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COMMENTARY

SCREENING FOR ANTI-CANCER AGENTS; THE RELATIVE MERITS OF *IN VITRO* AND *IN VIVO* TECHNIQUES

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A large number of tests, ranging from simple biochemical determinations to studies using animals with spontaneous tumours, have been investigated as potential screening methods in the search for new anti-cancer drugs. The most successful systems to date have been those using transplanted animal tumours and these have been responsible for the discovery of most clinically useful anti-cancer agents. Although anti-cancer agents come from many different classes of chemical and act by different mechanisms, the majority are specifically toxic to cells in cycle and their biological properties are broadly similar. It has been proposed that fundamentally different types of anti-cancer agent will only be found by using human cancers as the test system. *In vitro* studies using malignant cells of human origin in short or long term culture have been well investigated and while they have some advantages over transplanted tumour systems, they also have the many disadvantages inherent in an *in vitro* system. A method now being developed combines the best features of animal models, yet uses cells of human origin. In this system, human cancers are transplanted to 'immunologically deprived' mice. These transplanted tumours, which can be readily obtained from most human cancers, appear fairly stable for at least a few transplant generations and maintain many of the properties of the human cancer from which they were derived. In preliminary tests their response to chemotherapy is in accord with clinical experience. It is suggested that these tumours provide an essentially different model for the screening of new anti-cancer agents and may pick out drugs with greater selectivity for solid cancers in man.

Testing against animal cancers

The majority of the chemicals used today in the chemotherapy of cancer were first selected for clinical trial mainly because of their ability to inhibit the growth of one or more animal tumours *in vivo*. However, quite often these animal models select new classes of agent which are subsequently shown to have no clinical value. While this may be due to one of many reasons, such as the occurrence in man of unacceptable side-effects which are not predicted by pre-clinical pharmacology, more often than not it is simply because the drugs have no tumour inhibitory properties in man. Besides selecting such 'false positive' agents, the experimental systems probably produce 'false negatives', that is, chemicals which would be active against human cancer but which have no activity in the experimental systems used. Clearly there is no way of quantitating the 'false negatives'that have been missed over the years, since agents without activity against animal tumour models are rarely tested in the clinic. However, some very useful anti-cancer agents in man, such as prednisolone and busulphan (myleran) are virtually inactive against the commonly used animal tumour models and probably would not have been selected for trial if the present day protocols for drug screening had been in use at the time of their discovery.

It is now taken for granted that an agent having an effect on one type of animal tumour will not necessarily be active against another tumour. It is also well recognised that these differences can be the result of many separate factors such as differences in tumour size and location, differences in cell kinetics, metabolism, vascularity and antigenicity or from the acquisition of drug resistance. Even if all these variables could be controlled, however, different types of cancer would still differ in their response to a chemotherapeutic agent since selective cytotoxicity depends fundamentally on the biochemical make-up of cells and in the ways in which this differs from one cell type to another.

Asparaginase, which can cure animals transplanted with the Gardner (C3HED) lymphosarcoma at dose levels well below the maximum tolerated, is an example of a selective anti-cancer agent which is exploiting a biochemical property of a tumour (lack of asparagine synthetase) not found in normal cells. This agent is only moderately useful in man simply because most human cancers do not have this biochemical difference, a result which might have been predicted since the vast majority of animal tumours in common use are also insensitive to asparaginase.

Some idea of the variable responses of animal tumours to chemotherapy is shown in Table 1. From the results obtained using four transplanted tumours it can be seen that whether or not an anti-tumour effect is observed depends both on the tumour system employed and the drug under investigation. Thus cyclophosphamide would be selected as highly active by the Walker and PC6 tumours but rejected as a 'false negative' by the TLX5 lymphoma. Conversely methotrexate, which like cyclophosphamide is a clinically useful agent, would be missed by the PC6 tumour and selected as active by the TLX5 lymphoma. Asparaginase was

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Table 1. Effect of four different types of anti-cancer agent on four transplanted tumours

	Walker (TI)	PC6 (TI)	TLX5 (ILS)	Gardner (TI)
Cyclophosphamide	30	136	inactive	
Methotrexate	1·7	inactive	55	
Asparaginase	inactive	inactive	40	7000
CB 1954	75	inactive	inactive	inactive

TI = Therapeutic index.
ILS = Increase in life span.

discovered when it was used fortuitously (as a constituent of guinea-pig serum) against the Gardner lymphosarcoma. Had it been submitted for test against the more commonly used tumours it would have been rejected. CB 1954 is the most active compound yet tested against the Walker tumour yet it has no activity against the other tumours shown in Table 1. It may be that there are human tumour types which are also sensitive to CB 1954 but no means exists at present of identifying them.

The obvious limitations of these models have led to many attempts to improve upon them by using, for example, 'spontaneous' or chemically or virally induced tumours which may be more related to human cancer in their vascularity and in their association with host connective tissues, and transplanted tumours with cell kinetic properties resembling solid cancer in man. However, there has been no indication that these animal models are any better in selecting new anti-tumour agents than rapidly growing transplanted tumours such as the L1210 and P388 leukaemias which respond to a broad range of anti-cancer chemicals. Because of the individual properties of each cancer or type of cancer, it may well be that truly selective agents can only be found by using each type of human cancer as its own test system.

Testing against human cancer in vitro

Human cancers can be grown in animals in immunologically-privileged sites such as the hamster cheek pouch[1], anterior chamber of the eye, in skin islands[2] and in millipore diffusion chambers[3]. Some of these methods have been used to asssess the sensitivity of human tumours to known agents and all have given much information on human tumour biology. However, none of these techniques has yet been adapted to the routine screening of drugs for anti-cancer activity.

Another approach which has been studied fairly intensively over the years and which appears to be particularly useful for certain types of cancer, involves the study of short term tissue cultures of human cancers removed by biopsy or at operation. In ovarian cancer for instance, cells may be readily obtained, especially from malignant effusions and can be grown as monolayers with a take rate of greater than 80% under the best conditions[4].* For different cell types which rapidly establish a monolayer, it has been shown that only a small percentage of the cells are in cycle and thus they reflect more accurately the behaviour of human solid cancer *in situ* than do transplanted animal tumours[5]. However, tests of this sort are primarily used to determine the sensitivity of individual tumours to a range of available drugs, thus allowing tailoring of the drug regimens for each patient's cancer. When accurate methods are used for assessing the anti-tumour effectiveness of various agents it has been claimed that these models are useful, that is, they correlate with the response observed in the patient in more than 50% of cases[6, 7]. Not all investigators agree that this method is highly predictive[8] but there is sufficient evidence to suggest that under the right conditions it may be useful for particular patients. What is still required is a large scale clinical trial to determine whether, under strictly controlled conditions, this technique can be useful in selecting the appropriate drug combinations for individual patients for treatment of their primary cancer or of subsequent recurrences. Although it might be possible to adapt such a system to large scale drug screening, it would still be subject to many of the objections to *in vitro* drug testing mentioned below.

A simple alternative to animal testing which uses human cancer as the test system would appear to be the establishment of cell lines in culture which are derived from different types of human cancer. It has been argued that the use of such *in vitro* systems has several advantages over animal tumour models[9]. They are rapid and simple methods, economising on animals and space and allow a good control of the experimental conditions. Uniform material can also be selected and careful and continuous observations of the effects of the drug can easily be made during the course of the experiment. Certainly these assay systems are the only practical means of screening drugs which are only available in milligram amounts, a situation which nowadays occurs more and more frequently—a consequence of the development of microtechniques for drug synthesis and analysis. They are also of especial use in the rapid monitoring of fractions during the isolation of active constituents of natural products. Nevertheless, while tests using human cancer cells *in vitro* are a useful complement to the more commonly used transplanted animal tumours, and while they may overcome some of the recognised limitations of animal tumours, they also create many problems of their own.

Cell selection and cell kinetics

The rationale for using human cells in culture is that they are biochemically identical with the cancer one wishes to treat. However, for many cancers it is not always easy to obtain a viable cell culture. Even if uniform material (from biopsy or at operation) can be obtained consistently, there is evidence that often the cells that can be grown constitute only a small number of the cells present in the original sample, being those which are particularly well-suited to the culture conditions used. Such cells may not be representative (in their biochemistry) of the malignant cells that composed the tumour *in situ*, and thus even if stable cell lines

* However, for many other cancer types the take rate is much lower.

are eventually obtained from the culture, they may prove to be far removed from the original situation. Some transplanted animal tumours are known which remain constant in their response to chemotherapy for many years as long as they are continually passaged in animals. When they are adapted to growth in culture and then transferred back to animals their response to chemotherapy may change radically even though their time in culture is short. There are many other examples showing that a considerable amount of cell selection may take place when animal tumours are adapted to growth in culture, and there is no reason to assume these same problems do not apply to the growth of human cell lines.

Cell biologists are well aware of the possibility of contamination of their cell cultures by bacteria and fungi, but do not always pay the same attention to contamination by other cells, despite the fact that the intraspecific contamination of cultures and the difficulties of showing that this has taken place have been recognised for a number of years[10]. Quite recently, evidence from both chromosomal and isoenzyme studies has strongly suggested that many established lines of human cells are in reality cultures of HeLa cells[11]. There is evidence that this contamination has taken place even in laboratories where special precautions have been taken to isolate cell lines one from another, and thus it seems essential to check continually the identity of cell lines used in screening. Chromosome banding and isoenzyme measurements could probably be used to ascertain that cell lines remained uncontaminated, but the introduction of such tests would certainly detract from the advantages of *in vitro* tests, whose very simplicity is one reason why they are considered useful.

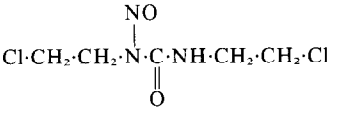
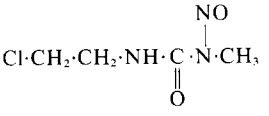
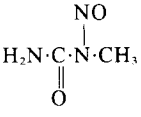
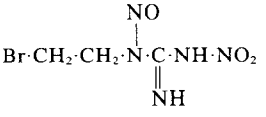
A major objection to using transplanted animal tumours has been that in their cell kinetics they are quite different from most human cancers, particularly of the solid type. The most frequently used animal tumours have short doubling times and a large proportion of cells in cycle with short generation times. In most human cancers, intermitotic times are much longer. It has also been claimed that many cells are out of cycle, but that they can still divide and repopulate tumours after cytotoxic therapy. Differences in cell kinetics make large differences in response to chemotherapy even with similar cell lines and it has in fact been demonstrated that some cells, which in the resting stages are insensitive to cytotoxic agents, become very sensitive to the same drugs when in cycle[12]. Transplanted tumours may therefore only select as active, agents specific for dividing cells rather than for cancer cells. Certainly the vast majority of chemicals selected by these models are cycle-specific agents[13], and it has even been proposed that anti-cancer agents should be classified not in terms of their chemical properties, but in terms of their effects on the cell cycle[14]. Clearly a system which enabled one to screen drugs for their effects on the G_0 cells of human tumours would be welcomed. Unfortunately there is no reason to suppose that any cell line, once adapted to growth in culture, is any more related in cell kinetics to the human situation than animal tumours. Once adapted to tissue culture, cells usually grow rapidly until a certain cell density is reached, and inter-mitotic times are short. They are just as likely as animal tumours to select as active, agents acting against cycling cells rather than agents specifically active by virtue of their other biochemical properties.

Anti-tumour selectivity

Given a high enough intracellular concentration, any chemical will kill all cell types. The object of cancer chemotherapy is to discover drugs which will be irreversibly toxic to cancer cells (no matter what their cell-kinetic status is) at dose levels which are tolerated by the host. For this reason drug activity in a screening test is defined in terms of a therapeutic index. Usually this is some ratio between a lethal dose and a tumour effective dose, since both are simple to estimate. In more refined tests, the effect on the stem cells of the most sensitive host tissue is compared to the effect on the stem cells of the tumour. Tests *in vitro* cannot easily provide this information on selectivity unless 'normal' cell lines (which are difficult to maintain in culture) are grown side by side with the cancer cell line. Thus one major disadvantage of *in vitro* tests is that they only compare the potency of drugs, the assumption often being made that the most potent chemicals are the best anti-tumour agents. This assumption is justified to a certain extent by the results of experiments where activity against human cell lines has been compared with the activity obtained against *in vivo* systems, but there is also ample evidence that many clinically useful agents are not necessarily very toxic to mammalian cells in culture. Cell cultures, like animal tumour models, will therefore also produce 'false negatives'. Table 2 shows the results of testing a closely related series of nitrosoureas against a mouse lymphoma which can be readily transplanted to animals or grown in tissue culture. Even with this closely related series there is no correlation between potency (cytotoxicity) and the anti-tumour effect obtained in whole animals, some potent compounds being inactive *in vivo* and one of the least toxic members of the series having good activity *in vivo*.

In whole animals the anti-tumour action of a drug can be measured in a number of ways, depending on the tumour employed and the site of its transplantation, but generally, because it is the simplest, some form of measurement of tumour volume or weight is used. Certain tumours show a good inverse relationship between survival time and number of tumour cells originally transplanted, and survival time can be used as a measure of tumour cell kill (provided treatment has not drastically altered the kinetics of surviving cells). However, none of these methods is particularly sensitive and relatively large and important effects obtained with a new class of chemical might be missed. In the case of solid tumours, for instance, a drug may kill a considerable proportion (greater than 99%) of malignant cells yet there may be only a small reduction in tumour volume. This masking of the anti-tumour effect may be due to the presence in the tumour of large amounts of

Table 2

	% ILS *in vivo*	Optimal* dose *in vivo* (mg/kg)	Dose *in vitro* to give 99·9% cell kill (μg/ml)
	>300	40	4
	inactive	12	3
	50	160	64
	inactive	200	80

* The optimal dose is the dose that gives the greatest extension in survival time. For inactive compounds the dose given is the maximum tolerated.

connective tissue not affected by the drug, because of the drug-induced induction of oedema or because the killed cells are only slowly removed from the tumour mass. If a rapid regrowth of the surviving cells occurs before the dead cells are removed, there may be little or no change in tumour volume. In order to detect these effects *in vivo*, tedious clonogenic assays are required. This is not necessary in *in vitro* tests where cell lines in exponential growth are used. If the growth of the cultures is followed continuously then cytotoxicity representing 50% cell kill or less can be easily monitored. However, if cells in plateau phase or other *in vitro* cultures where the cells are non-dividing are used, the accurate assessment of anti-tumour effect becomes difficult. In these systems morphological criteria or the uptake of dyes have been used as measures of cytotoxicity but neither is particularly quantitative. Some cytotoxic agents kill cells rapidly (as measured by transplantation tests) at dose levels which have little or no effect on cell morphology or the ability to exclude dyes for several hours or even days after treatment. Thus, when these parameters are used to measure cytotoxicity, the cells must be monitored for several days and this adds to the complexity of *in vitro* experiments. A more quantitative method has been to estimate the incorporation of radioactively labelled precursors into nucleic acid and protein fractions but even here there is not necessarily a direct relationship between uptake of label and cytotoxicity. The uptake of tritiated thymidine into DNA for example, will depend on a number of factors such as the amount of unlabelled thymidine in the cell culture medium, the ratios of thymidine kinase and thymidine catabolising enzymes in the cells and the activity of the *de novo* synthetic pathway to thymidylic acid. An agent which kills cells by preventing the *de novo* synthesis of thymidylic acid may actually cause initially an increased thymidine incorporation. It has also been quite clearly shown in some systems that there is no clear relationship between incorporation of thymidine into DNA and DNA synthesis[15]. The only true measure of drug effectiveness is the demonstration that cells no longer have the ability to divide indefinitely and to invade tissues and metastasise, and this can only really be proved by following the properties of drug-treated cells *in vivo*. Tissue culture, for instance, would not uncover agents that were not directly cytoxic but which prevented the tumours' ability to invade and metastasise.

Tumour-host relationships

Whether or not a chemical selectively inhibits the growth of a cancer *in vivo* sometimes depends more on the host than on the cancer. Thus brain tumours may be inherently sensitive to a particular drug, but not respond *in vivo* because the drug in question does not penetrate the blood brain barrier. This is well enough known to be allowed for in an *in vitro* system using brain cancer cells, but more subtle differences probably exist where the location of the tumour *in vivo* may greatly influence the tumour's response to drugs. Some animal tumours, for instance, are very sensitive to alkylating agents when transplanted to the flank but do not respond to the same agents if growing in the lung[16]. It is not inconceivable that the location of many tumours influences the amount of drug that reaches them and there is no reason to suppose that this does not also occur in man, particularly when metastases have formed. In testing *in vitro*, it could

be that chemicals only moderately cytotoxic are really the agents of choice, because they have the ability to reach the cells in high concentration *in vivo*.

An important problem *in vivo* which is certainly not seen *in vitro* where free cells are used, concerns tumour vascularity and the presence of ischaemic regions in solid tumours which contain viable cells probably out of cycle. A high concentration of drug would not be expected to penetrate these ischaemic areas, and the cells they contain, even if inherently sensitive to the drug used, might not be affected by treatment, yet provide the basis for tumour regrowth after the majority of the malignant cells have been killed. There seems at present no way of simulating this phenomenon in cell culture, unless the multi-cell spheroid system recently described can be adapted as an *in vitro* test[17].

Different species respond in a variable manner to each pharmacological agent. This can often be shown to be due not to basic differences between species, but simply to the persistence of the drug, that is, the period of time for which the drug is present in the animal at its effective dose[18]. Drug persistency is a function of many host factors such as rate of absorption from the injection site, metabolism and excretion, protein binding and the occurrence of tissue depots of the active drug, all of which determine the plasma concentration attained and the time for which the drug persists at effective dose levels. These functions all vary depending on the drug administered and clearly in testing novel structures *in vitro* it would be impossible to allow for all these variables.

Another important host factor is the immune response and there is ample evidence that in animal systems, a drug-induced tumour regression may have two components, a direct effect of the drug on the cell and a host-mediated immune response. Sometimes the direct cytotoxicity of the drug may be relatively small and the immune response large so that *in vitro* the effectiveness of the drug might be overlooked. In the extreme case the agent may act exclusively by stimulation of some part of the immune response and so would be missed by simple tissue culture testing.

Drug metabolism

A well recognised advantage of *in vivo* tests is that they can detect effective anti-cancer agents which are themselves biologically inert and only act after some form of metabolism. If this metabolism can be performed by malignant cells, such as the conversion of purine and pyrimidine analogues to their nucleotides, then cytotoxicity will be seen *in vitro*. However, there are many examples, such as in the dimethyltriazene series, where the essential enzymatic activation step takes place predominantly in the liver. Cyclophosphamide is considered to be one of the best alkylating agents in clinical use, yet it is hardly cytotoxic *in vitro* compared with agents of the same type (Table 3). For the other agents shown in the table, the *in vitro* toxicity is always greater than the *in vivo* toxicity, but all are related by the same conversion factor—which probably reflects the fact that the product of drug

Table 3. Effect of different nitrogen mustards on Walker tumour cells *in vivo* and *in vitro*

	Walker tumour cell ID_{90} (μg/ml)		
	In Vitro	*In Vivo*	*In Vitro In Vivo*
Chlorambucil	1·2	4·5	0·27
Melphalan	0·2	0·6	0·33
Merophan	0·07	0·25	0·28
HN2	0·025	0·10	0·25
Cyclophosphamide	8000	7·5	1067
Cyclophosphamide + Microsomes + cofactors	1·82	7·5	0·24

concentration and time in the *in vitro* system is greater than the same product in the *in vivo* system where the drugs are excreted and possibly deactivated in the liver. Cyclophosphamide does, however, fit into the series when it is incubated *in vitro* with liver microsomes and an NADPH generating system. Potential carcinogens which require conversion to electrophilic reactants, are now routinely screened in simple *in vitro* systems which incorporate drug metabolising enzymes.

In order not to reject 'pro-drugs' in screening agents for anti-cancer activity, a system of the type illustrated in Fig. 1 would need to be employed (using cyclophosphamide as an example). Besides comparing the growth rate of cells in the presence of several concentrations of the drug with a control, there should also be cells which are incubated with drug metabolising liver fractions in the presence of the drug under test. To be done properly, with all the necessary controls, the system would become much more complicated than a simple *in vitro* test but it would prevent the rejection of at least some of the false negatives that pass through *in vitro* systems.

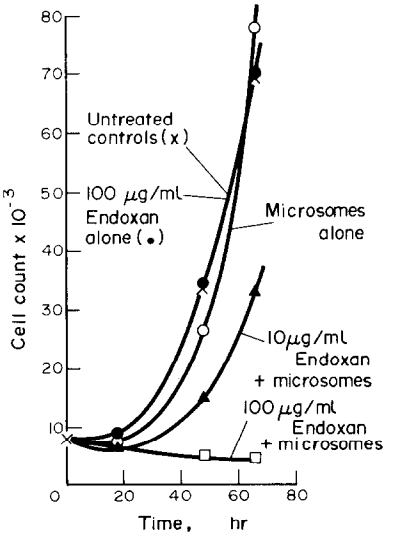


Fig. 1. Activation of cyclophosphamide (Endoxan) *in vitro*. Cyclophosphamide (100 μg/ml) or microsomes with cofactors have no effect individually on the growth of Walker carcinoma cells *in vitro*. Microsomes and cofactors added in combination with cyclophosphamide cause significant growth inhibition at 10 μg/ml of the alkylating agent and are completely lethal at 100 μg/ml.

In vitro tests for detecting new anti-tumour agents may thus be useful in certain well-defined circumstances where it is not possible to use animal tumour models, but they cannot, in general, be considered as replacements for tests using tumour-bearing animals, even if human tumours are used as the source of the tissue culture material.

Heterotransplantation of human tumours

It has now been shown that the majority of human cancer types can be grown in either rodents which have non-functional T-lymphocytes ('nude athymic' mice) or whose T-lymphocytes have been greatly reduced by a process of adult thymectomy, lethal irradiation followed by syngeneic bone marrow protection with or without anti-lymphocyte serum[19–25]. In a number of independent laboratories, carcinomata of the lung, ovary, colon, kidney and pancreas, as well as various lymphomas, have been grown directly from human tumours and serially transplanted. Breast cancers have proved the most difficult to grow. At one time they could only be obtained after being adapted to growth *in vitro*, which probably allowed considerable cell selection to take place. However, breast tumours have recently been successfully xenografted directly to athymic mice. In some early tests on the Walker rat carcinoma heterotransplanted to CBA mice, artificially deprived of T-lymphocytes, it was shown that the response of the tumour to chemotherapy was exactly the same as its response in the rat, thus demonstrating that, at least in this case, the prediction of drug sensitivity was not affected by xenografting[26]. A similar conclusion had already been reached from studying the response of the mouse L1210 leukaemia in its normal host and after transplantation to the hamster cheek pouch[27]. An investigation of some of these human tumours xenografted to mice has already shown that the cells retain their morphology[20], chromosome content and isozyme levels[28] at least for a small number of transplant generations, and in other studies there is also evidence that there is no alteration in cell kinetics during early serial passages[29]. Much tumour biology remains to be done in these systems to show (a) that they are representative of the human tumours from which they were derived (that is, that there was no selection of cells on the first transplantation); (b) that they contain only human chromosomes (proving no cell hybridisation has taken place); and (c) that there are no alterations in cell kinetics or biochemical properties for at least a few transplant generations. One would then need to know whether the response of these tumours to known chemotherapeutic agents was reminiscent of the human situation. Already from the results of a few chemotherapeutic studies it appears that the susceptibility of human tumour xenografts to known agents is in agreement with clinical experience[30–32]. DTIC, for example, causes regression of a melanoma, Burkitt's lymphoma is markedly susceptible to cyclophosphamide, while this compound also holds up the growth of an oat-cell carcinoma. Carcinoma of the colon grows readily in thymus-deprived and in nude mice, but only responds poorly to chemotherapy, which is in accord with clinical experience. It is also possible that these tumour models may select compounds not active against animal tumours but effective in man. Some evidence for this comes from the response of a human lung xenograft to hexamethylmelamine (Fig. 2). This drug has a definite effect on human lung cancer but is quite inactive against the most frequently used transplanted tumours[33]. The lung tumour is very sensitive, undergoing complete regression at two well-tolerated doses and thus predicts as active a compound which might have been missed by present day drug screening.

Before these systems (which are much more difficult to operate than transplanted tumours) can be accepted, far more data are required concerning their behaviour, especially to chemotherapy. Because they only grow slowly on transplantation, it is not likely that xenografts can be used for selecting treatment for individual patients, but they may be of use if the tumour recurs. However, their major role, if they really prove to be more predictive models than transplanted animal tumours, will be to form the basis of large scale screening programmes for the detection of new agents. Such systems may combine the advantages of the transplanted animal tumour system with the advantages of using cells, biochemically identical to the cancer type one wishes to treat. The hope is that they will prove useful in selecting new classes of chemical agents which are not just anti-proliferative agents but selectively cytotoxic to a particular cancer cell type.

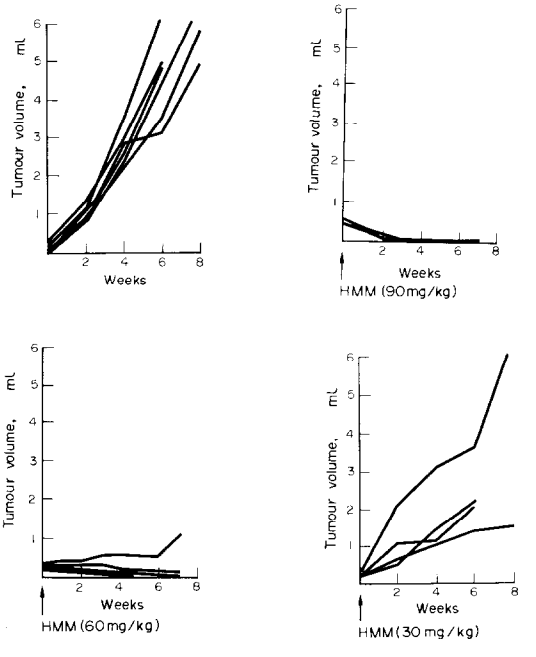


Fig. 2. Effect of hexamethylmelamine (HMM) on a human lung tumour xenografted to CBA/LAC mice artificially deprived of T-lymphocytes. Treatment commences only when the tumours are palpable and their growth rate is calculated by caliper measurements. HMM given by two courses of 5 daily intraperitoneal injections (separated by a 1-week interval) causes complete tumour regression at 90 mg/kg, completely holds up the growth of the tumour at 60 mg/kg and retards tumour growth at 30 mg/kg.

REFERENCES

1. D. M. Goldenberg and H. J. Hansen, *Science* **175**, 1117 (1972).
2. M. M. Ziegler, E. E. Miller and C. F. Barker, *Surg. Forum* **23**, 127 (1972).
3. E. Capalbo, J. F. Albright and W. E. Bennett, *J. Immunol.* **92**, 243 (1964).
4. C. N. Hudson, *Lancet* **1**, 1116 (1972).
5. J. S. Mitchell, P. P. Dendy, M. P. A. Dawson and T. K. Wheeler, *Lancet* **1**, 955 (1972).
6. S. Tanneberger, in *Aktuelle Probleme der Zellzüchtung* (Ed. B. Mauersberger), p. 417. Stuttgart (1971).
7. J. C. Wright, J. P. Cobb, S. L. Gumport, D. Safadi, D. G. Walker and F. M. Golomb, *Cancer* **15**, 284 (1962).
8. E. Hirschberg, *Cancer Res.* **18**, 869 (1958).
9. G. Penso and D. Balducci, *Tissue Cultures in Biological Research*, p. 360. Elsevier, Amsterdam (1963).
10. C. S. Stulberg, in *Comtamination in Tissue Culture* (Ed. J. Fogh), p. 2. Academic Press, New York (1973).
11. W. A. Nelson-Rees, R. R. Flandermeyer and P. K. Hawthorne, *Science* **184**, 1093 (1974).
12. R. F. Pittillo, F. M. Schabel and H. E. Skipper, *Cancer Chemother. Rep.* **54**, 137 (1970).
13. H. Madoc-Jones and T. Mauro, in *Anti-neoplastic and Immunosuppressive Agents. I* (Eds. A. C. Sartorelli and D. G. Johns), p. 205. Springer, Berlin (1974).
14. L. A. Price and B. T. Hill, *Lancet* **2**, 172 (1974).
15. H. Grunicke, F. Hirsch, H. Wolf, U. Bauer and G. Kiefer, *Exp. Cell. Res.* **90**, 357 (1975).
16. G. M. Conzelman and K. Springer, *Cancer Chemother. Rep.* **53**, 105 (1969).
17. R. M. Sutherland, J. A. McCredie and W. R. Inch, *J. Natn. Cancer Inst.* **46**, 113 (1971).
18. L. B. Mellet, in *Anti-neoplastic and Immunosuppressive Agents. I* (Eds. A. C. Sartorelli and D. G. Johns), p. 830. Springer, Berlin (1974).
19. J. Rygaard and C. O. Povlsen, *Acta Path. Microbiol. Scand.* **77**, 758 (1969).
20. L. M. Cobb, *Br. J. Cancer* **28**, 400 (1973).
21. J. E. Castro, *Nature New Biol.* **239**, 83 (1972).
22. B. Phillips and J. C. Gazet, *Br. J. Cancer* **24**, 92 (1970).
23. B. C. Giovanella, J. S. Stehlin and L. J. Williams, *J. Natn. Cancer Inst.* **52**, 921 (1974).
24. P. Arnstein, D. O. N. Taylor, W. A. Nelson-Rees, R. J. Huebner and E. H. Lennette, *J. Natn. Cancer Inst.* **52**, 71 (1974).
25. B. Sordat, R. Fritsche, J. P. Mach, S. Carrel, L. Ozzello and J. C. Cerottini, in *Proceedings of 1st International Workshop on Nude Mice* (Eds. J. Rygaard and C. O. Povlsen). Gustav-Fischer, Stuttgart (1974).
26. C. E. Sheard, J. A. Double and M. C. Berenbaum, *Br. J. Cancer* **25**, 838 (1971).
27. D. M. Goldenberg, K. T. Schricker, J. Emde and H. H. Sogtrop, *Arzneim.-Forsch.* **18**, 712 (1968).
28. C. O. Povlsen, P. J. Fialkow, E. Klein, J. Rygaard and F. Wiener, *Int. J. Cancer* **11**, 30 (1973).
29. R. G. Pickard, L. M. Cobb and G. G. Steel, *Br. J. Cancer* **31**, 36 (1975).
30. M. C. Berenbaum, C. E. Sheard, J. R. Reittie and R. V. Bundick, *Br. J. Cancer* **30**, 13 (1974).
31. L. M. Cobb and B. C. V. Mitchley, *Cancer Chemother. Rep.* **58**, 645 (1974).
32. C. O. Povlsen and J. Rygaard, in *Proceedings of 1st International Workshop on Nude Mice* (Eds. J. Rygaard and C. O. Povlsen). Gustav-Fischer, Stuttgart (1974).
33. B. C. V. Mitchley, S. Clarke, T. A. Connors and A. M. Neville, *Cancer Res.* **35**, 1099 (1975).