QUANTITATION OF ASBESTOS-INDUCED HYPERPLASIA IN HAMSTER TRACHEA EPITHELIUM MAINTAINED IN ORGAN CULTURE

by

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ABSTRACT

QUANTITATION OF ASBESTOS-INDUCED HYPERPLASIA IN HAMSTER TRACHEA EPITHELIUM MAINTAINED IN ORGAN CULTURE

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Advisor: Professor Irving J. Selikoff

Exposure to asbestos has been associated with the development of cancer, especially bronchogenic carcinoma and mesothelioma. Epidemiologic and laboratory studies have confirmed that neoplasia develops after exposure to all forms of asbestos. For the development of bronchogenic carcinoma a synergism exists between cigarette smoking and asbestos exposure.

Although there are many *in vitro* laboratory models to investigate biological effects induced by asbestos, few have been able to quantitate changes noted. This study investigates and quantitates an effect of asbestos on respiratory epithelium, and relates the changes to the development of cancer.

Hamster tracheas were maintained in a chemically defined modified CMRL-1066 medium and were exposed to

asbestos and other materials. Most specimens were embedded in plastic, sectioned at 2u, and the cell number, as a function of tissue length or volume, was counted with image processing instruments. Consideration was given to possible sources of error in the techniques used, and data are presented to show that only the presence of asbestos explains the statistically significant difference in cell number noted. Amosite and chrysotile asbestos at a concentration of 1.0 mg/ml in the culture medium induced basal cell hyperplasia in respiratory epithelium within twenty-four hours after first exposure. Activated carbon and celite, a diatomaceous earth preparation, at 1.0 mg/ml, and lower doses of asbestos did not induce hyperplasia. Talc at 1.0 mg/ml induced hyperplasia but only after delay, when compared to asbestos. Variable doses of vitamin A did not modify the response to asbestos challenge. The tissue response to 1.0 mg/ml of amosite was noted to be consistent over a series of experiments.

Basal cells are the only cell type in respiratory epithelium capable of division, and must be considered as the precursors of any neoplastic cells that develop in this tissue. Hyperplasia often precedes neoplasia, and the hyperplasia induced by asbestos may aid the development of cancer.

FOREWORD

After some scientific training at the Environmental Sciences Laboratory of the Mount Sinai School of Medicine, the author joined the staff of the Lung Cancer Branch of the National Cancer Institute. He had the opportunity to investigate aspects of an environmental health problem. A colleague in the laboratory had recently developed a technique to maintain in short-term organ culture the tracheas of hamsters. The author wondered about what effects might result from exposure of these cultures to asbestos, especially since this kind of exposure in respiratory tract organ cultures had never been studied. The initial answer was soon clear; the problem then became to devise methods that allowed quantitation of the change noted. This study presents techniques developed to investigate this problem and attempts to relate the results to understanding the development of cancer.

ACKNOWLEDGMENTS

Scientific research requires help from many sources. I would like to thank Dr. Irving J. Selikoff for his guidance in the problems of science related to environmental exposure. This study has had the benefit of conceptual guidance from Dr. E. Cuyler Hammond. Much of this research was done while the author was on the staff of the National Cancer Institute, an opportunity for which he remains grateful. While there, the author was the recipient of kind and encouraging help from Dr. Lewis Lipkin and his staff, especially Morton Schultz and Marta Wade. Dr. Steven Geller of the Department of Pathology at the Mount Sinai School of Medicine was gracious in sharing his equipment and expertise. The library staffs of both the National Institutes of Health Library and the Levy Library at Mount Sinai were ever helpful in providing even the most unusual references. There have been many others who have given support and guidance, and I am individually grateful to them all. No acknowledgment would be complete without mention of the support and understanding of my wife, and also my mother.

DEDICATION

This work is dedicated to my mother, for those reasons she understands best.

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LIST OF ABBREVIATIONS

BP	3,4 Benzo(a)pyrene
Ci	Curie
DMBA	Dimethylbenzanthracene
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic Acid
EDTA	Ethylenediaminetetracetic Acid
H&E	Hematoxylin and Eosin
MCA	Methylcholanthrene
mg/ml	milligram per milliliter
ml	milliliter
mm	millimeter
mM	millimolar
mmole	millimole
PVPNO	Polyvinylpyridine-N-oxide
S.D.	Standard Deviation
S.E.	Standard Error of the Mean
UICC	Union Internationale Contre Le Cancer
u	micron
uCi	microcurie
ug/ml	microgram per milliliter
ul/ml	microliter per milliliter
$\bar{X}$	Mean

I. Use of Laboratory Models to Study Associations of Environmental Agents and the Development of Cancer

Epidemiological investigation of "experiments of nature" have been valuable in identifying environmental agents associated with neoplastic development in humans. Basic principles of cancer biology, such as dose-response relationships, latency periods, and age or sex differences, can be investigated with these techniques. For an understanding of some problems, such as the mechanism of disease production, laboratory models complement human population studies. A wide range of chemical and physical agents has been associated with neoplasia in man. In laboratory animals many of these agents, and in addition some viruses, have been related to the development of cancer.

Exposure to asbestos has been associated with the development of cancer in humans. Bronchogenic carcinoma and mesothelioma are the cancers particularly related to asbestos exposure, although it may also influence the development of cancer in other organs. Among individuals who smoke cigarettes and are occupationally exposed to asbestos, the risk of developing bronchogenic carcinoma is many times that of an individual who neither smokes

nor works with asbestos. The effect of smoking and asbestos exposure is not simply additive, since among insulators exposed to asbestos the risk of developing lung cancer is greater than the sum of the effects of either smoking or asbestos exposure alone, and smoking alone appears more strongly associated with the development of lung cancer than exposure to asbestos alone. For mesothelioma there does not appear to be a relationship to smoking. These associations and other problems of human neoplasia can be investigated with laboratory models.

Laboratory studies may be useful in understanding how changes in morphology may be related to the subsequent development of cancer. The use of culture models in the study of cancer development after exposure to some chemicals has been reviewed by Heidelberger (1973). He discusses the use of individual cell cultures and the use of organ cultures. For the purpose of this report the definition of Paul (1970) will be used to define the term organ culture. He states that this term is "usually employed to /mean/ small pieces of organ in vitro." He continues, "The object of organ culture technique is to maintain the architecture of the tissue."

This study utilizes organ cultures of hamster tracheas to investigate effects of asbestos on respiratory epithelium.

Studies of other agents have shown that some changes occurring soon after exposure may be associated with the development of neoplasia. This culture model facilitates investigation of some of the earliest changes induced by asbestos in respiratory tract tissue. Repeated tissue sampling after asbestos exposure in animals is impractical, and such sampling in humans is not possible. Controlling the amount of exposure is difficult in intact animals. This system allows for sampling over time with control of dosage, and quantitation of change was possible.

At the initiation of this study the author was unaware of prior work using tracheal cultures to investigate biological effects of asbestos, and to date there appears to be only a single publication (Mossman et al., 1977) which studies the effect of a type of asbestos not investigated with this system. It is not the purpose of this paper to review all laboratory models used to study the problems of neoplasia, but background in the use of organ cultures and of laboratory models used to study biological effects of asbestos is appropriate.

A. Organ Culture

Organ culture as a laboratory technique was first developed in Great Britain in the 1920's (Strangeways and Fell, 1926; Fell and Robison, 1929). It is becoming

an increasingly more common technique for the study of agents associated with the development of cancer.

Organ culture allows exogenous addition of materials which can then interact with the cell types under study in a manner that maintains anatomic and morphologic integrity of the tissue. Cell cultures allow investigation of one cell type isolated from others. With both culture systems one makes observations on cells that are independent of normal systemic circulation, endocrine influences, and other effects that require an intact animal. This may be disadvantageous if one wishes to study sequential patterns of enzyme activation, or hormonal influences mediated through the circulation, but can be useful in assessing changes resulting from direct interaction of tissues and materials under study.

Organ cultures are not sufficiently long-lived to allow for development of neoplasia after exposure to agents that may cause cancer. This difficulty may be overcome by reimplantation of tissue into whole animals. An advantage of the organ culture technique is the rapid assessment of biological changes induced by some materials or modifying agents.

B. Specific Organ Culture Models

1. Respiratory Tract Tissue

a. Lung. Early use of organ cultures to investigate the effects of agents associated with the development of cancer was the work of Lasnitzki (1956), who studied the effect of BP on human fetal lung. She demonstrated epithelial hyperplasias and metaplasias which she regarded as precancerous. Other of her studies have looked at the effect of cigarette smoke condensate on human fetal lung (Lasnitzki, 1958). Other investigators, using embryonic mouse lung, demonstrated that pieces incubated with MCA showed growth of adenomata and transplantable adenocarcinomata at the site of implantation on subsequent placement in isologous mice (Laws and Flaks, 1966; Flaks and Laws, 1968). Shabad (1971) has used embryonic lung tissue for transplacental studies.

b. Trachea and Bronchus. Trachea and bronchial tissue from several species of laboratory animals have been successfully maintained in organ culture. These include mouse (Lasnitzki, 1968), suckling rat (Crocker et al., 1965), and adult rat (Palekar, 1968).

Hamster tracheal cultures have been developed and used by several investigators. Clamon (1974) developed

a set of culture conditions, utilized in the present study, to investigate the effects of retinoids on cell differentiation. Mass and Lane (1976) looked at the effects of chromates on rat trachea, and Adalis (1977) studied the effect of cadmium on hamster trachea cultures. Mossman (1975) has investigated the use of different culture media and has examined the effects of crocidolite asbestos in such cultures (Mossman et al., 1977).

Bronchial tissues from several species have been grown successfully in organ culture and used for study. O'Donnell (1973) cultured human bronchial mucosa obtained at lobectomy and from bronchoscopy specimens, and studied its morphology. Harris (1974, 1976) and co-workers (Trump et al., 1975; Barrett et al., 1976) have cultured human bronchus obtained at surgical resection or "immediate" autopsy, studied its morphology both at the time of collection and after long-term culture, and have shown that BP binds to the DNA of human respiratory epithelium. In addition, bovine bronchial tissue has been cultured by this group in a similar manner.

The usefulness of these cultures is evident when correlations are made with the in vivo changes seen after exposure to cancer causing agents.

There have been reports of changes in human bronchial

tissues after cigarette smoking (Auerbach et al., 1961), and changes have been noted in hamsters after exposure to polycyclic hydrocarbons (Saffioti et al., 1968). Hilding (1962) has shown in isolated, but not cultured, bronchial tissue from freshly slaughtered cows and calves that ciliary activity may be affected by cigarette smoke.

c. Pleura. Rajan (1972) has cultured pieces of human pleura and exposed these to asbestos. He has reported proliferation of mesothelial cells after such exposure.

2. Urinary Tract Tissue

Culture of prostatic tissue has been used by several investigators. Lasnitzki (1957, 1963), among others, has cultured mouse prostate gland tissue and studied the effect of polycyclic hydrocarbons. Using this technique, Röller and Heidelberger (1967) were able to induce cancers in animals implanted with these exposed tissues, and Heidelberger and Iype (1967) were able to produce sarcomata in animals injected with cells from lines established from morphologically altered organ cultures that had been exposed to hydrocarbons. More recently Schrodt and Foreman (1971) have grown human prostatic tissue in organ cultures.

Fetal mouse kidney has been cultured by Shabad (1972) for use in transplacental studies; this has also been done

by Bogovoski and Sorokina (1973). Other studies have involved culture of rat bladder.

3. Breast Tissue

Dao (1972) has cultured rat mammary tissue, exposed these organ cultures to DMBA, and has obtained adenocarcinoma when these tissues were transplanted into isologous animals. Wellings and Jentof (1972) have maintained human breast tissue in culture.

4. Other Organs

Other tissues have been maintained in organ culture. These include skin (Lasnitzki, 1974), digestive system tissue including pieces of the esophagus (Lasnitzki, 1963), gastric tissue (Hayashi et al., 1975), and rectal mucosa (Eastwood and Trier, 1973), tissue from the reproductive system including the ovary (Jull et al., 1968) placenta (Hou et al., 1968), and human digits (Rajan and Hopkins, 1970). The development of organ culture techniques has been reviewed by Gaillard (1953).

II. Laboratory Models for the Study of the Biological Effects of Asbestos

The first association of lung cancer and asbestos exposure was made by Lynch and Smith (1935), although asbestosis was known and compensable in Great Britain prior to this report. The asbestos body had been described by Marchand (1906), although not so designated at the time. The firm association of asbestos exposure and the development of lung cancer was made by Doll (1955), and subsequently numerous reports have detailed this relationship. The association of asbestos exposure and the development of mesothelioma was emphasized by Wagner (1960), and the important synergism between asbestos exposure, smoking, and the development of lung cancer was made clear by Selikoff and associates (Selikoff et al., 1968).

Each type of commercially important asbestos has been associated with the development of human neoplasia. This includes amosite (Selikoff, 1972), anthophyllite (Kiviluoto and Muerman, 1970), chrysotile (McDonald et al., 1974), and crocidolite (Webster, 1973). The association of asbestos exposure and the production of many human diseases has been reviewed by Becklake (1977).

A. In Vivo Laboratory Models

Wagner and his colleagues (1974) produced both bronchogenic carcinoma and mesothelioma in rats after inhalation of asbestos. Exposure to all five forms studied resulted in the development of bronchogenic carcinoma, and all but one produced some mesotheliomata. No such cancers were noted in the unexposed controls. Length of exposure ranged from one day to twenty-four months. As little as one day's exposure yielded both lung cancers and mesotheliomata. Using instillation techniques, others have produced cancers in animals with asbestos, including Smith (1965) and Gross (1967). Several investigators, including Saffioti (1968) and Shabad (1970), have produced lung tumors in hamsters and rats without using asbestos. These investigators used BP and a carrier dust such as iron oxide or carbon black.

In addition to investigating the development of neoplasia, there have been studies of a descriptive nature reporting on morphological changes induced in the lungs or pleura of animals after exposure to asbestos. Suzuki and Churg (1969) have detailed the formation of the asbestos body, as has Davis (1970). Botham and Holt have

looked at changes in cell structure after instillation of various forms of asbestos and glass fibers in guinea pigs and rats (Botham and Holt, 1968, 1971a,b, 1972a,b).

The development of mesotheliomata and mesothelial cell alterations have also been studied after intrapleural or intraperitoneal injections. Wagner and Berry (1969) produced mesotheliomata after intrapleural injections of asbestos, notably after removal of contaminating polycyclic hydrocarbons. Stanton (1972) has extended this work by reporting the development of intrapleural tumors following injection of asbestos or fibrous glass. Asbestos has been injected intraperitoneally with the subsequent development of mesotheliomata (Shin and Firminger, 1973; Engelbrecht and Burger, 1973; Davis, 1974). Harington (1975) has reviewed both these *in vivo* and many *in vitro* studies of asbestos-induced biological alterations.

B. *In Vitro* Laboratory Models

In vitro laboratory models have been utilized to assess biological interactions of cells and some mineralogic materials. The first such investigation in the area of pneumoconiosis research was by Fenn (1921a,b), who examined the phagocytosis of quartz and coal dust by rat exudate leukocytes. Since then, several kinds of models have been used.

1. Hemolysis

The study of hemolysis of erythrocytes is a simple and rapid method of studying the effects of various materials on membranes and possible modifications of these effects. This technique has also allowed prediction of cytotoxic effects induced by asbestos and other dusts in other cell systems. First used by Harley and Margolis (1961), then by Stadler and Ströber (1965) to study the effects of silica, MacNab and Harington (1967) used this technique to study the effects of several forms of asbestos and some related materials and also the effects of certain compounds such as EDTA and PVPNO to modify these interactions. This subject has been well reviewed by Schnitzer (1970,1972) and Harington (1975).

2. Macrophage Cultures

A widely used technique to study biological effects of asbestos and related materials has been macrophage cultures. Most often these are freshly prepared from a wide range of animals, including mouse (Allison, 1970; Pernis and Castano, 1971), rat (Koshi et al., 1969), guinea pig (Parazzi et al., 1965), and hamster (Bey and Harington, 1971), and cultured in serum supplemented media. These cells are no longer capable of cell division and die

out over time with a continuously decremental growth curve. Rabbit macrophages have been used to study metal toxicity in culture (Waters et al., 1975). There is a report of a macrophage-like cell, $P388D_1$, which was used to study asbestos effects in an established, regenerating cell population with phagocytic capacity (Wade et al., 1976).

Taken together, these studies have allowed observations of cytotoxic effects of various forms of asbestos, silica, and other dust preparations, and have also yielded information on modification of such effects by materials such as PVPNO. With some exceptions, the data from these experiments were descriptive or were quantitated only by a subjective grading system, such as that employed by Marks (1956) or indirect quantitation using data from some chemical analysis (Parazzi et al., 1968; Miller and Harington, 1972).

3. Anti-Growth Effects

Other biologic effects have been associated with asbestos. Schnitzer (1972) has shown that under some circumstances asbestos has an antibiotic-like effect. Litterest (1970) has shown that growth of HeLa cells in a commercially prepared growth medium is inhibited if the medium is sterilized by passage through a Seitz filter.

4. Organ Cultures

a. Non-Trachea Cultures. There has been a small amount of work investigating the effects of asbestos in organ culture systems. Mention has already been made of human pleural cultures of Rajan (1972) and the increase in the thickness of mesothelial cells seen after asbestos exposure of these explants.

Some years ago Davis (1967a,b) published reports outlining a method for culture of guinea pig lung in organ culture and described the morphologic changes noted in these cultures. He continued by examining the effects of chrysotile on lung macrophages maintained in such cultures. Asbestos fibers were quickly engulfed by macrophages, were held in phagocytic vacuoles, and these fiber-containing macrophages formed giant cells within a short period of time.

b. Hamster Trachea Organ Cultures.

1. General Considerations. The use of hamster tracheas in organ culture systems follows naturally from the use of these animals for experimental lung cancer studies. Hamsters have several advantages over mice and rats; since their respiratory tract harbors fewer potential pathogens, they are more resistant to lung infections and

are less likely to succumb to lung diseases unrelated to the disease under study. One disadvantage of using hamsters is the scarcity of genetically identical strains, but for many studies the use of random-bred animals is appropriate. The laboratory model in the present study used such random-bred animals. The culture techniques utilized result, in part, from studies of potential anti-cancer agents. Using hamster trachea cultures derived from animals raised on a vitamin A deficient diet (Clamon et al., 1974), it was possible to show that addition of vitamin A to the culture medium reversed deficiency-induced metaplasias. The culture technique had been developed using a standard culture medium with testing of possible additives based on published data that some compounds added to standard media help maintain tissues in a more optimal state. Such compounds included insulin and steroids. One of the requirements of this research was the use of a completely chemically defined medium, without serum. This allowed exact quantitation of all constituents in the final medium, even after additions. Analogues of vitamin A were investigated for their ability to reverse metaplastic changes in these cultures (Sporn et al., 1974). In part because of these studies retinoids have been proposed as

anti-cancer agents. These compounds would be useful if they could be shown to reduce the risk of developing cancer, and the use of model systems is one method of examining how such compounds might alter biological activity after exposure to cancer causing agents.

2. Asbestos Studies. Subsequent to the initiation of this investigation a hamster trachea organ culture model was used to study the effects of crocidolite asbestos (Mossman et al., 1977). In that study crocidolite concentrations from 1.0 mg/ml to 40.0 mg/ml in the culture media were investigated. Tissues were sampled after periods from one hour to four weeks to evaluate cytotoxic alterations. The techniques used included light, scanning, and transmission electron microscopy and radioautography. The authors reported"percentage of cultures with epithelial changes." No data were presented after exposure of the cultures to 1.0 mg/ml of crocidolite. Changes were noted by transmission electron microscopy in cultures exposed for as little as one hour. Basal cell hyperplasia was noted as early as twenty-four hours after exposure to crocidolite. Phagocytosis of crocidolite by hyperplastic basal cell tissue was noted after sloughing of superficial necrotic epithelium. Fibers were also found in connective tissue of the submucosa.

III. Methods and Materials

A. Introduction

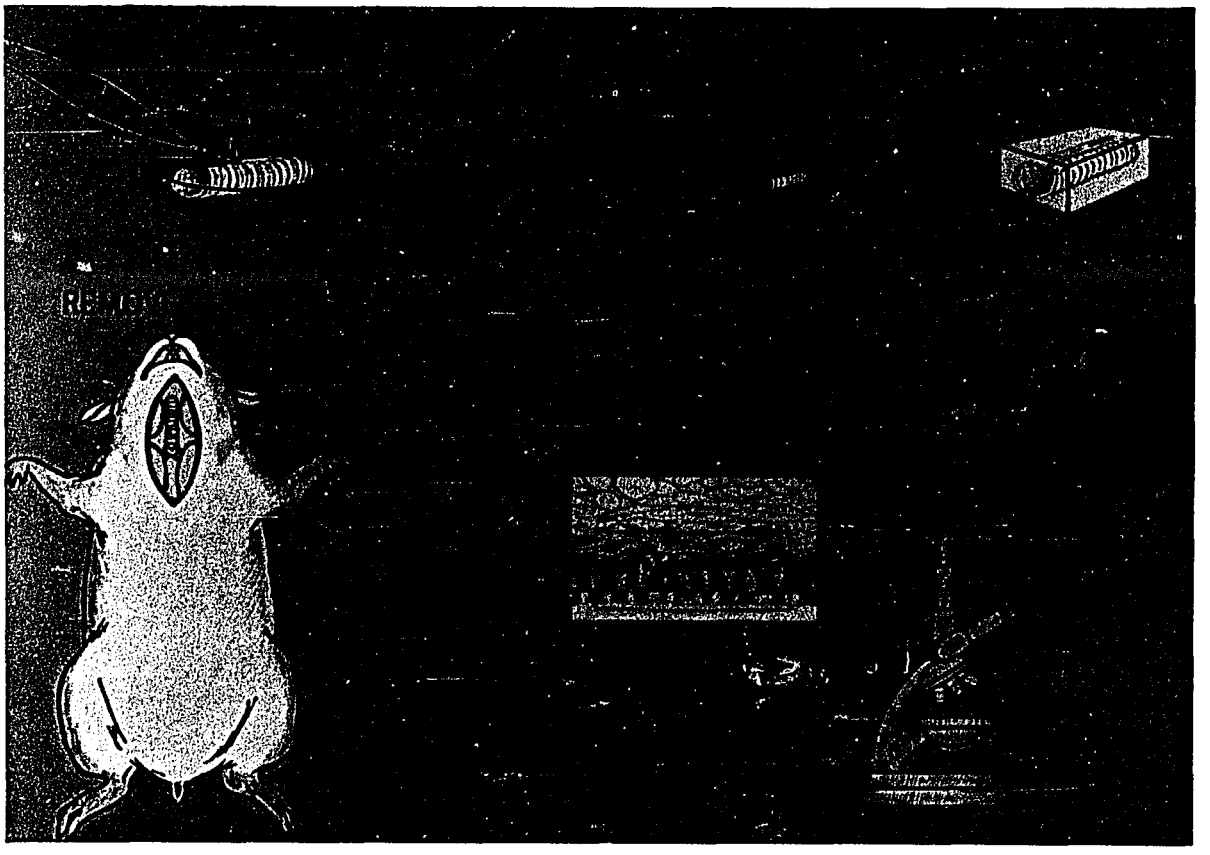
This research utilized some materials, procedures, equipment, and techniques that are used in many laboratories, and some that are limited to as few as a single facility. For evaluation of biologic change there was no need to employ a subjective grading system or use category quantitation or to rely solely on descriptive morphology, as useful as all these techniques may be. All data are direct and quantitative. Anticipated limitations of equipment and experimental design were considered, and appropriate investigations were conducted to eliminate potential sources of error. Manual procedures, although tedious and time-consuming, were used when equipment could not be relied upon to produce accurate results.

The basic scheme for this research is shown in Figure 1.

B. Animals

Tracheal cultures were established from male random-bred Syrian golden hamsters of approximately eight weeks of age. The animals had been maintained in isolation from any other animals with exposure to asbestos. Prior to

Figure 1



Diagrammatic scheme of research plan

killing they were maintained on a fully adequate pelleted hamster chow and given water ad libitum.

C. Preparation of Cultures

For preparation of the cultures groups of six to ten were killed by intraperitoneal injection of a lethal dose of freshly prepared sodium pentobarbital (Eli Lilly Co.). After swabbing the skin with alcohol the tracheas were removed using sterile techniques, which included changes of alcohol-bathed instruments, and were placed in a medium, designated the holding medium, used to maintain the tissue until final preparations were completed. Excision of the trachea was always preceded by exsanguination from the abdominal aorta, giving a relatively bloodless field, and care was taken not to damage the esophagus to reduce potential bacterial contamination. Each trachea took several minutes to excise, and no animal had been anesthetized for longer than thirty minutes.

After initial dissection, from just below the larynx to just above the bronchial bifurcation, each trachea was cleaned as well as possible from all connective tissue and then carefully cut open along its membranous portion using 8 mm blade scissors. This did not damage tissue within the trachea. A freshly prepared tracheal organ culture is pictured in Figure 2.

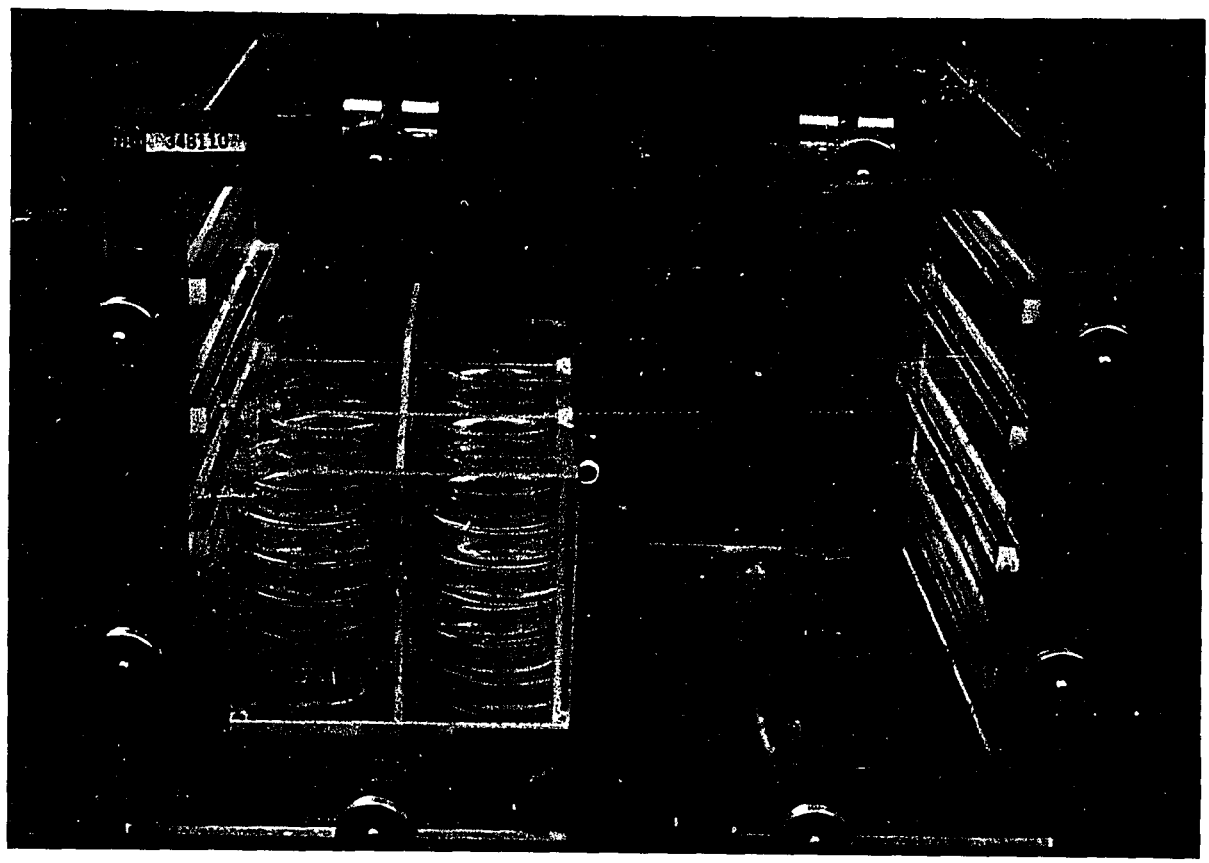
Figure 2

Close up of freshly prepared hamster trachea culture. Cut below larynx and above the bifurcation, and opened along its membranous portion.

The prepared trachea was placed in a sterile 60x15 mm Petri culture dish and two ml of prepared growth culture medium were added. After several such dishes had been prepared, they were transferred from the hooded work area to an incubator set at 37°C, in which a 5% carbon dioxide:95% air mixture was freely circulating. After all tracheas had been prepared, they were put in appropriate containers for the duration of the experiment. This involved stacking the dishes in a special plexiglass carrier, which was then placed into a gas-tight box (Bellco Glass Co.). Figure 3 illustrates this apparatus. Air in the box was evacuated and replaced with a special gas mixture of 5% carbon dioxide, 50% oxygen, and 45% nitrogen (Matheson Gas Co.). Boxes were placed on platforms which were set to rock back and forth twelve times per minute (Bellco Glass Co.). These rocker platforms were located in a large incubator with the temperature maintained at 36.5°C. Routine maintenance included checking the temperature at least twice each weekday; there was little variation from the pre-set temperature.

The culture medium was usually changed every other day for fresh medium or fresh medium containing materials under study. Cultures were always re-gassed before returning

Figure 3



Gas-tight box with stacks of culture dishes. The box was placed on a rocker-platform inside an incubator maintained at 36.5°C, and rocked 12/min.

them to the incubator. Media changes were accomplished by sucking off spent medium with a sterile Pasteur pipette. These changes were made in a sterile hood.

D. Culture Media

1. Holding Medium

When first removed, the tracheas were placed in warmed L-15 medium (Grand Island Biological Co.) (Lebovitz, 1963). After short-term holding and completion of more careful dissection the tracheas were placed in the growth medium. A holding medium was used to maintain nutrition for the tissue at all times, and L-15 was selected in part for its good buffering qualities in room air.

2. Growth Medium

In order to maintain complex tissues, such as these tracheal organ cultures, the growth medium was based on CMRL-1066 (Grand Island Biological Co.) (Parker et al., 1957). This is an enriched, totally chemically defined medium, and was supplemented with 2mM L-glutamine (Grand Island Biological Co.), 1.0 ug/ml insulin (crystalline bovine insulin, kindly supplied by Eli Lilly and Co.), 0.1 ug/ml hydrocortisone (hydrocortisone hemisuccinate, kindly supplied by the Upjohn Co.), an antibiotic mixture of 100 units/ml penicillin and 100 ug/ml streptomycin

(Grand Island Biological Co.), and 0.1 ug/ml of B-retinyl acetate (vitamin A, kindly supplied by Hoffman-LaRoche) which was dissolved in sterile DMSO (final concentration 1 ul/ml). In those experiments using a higher dose of vitamin A the final concentration of DMSO was maintained at 1 ul/ml.

E. Materials Studied

1. Asbestos

Asbestos is a general term used to describe several forms of fibrous, hydrated silicate materials. All share common properties of: (1) separability into thin fibers which may be woven or fabricated, (2) chemical resistance, and (3) suitability as electrical and thermal insulators. Chemical composition varies, especially with regard to MgO and FeO content, but the SiO_2 content of the commercially important forms is more constant (40-50%).

Although commercial asbestos is available from many sources, characterization of most materials would be difficult. For some investigations special forms of asbestos have been prepared in laboratories that are uniquely equipped to characterize such materials. Fortunately, there is a series of well-characterized specimens supplied to investigators throughout the world through the Union

Internationale Contre Le Cancer (UICC). These UICC specimens have been carefully prepared (Timbrell and Rendall, 1971/72) and well characterized (Timbrell, 1969; Rendall, 1972) in terms of size distribution, surface area, analysis of trace metals, and other factors. For some studies, such as investigating effects related to fiber size, they might be unsuitable, but there is great benefit in having well-characterized standard samples in sufficient quantity to allow for some degree of comparability between laboratories. At present one of the active areas of asbestos-related research is the study of fiber size as a determinant of disease, but this area is not addressed in this study.

There are five standard materials available as UICC samples: three amphiboles, amosite from a source in South Africa, Finnish anthophyllite, South African crocidolite, and two samples of serpentine material, that marked A, chrysotile from Rhodesia, and that marked B, chrysotile mixed from eight Canadian mines in rough proportion to their overall commercial production. UICC amosite and UICC chrysotile B were used in this study. These materials were shipped, and kept, in brown paper bags, minimizing any contamination from plastic or jute storage bags, and were stored in cardboard cylinders. For experimental use the

asbestos was steam sterilized at about 100°C for ten minutes and then dried.

These conditions are not known to alter the asbestos fibers. These dried, sterile specimens were weighed as needed, usually into sterilized bottles into which appropriate amounts of fresh media were added just before use in experiments. With the exception of two experiments, in which multiple doses of asbestos were used, the concentration of asbestos, or any other material studied, was 1.0 mg/ml of culture medium.

2. Other Materials

Other materials were used for comparison with asbestos-induced effects to determine if the changes first seen with asbestos were specific. Subsequent interpretation of results would be more meaningful if this were true.

a. Activated Carbon. For this experiment, Darco Brand activated carbon (Atlas Chemical Industries, Inc.) was handled in a manner similar to the asbestos materials. After steam sterilization it was prepared for addition to cultures in the same manner.

b. Celite. Celite (Johns-Manville Products Corp.) is a commercially available preparation of diatomaceous earth obtained from deposits at Lompoc, California.

Normally a laboratory filtering material, it was used in these investigations because of the similarity of chemical composition to, but difference in form from, asbestos.

c. Talc. In a like manner, talc was investigated because of its chemical similarity to asbestos, but difference in form. Laboratory grade Talcum Powder (Fisher Scientific Co.) was prepared and used in a similar manner to other materials. This material was listed as meeting U.S.P. specifications, but there was no analysis of possible asbestos contamination, known to be present in some talcs, nor was one available from the supplier.

F. Tissue Preparation

A culture experiment was terminated by rapid fixation in buffered neutral formalin (Sanders, 1972) and kept in this fixative for a minimum of forty-eight hours. Storage of specimens prior to additional processing was either in this solution or in 70% ethanol.

Initially, specimens were embedded and cut in paraffin blocks, then stained with either H&E, or with use of the Feulgen reaction. The Feulgen reaction involves hydrochloric acid, Schiff's reagent, and sodium bisulfite to stain the DNA of cell nuclei a purple color, with use of a Fast Green counterstain for contrast (Sanders, 1972).

This allows rapid identification of cells and facilitates counting. Paraffin sections were routinely cut at 5u. While acceptable, improvements were sought, and other processing methods were subsequently adopted.

Because of increased clarity, and subsequent simpler cell identification, plastic embedding was used. Although more tedious to process, and more difficult to cut than paraffin, this technique, using a methyl methacrylate polymer (Polysciences, Inc.), greatly improved the tissue appearance and better preserved morphological relationships. The blocks were prepared by mixing two solutions which caused a polymerization reaction to occur, hardening in and around the tissues. The plastic used was comparatively soft, unsuitable for electron microscopy, but quite good for "thin" sectioning (i.e., less than 5u). With this material, all routine sections were cut at 2u using a JB-4 rotary microtome (Sorvall Co.), using hand-made glass knives. Whenever possible two tracheas were embedded in the same block to facilitate handling of the tissue and reduce study time at the microscope. The methyl methacrylate allowed for staining with H&E, but did not accept a Feulgen stain. Thin sectioning was thought useful for autoradiographic work, facilitating the analysis of grains over individual cells.

G. Scanning Electron Microscopy

Some of the trachea cultures, with and without exposure to asbestos, were subjected to scanning electron microscopy. (This was kindly done by Dr. Curtis Port of the IIT Research Institute, Chicago, Ill.) A glutaraldehyde-based fixative was used for tissue preparation, and several tracheas were examined after six and eleven days of culture. This technique was thought useful to examine surface morphology and to assess adequacy of the culture technique and conditions of culture.

H. Autoradiography

Attempts at autoradiographic analysis of the cultures were made. Cell labelling was accomplished with tritiated thymidine (thymidine[methyl-^{3}H], specific activity 6.7 Ci/mmole, New England Nuclear) and incorporation was allowed to proceed over a four hour period immediately prior to fixation. The dose used was 50uCi/ml of culture media. This technique was used to demonstrate the selective capacity of basal cells to divide under the culture conditions.

Autoradiographs were prepared by dipping unstained slides into a photographic emulsion (NTB-2, Kodak Co.) made liquid by warming to 45°C. These dipped slides were then placed in light-tight boxes and held at 4°C for

a period of seven to ten days. Developing took place at 15°C so that the emulsion would not wash off the slides. After developing the slides were stained with H&E.

I. Analysis and Quantitation

1. General Considerations

Since more than simple descriptive morphology was deemed essential for this study, and since it was thought unsuitable to use a subjective grading system, proper quantitation of biologic changes became essential. Image analysis methodology was considered as one means of arriving at quantitative data. The status of this instrumentation has been reviewed by Lipkin and Lipkin (1975).

Unfortunately, it soon became apparent that an instrument was not yet available which could, without considerable error, match the complexity of the human eye and brain in identifying individual cells and tallying this information. The crux of this difficulty is the inability of image analysis instruments to recognize oddly shaped nuclei or to discriminate when two cells are overlying. The Image Processing Unit at the National Institutes of Health made available a unique apparatus designed for image analysis of biologic materials prepared on slides. (This instrument was kindly made available by Dr. Lewis Lipkin and his staff.)

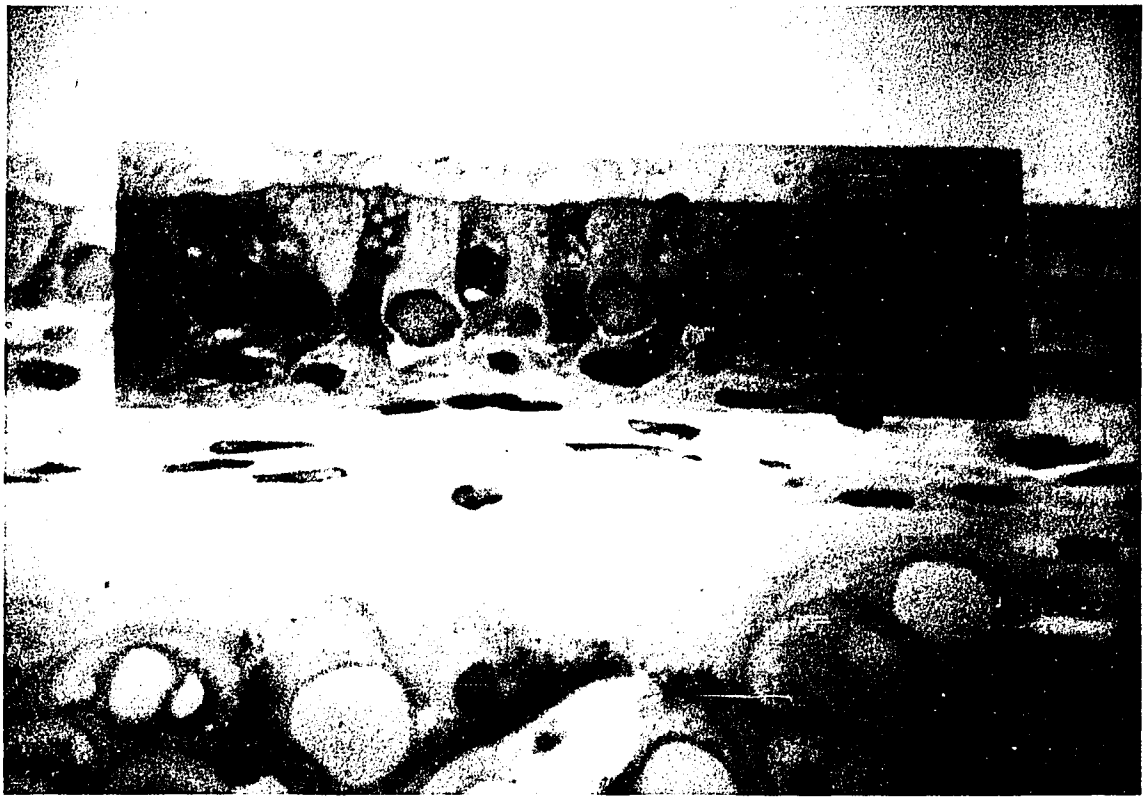
The apparatus (Lipkin et al., 1974) contained a trinocular eyepiece Zeiss microscope with a small television camera mounted on the single viewing tube. The camera transmitted an image to a screen in front of the observers' working console. The Quantimet imaging system (Imanco) and information storage was under the control of a PDP-8 computer (Digital) which contained programs that allowed various manipulations by the investigator. These maneuvers were carried out via controls at the working console and allowed fine and coarse adjustment of focus, automatic placement of various filters, and two-dimensional movement of the motorized stage by manipulation of a "joystick." Orientation of the tissue on the viewing screen in a direction that allowed for linear measurement was accomplished by manually rotating the television camera. Electronic imaging available within the system allowed for projection on the screen of any length of shading to be superimposed on the image of the tissue on the screen. This facilitated and standardized the cell counting. The standardized length along which the cells were counted was 0.1 mm, as measured from a standard microscope micrometer disk (Bausch and Lomb). Because of the great magnification used the naturally curved tissue appeared in a straight

line over this length. All cell counts were manually recorded. An illustration of the clear image available for this work is shown in Figure 4.

Suitable programs were available to determine if the instrument could match human abilities to identify individual cells. Studies showed this was not possible. When a commercially available automated imaging device was evaluated for the purpose of counting cells, it also did not produce accurate results. Even with the ability to determine up to sixty-four grey tones, and using shape analysis, these instruments do not allow accurate counting of cell number, even along short distances. All work was done with human evaluation and counting, the process facilitated by the sophisticated apparatus available for this work.

Some data were not collected with the system described. Equally suitable analysis was made with a simpler but adequate apparatus available in the Deparment of Pathology at the Mount Sinai School of Medicine (kindly made available by Dr. Steven Geller). This apparatus was simply a television camera mounted on a trinocular Balplan microscope (Bausch and Lomb) and projected onto a commercially available color television set (SONY). All control was

Figure 4



View of tracheal epithelium on the screen of the image processing instrument. Shaded area is bracketing a 0.1 mm length of tissue. Approximately 800x.

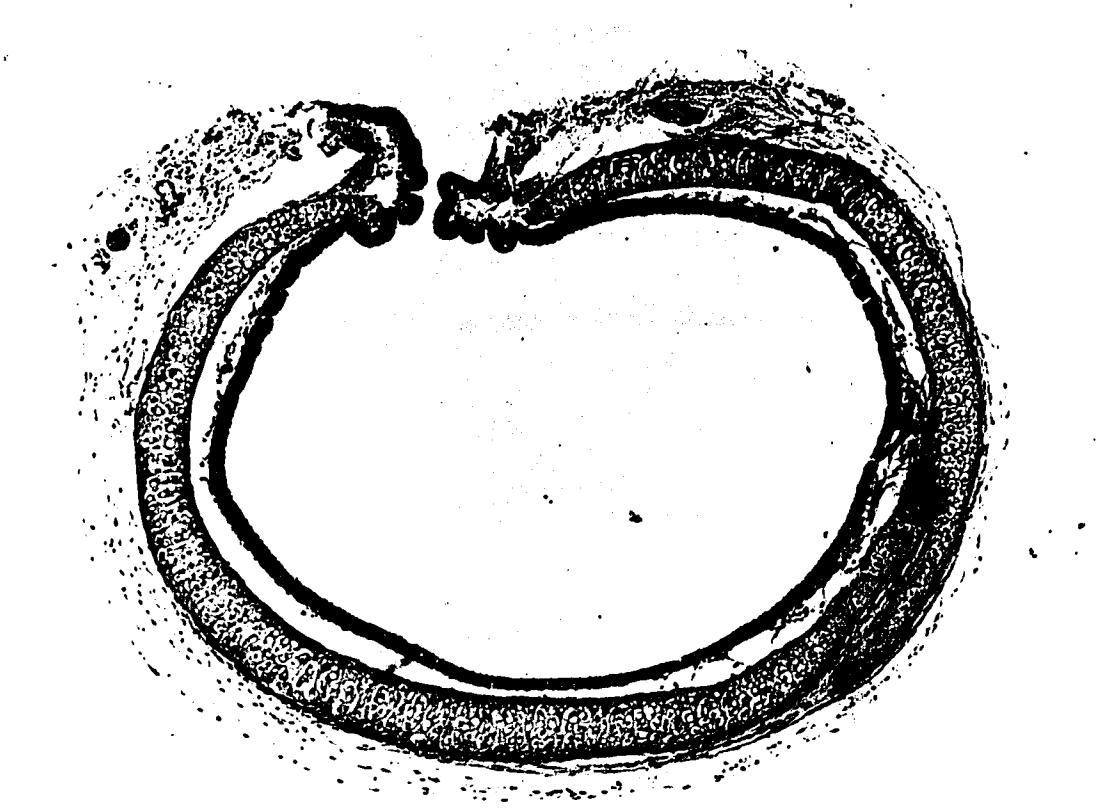
manual, and internal electronic imaging was not available for length projections. Tissue sections could be projected with sufficient magnification to straighten out the curvilinear nature of the tissue, but, since the magnification was somewhat lower, 0.2 mm lengths were counted at one time. Calibration was with the same micrometer disk, fixed lines being drawn directly on the screen surface for length measurements. Since the magnification was less and the color shading not as sharp as on the previously used system, the data obtained were not compatible with that from the other system. The values obtained on this second system were consistently lower than on the first. No experimental comparisons were made between data obtained on the two instruments.

There were checks on the data collection process, the same data being recollected at several different times, with evaluation of variability by the same observer at different times, and between the two machines. Counting the same slides at different times showed variability of about 2-3%. Although differences were noted between the two machines used, they were always consistent, and in one case the same set of data generated lines with virtually identical slopes, although the absolute numbers varied.

For evaluation of tissue changes a standardized technique was developed to collect data. After all slides from a single experiment were randomized, they were counted and records were made of the number of cells present per unit length of tissue on multiple sections of the same tissue. The slides were not classified as to experimental group until after all the slides had been counted. Although in some cases it was possible to identify asbestos or some other study material, there was no way of knowing the time point of any individual slide. No bias was thought to have resulted since complete identification of slides was not available until all slides were counted.

For each section of trachea to be examined, the area opposite the incision was identified for counting. This area was selected because it was a fixed point which could easily be identified and was far removed from the incision trauma so that regenerative activity at the cut edge would not be an artifact. From this sampling point, several adjacent areas were analyzed on several sections of the same trachea. A low-power view of a section, illustrated in Figure 5, demonstrates how this was done. Sections were discarded from analysis if there was any defect, such as a fold or tear in the area selected for potential

Figure 5



View of routine tissue section. Incision through membranous portion. Cell counts were made in area opposite this incision to eliminate trauma artifacts. 54x.

counting. The area counted for any trachea represented a small portion of the total circumference, or total length of the tissue. As described, sections were counted along 0.1 mm or 0.2 mm lengths depending on the apparatus used, and a total of between 0.2 mm to 0.4 mm were counted on any section. Data were usually collected from three sections of the same trachea, representing a total depth of 6u for those sections cut at 2u, or 15u for the 5u paraffin sections. Because of the problems in cutting plastic-embedded tissue sequentially, as one is capable of doing with "ribbons" of paraffin material, it was not possible in all cases to get exactly serial sections. However, the assumption can be made that the tissue obtained came from sufficiently adjacent areas to be considered a three-dimensional representation of the tissue at some point along the trachea. No specific area was selected for sectioning, but some standardization was achieved by trimming down the trachea to its approximate mid-portion and then obtaining sections for analysis. The data obtained by this technique are reported as either the mean cell number for any trachea along a 0.1 mm or 0.2 mm length, or as mean cell number in an equal "three-dimensional" volume of tissue. No differences were noted with either reporting method when analyzed by the statistical methods used.

2. Section Thickness

Since data were tabulated as mean cell number per unit length, or in an equal tissue volume, it was considered that the resultant numbers might vary proportionally with the thickness of individual sections. To evaluate this a single trachea was embedded in the usual manner in the plastic embedding material and sequentially cut in 0.5u steps from 2.0u through 5.0u and analyzed for number of cells per 0.1 mm as a function of increasing thickness of sections. All experimental tissues that had been embedded in plastic were cut at 2u. Although there is a known variation in the ability of microtomes to cut sections of uniform thickness--more of a problem with rotary than with slide microtomes--it was thought that any variation in large numbers of cut sections would be randomly distributed, especially since the blocks were cut randomly. In one instance, this potential variability was directly controlled by embedding within the same block a tracheal culture that had been exposed to asbestos along with one that had not. The counting of the tissues was done using parallel sections of tissue with matched sections used for each data point. This would eliminate as a source of difference any non-random variability in tissue sections cut by a particular microtome.

3. Differences in Total Tissue Length

Another anticipated source of potential error, using the technique employed, was if there was a difference in the total length of tissue used from which the small lengths were counted. Since age-matched animals were used for all experiments, it was thought unlikely that there was non-random variation in the size of the animals used. However, there might be some contraction or elongation of tissues in culture, depending upon whether they were exposed to a study material or not. Although this was considered unlikely, since the epithelial tissue under investigation grows fixed to a cartilagenous matrix, one explanation of differences might be as a result of contraction of the tissue causing a piling up of cells, or an elongation resulting in a thinning out of cells, reflected not in any portion of the length, but as a variable of the total length of the culture. A technique was devised to study this potential difficulty.

Using microprojection equipment (Nikon), several sets of experimental tissues were projected at a uniform distance, and tracings of the epithelial cell layer were made. The length of these sets of irregular curves was measured using a planimeter (Keuffel and Esser Co.) and

the data analyzed for differences in total epithelial tissue length. If the tissue lengths were similar for exposed and unexposed tissues, then variability in tissue length could be eliminated as an explanation for differences noted. Fixation and tissue preparation had always been done in parallel for all tissues from any single experiment.

4. Statistical Methods

Student's t-test was used to evaluate the differences in the means of samples of tissue of equal and unequal size (Snedecor and Cochran, 1967). This parametric test was used after F-test analysis of the variances allowed the t-test to be applied. For experimental comparisons using the t-test the p-values were determined and considered significant if $p \leq 0.05$. Inequality in the size of groups resulted from several factors. These included planned inequality of group size to maximize limited resources, loss of individual specimens through improper culturing or handling, or unsuitability of a specimen at the time of section analysis because of such factors as an obviously tangential cut, or folding or tearing of tissue in the area to be evaluated.

It should be noted that small numbers of samples were used, possibly making analysis of groups with small differences difficult. This has been overcome in this research.

Most data presented will show (1) the number of individual tracheas within a group, n, (2) the mean for that group, ($\bar{X}$), and (3) the standard error of the mean, S.E. P-values will be given for each comparison. Other statistical techniques were used as appropriate for evaluation of some of the data.

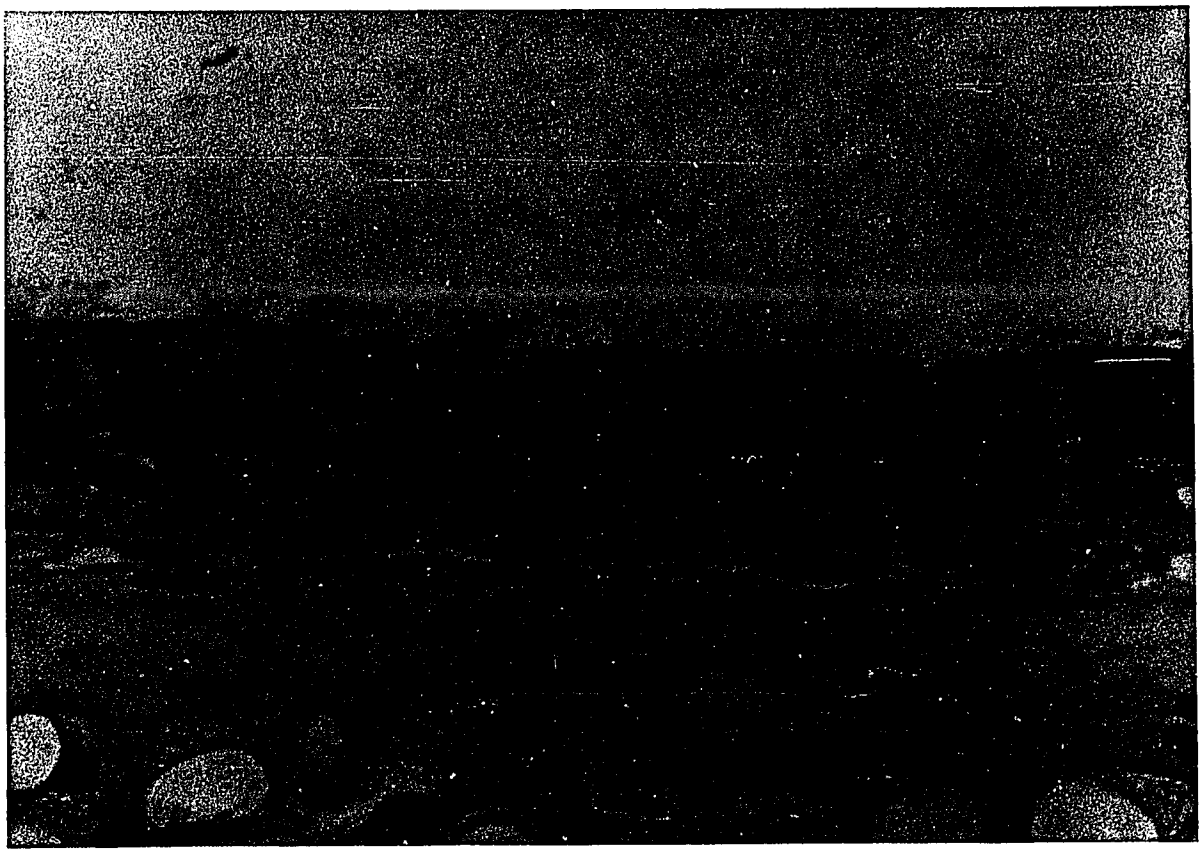
IV. Results

A. General Considerations

Addition of amosite or chrysotile asbestos caused hyperplasia of hamster trachea epithelium maintained in organ culture, under the conditions described. Non-asbestos materials when added at the same concentration did not produce this effect, demonstrating selectivity of the response. Directly recorded cell counts were analyzed for all experiments. The experimental design considered possible sources of error that might account for differences noted. The presence of asbestos appears to be the only variable that could account for increased cell numbers.

Normal tracheal tissue in the hamster consists of three cell types, generally present in two cell layers. The upper cell layer is composed of ciliated cells and mucin-producing cells, in a pattern ranging from simple columnar to pseudostratified columnar. The second layer is that of the basal cells. The ciliated and mucin-producing cells are generally oriented vertically, while the basal cells are more rounded and appear to be oriented horizontally. This normal morphology is illustrated in a four-day old culture in Figure 6a. The hyperplasia induced by asbestos primarily affected the basal cells,

Figure 6a



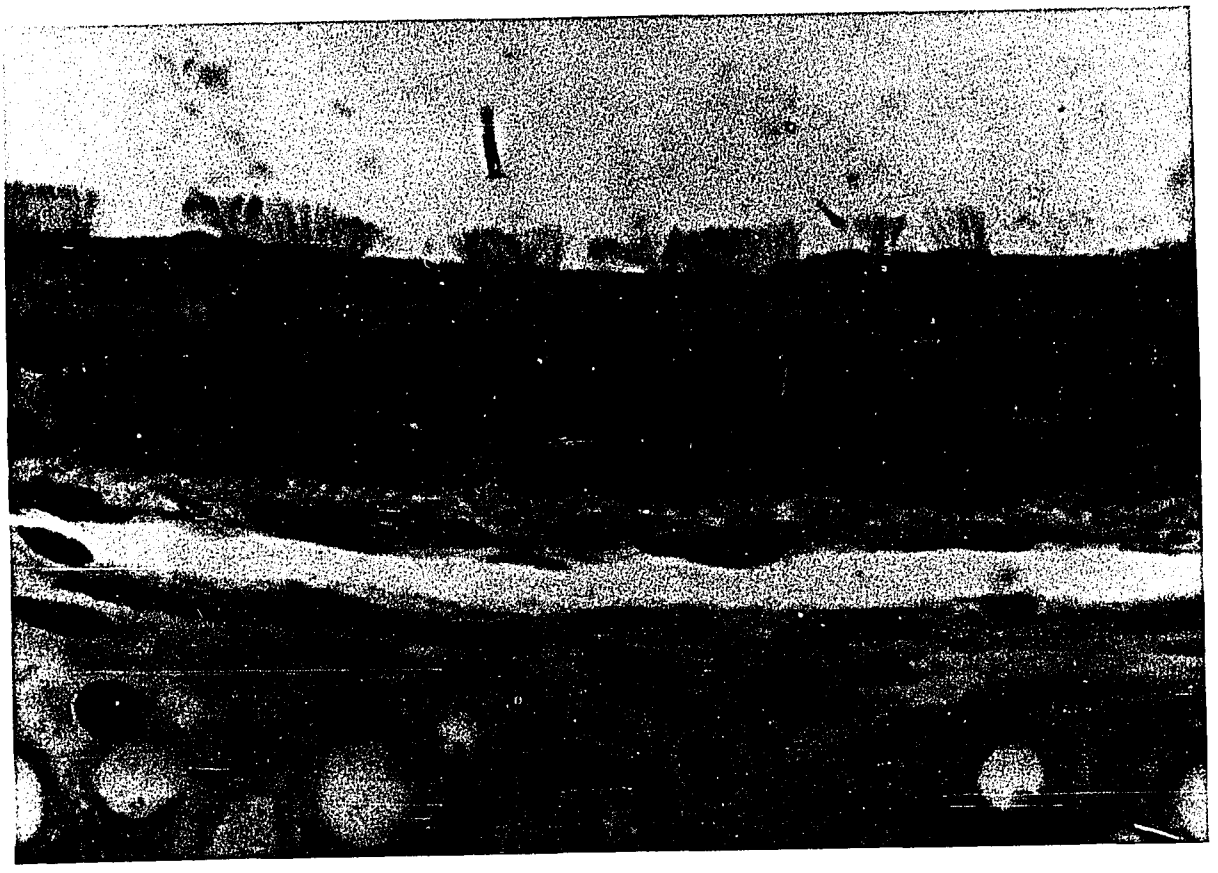
Four-day-old untreated culture. 2u section. Clearly visible is a layer of ciliated and mucin-producing cells, and a single layer of basal cells with no cell overlap. Approximately 500x, H&E.

causing a multiple layering that was not seen in unexposed tissues. This hyperplasia is illustrated in a six-day old culture after a two-day exposure to amosite asbestos, shown in Figure 6b. Although differential cell counts of all three cell types were not made, at no time did there appear to be an excess of ciliated or mucin-producing cells.

B. Standard Growth Curve

Table 1 shows the mean values of a representative growth curve of untreated cultures maintained under standard conditions. The initial increment in cell number, from Day Zero to Day 2, while not statistically significant, is a general feature of this technique and reflects the richness of the culture medium. Although not carried past ten days in this experiment, the downward trend in cell number reflects the degeneration of the cultures over time, starting after about ten days in culture. There is a continuing decrement in cell number after this time, and most observations were carried out in the most stable period between the second and eighth day in culture. Always noted was a variability of the individual tracheas. For this experiment, each value represents the average of ten one-tenth millimeter

Figure 6b



Six-day-old culture after treatment with 1.0 mg/ml amosite from Day 4 through Day 6. 2u section. In addition to a layer of ciliated and mucin-producing cells basal cell hyperplasia is illustrated by multiple layers of basal cells, with evidence of cell overlap. Photographed at same magnification as Figure 6a. H&E.

Table 1

Mean cell number per 0.1 mm for untreated tracheas at two-day intervals.

Day	n	Mean ($\bar{X}$)	S.E.
0	6	14.6	.808
2	6	16.15	.723
4	6	15.9	.298
6	5	15.8	.953
8	8	15.45	.502
10	6	14.3	.592

Analysis of variance generates F=1.29, demonstrating no effect by day of culture.

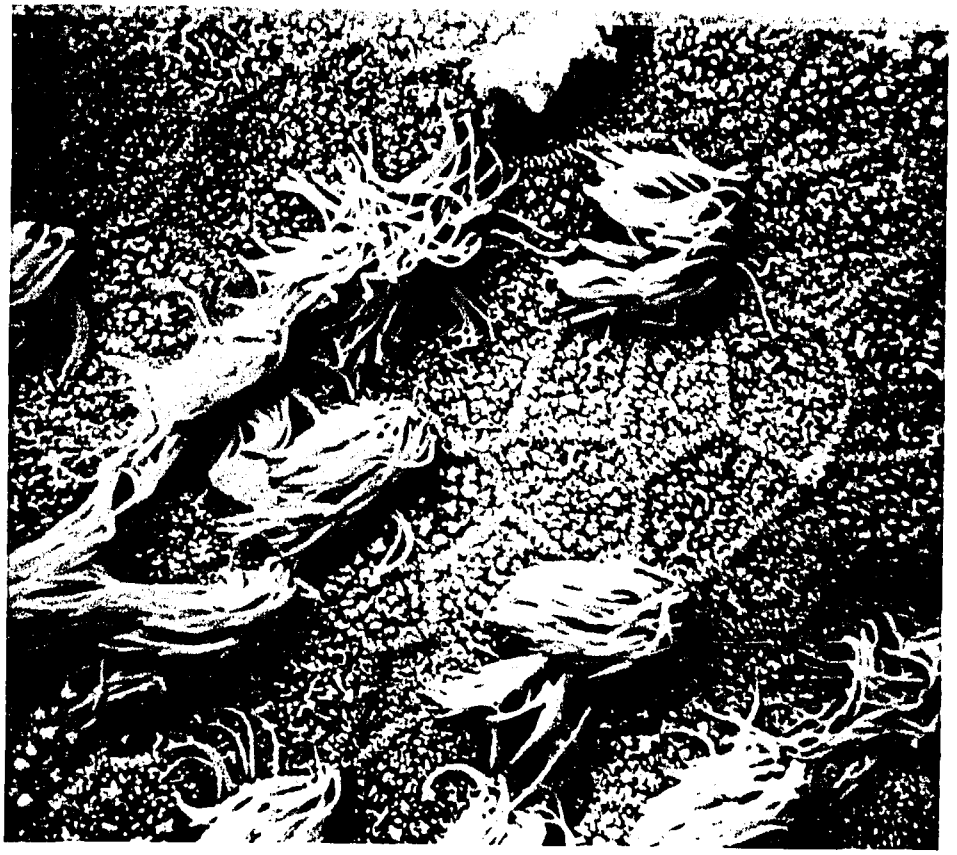
lengths, measured on three tissue sections of the same trachea, and representing, as well as possible with the techniques used, sequential slices. One is collecting a set of areas of tissue from the same area of each trachea, and presentation is as a linear, rather than volumetric, measurement displayed over time. In some cases data will be presented reflecting the volumetric nature of the data collection; this does not alter the final statistical values or their significance.

C. Scanning Electron Microscopy

To investigate the morphology of the tissue under study and to determine the relationship of asbestos to cells, scanning electron microscopy was done on several specimens selected from two time points in the same experiment.

Figure 7a is a scanning electron photomicrograph of a six-day old culture illustrating the epithelial cell surface; ciliated cells are prominent. Indirect evidence of culture viability is that at the instant of fixation all the cilia appear to have been beating in unison. Figure 7b shows a similar surface from an amosite asbestos exposed culture, and one notes the amosite fiber on the surface of the tissue. This illustrates the close proximity

Figure 7a



Scanning electron photomicrograph of 6-day-old untreated culture. Ciliated cells are prominent, with cilia moving in one direction. No cellular degeneration. 2000x.

Figure 7b

Scanning electron photomicrograph of 6-day-old culture treated with amosite asbestos. Ciliated cells are prominent, with cilia moving in one direction. Rod-like structure is an amosite fiber. 2000x.

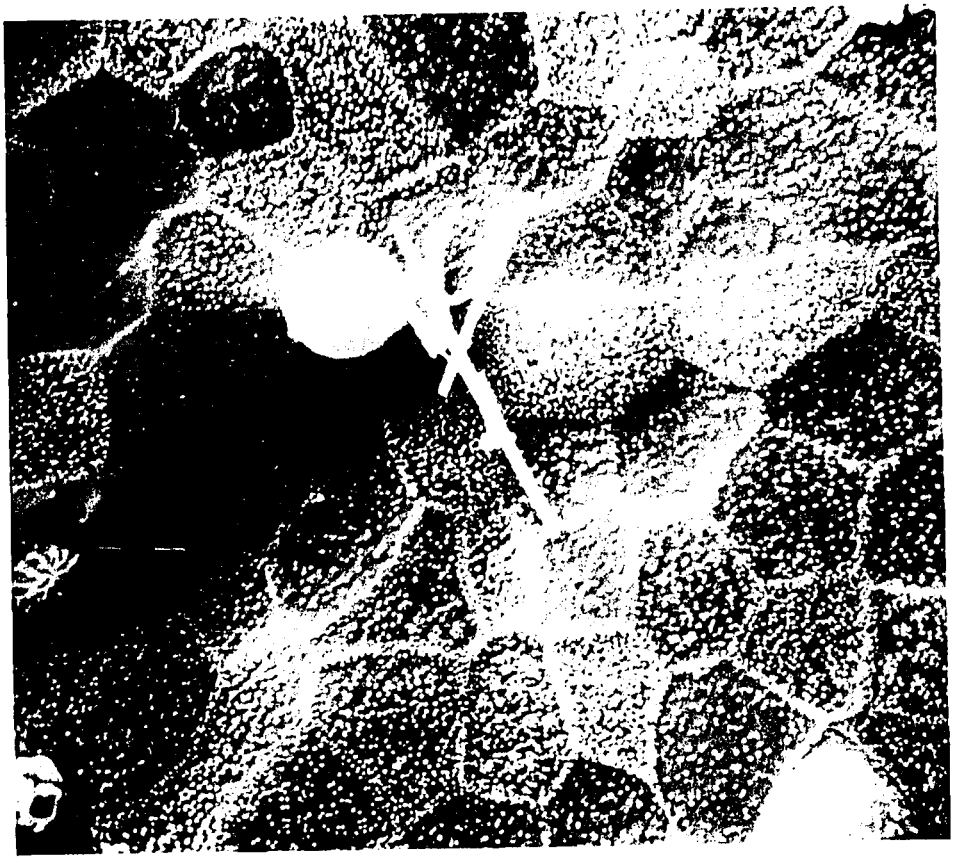
of the asbestos to the cells that respond to its presence.

Figure 7c shows an asbestos-treated trachea cultured for eleven days. The culture appears to be far less viable than the six-day old culture, with obvious cell death and loss of cilia, an effect independent of asbestos exposure. Cell death was a function of the length of time in culture.

D. Autoradiography

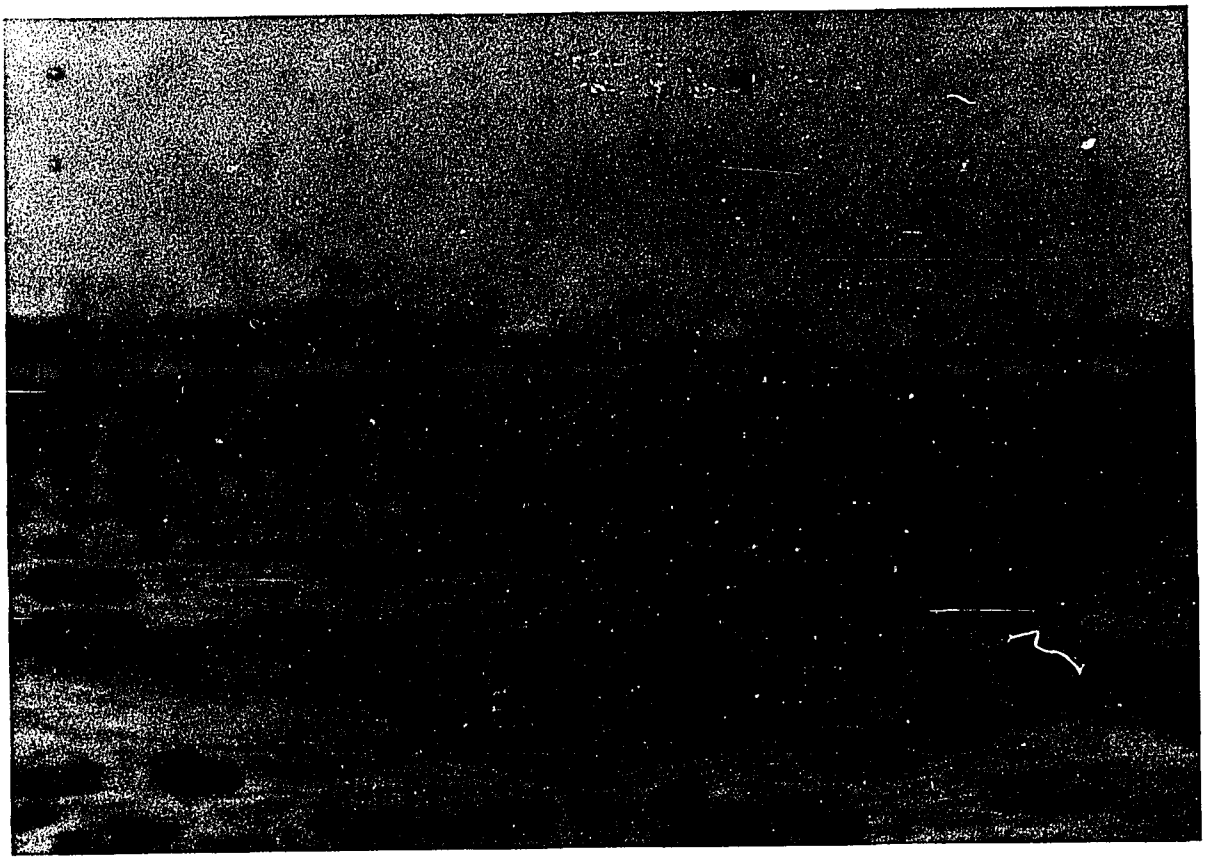
In hamsters, and other animals that have been studied, cell division in tracheal epithelium is possible only in basal cells which differentiate into ciliated and mucin-producing cells. Reproductive ability under these culture conditions was determined by use of tritiated thymidine to observe which cells incorporated this precursor of DNA, presumably in anticipation of cell division. Figure 8 is a radioautograph of a four-day old tracheal culture after a four-hour incubation with a 50uCi/ml dose of tritiated thymidine. Labelling occurs almost exclusively in the basal cells, consistent with the expectation that these cells are capable of dividing. Additional radio-autographic procedures would allow complex evaluation of cell turnover rates in these cultures.

Figure 7c



Scanning electron photomicrograph of 11-day-old culture treated with amosite asbestos. Loss of cilia is prominent, and disintegrating ciliated cells present. Loss of cell viability is not a function of asbestos exposure, but age of culture. 2000x.

Figure 8



Autoradiograph illustrating uptake of tritiated thymidine by basal cells. This slide was prepared after a 4-hour labelling of a 4-day-old culture with 50uCi/ml. Focus accentuates grains rather than cells. Approximately 500x, H&E.

E. Effect of Section Thickness

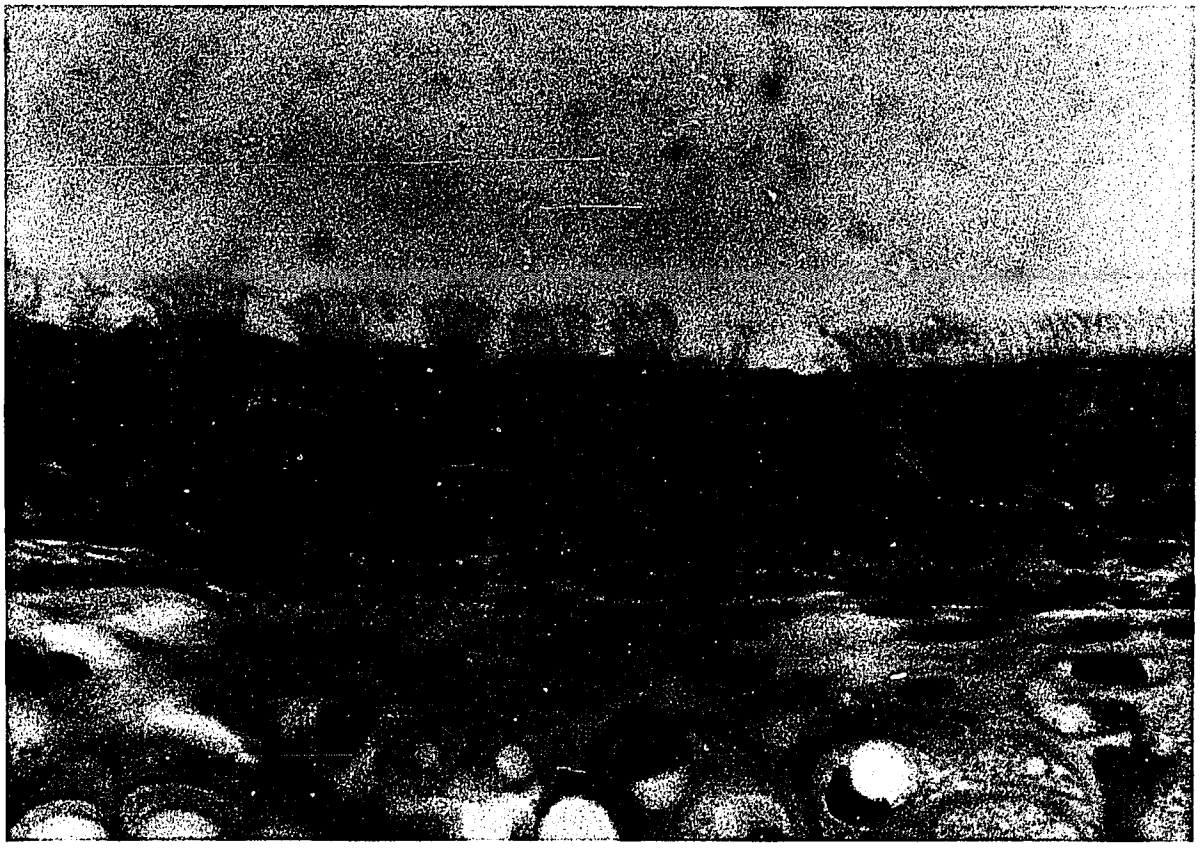
As previously discussed, uniformity of technique was a major goal for all procedures. One possibly important variable in this study might be variance in section thickness. A single trachea was embedded in plastic and cut in step-wise fashion on the microtome used for routine slide preparation in one-half micron steps from 2.0u to 5.0u. After randomization these slides were counted and plotted as mean cell number per 0.1 mm vs. section thickness. Not unexpectedly, the cell number per unit length increased as a function of increasing section thickness. While all slides were cut as uniformly as possible, other data will demonstrate that asbestos-induced effects did not result from variations in sectioning.

Using these varying thickness sections comparisons were made between the instruments available at the National Institutes of Health and the Mount Sinai School of Medicine. Lines with closely corresponding slopes were obtained. Two sections from the same trachea, shown in Figures 9a and 9b, illustrate differences in cell number per unit length as a function of section thickness.

Figure 9a

2u section of 4-day-old untreated culture. There is a layer of ciliated and mucin-producing cells, and a single layer of basal cells with little overlap of cells. Approximately 500x. H&E, plastic embedding.

Figure 9b



5u section of same untreated trachea illustrated in Figure 9a. At this section thickness there is basal cell overlap and more cells present per unit length than at 2u. Approximately 500x. H&E, plastic embedding.

F. Paraffin Embedded Studies

Initially, tissues were embedded in paraffin and cut at 5u. These experiments compared only untreated and amosite-treated trachea cultures. Table 2 shows data from tissues embedded in paraffin, cut at 5u, and stained with H&E. The experiment covers a total period of ten days, with data collected on Days 2, 3, 7, and 10. Amosite in a concentration of 1 mg/ml had been added on Day 1. As described earlier, the untreated cultures exhibit a slight, but statistically insignificant, increment in cell number per unit length during the earliest days in culture. The asbestos had been added one day prior to the first tissue sampling and was noted to have caused an increment in cell number during the first day of contact. This finding was noted repeatedly.

These data illustrate that the hyperplasia, once induced by the asbestos, is maintained throughout the life of the cultures. The response to asbestos does not appear to have a cytotoxic component after the initiation of hyperplasia.

Some experiments considered other staining techniques that might improve the ease of tissue analysis. A Feulgen stain was utilized in some paraffin embedded sections,

Table 2

Mean cell number per 0.1 mm for Days 2, 3, 7, and 10 after addition of amosite (1.0 mg/ml) on Day 1 through Day 3, and statistical analysis of differences.

Day	Untreated			Treated			p-value
	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.	
2	5	26.4	1.13	5	37.2	1.72	<0.001
3	5	29.4	1.04	5	36.8	2.01	<0.025
7	5	28.6	1.64	5	34.6	1.63	<0.05
10	5	28.5	.420	5	33.7	1.82	<0.0025

the nucleus staining a deep purple, the rest of the cell a pale green. Such a section is illustrated in Figure 10. This did facilitate cell counting.

Data from one such experiment are presented in Table 3. A sample of tracheas had been removed from culture and fixed on Day 4 after which amosite, in a concentration of 1.0 mg/ml, was added to half the remaining specimens with additional sampling on Day 6 and Day 7. The asbestos was present through Day 6, when all remaining cultures were changed with an asbestos-free medium. The asbestos-treated cultures showed an increase in cell number per unit length on both Day 6 and Day 7.

G. Plastic Embedded Studies

After attempts using paraffin embedded specimens all work was done with plastic embedding. The plastic 2u, rather than paraffin 5u, sections better maintained morphology, and the thinner sections facilitated cell counting. Using plastic sections, the question of variability in the section thickness as an explanation of cell number differences was dealt with at this point.

1. Parallel Embedding After Amosite Exposure

a. Differences in Cell Number. In this experiment two tracheas were embedded in each plastic block. Those

Figure 10

View of tracheal section illustrating use of Feulgen stain, highlighting cell nuclei. 54x.

Table 3

Mean cell number per 0.1 mm for Days 4, 6, and 7 after addition of amosite (1.0 mg/ml) from Day 4 through Day 6, and statistical analysis of differences.

Day	Untreated			Treated			p-value
	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.	
4	10	28.9	1.01	--	--	--	--
6	5	28.2	.882	5	36.3	1.96	<0.025
7	10	27.5	.897	10	34.8	1.21	<0.001

specimens that had been in culture for the same amount of time were embedded together. When possible, each block contained an untreated and an amosite-treated trachea. These tissues were then carried through all additional processing in parallel. Thus, after sectioning in the usual manner any variability due to varying section thickness would be eliminated from the final results as an explanation of any differences noted. Though multiple sections were present on each one slide, only parallel sections were counted for analysis; if one of the pair had to be eliminated for technical reasons, its mate was likewise discarded. The results, shown in Table 4, reflect the hyperplasia induced by the amosite. This table shows the parallel data for specimens with and without asbestos treatment from Day 6, and data from Day 4, prior to the addition of asbestos. In all cases but one the treated tracheas had more cells per 0.1 mm than the untreated.

b. Differences in Total Epithelial Length. Using this set of slides another possible source of variability was investigated. Although there was no reason to think that exposure of epithelial tissue in culture to asbestos, or any other material, should alter the linear relationship of this tissue, one might argue that exposure to

Table 4

Mean cell number per 0.1 mm for Day 4 and Day 6 after addition of amosite (1.0 mg/ml) from Day 4 through Day 6. Tracheas on Day 6 sectioned in parallel.

Day 4--Untreated	Day 6--Untreated	Day 6--Treated
22.08	21.25	27.16
22.16	17.83	27.16
20.42	22.50	25.75
19.00	24.00	24.83
22.75	23.08	22.92
20.83	28.25	29.00
18.00	19.25	24.08
25.75	19.75	21.56
25.83	21.5	23.42
	21.5	29.25

Day	Untreated			Treated			p-value
	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.	
4	9	21.9	.840	--	--	--	--
6	10	21.9	.916	10	25.5	.822	<0.005

asbestos either caused the tissue to contract, giving the appearance of increased cell number per unit length due to a piling up of cells, or might prevent the tissue from expanding in culture as compared to untreated tissues. Several techniques might be used to study this potential problem. These include complete kinetic studies of the growing tissue, or comparison of differential cell counts per unit length for all cell types. The impression, of course, was that no contraction of tissue was taking place since only basal cells, and not ciliated or mucin-producing cells, were increased in number. In addition, the impression gained after viewing many specimens was that asbestos-exposed cells appeared larger than the un-exposed cells, possibly making cell counts per unit length artificially lower than if cell size had remained the same. The increase in cell number after asbestos exposure appeared to result from an increase in basal cell layers.

This theoretical consideration was analyzed by measurement of total epithelial length of individual tracheas from several experiments, and comparison of the means of the groups that had been exposed and not exposed to study materials.

One evaluation was of the set of slides embedded and

cut in parallel. Using projection equipment, tracings of total tissue lengths were taken, measured with a planimeter, and the data analyzed with Student's t-test. Table 5 presents these data and shows that there was no statistically significant difference in the length of tissue from asbestos-exposed tracheas when compared to unexposed cultures. This eliminated linear changes in the tissue length as the source of difference noted after asbestos exposure. In light of the above studies, it would thus appear that observed changes in basal cell number were reliably the direct result of interaction of the cultured tissue and asbestos, rather than an artifact.

2. Additional Asbestos Studies

Change induced by 1.0 mg/ml of amosite asbestos was used as a standard of comparison, and in all subsequent experiments save one amosite was added to some of the tracheas to serve as a reference control.

a. Chrysotile. Chrysotile asbestos was also evaluated for its ability to induce changes in these cultures. Table 6 shows the results from an experiment in which both amosite and chrysotile were added to some cultures, in a concentration of 1.0 mg/ml. Data were collected at two sampling

Table 5

Total length of epithelial tissue of each trachea, reported as revolutions of planimeter dial, and statistical analysis.

Day 4--Untreated	Day 6--Untreated	Day 6--Treated
12.18	13.86	14.97
15.20	13.54	10.56
11.36	14.45	11.00
14.57	15.31	14.20
13.40	14.74	13.17
15.80	12.06	12.17
11.26	13.90	14.64
12.97	13.97	11.89
13.22	13.06	14.69
13.11	12.35	13.62

Day	Untreated			Treated			p-value
	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.	
4	10	13.31	.471	--	--	--	--
6	10	13.73	.324	10	13.09	.506	>0.20

Table 6

Comparison of both amosite and chrysotile treated (1.0 mg/ml) cultures with unexposed tracheas. Addition of asbestos from Day 2 through Day 4. Data reported as mean cell number per 0.2 mm, and statistical analysis of differences.

Day	Untreated			Amosite			Chrysotile		
	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.
4	7	29.4	.789	8	32.3	.798	6	34.1	1.98
6	8	27.8	.331	6	33.9	.591	6	32.3	1.09

Day 4	p-value
Untreated vs. Amosite	< 0.025
Untreated vs. Chrysotile	< 0.05
Day 6	
Untreated vs. Amosite	< 0.001
Untreated vs. Chrysotile	< 0.005

times for both forms of asbestos and compared to unexposed cultures. Both types were noted to induce significant hyperplasia.

b. Effect of Variable Dose of Asbestos. Both amosite and chrysotile were shown to induce measurable hyperplasia at a concentration of 1.0 mg/ml. Investigations were made to determine if the response was dose dependent. From the following data it appears that a"threshold"does exist for both materials, since concentrations less than 1.0 mg/ml did not induce any measurable increase in cell number.

1. Amosite. Table 7 shows the results of exposure to no asbestos and 0.1, 0.5, and 1.0 mg/ml of amosite. These data were collected as mean cell number per equal tissue volumes, reflecting the three-dimensionality of the area of tissue under study and takes into account cells in a block of tissue 0.2 mm wide and 6u deep. Statistically, this has no effect on the possible significance of the data.

2. Chrysotile. The same doses of chrysotile were tested in a similar manner. A different time frame for sampling was employed compared to the amosite experiment. The results are similar, with no effect demonstrated below 1.0 mg/ml. Table 8 presents the data and statistical analysis.

Table 7

Mean cell number in equal volumes of tracheas exposed to no asbestos, 0.1, 0.5, and 1.0 mg/ml of amosite, added Day 2 through Day 4, and statistical analysis of differences.

Day	Untreated			0.1 mg/ml			0.5 mg/ml			1.0 mg/ml		
	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.
2	9	92.2	2.05	--	--	--	--	--	--	--	--	--
6	10	88.2	1.86	6	86.6	2.16	6	90.2	3.21	9	102.9	3.39

Day 6	p-value
Untreated vs. 0.1 mg/ml	>0.50
Untreated vs. 0.5 mg/ml	>0.50
Untreated vs. 1.0 mg/ml	<0.005

Table 8

Mean cell number in equal volumes of tracheas exposed to no asbestos, 0.1, 0.5, and 1.0 mg/ml of chrysotile, added Day 4 through Day 6, and statistical analysis of differences.

Day	Untreated			0.1 mg/ml			0.5 mg/ml			1.0 mg/ml		
	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.
4	8	95.6	2.97	--	--	--	--	--	--	--	--	--
6	7	89.7	1.72	7	94.6	2.70	7	92.9	2.29	8	98.9	2.60

Day 6	p-value
Untreated vs. 0.1 mg/ml	>0.10
Untreated vs. 0.5 mg/ml	>0.20
Untreated vs. 1.0 mg/ml	<0.025

3. Non-Asbestos Materials

In addition to asbestos other particulate materials had been selected for testing. Activated carbon had no morphologic or chemical similarity to asbestos. Celite and talc were chosen for their chemical relationship to asbestos, but different structure.

a. Activated Carbon. With amosite as a reference control, an equivalent dose of activated carbon, 1.0 mg/ml, was added to a portion of tracheas in culture and sampled at several time points. The activated carbon did not cause any change in cell number. Reported as mean cell number in an equal volume of tissue the data and statistical analysis are shown in Table 9.

Again there is evidence of a small increment in cell number from zero-time samples to the first sampling point in the untreated group.

With this experiment it was decided to recheck the earlier finding that variability in tissue length of individual tracheas did not account for any differences noted. Both activated carbon and amosite-treated specimens were compared to untreated cultures. Table 10 shows the analysis of these data and confirms that variability in total tissue length did not account for differences noted.

Table 9

Comparison of values obtained after no treatment and exposure to activated carbon and amosite (1.0 mg/ml), reported as mean cell number is equal volumes of trachea, and statistical analysis of differences. Activated carbon and amosite added Day 4 through Day 6.

Day	Untreated			Activated Carbon			Amosite		
	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.
0	8	89.75	2.24	--	--	--	--	--	--
4	6	93.0	2.26	--	--	--	--	--	--
5	6	88.0	1.71	6	88.9	3.39	5	105.6	2.84
6	6	90.5	3.14	5	93.6	.500	4	102.25	1.66

Day 5	p-value
Untreated vs. Activated Carbon	>0.50
Untreated vs. Amosite	<0.001
Day 6	
Untreated vs. Activated Carbon	>0.50
Untreated vs. Amosite	<0.025

Table 10

Total length of epithelial tissue, reported as revolutions of planimeter dial, and statistical analysis.

Day	Untreated			Activated Carbon			Amosite		
	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.
5	6	12.23	.539	6	11.70	.177	5	13.15	.303
6	6	12.88	.520	6	11.70	.456	6	12.36	.628

Day 5	p-value
Untreated vs. Activated Carbon	> 0.20
Untreated vs. Amosite	> 0.10
Day 6	
Untreated vs. Activated Carbon	> 0.05
Untreated vs. Amosite	≥ 0.50

b. Celite. Celite, a commercial preparation of diatomaceous earth, was compared to untreated and amosite-treated cultures to determine if material of similar chemical composition, but different shape, would induce similar changes. Table 11, with data analyzed as differences in mean cell number per equal volumes of tissue between groups, shows that celite does not cause a hyperplastic response at a similar dose of 1.0 mg/ml.

c. Talc. Although a hydrated silicate, like asbestos, talc is not a fibrous mineral. Talc was added in the same dose as amosite, 1.0 mg/ml, and the response quantitated. Table 12 presents the statistical analysis of the data. Of note is that at the second of the two time points sampled, after forty-eight hours of exposure, the talc produced a statistically significant response in the tissue as measured by this technique. Collected as mean cell number per unit volume in this experiment a larger volume of tissue was analyzed. Again, three sections were studied, but for a total length of 0.4 mm on each section, and a total volume of 0.4 mm by 6u. The data analyzed both on a volume basis and by mean cell number per 0.1 mm showed no difference in the significance of results.

Table 11

Comparison of values obtained after no treatment and exposure to amosite and celite (1.0 mg/ml), reported as mean cell number in equal volumes of trachea, and statistical analysis of differences. Amosite and celite added Day 4 through Day 6.

Day	Untreated			Amosite			Celite		
	n	Mean (X)	S.E.	n	Mean (X)	S.E.	n	Mean (X)	S.E.
4	3	89.3	2.96	--	--	--	--	--	--
6	7	88.1	3.27	7	97.4	2.24	7	88.6	3.56

Day 6	p-value
Untreated vs. Amosite	< 0.05
Untreated vs. Celite	> 0.50

4. Effect of Variable Dose of Vitamin A

Because of interest in vitamin A and its analogues as potential anti-neoplastic agents investigations were made into the response induced by amosite in the presence of higher concentrations than that normally used in the growth medium.

In addition to the usual concentration of 0.1 ug/ml, the effect of a tenfold increase in this concentration, 1.0 ug/ml, was studied. All other factors were held constant, including the final concentration of DMSO, the solvent vehicle for the vitamin A. This increase in retinoid concentration did not prevent hyperplastic tissue response after challenge with amosite asbestos. This does not represent any final answer on the matter of retinoids, but in this system the hyperplastic reaction of tracheal epithelium was not altered. This is illustrated by the data and analysis in Table 13 which presents the results of duplicate experiments at these two doses of vitamin A.

Although the data from these duplicate experiments were quite consistent, the inherent variability in this technique required untreated controls for each experiment, rather than the establishment of some absolute value for untreated cultures.

Table 12

Comparison of values obtained after no treatment and exposure to amosite and talc (1.0 mg/ml), reported as mean cell number in equal volumes of trachea, and statistical analysis of differences. Amosite and talc added Day 2 through Day 4.

Day	Untreated			Amosite			Talc		
	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.	n	Mean ($\bar{X}$)	S.E.
3	5	174.4	3.54	6	206.2	8.94	6	177.8	5.50
4	4	165.75	4.75	4	211.0	11.12	5	197.4	4.70

Day 3	p-value
Untreated vs. Amosite	< 0.025
Untreated vs. Talc	> 0.50
Day 4	
Untreated vs. Amosite	< 0.01
Untreated vs. Talc	< 0.01

Table 13

Comparison of the effect of increased dose of vitamin A in the culture media as a possible modifier of hyperplastic response after exposure to amosite. Duplicate experiments. Data reported as mean cell number per 0.1 mm. Amosite (1.0 mg/ml) added Day 2 through Day 4.

	Day	Untreated			Treated			Untreated			Treated		
		n	Mean (X)	S.E.	n	Mean (X)	S.E.	n	Mean (X)	S.E.	n	Mean (X)	S.E.
0.1ug/ml vitamin A	2	5	23.2	1.04	--	--	--	5	20.4	.444	--	--	--
	6	6	21.5	.999	6	24.7	.662	6	20.9	.723	6	24.3	.923
				p-value						p-value			
Untreated vs. Amosite				< 0.025				Untreated vs. Amosite		< 0.025			
1.0ug/ml vitamin A	2	5	21.6	.517	--	--	--	5	23.3	1.05	--	--	--
	6	6	20.8	.560	5	24.5	.519	6	20.2	.851	6	24.5	.873
				p-value						p-value			
Untreated vs. Amosite				< 0.001				Untreated vs. Amosite		< 0.01			

5. Consistency of the System

Amosite and chrysotile induce hyperplasia in respiratory epithelium maintained in organ culture. The system as designed allows for quantitation of the changes induced by these materials. One difficulty with this system is the variability from trachea to trachea, although these differences did not preclude that appropriate statistical analysis could demonstrate that asbestos did induce hyperplasia, while other materials did not.

A virtue of this system is the consistency with which the same material induces the same degree of response. When measuring the percent difference in cell number after exposure to 1.0 mg/ml of amosite in multiple experiments, disregarding the units of data collection, the range is narrow. The percent difference is between 10.5 and 21.3 percent, with a mean about 16. Table 14 shows the percent difference between untreated and amosite-treated cultures from comparable data points from several experiments with use of plastic embedding and 2u sections. In each case the greater number was in the amosite-treated group, and all differences were found to be statistically significant in the separate experiments.

Table 14

Percent difference in cell number induced by amosite at 1.0 mg/ml from multiple experiments. In all cases treated tracheas had greater values than untreated.

Source of Data	Percent Difference
Table 4	16.05
Table 6	11.5
(2 points)	21.3
Table 7	16.6
Table 9	13.0
(2 points)	20.0
Table 11	10.5
Table 12	18.5
Table 13	15.0
(2 points)	15.9
Mean Difference	15.9 $\pm$ 3.5% (S.D.)

V. Discussion

A. Relevance to the Development of Cancer

This study demonstrates the selective ability of asbestos to induce hyperplasia of basal cells of respiratory epithelium. This change in cell number has been directly quantitated. Basal cells are the only cells capable of division and must therefore be considered the precursors of any malignant cells that develop in this tissue. Not all hyperplasia results in neoplasia, but hyperplasia commonly precedes neoplasia; it often is part of the continuum of morphologic change from normal to neoplastic. Cancer of the skin may develop after exposure to polycyclic hydrocarbons, and the neoplastic process is aided by application of croton oil, which alone is thought only to produce hyperplasia. In a similar manner asbestos may aid in the development of cancer in respiratory epithelium with exposure to cigarette smoke.

Neither activated carbon nor celite produced hyperplasia; talc did, but in a delayed manner when compared to asbestos. To date, activated carbon and diatomaceous earth have not been associated with the development of cancer in man. Kleinfeld (1974) and others have suggested the possibility that talc may be related to the development of

bronchogenic carcinoma in occupationally exposed human populations. Complicating this possibility is the presence of asbestos in some talcs. Questioning of the supplier of the talc utilized in this study revealed that the source of this material may be from a deposit known to be contaminated with tremolite, but no analysis was available. Electron microscopic analysis of the material used would be required to confirm such possible contamination. Such contamination may only in part explain the effect noted.

Although organ cultures are not sufficiently long-lived to demonstrate neoplastic changes, neoplastic potential after exposure to such agents as polycyclic hydrocarbons has been confirmed by implantation of altered tissue into whole animals.

B. Comparisons with Other Culture Models

The results obtained in this study are compatible with findings of previous investigations on the biological effects of asbestos.

1. Cell Cultures

Macrophage cultures, and hemolysis assays, have been used to study interactions between membranes and asbestos. Most evaluation has been descriptive, although some indirect

measurements, such as enzyme release into culture media after exposure to asbestos, have been used to quantitate change. A subjective quantitation was used to study the toxic effects of dusts on guinea pig exudate cells (Marks et al., 1956); a + to +++ "quantitation" was used, + representing "considerably fewer cells [adhering] to glass than in controls." Except for the study by Wade (1976) directly quantitating the effects of amosite and chrysotile on $P388D_1$ cells, a long-term cultured macrophage-like cell line, there has been little direct quantitation of asbestos effects on cells.

Although this study showed a "threshold" effect on the induction of hyperplasia with no significant change in cell number below 1.0 mg/ml, the investigation of Wade and others has demonstrated significant biological effects from doses of asbestos of 100ug/ml and less in culture media. Cytotoxic effects on macrophages are commonly used to evaluate asbestos-induced change in cell cultures, but information is now available on the effects of asbestos in a culture of non-macrophage cells. A human lung fibroblast cell strain, WI-38, has been exposed to amosite and chrysotile. While there were morphologic alterations in the cells, the asbestos, which at the same dose of 50ug/ml had been cytotoxic to $P388D_1$ cells, did not cause cell death

(unpublished data, Wade, Lipkin, and Frank). Increased cell death was not evident in the tracheal cultures of this study, and this parameter of cellular activity need not be the only criteria used to evaluate biological change induced by asbestos. It may be that the development of cancer is better studied by systems that evaluate growth.

2. Organ Culture

There are few comparisons that can be made to asbestos-related effects in organ culture because of the scarcity of such work. Rajan (1972) showed that the addition of asbestos to pleural cultures induced hyperplasia. Only the study of Mossman (1977) has used cultured hamster tracheas for the study of asbestos effects.

Mossman's study analyzed the ultrastructural and surfaces changes induced by crocidolite. The work demonstrated that "evidence of basal cell hyperplasia was noted as early as twenty-four hours." The present report contains the same observation. Enlargement of cell size was noted after exposure to crocidolite. This finding was reported in this study after exposure to amosite and chrysotile. Mossman recorded epithelial alterations as "Percentage of cultures with epithelial

changes." Hyperplasia was recorded as "Focal basal cell hyperplasia," and defined as "accumulation of 10 or more proliferating basal cells adjacent to deposits of crocidolite." The size of a "deposit" was not specified. Some changes noted in those experiments were not seen in the present study, most likely because of the large differences in doses used. The highest concentration of asbestos in these experiments was 1.0 mg/ml, and lower doses did not induce hyperplasia. There was no attempt to use higher doses. Mossman used from 1.0 mg/ml to 40.0 mg/ml, but reports on only those cultures exposed to 4.0 mg/ml or higher. Perhaps Mossman interpreted as no appreciable change the results at 1.0 mg/ml, since the evaluation technique may not have been sensitive enough to demonstrate small differences noted in the present study. Mossman reported the desquamation of 50% of the epithelial surface in some cultures; no such gross insult to tissue integrity was seen in the cultures of this study.

C. Effects of Culture Conditions and Potential Modification of This Model

Choice and alterations of media may effect characteristics of cultured tissues. Lasnitzki (1975) has demonstrated an effect of steroid hormones on growth

patterns of prostate organ cultures. Addition of retinoids to culture medium reverse effects of a vitamin A deficient diet, and vitamin A was used in the culture medium of this study to help maintain normal morphology. High doses of vitamin A did not prevent asbestos-induced hyperplasia under the culture conditions used. Mossman (1975) has reviewed the growth characteristics of several media in hamster tracheal cultures, including the basic medium used in the present study, CMRL-1066. Mossman investigated the suitability of these media for establishment of long-term cultures. With "rich" media such as CMRL-1066 Mossman described maintenance of normal epithelialization with some evidence of proliferation. In the present study it was noted that uncultured tracheas regularly had fewer cells per unit length than those in culture for a few days, although this was statistically insignificant. The use of small amounts of vitamin A may have modified the proliferation noted by Mossman. The hyperplasia induced by asbestos was identifiably separate from any effect of the medium.

Although the present system proved useful in quantitating asbestos-induced changes in respiratory epithelium, certain modifications in technique may have additional benefit. Since only a small area of each trachea is

analyzed, multiple cultures derived from a single trachea might reduce variability. The addition of serum to the culture medium might allow establishment of long-term cultures. This would allow longer observation periods and more experimental manipulations.

Since evaluation of biological changes in tissue samples can be difficult, improvement in image processing instruments would be welcomed. As noted, biological change is often evaluated without direct quantitation. Such quantitation offers opportunities to evaluate biological effects and then make accurate comparisons. Ideally one would like instrumentation that generates easily reproducible numerical data that would complement an investigator's description. The present study has illustrated some of the present difficulties in using image processing instruments.

D. Anticipated Developments

One might suggest additional experiments that could make use of this model system, and some have been initiated. Small increments in cell number may be increased by repeated exposures to asbestos. While doses smaller than 1.0 mg/ml had no effect, larger doses might increase the hyperplastic response. The effects of multiple agents on biological

changes could be studied. Potential modifiers of response could be evaluated. One could investigate if direct interaction of tissue and asbestos were required to induce change, as appears to be the case (unpublished data, Wade, Lipkin, and Frank). It might be possible to study tissue response as a function of fiber size or to study other inorganic fibers, such as fibrous glass. Combining in vivo and in vitro techniques, cultures exposed to asbestos could be implanted into whole animals to study the development of neoplasia. In addition to the study of asbestos, one might investigate the effects of other environmental agents that induce change in respiratory epithelium. These culture techniques may have use as short-term screening tests. It is anticipated that this laboratory model will be further utilized to investigate problems related to mechanism of disease development.

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