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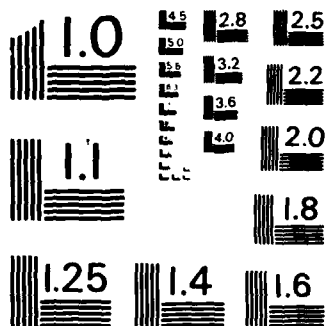
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The research project is concerned with the detection of premalignant and
malignant cells induced in vivo in a novel assay system the granuloma pouch
assay. Cells exposed to carcinogens in vivo can be studied for DNA damage,
chromosomal aberrations, specific locus mutations and cell transformations.
Various assays were developed to investigate the growth characteristics of
normal, carcinogen-exposed and transformed granuloma pouch cells.

These included: a) primary cloning efficiencies under optimal growth conditions,
b) growth in serum deficient and calcium depleted media,
c) growth in soft agar,
d) growth in athymic mice and rats,
e) growth with cocultured normal cells
f) growth under influence of growth factors.

Phenotypic alterations investigated were the DNA-dispersion in individual cells and the appearance of calcium binding proteins. Furthermore the genotoxic/cytotoxic activity of asbestos fibres was investigated.

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**CORRELATION OF MUTAGENIC, CARCINOGENIC AND COCARCINOGENIC EFFECTS OF CHEMICAL
SUBSTANCES (GRANULOMA POUCH ASSAY).**

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PREFACE

At the Institute of Toxicology, Swiss Federal Institute of Technology and University of Zurich in Switzerland, new methods for the detection and characterization of mutagenic and carcinogenic chemicals in mammals are developed. The AFOSR grant supported the part which deals with the detection of malignant and premalignant cells. The special feature of the procedure used ("granuloma pouch assay") is the treatment of defined cell populations in the intact animal, followed by the analysis of individual cells in various in vitro assays.

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TABLE OF CONTENTS

1. Introduction
 - 1.1. General concept
 - 1.2. Experimental design
2. Report of supported work performed
 - 2.1. Summary of work performed in the first and second year
 - 2.1.1. Culture conditions in vitro for neoplastic cells
 - 2.1.2. Selective growth conditons in vivo and in vitro for neoplastic cells
 - 2.1.3. Coculture of neoplastic cells with normal cells
 - 2.1.4. Analysis of preneoplastic cells
 - 2.1.5. Analysis of chromosomal rearrangements by flow cytometry
 - 2.1.6. Mutagenicity of asbestos
 - 2.2. Work performed in the third year
 - 2.2.1. Experimental design
 - 2.2.2. Analysis of preneoplastic cells by flow cytometry
 - 2.2.2.1. DNA- dispersion
 - 2.2.2.2. Effect on cell- cycle
 - 2.2.3. Calcium-binding proteins as marker for preneoplastic cells
 - 2.2.3.1. Evaluation and detection of tumor specific proteins
 - 2.2.3.2. Appearance of marker proteins in normal and preneoplastic cells
 - 2.2.4. Mode of toxic and genotoxic activity of asbestos
 - 2.2.4.1. Nonenzymic and enzymic lipid peroxidation
3. Conclusion

LIST OF ABBREVIATIONS AND SYMBOLS

CV : Coefficient of variation
EGF : Epidermal growth factor
FCM : Flowcytometrie
FGF : Fibroblast growth factor
GPA : Granuloma Pouch Assay
MNNG: N⁻-methyl-N⁻-nitro-N-nitrosoguanidine
NI : Neoplastic index
PAGE: Polyacrylamide gel electrophoresis
SCE : Sister chromatid exchanges
TPA : 12-O-tetradecanoyl-phorbol-13-acetate

1. Introduction

1.1. General concept

In the past a number of mechanisms were identified which lead to development of preneoplastic cells and ultimately to growth of a tumor. The best characterized events involved in the pathological activation of oncogenes are those at DNA- level (mutations). They can be detected in screening tests using microorganism and mammalian cells. However, evidence is increasing that chemicals often are not identified as carcinogen in in vitro systems, because of inadequate metabolic activation provided by the added subcellular fractions. The reason for this may be an unusual tissue specific activation, or the fact that activation of the chemical is mediated by a two step mechanism in different organs. Therefore, in vivo short term genotoxicity tests are developed, in the hope that the predictive value of the procedures can be improved.

Oncogenes might also be activated by indirect pathways. The post-transcriptional regulation of growth factor synthesis might be disturbed by chemicals interacting with endogenous or exogenous factors and receptors. This could stimulate the expression of spontaneously arrived or chemically induced alterations in the genotype. Furthermore, preneoplastic cells might be kept quiescent under the control of the surrounding tissue, and this control might be altered by xenobiotics. It can be assumed that these indirect pathways are specific for mammalian cells and requires corresponding analysis with in vivo/in vitro systems.

The basic approach used in our research is to expose a defined and rapidly dividing target cell population in vivo to known carcinogens. Subsequently cells are isolated and cultured in vitro. Genetic alterations can be determined immediately in vitro. Secondary insults are applied to the cells in vitro under defined conditions and the various phenotypic alterations characteristic for malignant transformation are monitored. From the data obtained so far, it appears that the extended latency period necessary in the standard long-term carcinogenicity bioassay might be drastically reduced by keeping cells in a proliferating state over prolonged culture time. It is hoped that the concept will result in a better understanding of mechanisms responsible for the expression of transformed phenotype in vivo and in vitro. Ultimately, the approach could lead to a short term in vivo test in which not only genotoxic but also nongenotoxic carcinogens can be identified.

1.2. Experimental design

Research was performed with the Granuloma Pouch Assay (GPA), an in vivo model developed at our Institute (Maier 1984). In this system, mutagenic events and in vivo transformed cells can be detected. Growth of target tissue (granulation tissue) is initiated by the application of 25 ml air subcutaneously between the shoulders of rats. The target cells (rapidly growing fibroblasts and endothelial cells) can be exposed directly if the chemical is applied into the air pouch or indirectly if treatment is given by parenteral injection or orally. Using a systemic application route, the pharmacokinetic characteristics of the test compound can be studied.

Cells isolated from the dissected granuloma tissue can be cultured in vitro with a high seeding and plating efficiency. They are accessible for analysis similar to that performed in established cell lines. So far tests for mutagenic events like DNA- strand breakage, chromosomal aberrations, sister chromatid exchanges and gene mutations were evaluated. In vivo transformed cells are obtained in long term experiments in which growth of fibrosarcoma can be detected at the site of application of the test compound. Cells isolated from these tumors can be cultured in vitro. They serve as reference cell populations for evaluation of growth characteristics and morphological and biochemical markers. Freshly isolated and subcultured "carcinogen-exposed" granuloma cells are investigated for the presence of similar changes in growth characteristics and appearance of markers. From these findings "preneoplastic" cells can be detected and their transformation to malignant cells can be observed.

2. Report of supported work performed

2.1. Summary of work performed in the first and second year

2.1.1. Culture conditions in vitro for neoplastic cells

Optimal culture conditions for neoplastic cells were determined. Malignant cell populations used were isolated from fibrosarcomas growing out

Maier, P., The Granuloma Pouch Assay, in: F.J. de Serres (Ed.), Chemical Mutagens, Principles and Methods for their Detection Vol. 9, Plenum, New York, pp. 233-260, 1984.

of granulation tissues treated in vivo with established carcinogens. Media, sera, pH and pO_2 were evaluated. The comparison with untreated isolated granuloma tissue cells, allowed a specific growth stimulus to be determined. Culture under low pO_2 (30- 40 mg Hg), as measured in subcutaneous tissues, stimulated growth of transformed as well as of nontransformed cells (Maier et al. 1986). The stimulus was 2- 5 fold in tumor cells and 1.2- 1.4 fold in normal cells. A selected set of growth modifying chemicals showed no further increase of the constitutive growth stimulus. Therefore, in the subsequent in vitro studies, cultures were performed under low pO_2 .

2.1.2. Selective growth conditions in vivo and in vitro for neoplastic cells

Optimal growth conditions in vivo of neoplastic cells were evaluated in nude mice and rats. Species, strain and inoculum size influenced initiation of growth and lag time of tumor development in the host. Of specific interest was that co-injection of normal cells with the transformed cells shortened the lag time of tumor growth or reduced the number of tumor cells required for tumor formation. (Holzer et al. 1984)

Selective growth conditions of neoplastic cells in vitro was investigated with in vivo transformed cells. Among 3 criteria tested (low- serum medium, Ca^{2+} -deprived medium, anchorage-independent growth in soft agar), growth in Ca^{2+} -depleted medium and anchorage independent growth in soft agar were the most reliable conditions. An excellent correlation was found between colony formation in soft agar and tumor formation in nude mice.

2.1.3. Coculture of neoplastic cells with normal cells

The growth stimulus found in coinjection experiments in nude mice, was further investigated. It was found that cocultures of normal cells with in vivo transformed cells stimulated their anchorage independent growth in soft agar. The stimulus was dependent on the number of normal cells added. Further analysis revealed that the cocultured cells do not divide but synthesize and

Maier P., Schawalter H.P. and Zbinden G., Tissue pO_2 stimulates growth and enzymic activity in freshly isolated mesenchymal rat cells, in preparation.
Holzer, C., Maier P., and G. Zbinden, Mesenchymal rat cells stimulate growth of their transformed counterpart in vivo and in vitro, *Experientia* 40, 626, 1984.

release an agar diffusible factor which stimulates growth of tumor cells. This effect could be substituted by high levels of fetal calf serum but not by newborn calf serum. The response of individual tumor cell populations to growth factors such as epidermal growth factor (EGF), Insulin and fibroblast growth factor (FGF) differed for each of the growth factors tested. With cocultures a general stimulating effect was observed in the different tumor cell populations. The conclusion was that the growth stimulus provided by normal cells is an optimal mixture of growth factors which could be further used to initiate or stimulate the selective growth of preneoplastic cells in vitro in soft agar tests (Holzer et al. 1986).

2.1.4. Analysis of preneoplastic cells

Based on this knowhow we tested whether we were able to accumulate and detect preneoplastic cells (cells exposed to a carcinogen) under the evaluated conditions optimal for in vivo transformed cells. Premeoplastic cells were believed to be present among freshly isolated cells exposed in vivo to the carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Doses applied were previously evaluated in mutagenicity and carcinogenicity tests. The exposure level chosen induced a high yield of mutations at the 6-TG locus (Maier et al. 1980) and furthermore induced in situ growth of subcutaneous fibrosarcoma (Zbinden et al 1980). Rats were subjected to three treatment schedules: Group I received the established tumour promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA), group II MNNG only and group III was without treatment. Two critical parameters were investigated, namely the interval necessary in vivo for the recovery of the highest yields of preneoplastic cells and the expression period necessary in vitro to express the transformed phenotype. Intervals between treatment and cell isolation were four hours, 2, 4, 6, 15,

Holzer, C., P. Maier and G. Zbinden, Clone formation of rat fibrosarcoma cells in soft agar is stimulated by cocultured normal cells, in preparation, 1986.

Maier, P., Manser P. and G. Zbinden, Granuloma Pouch Assay. II. Induction of 6 thioguanine resistance by MNNG and benzo(a) pyrene in vivo, Mutation Res. 77 165-173, 1980.

Zbinden, G., Maier P. and S. Alder, Granuloma Pouch Assay. III. Enhancement of the carcinogenic effect of MNNG, Arch. Toxicol. 45, 227-232, 1980.

21, 28 and 35 days. In vitro the expression period under optimal growth conditions were 7, 14 and 21 days. Freshly isolated and in vitro cultured cells were subjected to the three evaluated selective growth conditions (Ca^{2+} depleted medium and anchorage independent growth, growth in nude mice). Results obtained are:

Freshly isolated cells:

- a) The neoplastic index (NI) obtained in Ca^{2+} depleted medium (cells able to form colonies in Ca^{+2} deficient medium/ cells able to form colonies in normal culture medium) was below 0.11 in untreated cells. 4 hours after treatment, without culturing the isolated cells in vitro, the NI was 0.11 in group I, 0.23 in group II and 0.01 in group III.
- b) Anchorage independent growth differed between the three groups. In group II clearly more cells formed clones. However, clones were also found in cells from group I and III. Coculture with mouse cells enhanced, as expected, the cloning efficiency, but this occurred in all three groups.
- c) None of the freshly isolated carcinogen-exposed cell populations formed a tumor in nude mice. Therefore, growth conditions with highest selectivity for transformed cells did not allow growth of preneoplastic cells.

In vitro cultured cells:

The expression of the transformed phenotype after prolonged culture of the isolated cells was investigated. After 7, 14, and 21 days in culture, cells were subjected to the three growth conditions mentioned above. With increasing subcultures the NI increased in untreated cells (group III) to 0.4 and in group I and II the values ranged between 0.3 - 0.7. The differences observed with freshly isolated cells between the three groups disappeared. We concluded that the high NI obtained with freshly isolated cells is most probably an indicator of an acute membrane damage, and does not indicate a preneoplastic stage. Cultured cells formed clones in soft agar to a much lower extent than freshly isolated cells. No specific pattern related to the treatment of the cell population was observed.

In nude mice, none of the subcultured cell populations grew out to tumours. In conclusion, the culture procedure in vitro did not enhance the expression of the neoplastic phenotype.

2.1.5. Analysis of chromosomal rearrangements by flow cytometry

Based on the known correlation between the clastogenic and carcinogenic activity of a chemical, variations in the DNA content of individual cells should be detectable by flow cytometry. The method was introduced in our laboratory. The staining procedures and the analysis of data (soft ware) were evaluated. Furthermore we searched for the most suitable cell population to be used as an internal standard. The smallest detectable variations in the DNA content was found in rat thymocytes and was 2%. With cultured granulation tissue cells the coefficient of variation (CV) was between 3-4% (Maier P. and Schawalder H.P., 1985).

2.1.6. Mutagenicity of asbestos

As a carcinogen active by an unclear mechanism, we chose asbestos. Initially the mutagenic activity of crocidolite fibres was investigated. In a first series of experiments the cytotoxic activity of the fibres was determined in vitro. In mutagenicity tests in vitro we found enlarged nuclei, an enhanced frequency of sister chromatid exchanges (SCE) but no increase in the gene mutation frequency at the 6-TG locus. These results suggest (a) that different genetic endpoints have to be analyzed in order to detect the possible genotoxic activity of asbestos and (b) that a spindle disturbing (enlarged nuclei) or clastogenic activity (SCE frequency) might be responsible for the carcinogenic effect. Both mechanisms are well known to participate in the activation of oncogenes.

Maier P. and H.P. Schawalder, Application of flow cytometry to the detection of spindle poisons, Food and Chemical Toxicology, in press, 1985 .

2.2. Work performed in the third year

2.2.1 Experimental design

The conclusions from the previous studies were that in vivo treated cells, although carrying mutations in their genome, do not express a neoplastic phenotype detectable in vitro in an altered growth behaviour. The extended interval between cell treatment and isolation of cells or the subsequent culture in vitro did not enhanced the recovery of neoplastic cells. This was in contrast to results reported in the literature with established cell lines. Therefore, during the third year, endpoints were analyzed which are not directly related to growth behaviour of cells. In preneoplastic cells we observed changes in the dispersion of the DNA of individual cells and we looked for the appearance of tumor specific proteins. In addition we speculated that cells might require a second genotoxic insult in vitro in order to express their transformed phenotype in vitro.

Comparative studies were performed again with the three model cell populations derived from the subcutaneous granulation tissue. These are a) untreated, freshly isolated and cultured granuloma cells (normal cells) b) cells exposed in vivo, in vivo and in vitro or in vitro only to a carcinogen (preneoplastic cells) and c) in vitro cultured cells isolated from fibrosarcomas developed out of the granulation tissue after exposure to a carcinogen (malignant cells).

Investigations about the mechanism involved in the mutagenic activity of asbestos was focused on the primary interaction of the fibres with membranes (lipid peroxidation).

2.2.2. Analysis of preneoplastic cells by flow cytometry

The DNA- histograms of 12 malignant cell populations derived from fibrosarcomas were determined by flow-cytometry (FCM). All tumor cells expressed some degree of variation in the DNA content, and in all cases they were easily distinguishable from normal cell populations. Therefore, we assumed that changes in the chromosome set might occur already at an early stage of malignant transformation. By maintaining the cells after treatment in a proliferating state in culture, we hoped to detect the earliest sign of chromosomal instability due to selective growth of such preneoplastic cells. A

marker for the degree of aneuploidy was the dispersion of the DNA in individual cells expressed as an enlarged coefficient of variation (CV) of G_1 -phase cells in DNA- histograms.

2.2.2.1. DNA- dispersion

The experiments were carried out as follows: Cells were isolated from untreated animals and from animals treated with the established most efficient dose of MNNG. Half of both cell populations were treated in vitro again with MNNG. The dose chosen was known to induce a high mutation frequency. Half of the four cell populations were exposed to TPA. The resulting eight cell populations were cultured for 4 weeks. After each subculturing (7x) the DNA-content was determined by flow cytometry. The cell population without treatment at all was used as reference culture. At the beginning of the cultures the acute chromosome damage induced by MNNG was recognizable by an enhanced CV value. Cell populations treated with MNNG in vitro only expressed after three weeks a small increase in the CV. Cell populations exposed to MNNG in vivo only, became aneuploid after 1 1/2 week. The most pronounced effect was obtained with the in vivo and in vitro treated cell populations. Increased CV was observed after 2 1/2 weeks and to an extent higher than the other two cell populations. TPA always induced an additional increase in the CV.

2.2.2.2. Effect on cell cycle

The number of cells in the S- and G_2 - phase was calculated from DNA-histograms of the eight cell cultures subcultured 7 times. The values obtained were used as a marker for the proliferative capacity of the cell populations. A close correlation between the increase of CV and growth capacity was found. Clearly the FCM- analysis provided a tool not only to detect the acute disorders of the genome immediately after treatment with the carcinogen (genotoxic effect) but also to detect early instabilities in the genome most likely associated with advantage of growth of these cells within 20- 50 cell cycles.

2.2.3. Calcium-binding proteins as marker for preneoplastic cells

Cytosolic Ca^{+2} -ions and Ca^{+2} - binding proteins are detectable at elevated levels in some cancer cells and may have an impact on their uncontrolled proliferation. If certain Ca^{+2} - binding proteins were present in tumor cells but absent in their normal counterparts, they could also be used as marker for preneoplastic cells.

2.2.3.1. Evaluation and detection of tumor specific proteins

The appearance of Ca^{+2} -binding proteins was evaluated. Two of them namely protein A and oncomodulin, were suitable for our approach. They were absent in fibroblasts isolated from the subcutaneous tissue in rats without growth stimulation by an air pouch, but present in the malignant cell lines. Protein A has been described as a marker protein in cell lines from squamous cell carcinomas of the tongue and larynx (Pfyffer et al. 1984). Oncomodulin has been found in neoplastic cells and tissues as well as in human placenta. It was originally isolated from rat hepatomas (MacManus et al. 1982).

Protein A was identified on two- dimensional polyacrylamide gel electrophoresis (PAGE) according to the M_r 12,000, pI = 4.8 and by a distinct separation from parvalbumin in coelectrophoresis with ^{14}C - labelled parvalbumin (M_r 12,000 and pI = 4.9). Oncomodulin was pinpointed according to the M_r 11,500 and the pI = 3.9. In protein extracts of three malignant cell populations investigated, both proteins have been found to be heat stable and not precipitable when treated at 85°C for 30 min. Proteins were further characterized by HPLC analysis with regard to their hydrophobicities. The characterization of the two marker proteins by their molecular weight, their isoelectric point, heat stability and hydrophobicity suggests that these two proteins can be identified and might be used as marker proteins in preneoplastic rat cells. Further studies are going on to isolate tissue specific probes of the marker proteins for co-migration experiments and for the production of antibodies.

Pfyffer G. E., Heammerli G. and C.W. Heizman, Calcium binding proteins in human carcinoma cell lines, Proc. Natl. Acad. Sci. USA, 81, 6632-6636, 1984.

Mac Manus, J.P., Occurrence of low-molecular-weight calcium- binding protein in neoplastic liver, Cancer Research 39, 3000-3005, 1979.

2.2.3.2. Appearance of marker proteins in normal and preneoplastic cells

Analysis was then extended to the untreated granulation tissue induced by the subcutaneous application of air. As early as three days after pouch formation, both proteins were detectable. This result corresponds well with the clone formation capacity of freshly isolated untreated cells in soft agar (see 2.1.4.). Protein A and oncomodulin were not detectable in subcultured normal cells. Again this is in agreement with the findings that clone formation capacity in soft agar was drastically reduced after subcultures in vitro (see 2.1.4.). Control experiments with the freshly isolated cells in living hosts confirmed that they never grew out to tumors in the selected nude mice strain. In vivo, in long-term carcinogenicity studies, this observation finds its parallel. Small nodules were palpable in rats without treatment at the site of air pouch formation. They never grew out spontaneously to fibrosarcoma (Flückiger 1983). These results indicate that the selected tumor associated proteins are not aberrant, but their synthesis is aberrantly controlled in tumor cells.

Further investigations were focused on preneoplastic cells. Standard experimental conditions were chosen. Cells were treated in vivo/in vitro with MNNG or in vitro only with aristolochic acid, the most powerful mutagen so far tested in the GPA (Maier et al. 1985). From the treated cells freshly isolated, primary and secondary cultures were subjected to established soft agar conditions with and without addition of epidermal growth factor. So far from the secondary cultures (6 - 12 subcultures) clone formation was recorded. This result suggests, that a genotoxic insult in vitro is required for the expression of anchorage independent growth. For tumor specific marker protein analysis, cells were isolated from the soft agar and for further accumulation injected into living hosts. From the derived tumors, protein extracts were

Flückiger, B., Wachstum und chemisch induzierte maligne Transformation von Zellen eines subkutanen Granuloms unter dem Einfluss von Stimulatoren der Zellproliferation, Dissertation No. 7292, Swiss Federal Institute of Technology, Zürich, Switzerland, 1983.

Maier, P., Schawalder H.P., Weibel B. and G. Zbinden, Aristolochic acid induces 6-thioguanine-resistant mutants in an extrahepatic tissue in rats after oral application, Mutation Research 143, 143-148, 1985.

analyzed. In all 12 cases so far tested, the tumor associated proteins were detectable (Sommer et al. 1985).

2.2.4. Mode of toxic and genotoxic activity of asbestos.

In a pilot experiment antibodies against microtubuli were applied to cells treated with Vincristinesulfate a chemical disturbing the microtubule formation. This resulted in a diffuse expression of the tubulin polymers in the cytoplasm of the cells. After exposure toward asbestos fibres no changes were detectable by microscopical examination. Therefore, priority was set to characterize the cytotoxic action of asbestos.

2.2.4.1. Nonenzymic and enzymic lipid peroxidation

Initiation of carcinogenic/mutagenic events often occur by free radicals generated intracellularly (e.g. ionizing radiation). Free radical formation due to lipid peroxidation is a well known mechanism. Since Fe-ions catalyse peroxidation and some of the asbestos fibres carry iron in their structure, the catalytic activity of asbestos fibres was investigated. Degradation products formed after lipid peroxidation are malondialdehyde, pentane and ethane. They were used to determine lipid peroxidation (Lang et al. 1985).

First, an in vivo model was established consisting of arachidonic acid as a substrate, cumenehydroperoxide as the inducer of the peroxidation and Fe III or crocidolite with the expected catalytic activity. Clearly with and without inducer, the amount of malondialdehyde formed increased with increasing dose of Fe III or crocidolite. Using liver homogenates as substrate, again Fe III and crocidolite stimulated lipid peroxidation. The reaction was inhibited in a dose-dependent way by butylhydroxytoluene and was inducible at an optimal concentration of ascorbic acid. The reaction was dependent on the pO_2 in the

Sommer, E.W., Baer, M., Heizman C.W., P. Maier and G. Zbinden, Tumor associated Ca^{+2} -binding proteins in chemically transformed rat fibroblasts, The Journal of Cell Biology, in press 1985.

Lang, B., Maier P. and G. Zbinden, Ethane and pentane formation measured by capillary gas chromatography, Experientia 41/6, 822, 1985.

Wydler, M., Maier P. and G. Zbinden, Stimulation of lipid peroxidation by asbestos, Experientia 41/6, 826, 1985.

incubation medium. Highest values were obtained under hypoxic conditions similar to those found in the subcutaneous tissue (Maier et al. 1986).

Furthermore, the different types of fibres were compared. In the model system using arachidonic acid, the efficiency of Fe III and the fibres were ranked as follows: Crocidolite > amosite > Fe III > anthophyllite > chrysotile. With the liver homogenate it was FeIII > crocidolite > amosite. No activity was found with anthophyllite and chrysotile. Further experiments were performed with freshly isolated hepatocytes. Lipidperoxidation takes place also in intact cells and therefore might be responsible for the observed cytostatic/cytotoxic activity observed in vitro. Since microtubules are involved in chromosome movement and derive from membrane fragments, it can be speculated that asbestos fibres in intimate contact with membrane-lipids catalyze the formation of radicals reacting with microtubules or directly with the genetic material.

3. Conclusion

In granuloma cells transformed in vivo, typical phenotypic alterations and growth characteristics were investigated. These markers were then used to detect premalignant properties of in vivo carcinogen-exposed cells isolated from the granuloma pouch.

When cultured in vitro, preneoplastic cells express alterations at the transcription/translation step. Discrete DNA- alterations are detectable within 2-3 weeks. The aberrant synthesis of calcium-binding proteins occurs after 2-4 weeks. Alterations which affect growth control at intercellular level (e.g. anchorage independence) require a second genotoxic insult. Additional growth stimuli provided by low pO_2 , by normal cocultured cells, by tumor promoters and growth factors shorten the the duration of culture time in vitro.

The genotoxic activity of asbestos fibres is most likely due to the catalytic stimulation of peroxidative processes.

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