status of the use of LAI techniques in cancer diagnosis.

D.M.P. Thomson (from Montreal, Canada) reviewed some of the earlier work with the LAI technique and discussed the physiological phenomenon underlying the response. By the use of different mouse strains he has determined that the responses obtained correlate with immunocompetence and chemotaxis. He further described research on a new organ-specific cancer neoantigen (OSN). This antigen is shed by tumour cells and was isolated from spent tissue culture medium of a human lung cancer cell line and it has also been found in the human foetus. The antigen has a molecular weight of 40,000 Daltons. Similar antigens appear to be present also in tumours from other organs and evidence was presented that this is a general tumour associated antigen with organ specificity. Dr. Thomson's findings may provide more specific antigens for use in the LAI test and provide new insights concerning tumour antigens.

V. Holan et al (Prague, Czechoslovakia) studied patients with carcinoma of the larynx and found that the leukocytes from some patients only reacted with one or a few extracts of individual tumours. The pattern of reactivity was different in individual patients suggesting that histocompatibility antigens could play a role. A significant difference in adherence was observed in experiments where leukocytes from normal non-immunized rats were tested with syngeneic or allogeneic tissue extracts and it was concluded that a significant proportion of leukocytes recognize allogeneic histocompatibility antigens without previous sensitization.

Several reports were devoted to LAI measurements on leukocytes from patients with different types of cancer. D. Eljuga et al (Zagreb, Yugoslavia) found that most of their patients with breast cancer responded in the LAI assay against breast cancer extracts, while no responses were obtained with extracts from benign breast tumour tissues (fibroadenoma) or a colorectal carcinoma. T. Sanner et al (Oslo, Norway) also reported a high percentage of responses in patients with cancer of the breast. Some of their patients have now been followed for more than seven years after initial LAI measurements and the start of treatment. No correlation was found between the results of the LAI assay prior to diagnosis and the subsequent development of the disease. F. Kalafut et al (Bratislava, Czechoslovakia) followed the LAI response in patients with malignant melanoma of the uveal tissue. They found that the test was positive in about 85% of their patients. Of interest, when the test was repeated 3 to 6 months after excision of the tumour from the eye, the LAI response had dropped in about 50% of the patients. All these patients have survived, and after a mean time lapse of 3 years, no metastases have been observed. These results suggest that the LAI response measured after a certain time following treatment may have prognostic value.

H. Kotlar and T. Sanner (Oslo, Norway) discussed a new test for the detection of an antitumour immune factor present in the serum of cancer patients. The experimental procedure of this test is similar to the original LAI test and it has been called the humoral leukocyte adherence inhbition (H-LAI) test. In the H-LAI assay trypsinized leukocytes from control persons are used as indicator cells and the effect of addition of serum from the patient under study and the relevant antigen on the adherence of the indicator cells are determined. With the H-LAI test responses were obtained in 70 to 90% of patients with breast, ovary and lung cancers. These response rates agree well with the results obtained in the original LAI test. The advantage of the new test is that it can be performed on small amounts of serum which can be frozen and stored for long periods. H. Kotlar also reported results from a small retrospective study using coded serum samples from persons who have later developed lung cancer. In this study, responses were found 1 to 5 years prior to the clinical diagnosis of the disease. It has been found that the serum factor responsible for the observed reaction in the H-LAI test is a glycoprotein with molecular weight of about 70,000 Daltons. Mechanistic studies indicate that the T8-subpopulation of the T-lymphocytes is essential in the H-LAI reaction. This new LAI assay appears to be the humoral counterpart to the original cellular LAI test.

Three studies from Budapest, Hungary which confirmed the usefulness of the H-LAI test were presented. G. Kövesi and B. Fekete used myelin basic protein as a general cancer antigen and found that 97% of their patients with cancer in the head and neck region reacted while no false positive results were obtained among 27 control persons. M. Horvath and B. Fekete reported data on lung cancer patients and found that they all responded in the H-LAI assay. The response decreased to normal values 7 to 20 days after operation. T. Kubasova et al discussed an interesting modification of the H-LAI assay where they used a radioactive amino acid mixture to label the indicator cells. The H-LAI index was calculated from the radioactivity of adherent cells. The results showed high specificity and reproducibility. Hopefully, this seminar will stimulate further research upon the use of the LAI system which may contribute to a better understanding of tumour immunology in general and to improved methods in early cancer detection.

Cellular Transformation in Vitro

Reported by: N.E. FUSENIG
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In this seminar the presentations and discussions concentrated on the phenomenon of spontaneous malignant transformation in cultures of rodent and human cells. Particular emphasis was placed on the analysis of phenotypic and genetic changes occurring during the transformation process and on the elucidation of their possible causal relationship with certain stages of the development of tumourigenic cells.

Special interest was focussed to the demonstration of activated oncogens and their significance for the induction and maintenance of the malignant state.

"In vitro carcinogenesis" has gained increasing interest and importance in recent years and particularly during the two years since the last meeting. This is mainly the consequence of three developments and new discoveries:

1. The augmentation of this research area by the expansion of the biological in vitro systems available for these studies improving from rather undefined rodent mesenchymal cells to differentiating human cells from various tissues.

2. The rapid development of the oncogene field which nearly exclusively was achieved using cell culture systems, bringing together the fields of chemical and viral carcinogenesis and uniting their hypotheses to a fascinating common postulated mechanism of cell transformation.

3. The increasing importance of cytogenetic studies as a consequence of the discovery of specific chromosomal aberrations in distinct malignancies and the evidence of their causal significance in the transformation process via (proto-) oncogene activation.

The further elucidation of the correlation of cytogenetic alterations with altered gene expression in the transformation process might best be achieved in cell transformation assays functioning without genotoxic mediators, such as the so-called spontaneous cell transformation in vitro. These systems might also be biologically closer to the situation of tumour development in man, than the known carcinogenesis assays involving toxic carcinogen doses leading to unspecific alterations at the phenotypic and cytogenetic level. Spontaneous transformation in long term cultured rodent fibroblasts has been a well known phenomenon for many years. The malignant conversion without known interference of carcinogenic factors has been mainly attributed to the labile genome of these cells which is obviously very sensitive to exogenous influences (from the tissue culture environment) and/or only partially effective to maintain the integrity of the genetic constitution.

A new cell culture model of mouse skin keratinocytes with a high frequency of spontaneous transformation after distinct latency periods, depending on culture conditions, was reported and discussed by N.E. Fusenig and his co-workers (Heidelberg, F.R.G.). In this reproducible system of long term primary cultures of epidermal cells growing under different substratum- and medium conditions, the first stage of transformation is indicated by the ability of cells to continue growth in serial subcultures. The latency periods of 2 to 6 months after plating depended on the particular growth conditions and were obviously linked to the actual proliferative rate of the cells. Following this stage of "adaptation" with altered growth regulation, cells acquire "immortality" with further passages. In subcultured cells differentiation control is lost to variable degrees (depending on the particular culture conditions) ranging from disarranged morphogenesis, and lack of expression of differentiation specific keratins, to alterations in the topological expression of specific differentiated functions, comparable to stages of epithelial dysplasia in vivo.

The spontaneously developed cell lines were either malignantly transformed at their earliest passages (<10), indicated by invasiveness in transplants and tumourigenicity after injection, or acquired these abilities with continued passages (>20). The maintained (and expressed) degree of differentiation obviously had no immediate influence on the evident degree of malignancy since early passage cells of highly keratinizing lines were even metastatic while those from poorly differentiating other lines only become tumourigenic with later passages. Most cell lines grew in soft agar but no real correlation to tumourigenicity was noted.

At the cytogenetic level all mouse keratinocyte cell lines had acquired specific and individual marker chromosomes (duplications, deletions, translocations) whether cells were transformed in vitro (spontaneously or after carcinogen treatment) or, for comparison, derived from carcinogen-induced mouse skin carcinomas. The common feature of all cell lines, however, was their heteroploidy (tri- to tetraploid) with the consistent overrepresentation of chromosome number 5 and 6 while numbers 7 and 14 were in all or most cell lines underrepresented. During the process of spontaneous transformation chromosomal alterations (numerical and structural) started early during the first days in culture and increased with culture time. At early stages, with respect to subculturability ("adaptation" to "immortalization") the majority of cells had near tetraploid karyotypes which were characteristic for early subcultures. The appearance of cell line specific marker chromosomes is supposedly correlated with the acquisition of malignant growth behaviour and both were either achieved at early subcultures (<10) or acquired during subsequent passages (>20).

Within this spontaneous transformation system certain stages and possible mechanisms of the transformation process of mouse keratinocytes could be postulated: under appropriate culture conditions, enabling cells to accomplish a certain number of replications, structural and numerical chromosomal aberrations accumulate (due to the "stressful" culture conditions or incomplete control of chromosomal integrity). This chromosomal constitution causes quantitatively and/or qualitatively altered gene expression with the consequence of "adaptation" of cells to continued growth under in vitro conditions. Due to the stabilization of this chromosomal constitution (predominantly numerical changes with the consequence of gene imbalance) some cells acquire "immortality" and can be continuously passaged. Concomitantly, alterations in the regulations of keratinization occur ranging from smaller to severe changes in epidermal differentiation. By a second alteration, probably mediated by specific structural chromosomal changes, malignant growth ability is achieved, demonstrated by invasion and tumourigenicity. However, this stage can either be completed at the earliest subcultures or achieved only at later passages.

The earliest stages of spontaneous transformation of rodent cells, the acquisition of "immortality" and the significance of changes in proto-oncogene expression were the main topics of a further contribution (S. Shall, Brighton, U.K.). The process of cellular mortalization, that is the loss of reproductive ability in normal rodent cells, is a well-regulated biological process which almost

certainly has a genetic basis. In addition, it has been shown that a number of viral oncogenes can confer on primary cultures the phenotype of cellular "immortality"; amongst these genes are the large T-genes of both polyoma and SV40-viruses, and the E1A-genes of human adeno-viruses as well as genes from Epstein-Barr virus. Moreover, the v-myc-oncogene of the myelocytomytosis virus, MC29, is apparently required for the successful transformation of primary rodent cells by activated ras-oncogenes.

The cellular homologue of MC29-oncogene, c-myc, is normally expressed at a very low level in mortal, untransformed Swiss mouse embryo cells in primary and early subcultures. This level is unchanged whether cells grow at high or low proliferative rates (up to the 6th passage level). Upon spontaneous cellular "immortalization", however, these rodent fibroblastic cells substantially and specifically increase the steady state level of c-myc-mRNA expression (2 to 3 fold), while mRNA expression from β-actin and v-ras was unchanged. Neither amplification nor apparent translocation of the c-myc gene could be observed. It is concluded that the regulation of c-myc expression is permanently altered in immortal, transformed cells suggesting that the c-myc gene plays a significant role in the process of cellular immortalization.

An additive or synergistic action of c-ras and c-myc-oncogenes and chemical carcinogens on malignant transformation of rodent fibroblasts was discussed by Denner and co-workers (Berlin-Buch, G.D.R.), who used both mouse 3T3 cell lines and Fisher rat embryo fibroblasts in primary cultures. As known from other studies, transfection of Ha-ras$^{(EJ)}$ and c-myc oncogenes alone or in combination did not lead to focus formation of the transfected cells nor to formation of colonies in soft agar. Oncogene-transfected cells were subsequently treated with chemical carcinogens (3-methylcholanthrene and N-methyl-N-nitro-N-nitrosoguanidine) and analyzed for altered morphology and colony formation in agar. Preliminary results indicated altered growth behaviour of the oncogene-transfected and carcinogen-treated cells but so far no colonies in agar were observed.

The phenomenon of spontaneous cell transformation to malignancy is obviously not restricted to rodent cells but also possible with human cells, although at a much lower frequency and after longer latency periods. Observations of the spontaneous malignant transformation of a human monocytic cell line (CM-S) derived from the bone marrow of a child with congenital hypoplastic anemia (Diamond-Blackfan) was reported by R.P. Revoltella (Rome, Italy). Cells grow in suspension culture as undifferentiated mononuclear cells, although they constitutively and, following stimulation, express some antigens, enzymes and functional properties commonly present in the precursors of the monocyte-macrophage lineage. With successive passages in vitro, CM-S cells spontaneously transformed to tumourigenic cells. During earlier passages cells retained the euploid karyotype and did not (without stimulation) form colonies in agar nor grow to tumours in nude mice. On prolonged passages, CM-S cells gradually increased their growth capacity and became capable of forming colonies in agar and tumours in nude mice. These changes correlated in time with the appearance of specific numerical and structural chromosomal changes. Furthermore, the expression of differentiated traits markedly changed as a function of time in that the transformed cells appeared blocked at the monocyte precursor stage and no longer responded to the inducing agents active on CM-S cells of early passages.

In transfection experiments DNA from these later CM-S cells were capable of transforming NIH 3T3 mouse cells with high efficiency, while DNA from earlier CM-S cells or from fibroblasts of the same donor were non-transforming. The acquisition of non-random chromosome abnormalities and a gradual increase in transcription of myc, myb and ras proto-oncogenes correlated with the acquisition and progression of the transformed phenotype. The expression of myc, however, was not changed whether quiescent or actively proliferating (synchronized) CM-S cells were analyzed. It was postulated that an initial DNA-rearrangement detected early in culture (but not found in marrow fibroblasts of the same donor) initiated a series of changes which led to the preneoplastic stage of CM-S cells. The enhanced intracellular accumulation of multiple proto-oncogene products may then have contributed to significantly potentiate the genetic disarray, and by this, increased the likelihood of a malignant hit in these predisposed cells leading to stable alterations of the genome and resulting in the expression of a more malignant phenotype.

Another group also reported on spontaneous changes within human epithelial cell lines to malignancy growing in culture and the concomitant increased expression of oncogenes (J. Skouv and co-workers, Copenhagen, Denmark). A number of cell lines from human urothelium were established and characterized with respect to several biological properties such as morphology, cytogentics, antigen-expression, growth pattern, tumourigenicity, and invasiveness. According to these properties the cell lines had been classified into four transformation grades (TGrO to TGrIII), that most likely represent different steps in bladder oncogenesis. Two cloned cell lines of TGrII apparently had spontaneously developed to a tumourigenic and invasive TGRIII phenotype on various occasions during propagation in vitro. While cell lines of TGrO and TGrI had no detectable expression of the proto-oncogenes c-myc, Ha-ras, and Ki-ras, type II- and III-cells contained and expressed the activated form of oncogenes. However, no difference in the level of expression was observed between cell lines of TGrII as compared to those of TGrIII, indicating that this progression in transformation of these cell lines was not correlated with the higher expression of one of these oncogenes.

The last contribution by F.D. Tóth and co-workers (Debrecen, Hungary) dealt with the analysis of the expression of different oncogene products (myc, myb, Ha-ras, sarc) by immunofluorescence techniques using appropriate antibodies in different fresh human leukaemia and lymphoma cells in smears and short term cultures. The results indicated that more than one oncogene product was obviously synthesized in all of the cells examined with the clustering of some of the oncogenes to distinct types of leukaemias. When DNA from these cells was transfected into NIH 3T3 cells, a high transfection frequency was observed with following expression of more than one oncogene in the transfected cells. The possible significance of these data was discussed, although the analysis of oncogene expression by indirect immunofluorescence only was not considered sufficient both on a

quantitative and qualitative level.

In general, the contributions elucidated that both in rodent and human cells in vitro the process of spontaneous cell transformation becomes increasingly interesting for the analysis of phenotypic alterations related to differentiation features, cytogenetic changes and the expression of oncogenes in relation to the transition from normal to "premalignant" and finally to malignant cells. In these assays the elimination of the specific carcinogenic factors was considered advantageous for the undisturbed analysis of phenotypic and genotypic alterations and their correlations to the changes from a normal to a tumour cell.

Tumour-Host Interaction - Mechanism of Invasion

Reported by: A. VAHERI
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Several of the reports in this seminar dealt with the regulation of proteolysis, a process considered to be critically involved in invasion. This was discussed by A. Vaheri (Helsinki, Finland) in the introductory remarks. He emphasized that invasiveness and metastasis, distinguishing properties of malignant cells, involved penetration through components of the extracellular matrix. Enzymatic degradation of matrix components appears to be involved in these phenomena. In collaboration with S. Barlati's laboratory (Brescia, Italy) it was found that defined gelatin-binding fragments of fibronectin have transformation-promoting activity in experimental conditions. This activity is shared by tissue type plasminogen activator t-PA. Interestingly, t-PA is bound by immobilized fibronectin and immobilized laminin, a process possibly involved in "directional proteolysis" by the cell. Fibronectin fragments detected in body fluids of tumour patients may serve as markers for tumour progression. Pericellular proteolysis appears to be regulated in part through α_2-macroglobulin (α_2M), plasminogen activators and their inhibitors, as detected in cultures of various types of normal and malignant human cells. Proteolytic targets of the pericullular matrices of cells in culture include fibronectin and an M_r 66,000 matrix-associated protein. Interestingly, in cultures of human sarcoma cells u-PA is enriched at cellular adhesion sites where also fibronectin is found.

In previous studies production of α_2M has been confined to normal cells; apart from liver cells in vivo, in culture conditions macrophages and lung fibroblasts synthesize this wide-spectrum protease inhibitor. J. Bízik (Bratislava, Czechoslovakia) now reported that several human sarcoma and melanoma cell lines synthesized and secreted to the culture media a high molecular weight glycoprotein identified by immunological criteria, molecular size and NH_2-terminal sequence analysis as human α_2M. In immunoperoxidase staining some but not all human melanomas in vivo were positive for α_2M. J. Tőzsér (Debrecen, Hungary) reported on plasminogen activator levels in blood of mice bearing leukaemia induced by chemical carcinogens. Increased levels were detected in the blood cell extracts while in plasma the overall levels were unaltered or even decreased. The latter finding suggested that analysis of the molecular forms (size, pI) by zymography might be of interest.

The approach by K. Dingemans (Amsterdam, The Netherlands) was primarily a morphological one: ultrastructural analysis of the invasion of lung parenchyme by bronchogenic squamous cell carcinomas. At the sites of initial contact the tumour cells appeared to have inserted themselves between the alveolar epithelial cells and the tissue retained an essentially intact basal lamine that persisted relatively long. A noticeable feature in the deeper tumour areas was a prominent connective tissue reaction, an interesting and conspicuous process the mechanism of which remains to be defined.

What makes some tumour cells metastatic and how can this be correlated to cellular parameters in culture is being studied by a number of laboratories today. The clonal evolution and selection of tumour cells created obviously considerable problems in any such analysis. P. Veselý (Prague Czechoslovakia) reporting on characteristic patterns of in vitro behaviour of sarcoma cells with different metastatic capacities made an interesting comment about the pH of the study conditions. At the acid pH 6.7 (rather than at pH 7.4) a correlation between the increased locomotory activity and capability of metastasizing was found. K. Lapis (Budapest, Hungary) and collaborators are in the process of a multiparameter analysis of in vivo selected highly metastatic variants of Lewis lung tumour cells. Several cell surface properties (more microvilli, more fibronectin, highly sulphated heparan sulphate, altered lectin-binding sites) were characteristic of the highly metastatic cell line. Perhaps of more interest was the finding that these cells did not attract macrophages to the same extent as the parental tumour cells. The most provocative part in the report was, however, the result that 5-hexyl-2'-deoxy-uridine inhibited the highly metastatic cells in vivo without being cytotoxic.

Destruction of metastatic tumour cells by immunological means was the subject of the report by K. Yokoro (Hiroshima, Japan) using a reticulum cell sarcoma line originating from a C57BL/C3H mouse. Mice receiving spleen cells from specifically immunized donors were protected from metastasis. The observation that after anti-Thy 1 serum treatment the spleen cells lost their protective capacity indicated that specifically activated T-lymphocytes were responsible for metastatic cell destruction. R.Heicappell (Heidelberg, F.R.G.) reported on another approach for experimental prevention of metastatic spread. The highly metastatic murine lymphoma ESb cannot be curatively treated with surgery or chemotherapy alone. Postoperative metastatic spread could, however, be prevented with a combination of surgery and immunotherapy using