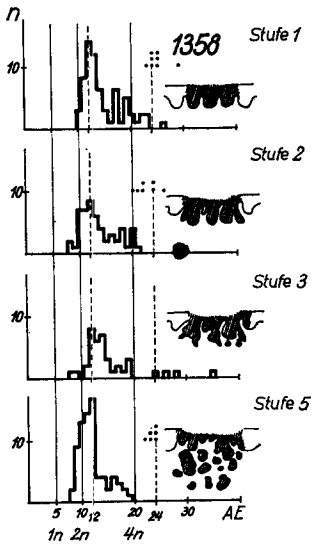


Fig. 6. *Different stages of progression in a carcinoma* in situ *and the DNA stem line.*

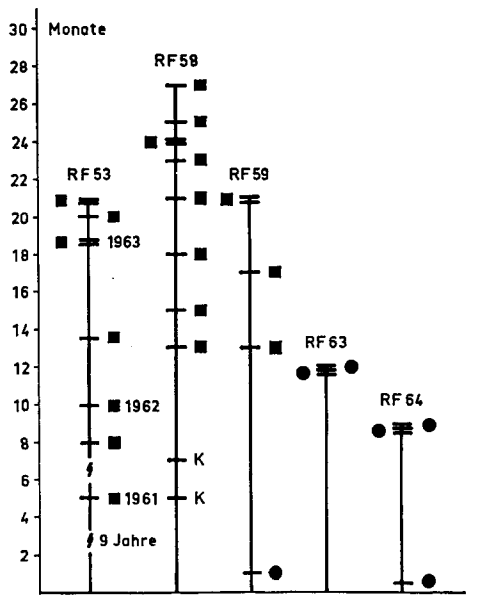


Fig. 8. *Scheme of the culture times of the cell stems, giving the DNA measuring time (single cross-line) and the time the chromosomes were counted (double crossline). The symbols on the left side of the line stand for the chromosome counts and those on the right of the line for DNA measurements. Round spot = diploid, square area = hypotetraploid, K = no stem line.*

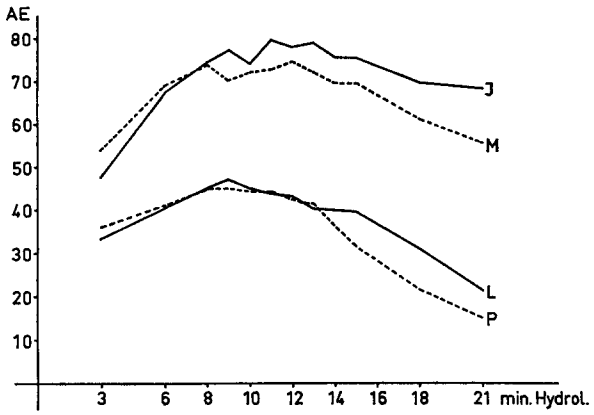


Fig. 9. Hydrolysis time curve of Feulgen reaction.
I = interphase cell of mouse acites tumour cells
M = mitosis of mouse ascites tumour cells
L = lymphocytes
P = mesothelial cells from peritoneum

cell populations by an aneuploid DNA stem line and a wide scattering of DNA values. However, tumours are also found having a regular DNA distribution. The question arises whether these are pseudodiploid tumours. Measurements of early cancerous lesions (e.g. carcinoma *in situ*) show that an abnormal DNA distribution pattern is already found with intra-epithelial atypical cell formations and that the DNA distribution remains the same during the invasive phase (realisation of the carcinoma). This leads to the conclusion that with regard to the DNA, the carcinoma *in situ* already represents a carcinoma and that the invasion depends on other factors than the atypical DNA distribution.

The amount of DNA by itself presents interesting information. However, there is the question to what extent there are also qualitative differences in the nucleo-proteins of normal and tumour cells. Measurements made during the Feulgen hydrolysis (time curve) have shown that contrary to normal cells, mouse ascites tumour cells exhibit an atypical hydrolysis curve with an inflection at 9 min [8] (see Fig. 9). We believe that this atypical curve results from a differential acid sensitivity of the eu- and heterochromatin. When the hydrolysis time (HCl, pH 1·2, 37°) is extended, we also see in the case of normal cell nuclei a curve [9] with an inflection at about 20–22 hr (Fig. 10). Recent data suggest that in the first part of hydrolysis (up to 20 hr) the aldehyde groups of the euchromatin are set free, while the heterochromatin is less acid sensitive.

Further investigations still need to be made to resolve this relationship.

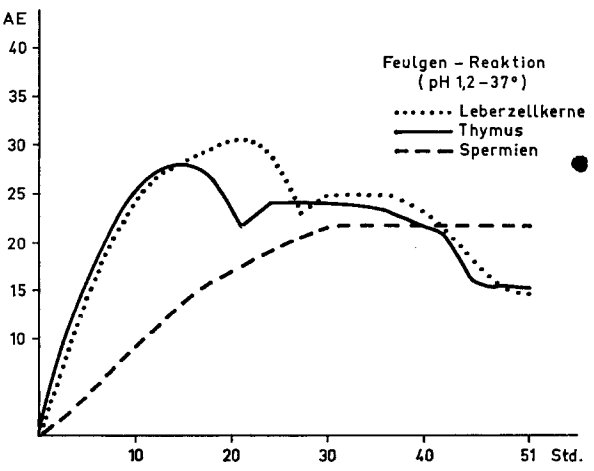


Fig. 10. Extended Feulgen hydrolysis curve of liver cell nuclei, thymus lymphocytes and sperm.

RESUME

L'auteur donne un bref aperçu de ses travaux sur la cytophotométrie des tumeurs malignes, notamment à la phase précoce de l'affection. Il fut possible de montrer que les tumeurs humaines présentent une distribution anormale de l'ADN.

Le carcinome in situ *du col utérin montre la même distribution de l'ADN que dans la tumeur invasive.*

Ce phénomène a aussi pu être suivi aux différents stades d'invasion des tumeurs. Le processus atypique de l'hydrolyse, dans la réaction de Feulgen, au niveau des cellules tumorales, peut être mis en relation avec une sensibilité différentielle, a l'égard des acides, de l'euchromatin et de l'hétérochromatin.

SUMMARY

A short account of cytophotometric investigations on malignant and on early cancerous lesions is given. It was possible to show, that human tumours exhibit an atypical DNA distribution pattern (DNA stem line). The carcinoma in situ *of the exocervix shows similar as in malignant tumors a DNA stem line. This phenomenon can be traced also in the stage of invasion. The atypical course of Feulgen hydrolysis in tumour cells is related to a differential sensivitity towards acids of eu- and heterochromatin.*

ZUSAMMENFASSUNG

Es wird ein kurzer Uberblick der eigenen cytophotometrischen Messungen an malignen Tumoren und Krebsvorstadien gegeben. Es wurde gezeigt, daß die meisten menschlichen Tumoren ein atypisches DNS-Verteilungsmuster aufweisen (DNS-Stammlinie). Das Carcinoma in situ *der Portio zeigt ebenfalls DNS-Stammlinien, die auch bei der Invasion nachzuweisen sind. Atypische Feulgenhydrolysekurven bei Tumorzellen werden auf eine unterschiedliche Säureempfindlichkeit von Eu und Heterochromatin bezogen.*

REFERENCES

1. W. Sandritter, D. Müller and O. Gensecke, UV-mikrospektrophotometrische Messungen des Nukleinsäuregehaltes von Spermien und diploiden Zellen. *Acta Histochem.* **10,** 139–154 (1960).
2. N. B. Atkin, Nuclear size on carcinoma of the cervix: Its relation to DNA content and to prognosis. *Cancer* **17,** 1391–1399 (1964)
3. W. Sandritter and D. Kleinhans, Uber das Trockengewicht, den DNS- und Histonproteingehalt von menschlichen Tumoren. *Z. Krebsforsch.* **66,** 333–348 (1964).
4. W. Sandritter, A. Seidel, D. Kleinhans, I. Paddags and W. Dontenwill, Zytophotometrische Messungen des DNS-Gehaltes an menschlichen und tierexperimentellen Bronchialmetaplasien. *Z. Krebsforsch.* (In press).
5. W. Sandritter, Cytophotometrische Untersuchungen am Portiocarinom und seinen Vorstufen. *Verh. Dtsch. Ges. Path.* 48. Tgg. Salzburg, 34-43 (1964).
6. A. Seidel and W. Sandritter, Cytophotometrische Messungen des DNS-Gehaltes eines Lungenadenomas und einer malignen Lungenadenomatose. *Z. Krebsforsch.* **65,** 555–559 (1963)
7. W. Sandritter, I. Hillwig, K. Engelbart, G. Kiefer and R. Kiefer, Versuche zur Carcinogenese *in vitro.* Chromosomenanalysen und cytophotometrische Messungen des DNS-Gehaltes *Z. Krebsforsch.* (In press).
8. W. Sandritter and N. Böhm, Atypische Hydrolysekurven bei der Feulgenreaktion von Mäuseascitestumorzellen. *Naturwissenschaften* **51,** 273 (1964).
9. W. Sandritter, K. Jobst, L. Rakow, and K. Bosselmann, Zur Kinetik, der Feulgenreaktion bei verlangerter Hydrolsezeit. Cytophotometrishce Messungen im sichtbaren und ultravioletten Licht. *Histochemie* **4,** 420–437 (1965).

Europ. J. Cancer Vol. 1, pp. 309–312. Pergamon Press 1965. Printed in Great Britain

An Electron Microscopic Study of Burkitt Tumour Biopsies

R. DOURMASHKIN

Division of Experimental Biology and Virology
Imperial Cancer Research Fund, London.

IN RECENT years, medical observers in Africa have described a syndrome, occurring in children, of tumours arising in the jaw. They are lymphomas histologically, but a striking feature is the lack of lymph node involvement. Other sites for the occurrence of the tumour syndrome are long bones, ovaries and thyroid. Because of the syndrome's particular anatomical distribution, age incidence and geographical distribution, it is recognised as a distinct clinical entity and is now known universally as 'Burkitt's tumour.' Burkitt's tumour is particularly common in the region of Africa with relatively low altitude and high rainfall. One of the suggested hypotheses for its aetiology has been that it is caused by a virus, spread by one of the arthropod vectors of the area. Consequently, a search has been carried out by several investigators, in order to identify a causative agent. Using tissue culture techniques, Simons and co-workers [1–3] isolated herpes virus from patients with Burkitt's tumour; however, they also found herpes virus in the throats of normal control individuals. Using a similar approach, Bell *et al.* [4–5] isolated reovirus type 3 from several cases. Dalldorf [6] isolated unidentified filtrable agents, which have since been rumoured to be mycoplasmas. Epstein [7] cultured tumour cells from biopsies of Burkitt's tumour, and found particles resembling herpes virus by electron microscopy of the cultured cells.

Wright [8] has described the histology of the Burkitt tumour. There are several points characterizing this syndrome from other types of lymphoma. In any one biopsy, a variation of the tumour cells is observed, from undifferentiated reticulum cells to more or less mature lymphoblast-like tumour cells. These cells show vacuoles that stain for fat. What is remarkable about Burkitt's tumour is the 'starry sky' appearance, which, on close inspection, proves to consist of fat-laden macrophages that in some sections appear to be phagocytosing necrotic cells. The 'starry sky' appearance was formerly thought to be a distinguishing characteristic of reactive follicular hyperplasia, as opposed to malignant lymphoma.

In the United States, O'Connor *et al.* [9] and Dorfman [10] both reported that although lymphosarcoma in American children is rare, some cases of Burkitt's tumour do occur. These cases showed the typical location in the jaw and the 'starry sky' appearance on histology. In addition, other American cases of the usual type of childhood lymphosarcoma, largely originating in lymph nodes, were shown by Diamandopoulos [11] to have the 'starry sky' histology. It appears, then, that the microscopical appearance of Burkitt's tumour is characteristic of childhood lymphosarcoma generally, and that Burkitt's tumour in Africa is remarkable in that it has a much higher incidence than other types of lymphoma or any other childhood tumour in Africa.

With the help of the group from the Imperial Cancer Research Fund in Entebbe, we have examined thin sections of 67 Burkitt tumour biopsies by electron microscopy. Most were

fixed in osmic acid and embedded in methacrylate at Entebbe, but some were fixed in glutaraldehyde and sent refrigerated to Mill Hill, where they were post-fixed in osmic acid and embedded in Epon. Either method of fixation proved successful in most cases. A classification of the cases is given in Table 1.

Table 1. *General description of biopsies of African lymphomas studied by electron microscopy* 1961–1965.

Jaw tumours	43
Skeletal tumours (other than jaw)	12
Visceral tumours	12
Total of Lymphomas	67

The results of the examination of the biopsies by electron microscopy of thin sections will be reported here briefly. A more detailed report will follow.

It was possible to correlate the histological observations summarized above, with the findings obtained by electron microscopy. Cells were found that varied in appearance from undifferentiated reticulum cells to mature lymphoblasts. Many of these cells were found in various stages of degeneration. Degenerating cells were found either singly or in small groups, and were situated close to cells having a normal appearance (Fig. 1). This would suggest that cellular degeneration was not the result of ischaemic necrosis. The early stages of degeneration were more noticeable in preparations for electron microscopy than in histological sections. This may be due to the margination of chromatin that is normally seen in formalin fixed and paraffin embedded sections.

Special attention was paid to the possibility that cell degeneration was due to the cytopathic effect of a virus. However, with the exception of one biopsy mentioned below, no virus-like particles were found in the degenerating cells. Another hypothesis might be suggested, then, for the finding of tumour cells in the process of disintegration: that they might represent a partially successful immunologic reaction on the part of the host.

The other main type of cell that was found, corresponded to the cell type that has been termed a macrophage. The macrophages were a constant feature of the Burkitt tumour biopsies; they give rise to the 'starry sky' appearance in paraffin sections. In the electron microscope, they were often seen with degenerating tumour cells in their cytoplasm. Many osmiophilic lipid droplets were also seen in the cytoplasm of these cells. In one case of Burkitt lymphoma, particles suggesting virus were found in cytoplasmic vacuoles of the macrophages. These particles were 40–50 mμ in diameter, and were also seen occasionally in the degenerating cells (Fig. 2). However, no supporting evidence that these may represent virus could be obtained. Furthermore, similar particles could not be found in any of the

Table 2. *Density of some viruses.*

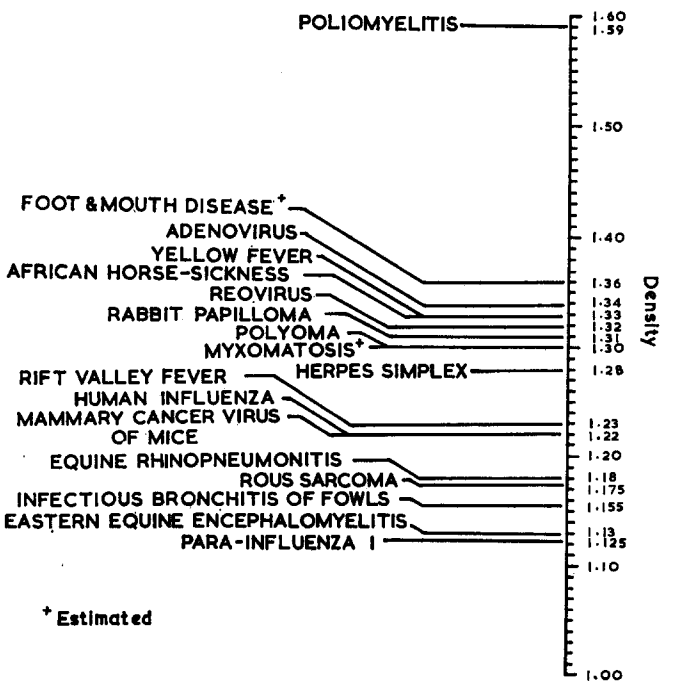




Fig. 1. *Electron micrograph of a section from a biopsy of Burkitt's lymphoma. In the centre of the micrograph, portions of H3 degenerating cells are seen (D). These cells are partially surrounded by the cytoplasm of a macrophage. The boxed portion is shown at higher magnification in Fig.* 2. *Other tumour cells in the field are well preserved. There is a cell in mitosis (M).* ×11,000.

(to face p. 310)

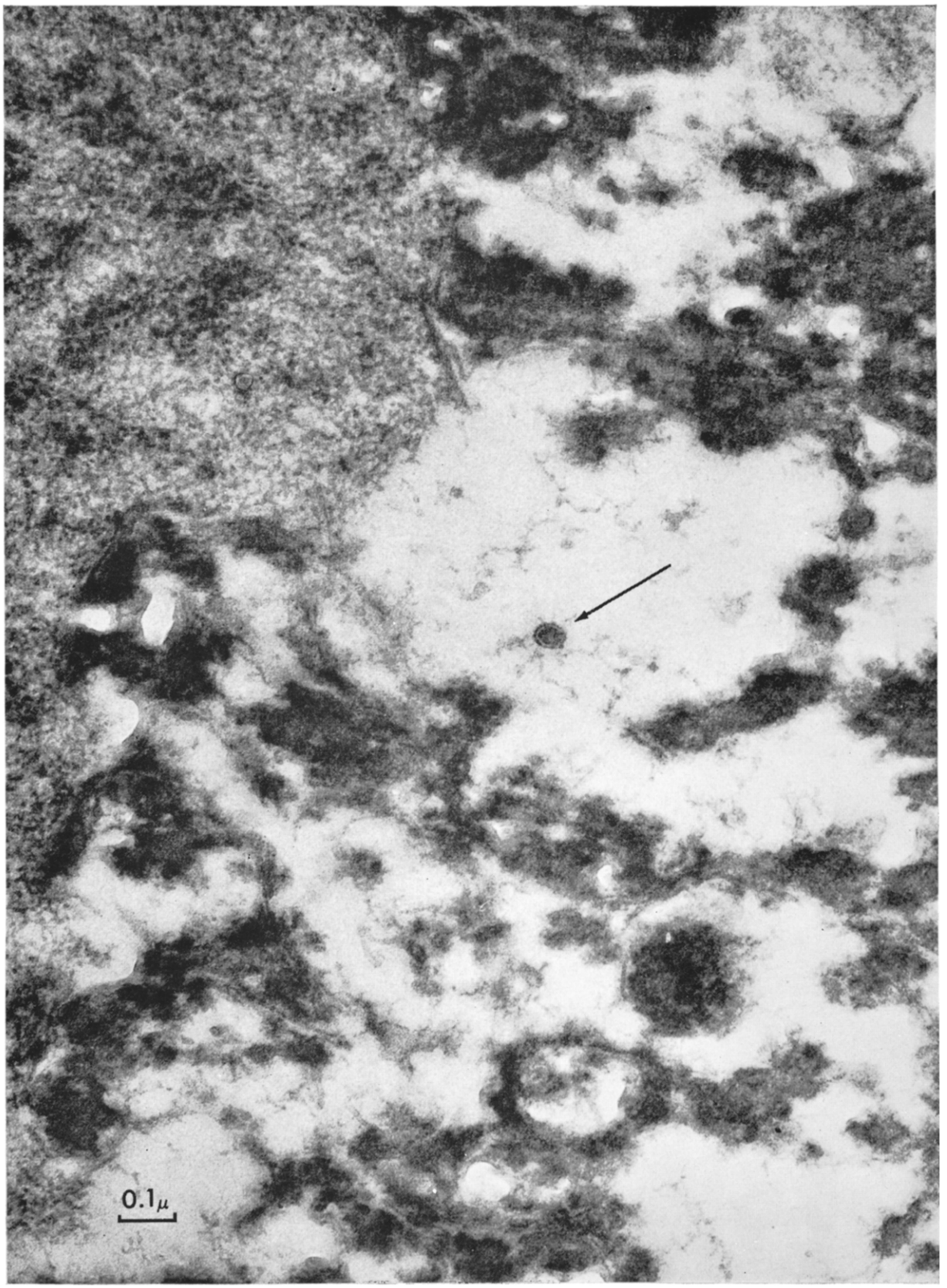


Fig. 2. A particle (—→) is shown in a degenerating cell, magnified from Fig. 1. It must be emphasized that this was the only biopsy in which such particles were found. ×100,000.

other biopsies studied, nor could particles be seen resembling reovirus or herpes (including the particles described by Epstein).

A complicating factor was the frequent finding of bacteria in the biopsies from the jaw.

In addition to the study of thin sections of Burkitt tumour biopsies, an attempt was made to separate and concentrate virus particles from frozen tumour tissue. Biopsy material was homogenised and sonicated, enzymatically digested to remove ribosomes and collagen, treated with receptor-destroying enzyme, differentially centrifuged, and the concentrate of small size particles was placed on a caesium chloride equilibrium density gradient.
Fractions from the gradient were diluted and recentrifuged to remove the caesium salt, and the sediment was resuspended in a very small volume of negative stain and examined for virus-like particles by electron microscopy. Not enough biopsies have been examined in this way to report a result, but those studied up to now have been negative.

In choosing the range of densities for the gradient, it must be borne in mind that the densities of viruses differ greatly. A table derived from Andrewes [12] shows some types of viruses arranged on a scale according to their density (Table 2).

In conclusion, our search for virus particles in Burkitt tumour biopsies has been negative so far. The electron microscopic findings, which are incomplete, neither support nor refute the virus theory for the aetiology of this tumour.

Acknowledgements — I would like to thank Dr. R. J. C. Harris for his encouragement, advice and help, Drs. T. M. Bell, P. J. Simons and their collaborators for preparing and sending the biopsies from Entebbe, and Mr. R. Parekh, Mrs. J. Watson, Mrs. H. Clough, and Mr. C. N. Schofield for their able technical assistance.

RESUME

Soixante-sept biopsies du lymphome de Burkitt ont été examinées au microscope électronique, en utilisant la technique des coupes fines. Des particules ayant l'aspect de virus ont été observées dans un seul cas. En conclusion, cette étude n'apporte pas d'élément en faveur de l'hypothèse de l'étiologie virale du lymphome de Burkitt.

SUMMARY

Sixty-seven biopsies of Burkitt's lymphoma were examined by electron microscopy, using thin sectioning technique. In only one case were particles found suggesting virus. In general, no support was obtained for the virus theory in the aetiology of Burkitt's lymphoma.

ZUSAMMENFASSUNG

Siebewundsechzig Biopsieen von Burkitt'schen Lymphomen wurden elektronenmikroskopisch unter Anwendung der Feinschnittechnik untersucht. Nur in einem Falle konnten virusähnliche Körper gefunden werden. Im Allgemeinen konnte kein Beweis dafür erbracht werden, dass die Aetiologie des Burkitt'schen Lymphoms mit einem Virus im Zusammenhang steht.

REFERENCES

1. P. J. Simons and M. G. R. Ross, The isolation of herpes virus from Burkitt tumours. *Europ. J. Cancer.* In press.
2. P. J. Simons and M. G. R. Ross. *The Annual Report of the Imperial Cancer Research Fund.* p. 48 (1963).
3. J. P. Woodall and A. J. Haddow. *East African Virus Research Institute Report.* p. 30 (1962).
4. T. M. Bell, A. Massie, M. G. R. Ross and M. C. Williams, Isolation of a reovirus from a case of Burkitt's lymphoma. *Brit. Med. J.* **1,** 1212 (1964).
5. T. M. Bell, *The Annual Report of the Imperial Cancer Research Fund.* p. 38 (1964)
6. G. Dalldorf, and F. Bergamini, Unidentified filtrable agents isolated from African children with malignant lymphomas, *Proc. Nat. Acad. Sci.* **51,** 263, (1964).
7. M. A. Epstein, B. G. Achong and Y. M. Barr, Virus particles in cultured lymphoblasts from Burkitt's lymphoma. *J. Nat. Cancer Inst.* **34,** 231 (1965).

8. D. H. WRIGHT, Cytology and Histochemistry of the Burkitt lymphoma. *Brit. J. Cancer* **17,** 50 (1963).
9. G. T. O'CONOR, H. RAPPAPORT and E. B. SMITH, Childhood lymphoma resembling "Burkitt tumor" in the United States. *Cancer* **18,** 411 (1965).
10. R. F. DORFMAN, Childhood lymphosarcoma in St. Louis, Missouri, clinically and histologically resembling Burkitt's tumor. *Cancer* **18,** 418 (1965).
11. G. TH. DIAMANDOPOULOS and E. B. SMITH, Phagocytosis in reticulum cell sarcoma. *Cancer* **17,** 329 (1964).
12. Sir. C. ANDREWS. *Viruses of Vertebrates.* Balliere, Tyndall and Cox, London (1964).

Europ. J. Cancer Vol. 1, p. 313. Pergamon Press 1965. Printed in Great Britain

The Investigation of Metastasis of Endocrine Tumours in Bone

D. C. WILLIAMS and D. M. ROBERTSON

The Marie Curie Memorial Foundation, Research Department, Harestone Drive, Caterham, Surrey, England.

THE PURPOSE of our experiments was the study of the possible influence on calcium metabolism of substances liberated into the blood by mammary tumours. *In vitro* experiments were designed to simulate known *in vivo* reactions.

Bone was incubated in a serum substitute of defined chemical composition with tumours, serum or preparations from tumours. Release of calcium into the medium was followed chemically as was the appearance of various enzymes. In this way it was possible to show that the medullary soft tissue, the bone matrix and the blood all play important parts in the action of the tumour. Bone and marrow from rats with a tumour released less calcium into the medium than normal bone and marrow. A tumour progressively impairs the action of the calcium-mobilising elements in the bone marrow to about one-half of the normal level.

Dialysate from the tumour contained a substance having a direct effect on calcium exchange of bone *in vitro* but it is probable that the tumour also provokes a reaction elsewhere in the body. Parathyroid hormone caused a marked calcium release which was greatly enhanced by vitamin D although vitamin D alone gives very little increase. The addition of proteolytic enzymes such as fibrinolysin also caused calcium release. Experiments involving dialysis and gradient electrophoresis suggest that at least one active substance is a polypeptide of low molecular weight.

Table 1.

	Control Ca (mg%)	Test Ca (mg%)
Parathyroid hormone	58	68
Parathyroid Hormone† Vitamin D	58	78
Vitamin D	65	66
Fibrinolysin (50 units)	80	96

Table 2.

No. of patients	Average age	Average serum Ca	Increase (%)
15 (control)	45	12	0
6	57	13	0
5	55	13	11 (7–16)
4	49	14	64 (28–125)

Serum from patients with advanced cancer of the breast (kindly supplied by Mr. R. W. Raven) was tested and the results have been divided into three groups: (1) those giving no increase in calcium level over normal controls; (2) those producing a small increase (7–16%); and (3) those giving between 25 and 125% increase over control levels. There appears to be very little difference in initial serum calcium between the groups but clinically the patients having the highest increase in calcium levels were generally those with advanced cancer. The active substance present in human sera appears similar to that obtained from rat breast tumour.

Europ. J. Cancer Vol. 1, pp. 315–317. Pergamon Press 1965. Printed in Great Britain

Antigenic Properties of Human Tumours

"Tumour-Specific" Antigens

D. E. H. TEE, MILDRED WANG and J. WATKINS

Department of Pathology, King's College Hospital Medical School, London, S.E.5.

INTRODUCTION

THE LITERATURE on the antigenic properties of tumours is voluminous, complex and even contradictory. The results of workers in this field may be summarized as indicating the presence in at least a proportion of tumours of antigens which were absent from normal tissues. The loss of normal tissue antigens from tumours has also been demonstrated by some investigators. The possible importance of any conclusions concerning the antigenic differences between tumours and normal tissues suggested the need for further investigations.

Preliminary studies on the antigenic properties of human malignant tumours using heterologous antisera had shown the presence of antigens which were not detectable in normal tissues, and in addition some loss of normal tissue antigens [1]. This communication presents the findings of further investigations of tumours using additional antisera and including a greater variety of tumours.

METHODS

Ninety-two tumours from different individuals were used for this study. These tumours were collected mainly from surgical operations and a small number were obtained from autopsies. The tumours consisted of 82 carcinomas, 1 neuroblastoma, 2 lymphosarcomas, 2 sarcomas and 5 benign tumours. Twenty normal tissues were obtained from patients from whom tumour was also available and 60 normal tissues were obtained from 7 post-mortems in which there was no evidence of tumours. Soluble extracts of the various tissues were prepared by a cryostat method [2] using phosphate buffered saline, pH 7·6, as a solvent.

Antisera were prepared from individual and pooled cancerous and normal tissues from different sites. Rabbits were injected with a mixture of either individual or pooled tissue homogenates and complete Freund's adjuvant. After 1 month, immunisation was continued with multiple injections of the homogenates without adjuvant. Antisera were also prepared against protein fractions isolated from tumours by chromatographic methods.

The antisera were variably contaminated with antibodies which reacted with serum proteins, blood cells and blood group substances. These antibodies were removed by repeated absorption of the antisera with the corresponding antigens. The antitumour antisera were further absorbed with a large pool of normal tissues. After these absorptions, several antitumour antisera were obtained which reacted exclusively with tumours (Fig. 1). An antigenic analysis was made of the 92 individual tumours and 5 tumour pools with the Ouchterlony agar gel double diffusion technique and using the 6 absorbed antitumour antisera.

RESULTS

Confirmatory experiments showed that the "tumour-specific" antigens were not detectable

in non-malignant adult human tissues nor were they due to iso-antigenic differences between the cells of genetically different individuals. Some tumours showed loss of normal tissue antigens when reacted with anti-normal tissue antisera.

Each of the antitumour antisera reacted with a different number of tumours ranging from one to twenty-seven. All the tumours giving a positive reaction with a particular antiserum were then retested using the same antiserum to determine whether the precipitin reactions were identical. These experiments showed that three of the antisera gave a single precipitin line, which was common only to those tumours which reacted with a particular antiserum. The other three antisera gave two precipitin lines, one of which was common to all the tumours reacting with the respective antiserum, while the second precipitin reaction occurred only with a few tumours.

Each antitumour antiserum gave a different pattern of positive reactions with the various tumours, thus suggesting that the antisera were reacting with different antigens. Further experiments showed that the six antisera contained antibodies reacting with nine different "tumour-specific" antigens. These antigens were arbitrarily designated A to I, as shown in Table 1. "Tumour-specific" antigens were detected in 45 individual tumours and in three tumour pools. The number of different antigens detected in each of the tumours ranged from none to four as shown in Table 2. Approximately half of the tumours contained no detectable "tumour-specific" antigen. The majority of the tumours showing positive reactions contained only one antigen, and very few were shown to contain two or more antigens.

Table 1. Classification of tumour-specific antigens

Antigen code	Source of antigen	
A, B	Breast	15 carcinomas (pooled)
C	Caecum, colon, rectum	18 adenocarcinomas (pooled)
D	Pancreas	4 carcinomas (pooled)
E, F	? ovary	1 adenocarcinoma
G, H	Muscle	1 leiomyoma
I	Thyroid	1 carcinoma

Table 2. Variation in number of antigens in different tumours

Number of antigens	Number of tumours
0	49
1	36
2	9
3	2
4	1

The distribution of the different antigens in the various tumours is shown in Table 3. A more detailed analysis of these antigens is being undertaken and the results, so far, can be summarized as follows. A and G were the commonest, and were present in tumours from a number of different sites. Antigens B and D were detected only in the tumours used for immunisation, while antigen H was present in one tumour other than the immunising specimen. Antigen C was found only in tumours from the same sites as the immunising tumour pool. The combination of antigens A, E and I was present only in ascitic and pleural fluids obtained from cases with malignant tumours. Only one antigen, G, was found in one sarcoma from a total of two sarcomas and two lymphosarcomas examined. No "tumour-specific" antigen was detected in the single neuroblastoma investigated.

Table 3. Distribution of serologically different antigens in tumours

		Individual tumours	Tumour pools
No. of tumours examined		92	5
No. of tumours *with* antigens		45	3
No. of tumours with antigens:	A	15	0
	B	1	0
	C	7	1
	D	0	1
	E	4	0
	F	2	0
	G	25	1
	H	2	0
	I	5	0

DISCUSSION

The fact that there are different antigens in tumours has been shown, also, by other workers. Bjorklund [3], using the gel diffusion technique, found in a tumour pool four different cellular antigens, which he designated 1 to 4. However, he found that these antigens were not

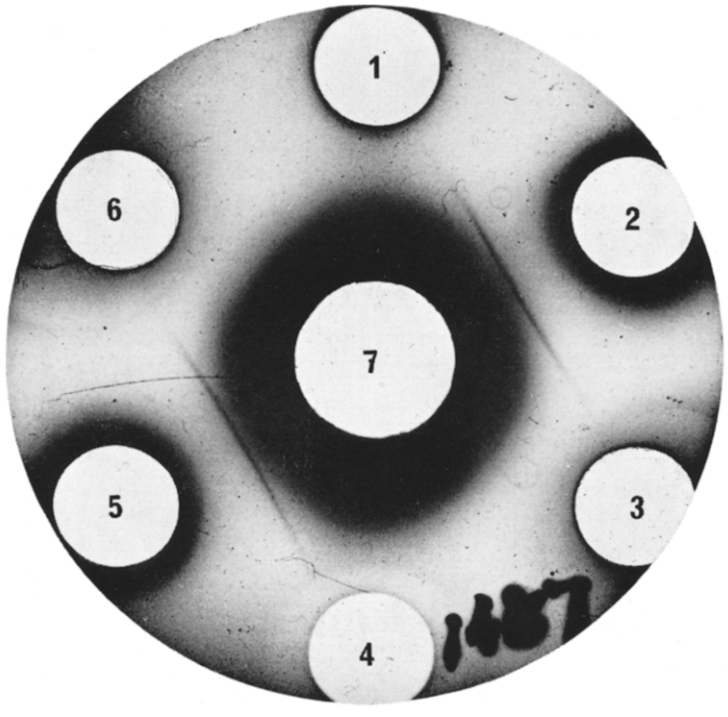


Fig. 1. *Ouchterlony plate showing "tumour-specific" reaction.* 1, *normal tissue extract;* 2 *and* 5, *tumour extract;* 3 *and* 6, *serum;* 4, *leucocyte extract;* 7, *antitumour antiserum.*

(to face p. 318)
Refer to p. 316

specific to tumour tissue; antigen 1 was found in some normal tissues and was absent from others, while antigens 2, 3 and 4 were generally distributed. Kosyakov and Korosteleva [4] showed that tumours possessed a specific antigen which was absent from normal tissues, and were able to distinguish between the cancer antigens of different human tumours. In addition they showed that there was no specific antigen common to all tumours.

Thus, some of our findings on the presence of different antigens in human tumours have been substantiated by other workers. However, despite the increasing volume of literature on tumour specific antigens, there has been very little work reported on the immunological classification of tumour antigens.

Our studies have revealed a complex antigenic pattern in human tumours. No specific antigen common to all tumours has been detected, but so far 9 serologically different tumour antigens have been found. These antigens, which have been designated "tumour-specific," may be present in normal tissues in concentrations so low that they were not detected by the double diffusion technique. However, absorbing the antitumour antisera with a large excess of pooled normal tissues failed to remove the antitumour antibody. The fluorescent labelled antibody technique is being used as an additional method in order to check the tumour specificity of these antigens.

RESUME

En utilisant des antisérums hétérologues, les auteurs ont mis en évidence, dans des tumeurs humaines, la présence de plusieurs antigènes sérologiquement distincts, non décelables dans des tissus normaux. Il ne fut pas trouvé un seul antigène commun à toutes les tumeurs étudiées. Les tumeurs avaient perdu certains antigènes des tissus normaux.

SUMMARY

Studies on human tumours using heterologous antisera have shown the presence of a number of serologically distinct antigens which were not detectable in normal tissues. No single additional antigen common to all tumours was found. The tumours also showed some loss of normal tissue antigens.

ZUSAMMENFASSUNG

Untersuchungen an menschlichen Tumoren, unter Anwendung von heterologen Antisera, zeigten die Anwesenheit einer Anzahl von serologisch verschiedenen Antigenen, welche in normalen Geweben nicht zu finden waren. Kein einzelnes, zusätzliches, allen Tumoren gemeinsames Antigen konnte gefunden werden. Die Tumoren zeigten ausserdem den Verlust von einigen Antigenen, welche in normalen Geweben zu finden sind.

REFERENCES

1. D. E. H. Tee, M. Wang and J. Watkins, Antigenic properties of human tumours. *Nature, Lond.* **204,** 897 (1964).
2. D. E. H. Tee, M. Wang and J. Watkins, A cryostat method for the extraction of soluble tissue components. *Nature, Lond.* **204,** 682 (1964).
3. B. Bjorklund, Antigenicity of malignant and normal human tissue by gel diffusion techniques. *Int. Arch. Allergy* **8,** 179 (1956).
4. P. N. Kosyakov and V. S. Korosteleva, The antigens of cancerous tumours in man. *Acta. Un. Int. Cancr.* **19,** 158 (1963).

Europ. J. Cancer Vol. 1, p. 319. Pergamon Press 1965. Printed in Great Britain

Some Biological and Biochemical Aspects of Human Tumour Growth

R. A. Q. O'MEARA

School of Pathology, Trinity College, Dublin, Eire

THE FREQUENCY of tumours and the predominance of carcinomas in the Irish Republic (85–90 per cent) are reviewed. Forty-five per cent of those registered are of the skin and lower lip and 40 per cent of cancer deaths are due to carcinomas of the alimentary tract. Experiments over a number of years with human carcinomas are considered: they deal mainly with the presence in and escape from the site of malignancy of a labile cell component with coagulative properties. It has not been found in detectable amounts in extracts of adult tissues except colon though it diffuses readily from the foetal cells of amnion, chorion, and placenta. Carcinomas commonly lack the fibrinolytic activity of normal tissue or only possess it to a limited degree. Thus fibrin formed in or near a carcinoma tends to persist and to become organised by the growth into it of blood and lymphatic vessels and connective tissue cells.

At present the nature of one of the intracellular components which leak from cancers and occur in chorion, namely the coagulative factor, is investigated (Clarke, 1965 [1]; Clarke and O'Meara, 1965 [2]. Its composition and the plasma components with which it reacts is being studied by Boggust *et al.* (see bibliography O'Meara *et al.*, 1958 [3, 4], 1960 [5], 1961 [6, 7], 1962 [8], 1963 [9] 1964 [10]; Boggust *et al.*, 1963) [11].

REFERENCES

1. N. P. G. CLARKE, Intracellular location of tissue thromboplastin and possible relation to fibrin deposits in human neoplasms. *Nature* **205,** 608 (1965).
2. N. P. G. CLARKE and R. A. Q. O'MEARA. 1965. Unpublished observations.
3. R. A. Q. O'MEARA, Coagulative properties of cancers. *Irish J. med. Sci.* **394,** 474 (1958).
4. R. A. Q. O'MEARA and R. D. JACKSON, Cytological observations on carcinoma. *Irish J. med. Sci.* **391,** 327 (1958).
5. R. A. Q. O'MEARA, The growth-pattern of carcinomas. *Arch. De Vecci Anat. pat.* **31,** 365 (1960).
6. R. A. Q. O'MEARA and R. D. THORNES, Some properties of the cancer coagulative factor. *Irish J. med. Sci.* **424,** 106 (1961).
7. R. D. THORNES and R. A. Q. O'MEARA, A method of detecting and estimating inhibitors of the cancer coagulative factor. *Irish J. med. Sci.* **428,** 361 (1961).
8. R. A. Q. O'MEARA, A systematic approach to the study of the morbid changes induced by spontaneous cancers and their precursors in man. In "*Morphological Precursors of Cancer,*" Perugia, 21–34, (1962).
9. R. A. Q. O'MEARA and M. J. O'HALLORAN, Protamine derivatives in advanced breast cancer. *Lancet* II, 613 (1963).
10. R. A. Q. O'MEARA, Fibrinolytic treatment of cancer. *Lancet* II, 963 (1964).
11. W. A. BOGGUST, D. J. O'BRIEN, R. A. Q. O'MEARA and R. D. THORNES, The coagulative factors of normal human and human cancer tissue. *Irish J. med. Sci.* **447,** 131 (1963).

Europ. J. Cancer Vol. 1, pp. 321–325. Pergamon Press 1965. Printed in Great Britain

Anticytoplasmic Auto-Antibodies In A Patient With Signs Of Tumour Resistance

PH. RÜMKE

Department of Immunology,
The Netherlands Cancer Institute, Amsterdam, The Netherlands

INTRODUCTION

IT HAS been supposed that under certain conditions the formation of auto-antibodies may be induced by self-constituents, which have been altered e.g. by burning, irradiation, infection denaturation or enzyme action, and so have become foreign and therefore antigenic [1]. Antibodies induced by such an altered self-antigen cross-react with the unaltered parent substance, therefore they are auto-antibodies. Examples of such a mechanism have been found by Cinader [2] who injected rabbits which were made tolerant for human albumin at birth, with azoalbumin after which they first developed antibodies to the azo-group of the molecule and later to the unaltered determinants of albumin. Weigle [3] induced the formation of auto-antibodies to native thyroglobulin by injecting rabbits with diazotised thyroglobulin. A similar mechanism of auto-antibody formation can be found in the work of Asherson and Dumonde [4], who used tissues from other species in order to induce auto-antibodies to the similar autologous tissues and by Ishizaka, Campbell and Ishizaka [5] who demonstrated that 'hidden' internal antigenic determinants in bovine serum albumin could become effectively antigenic, and that the antibodies reactive with them would also be capable of reacting with unaltered native antigen, thus being auto-antibodies.

If spontaneous tumours have tumour antigens, it is to be expected that in most cases these would be only slightly different from host antigens [2]. In that case they would not be recognized as foreign when they come into contact with the R.E.S. of the host. However, an immune response can be expected if the tumour antigen and its native counterpart are not too closely related, unless of course the host has an impaired immune capacity or a specific tolerance induced by the tumour antigen. Such an immune response may be expressed by the induction of circulating antibodies to the tumour-specific determinants. In most cases, these antibodies would be difficult to detect with our present methods, because titers of antibodies might be too low and the amount of tumour antigens too small. Moreover, each tumour might have its own specific tumour antigen, in the way chemically induced tumours, even when they are induced in the same animal by the same carcinogen, may differ in antigenecity. This would necessitate testing each serum sample with the corresponding tumour of the host, which would not be a very practical method.

However, the foregoing examples of the induction of auto-antibodies show that tumour antigens, if they are altered native antigens, may induce antibodies cross-reacting with their normal counterparts. If this happens indeed, a more convenient way of detecting such auto-antibodies would be testing all sera of cancer patients on the same normal tissue. Moreover, when the overlap of the antibodies to 'normal' has occurred, in some cases a self-perpetuating mechanism of antibody formation might be expected, which would cause a high titer and a

strong affinity of the antibodies to the unaltered antigen. An example of such a mechanism was given by Weigle [3], who demonstrated that rabbits, after having lost their natural tolerance to native thyroglobulin by injections of diazotised thyroglobulin, responded to subsequent injections of native thyroglobulin by producing in some cases very high levels of circulating antibody to native thyroglobulin. Of course, the presence of autoantibodies to normal 'self' constituents in cancer patients does not *prove* that tumour antigens have provoked their formation; other explanations for their development could be given as well. But especially when there are features of tumour resistance, the existence of these autoantibodies might be an indication for the former presence of a tumour specific antigen.

In our study we attempted to detect such auto-antibodies in the serum of patients with breast cancer. For routine purposes we used the fluorescent antibody technique on normal mammary tissue sections, to test the sera for the presence of complement fixing antibodies to components of this tissue.

MATERIALS AND METHODS

Patients

Mammary carcinoma — 484 serum samples of 195 patients with mammary carcinoma at several stages were tested. Of these, 27 patients had no metastases at all, or metastases in the regional lymphnodes only at the time of radical surgery and no symptoms of metastases since; of the 168 patients with metastases at the time of bloodsampling, 105 patients had symptoms less than 5 years, 43 from 5 to 10 years, 15 from 10 to 15 years and 5 patients had symptoms longer than 15 years. About half of the patients of each group died within 2 years after serum sampling. Ages were: younger than 50 years: 24 per cent, 50–59 years: 28 per cent, 60–69 years: 26 per cent, 70 years and older: 22 per cent of the patients.

Controls — Sera of 378 unmatched controls were tested, ranging in age from 24 to 80 years. These controls included 44 healthy pregnant women, 31 shortly after delivery, 44 males with lung cancer, 25 patients with various tumours other than breast or lung carcinoma, 25 patients with non-malignant pulmonary diseases, 46 with diseases of the urogenital tract and 56 patients with various auto-immune syndromes, 107 miscellaneous.

Serological methods

Fluorescent antibody tests — The indirect fluorescent antibody technique was used throughout. 3–5 μ unfixed cryostat sections of a biopsy of the normal mammary gland of a 43 years old patient with mammary carcinoma were used as a routine. Unfixed and fixed sections of mammary tissue of other donors, and of other organs (adenomatous goiter, fetal kidney and liver, monkey kidney and liver) were used for special purposes. Routinely, tests were performed with a fluorescein-conjugated rabbit anti-human-complement serum, which combines specifically with the β_{1A-C} and the β_{1E} components of human complement (the conjugate was supplied by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service). This conjugate appeared to give better clear-cut results than an anti-human-gamma-globulin conjugate, and was therefore chosen for screening purposes. The tissue sections were incubated in a moist box for 30 min at room temperature with the patient's serum, they were then briefly rinsed in phosphate-buffered saline pH 7·4 and incubated for another 30 min with fresh normal human AB serum as a source of complement. After a washing of 20 min the conjugate was applied for 30 min, the sections were then washed again for 20 min and embedded in buffered glycerol. Positive reactions indicate that complement fixation has taken place on the tissue section to which an antibody has become attached. The specificity of this reaction was proved by control experiments, in which the inactivation of both the patient's serum and the AB serum by heating for 30 min at 56°C, or the application of unlabelled anti-human-complement serum preceding the incubation with conjugate resulted in the abolishment of fluorescent staining.

Cell fractionation experiments — Subcellular fractions of monkey kidney and liver were prepared by Dr. W. S. Bont (Biochemical Department) according to the method of Appelmans *et al.* [6]. These fractions were used for complement-fixation tests.

Complement-fixation test — The complement-fixation test was kindly performed by Dr. F. Dekking, Virus Laboratory of the University of Amsterdam, according to a drop method (50 drops to 1 ml) in plastic plates. Sera had been inactivated by heating for 30 min at 56°C, the antigens had not been inactivated. Preserved sheep red cells (Alsever) and preserved complement (Richardson) were used. The complement was titrated by a 50 per cent haemolysis method, using an E.E.L.-colorimeter, and five

50 per cent units were used with overnight fixations at 4°C.

RESULTS

Fluorescent antibody tests

Using the normal mammary gland, roughly three types of fluoresecent staining of epithelial cells were observed (staining of stromal elements was discounted).

1. Course granular staining of the cytoplasm of all epithelial cells of ductuli and and lobuli.
2. Cytoplasmic staining of the myoepithelial cells of ductuli and lobuli.
3. Several types of nuclear staining (diffuse, speckled, nucleolar).

The latter two types were found with sera of both patients and controls; they did not seem to be associated with a special type of mammary carcinoma and were very often found when no tumour resistance was indicated. Coarse granular cytoplasmic staining was found with twenty two serum samples of three mammary carcinoma patients, designated vdH,Oos and Ste (see case reports). This type of staining was not seen at all in our control series (Table 1).

Table 1. *Various types of complement-fixing antibodies in the serum of breast cancer patients according to the fluorescent antibody tests on normal mammary tissue.*

Serum of	Number of patients tested	Fluorescent staining of: Cytoplasma of all epithelial cells	Cytoplasma of myoepithelial cells	Nucleus (several types)
Patients with mammary carcinoma	195	3*	10	14
Unmatched controls	378	0	18	28

*The one patient vdH with high titer (1 : 256) is the only patient with evidence of tumour resistance.

Serum samples of vdH were found to be positive on sections of normal mammary tissue of all fifteen donors, including the patient's own mammary tissue. This latter reaction proved the *auto* character of the antibodies. The fluorescent staining observed with serum Oos was weaker, but essentially the same. The pattern of fluorescent staining given by the serum of Ste was slightly less coarse than that of the two others. The sera of vdH and Ste gave in addition to cytoplasmic staining also fluorescent staining of nucleoli.

The sera of vdH and Ste were tested on sections of a number of other organs. Positive reactions were found with adenomatous goiter, stomach, fetal liver and kidney, and monkey kidney. Reactions with monkey liver were weak, and with monkey pancreas and bovine or rat kidney were negative. Serum of vdH, tested on mammary carcinomas, was found to be positive with 7 out of 8.

The course granular staining pattern of these sera, also found when tested on the adenomatous goiter tissue sections, stood in striking contrast to the finely granular staining given by sera containing thyroid specific antibodies.

On kidney sections, fluorescent staining of tubuli was more homogenous; the glomeruli were not stained.

The reaction of serum of vdH with sections of normal mammary tissue and of vdH and Ste with sections of monkey kidney was abolished when the sections were fixed with 100 per cent ethanol at −20°C before incubation; fixation with acetone at −20°C did not markedly inhibit the fluorescent staining reaction. On monkey kidney the titer of serum of vdH was 1 : 256, of Ste 1 : 16 and of Oos 1 : 8. With an anti-7S-human-gamma-globulin conjugate only serum of vdH gave an unequivocally positive reaction. Only a 7S-gamma-globulin fraction of this serum gave a positive reaction with the anti-human-complement conjugate. The auto-antibodies in serum vdH thus appeared to be of the 7S-gamma-globulin (IgG) type.

Complement fixation tests

In order to localize the non-organ specific cytoplasmic antigen to which the auto-antibodies of serum vdH and Ste are directed, complement fixation tests were performed with subcellular fractions of monkey kidney. Of the seven sera tested only serum vdH gave a reaction with the rough mitochondrial fraction, the titer being 1 : 1024. The titer with a relatively pure monkey liver mitochondrial fraction was 1 : 256. No significant reaction could be found with the nuclear, the microsomal, and ribosomal fraction or with the supernatant.

Case reports

Patient vdH. — Born 1900. 1948 mammary carcinoma and axillary metastases. Non-radical surgery; X-ray treatment during 8 months. 1952 tumour recurrence in muscle, axillary metastases. Attempt to radical surgery, X-ray treatment. 1963 lymphnode metastases

supraclavicular, regression after cyclophosphamide and X-rays. 1964 extirpation lymphnode metastases supraclavicular other side. X-rays for signs of compression Th7. Prednison since May 1964. Until now (May 1965) without major complaints. First supraclavicular lymphnode shows slow progression.

Histology: 1948 according to report: carcinoma simplex and axillary metastases. 1952 carcinoma solidum mammae; infiltration of stroma with granulocytes, lymphocytes and plasma cells, lymphnodes with metastases and plasma cells. 1964 lymphnode with metastases and many plasma cells. Bone marrow: plasmoid reaction. Sections of 1948 are not available for reinvestigation, so that it remains uncertain whether the primary tumour also was infiltrated with lymphocytes and plasma cells. Detection of high titer (1: 256) of cytoplasmic autoantibodies in 1964 and 1965.

No signs of liver disease. Wassermann reaction negative.

Patient Oos. — Born 1894. 1960 mammary carcinoma with supraclavicular and axillary metastases. X-ray treatment. 1962 X-ray treatment of skin and lymphnode metastases. 1963 metastases in skin, lymphnodes, bones and peritoneum. Hormones induce temporary improvement. Again X-rays. Since 1964 prednison and cyclophosphamide, 1965 deterioration.

Histology: exceptional case of mucoid producing mammary cancer, with 'signet' cells. No infiltration with lymphocytes or plasma cells. Cytoplasmic antibodies in low titer (1: 8), which disappeared during cytotoxic treatment.

No signs of liver disease.

Patient Ste. — Born 1901. 1960 nodule in axilla; refused treatment until 1963. From 1963 to 1965 several courses of X-ray, hormonal and cytotoxic treatment because of heavy tumour infiltration of the whole breast, nodules in both axillae, skin metastases and tumour mass in other breast. Died in 1965.

Histology: not available (no biopsies). Cytoplasmic antibodies in low titer (1: 16) detected since 1964, present until a few weeks before death.

No signs of liver disease.

DISCUSSION

We have found several types of complement-fixing auto-antibodies in the sera of patients with breast cancer. None of these types has been demonstrated to be present exclusively in sera of these patients. Anti-nuclear factors and myoepithelial antibodies were present in our control sera as well. Mitochondrial antibodies have been found recently by Walker *et al.* [7] in the serum of patients with biliary cirrhosis and in some patients with connective tissue disorders or active chronic hepatitis (our patients with mitochondrial antibodies did not have signs of any of these diseases).

It was the aim of this study to correlate the presence of auto-antibodies with tumour resistance. Arbitrarely, we consider tumour resistance present only if histological features of immune response have been found in a patient who lives longer than 10 years with metastases. Histological investigations should reveal infiltration of plasma cells and lymphocytes *into* (and not only around) the tumour and metastases.

In our group twenty patients lived longer than 10 years with their metastases. Histological data of seventeen patients were available Only one of these patients appeared to have a heavy infiltration of plasma cells and lymphocytes into the recurrence of the tumour and plasma cell infiltration in the metastasized lymphnodes. (Unfortunately histological sections of the primary tumour were not available for re-investigation). This patient who is living well for more than 17 years in spite of her metastases is the only patient with high-titered mitochondrial antibodies (patient vdH). It seems reasonable to presume a relationship between the presence of these auto-antibodies and the signs of tumour resistance, though it cannot be proved. If such a correlation exists we do not know whether true tumour antigens have triggered the auto-antibody formation to the normal mitochondrial antigens. Other explanations could be given as well. Unfortunately it is unknown whether the heavy infiltration with plasma cells and lymphocytes in the tumour existed before the start of X-ray treatment for 8 months. This treatment could have caused extensive necrosis or alteration of normal tissue components. Also normal 'hidden' components could have become accessable during this treatment, thus causing the auto-antibody formation. Anyhow the latter explanations have to be related also to the tumour resistance in ony way or the other. Of course our results are preliminary. More

cases should be found before a relationship between tumour resistance and auto-antibodies to 'normal' can be established. And after that, the way they are provoked, their initial specificity and their precise role in tumour immunity have to be evaluated, hoping that we can learn more about tumour antigens and tumour-host relationships in man.

Acknowledgements — The author wishes to express his thanks to Mrs. M. de Vries Reilingh-Linders for her skillful technical assistance, Prof. J. F. Hampe for the histological reports, Dr. F. Dekking for performing the complement fixation tests, Dr. A. S. Groen for one of the case reports, Dr. T. E. W. Feltkamp and Dr. K. W. Pondman for supplying the fluorescent conjugate, and many others: nurses, medical technicians, and colleagues of the Institute for their kind co-operation.

RESUME

Les antigènes de tumeurs aboutiraient non seulement à la résistance tumorale, mais aussi conduiraient à la formation d'autoanticorps contre les constituants des tissus normaux. Dans une série de 195 *malades atteints de cancer du sein, un seul patient, — qui avait une forte infiltration de lymphocytes et de plasmatocytes de la tumeur et des métastases, et qui avait une survie de plus de* 17 *ans, — présentait un titre élevé d'auto-anticorps contre une composante cytoplasmique sans spécificité d'organe (probablement un antigène mitochondrial.)*

SUMMARY

It is postulated that tumour antigens might not only lead to tumour resistance but also to induction of auto-antibody formation to normal tissue constituents.

The only patient in our series of 195 *breast cancer patients with heavy infiltration of lymphocytes and plasma cells into tumour and metastases, who is living well for longer than* 17 *years, appeared to be also the only patient with high-titered auto-antibodies to a non-organspecific cytoplasmic component (probably a mitochondrial antigen).*

ZUSAMMENFASSUNG

Es wird poniert dass Tumorantigene nicht nur zur Tumorresistenz führen können, sondern auch zur Induction Auto-Antikörper gegen normale Gewebebestandteile.

Der einzige Patient aus unserer Gruppe von 195 *Mamma-Carzinom Kranken, mit starker Infiltration von Lymphocyten und Plasmazellen im Tumor und in Metastasen, der nach* 17 *Jahren noch im Wohlbefinden lebt, war auch der einzige Patient mit einem hohen Titer an Antikörpern gegen eine nicht-organspezifische cytoplasmatische Komponente (wahrscheinlich ein Mitochondrien-Antigen).*

REFERENCES

1. B. H. Waksman, Auto-immunization and the lesions of auto-immunity. *Medicine (Baltimore)* **41,** 93 (1962).
2. B. Cinader, Acquired tolerance, auto-antibodies and cancer. *Canad. Med. Ass. J.* **86,** 1161 (1962).
3. W. O. Weigle, The relationship among acquired immunological tolerance and its termination and tolerance to self and auto-immunity. *Int. Symp. Immunological Tolerance*, Locarno, May (1965).
4. G. L. Asherson and D. C. Dumonde, Auto-antibody production in rabbits. II. Organ specific auto-antibody in rabbits injected with rat tissue. *Immunology* **6,** 19 (1963).
5. T. Ishizaka, D. H. Campbell and K. Ishizaka, Internal antigenic determinants in protein molecules. *Proc. Soc. Expl. Biol. (N.Y.)* **103,** 5 (1960).
6. F. Appelmans, R. Wattiaux and C. De Duve, Tissue fractionation studies. The association of acid phosphatase with a special class of cytoplasmic granules in rat liver. *Biochem. J.* **59,** 438 (1955).
7. J. G. Walker, D. Doniach, T. M. Roitt and S. Sherlock, Serological tests in diagnosis of primary biliary cirrhosis. *Lancet* **1,** 827 (1965).

Europ. J. Cancer Vol. 1, pp. 327–328. Pergamon Press 1965. Printed in Great Britain

Immunological Studies on the Uterus Epithelioma T-8 Guérin

J. GELZER, R. DEGUILLAUME and R. HESS

Research Laboratories, Pharmaceutical Department, CIBA Limited, Basle, Switzerland

THE PRESENCE of unique tumour constituents has been successfully demonstrated by various techniques [1]. Whereas antigens not present in normal tissue were conclusively shown in chemically induced tumors of inbred mice [2–4] and more recently in virus-induced neoplasms of various experimental animals [5–8] controversial findings were obtained in studies on tumours in allogenic animals [9–11] and on tumours of human origin [12–15]. The specificity of the antisera used for characterization of the latter type of such "tumour-antigens" was repeatedly shown to depend upon the total absorption of their non-antitumour components (i.e. normal tissue antigens) [16].

The effect of various procedures of absorption was studied by comparing two techniques. Fluorescent labelled rabbit anti-uterus epithelioma T-8 Guérin sera were tested (a) for staining specifically the cytoplasma of tumour cells and (b) for their behaviour in agar gel double diffusion experiments [19]. Microsomal fractions of saline perfused T-8 Guérin tumors [17] prepared according to the method of Schneider and Hogebom [18] were used in Difco complete Freund adjuvant for immunising rabbits as described by Herbeuval [10]. Ammonium sulphate globulin fractions [19] of pre- and postimmunisation bleedings, conjugated with fluorescein iso-thiocyanate [20], were absorbed with rat liver- and rat kidney/heart tissue powder [20]. No reactions were obtained with preimmunisation samples neither in agar double diffusion nor when the same microsomal tumor fraction was used for fluorescent staining of frozen sections of uterus-epithelioma T-8 Guérin tumour tissue. Antisera, although strongly crossreacting in agar with up to 1: 100 diluted normal rat serum, even after absorption with liver powder, appeared to stain almost exclusively the cytoplasma of the uterus-epithelioma T-8 Guérin cells suggesting the presence of antigenic material of tumour origin within the microsomal fraction used for immunisation as reported previously [10]. However, after absorption of the antisera with a powder mixture of normal rat kidney and heart tissue specific fluorescence was practically limited to numerous macrophages that are found in the outer zones of actively growing tumors. The same antiserum sample produced in agar gel one single line with the microsomal tumor preparation.

The use of at least one other parameter for evaluating the specificity of antisera against tumor cell fractions appears to be helpful for a cautious interpretation of immunohistological findings.

REFERENCES

1. F. Milgrom, A short review of immunological investigations on cancer. *Cancer Res.* **21,** 862 (1961).
2. L. Gross, Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. *Cancer Res.* **3,** 326 (1943).
3. E. J. Foley, Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. *Cancer Res.* **13,** 835 (1953).
4. A. Haddow, Immunology of the cancer cell: tumour-specific antigens. *Brit. Med. Bull.* **21,** 133 (1965).
5. K. Habel, Resistance of polyoma virus immune animals to transplanted polyoma tumors. *Proc. Soc. Exp. Biol.* (N.Y.) **106,** 722 (1961).
6. H. O. Sjögren, I. Hellström and G. Klein, Resistance of polyoma virus immunized mice to transplantation of established polyoma tumors. *Exp. Cell Res.* **23,** 204 (1961).
7. P. H. Black, W. P. Rowe, H. C. Turner and R. J. Hübner, A specific complement-fixing antigen present in SV 40 tumor and transformed cells. *Proc. Nat. Acad. Sci.* (Wash.) **50,** 1148 (1963).
8. J. L. Old and E. A. Boyse, Immunology of experimental tumors. *Ann. Rev. Med.* **15,** 167 (1964).
9. J. G. Kidd, A distinctive substance associated with the Brown–Pearce rabbit carcinoma. I. Presence and specificity of the substance as determined by serum reactions. *J. Exp. Med.* **71,** 335 (1940).
10. R. Herbeuval, J. Duheille and F. Bellut, Etude par immuno-fluorescence des propriéties antigeniques de l'épithélioma atypique de Rau T-8 de Guérin. *C.R. Acad. Sci., Paris* **258,** 2938 (1964).
11. J. Duheille, H. Herbeuval and M. Fourot, Cytodiagnostic immunologique des plasmocytes en leucoconcentration. *C.R. Soc. Biol.* **157,** 591 (1963).
12. J. B. Graham and R. M. Graham, Antibodies elicited by cancer in patients. *Cancer, N.Y.* **8,** 409 (1955).
13. B. Bjorklund, Antigenicity of malignant and normal human tissues by gel diffusion techniques, *Int. Arch. Allergy* **8,** 179 (1956).
14. S. De Carvalho and H. J. Rand, Antigens in human tumors revealed in suppressed rabbits rendered tolerant to normal human tissues. *Exp. Mol. Path.* **2,** 32 (1963).
15. P. Gold and S. O. Freeman, Demonstration of tumor-specific antigens in human colonic carcinomata by immunologic tolerance and absorption techniques. *J. Exp. Med.* **121,** 439 (1965).
16. C. M. Southam, Relationships of immunology to cancer: a review. *Cancer Res.* **20,** 271 (1960).
17. M. Guerin and P. Guerin, Epithelioma de l'uterus du rat, lymphotrope et transplantable. *Bull. Cancer* **23,** 632 (1934).
18. W. C. Schneider and G. H. Hogebom, Intracellular distribution of enzymes. V. Further studies on the distribution of cytochrome C in rat liver homogenates. *J. Biol. Chem.* **183,** 123 (1950).
19. E. A. Kabat, Kabat and Mayer's Experimental Immunochemistry (2nd ed.) Charles C. Thomas, Springfield, Illinois (1961).
20. A. Coons, E. H. Leduc and J. M. Connoly, Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J. Exp. Med.* **102,** 49 (1955).

Europ. J. Cancer Vol. 1, p. 329. Pergamon Press 1965. Printed in Great Britain

Tobacco Dosage and Lung Cancer in the Light of Experimental Carcinogenesis

L. KREYBERG

Department of Pathology, University, Oslo

SUMMARY

Accumulated occurrence of benign and malignant tar tumours are recorded, and it is stressed that if the number of animals in the series is large enough, the very earliest few tumours appear at the same time, namely close to the minimum time necessary for such tumours to develop, and regardless of the strength of the carcinogen.

The epidermoid lung carcinomas in man are next recorded in relation to different dose levels of tobacco smoking, and the similarity to the experimental curves shown.

The material of 449 *epidermoid carcinomas of the lung is then examined in relation to smoking methods (cigarette, pipe, mixed c.p.), as well as to smoking doses, and to the appearance of Group II Tumours (adenocarcinomas, Bronchiolo-alveolar cell carcinomas, carcinoids and salivary gland type tumours).*

It is shown that also pipe smoking is also responsible for a number of epidermoid carcinomas, but pipe smoking appears less dangerous than cigarette smoking. It is shown that part of the total difference is caused by lower dose levels, but qualitative differences are probably also present.