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Induction of Squamous Metaplasia in Organ Cultures of Hamster Trachea by Naturally Occurring and Synthetic Fibers[1]

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ABSTRACT

Asbestos exhibits many properties of classical tumor promoters. These characteristics include the ability to stimulate proliferation and inhibit normal differentiation of cells. In organ cultures of trachea, crocidolite and amosite asbestos stimulate squamous metaplasia, a pathological process in which a rapidly proliferating squamous epithelium replaces the normal epithelium. We hypothesized that the induction of metaplasia depends upon the fibrous nature of asbestos. Accordingly, several naturally occurring and synthetic fibrous materials and their nonfibrous analogues were assessed for their ability to induce metaplastic changes in tracheal mucosa of the Syrian hamster. Exposure to both crocidolite asbestos and fiberglass resulted in significant increases ($p < 0.05$) in squamous metaplasia over a range of dosages (1.0, 4.0, 16.0 mg/ml). Attapulgite (palygorskite) and both "long-" and "short-" fiber preparations of chrysotile asbestos had similar but less marked effects. Nonfibrous analogues of each material (riebeckite, antigorite, and glass particles) failed to produce metaplasia. Asbestos, and fibrous materials in general, appear to stimulate squamous metaplasia because of their fibrous geometry.

INTRODUCTION

Asbestos has enormous commercial importance (3, 21) because it is durable and resistant to both heat and fire. Asbestos acts as a cocarcinogen in the respiratory tract (6, 9, 24), and exhibits properties of classical tumor promotors (for review, see Refs 6, 17, and 28). These characteristics include the ability to stimulate proliferation of cells and alter their normal differentiation. For example, crocidolite and amosite asbestos induce squamous metaplasia in cultured tracheal epithelium (18). Although the chemical composition of asbestos minerals might influence pathogenicity (11), most experimental evidence indicates that physical parameters such as the length and diameter of fibers are important (10, 13, 26, 33).

Fiber geometry is critical in the experimental induction of mesotheliomas in rats (26). Intrapleural inoculation of materials comprised of fibers which are long and thin results in tumors, whereas short, thick fibers and particles are less tumorigenic. We hypothesized that fibrous morphology also might be important in the induction of proliferation and squamous metaplasia by asbestos. To test this, several different fibrous materials and their nonfibrous analogues were examined for their ability to induce these alterations in organ cultures of hamster trachea. We used chrysotile and crocidolite, the 2 types of asbestos of greatest commercial importance. Attapulgite, a naturally occurring fibrous clay mineral, and 2 forms of fiberglass also were examined.

MATERIALS AND METHODS

Preparation of Minerals. The sources of materials tested in these studies are listed in Table 1. Fibrous materials were prepared as follows. β-Fiberglass yarn was cut to varying lengths using a Sorvall TC-2 tissue sectioner. Code-100 fiberglass wool was ground gently in a tissue homogenizer since the random orientation of fibers made cutting ineffective. Attapulgite, crocidolite, and "long" and "short" chrysotile (Tables 2 and 3) were used without further modification. Samples of the minerals antigorite and riebeckite were prepared from rocks selected because of their mineralogical purity, and glass particles were produced by fusing code-100 fiberglass at 750°. These latter materials were ground in a ball mill for 15 min, yielding powders with heterogeneous size distributions. The large particles (>5 μm in diameter) then were separated by aqueous sedimentation (27). The suspensions of minerals were dried, sterilized (125° for 16 hr), and stored in powdered form.

Materials were dispersed in HBSS[3] (Grand Island Biological Co., Grand Island, N. Y.) by a 2-min bath sonication (Model B-22-4 Branson Ultrasonic Cleaner; Branson Cleaning Equipment Co., Shelton, Conn.) before addition to organ cultures. Chrysotile was not sonicated because this procedure alters the size of the fibers (25).

Characterization of Minerals. The mineralogical purity, surface charge, and size distributions of the particles were characterized. We used X-ray diffraction to assess mineralogical purity (Table 1). The electrophoretic mobility, an indication of net surface charge (22), was measured after suspending the particles in MEM (Grand Island Biological Co.) and the zeta potential (22) was assessed after dispersal of selected fibers in distilled H_2O (Table 1). Zeta potential and electrophoretic mobility are similar; however, the latter is applicable when particles are suspended in solutions containing organic compounds. When suspended in MEM, particles had no detectable net surface charge.

Size distributions were assessed using SEM (Tables 2 and 3). Suspensions of each material (10 μg/ml) were collected on Nucleopore filters (Nucleopore Filter, Pleasanton, Calif.) by pressure filtration. Regions from each filter, which appeared to represent the normal distribution of fibers, were photographed at magnifications ranging from ×100 to ×10,000. Photographs of adjacent regions were assembled as a montage to allow measurement of long fibers. A planimeter was used to calculate the length of long, curly fibers of chrysotile, and diameters of nonfibrous particles were determined by the technique of Cadle (5). Eight hundred fibers were counted, and particle size distributions were compiled after each experiment to standardize results.

Tracheal Organ Culture. The procedures used to prepare and maintain organ cultures of hamster trachea have been described previously (16). Briefly, random bred male Syrian hamsters between 6 and 10 weeks of age were sacrificed by an i.p. injection of 0.1 ml euthanasia solution (Taylor Pharmacal Co., Decatur, Ill.). Each trachea was isolated aseptically, and the adherent connective tissue was removed by dissection. The trachea was divided longitudinally at the cartilagenous discon-

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[3] The abbreviations used are: HBSS, Hanks' balanced salt solution; MEM, Eagle's minimal essential medium; SEM, scanning electron microscopy

y and then dissected into 14 to 16 explants measuring approxi-ly 1.5 x 2.5 mm.

xperimental Design. The study was carried out as 14 separate eriments because of procedural considerations. In each, 2 materials the appropriate controls were evaluated. Explants from 16 hamsters e divided randomly into groups and then transferred to 60- x 15-mm dishes (approximately 36 to 40/dish). Suspensions of each material BSS (1.0, 4.0, and 16.0 mg/ml; 2 ml total volume) were added to the dish and allowed to deposit onto the mucosal surface of organ res for 1 hr. The amounts of minerals used in our studies are tantially higher than those causing biological effects in monolayers ls. However, the mucociliary clearance of tracheal organ cultures is very efficient, and only a small fraction of the original amount remains on the surfaces of explants after 1 hr. We chose the dosages above on the basis of previous experiments using crocidolite which indicated that a 4.0-mg/ml dose was most effective in inducing metaplasia (18). For each type of dust, 12 explants were assessed at each concentration. All experiments were repeated twice.

Table 1

Source and net surface charge of materials

rous (A) and nonfi-us (B) analogues	Source	Net surface[a] charge
rocidolite asbes-tos	Union Internationale Contre Cancer	0 (−42.0 ± 3.2)[b]
iebeckite[c]	Wards Natural Science Est., Rochester, N. Y. (sample of fluor-riebeckite from El Paso county, Co.)	0
hrysotile asbestos		
Long" fibers	Manville Corp., Denver, Co. (samples from the Jeffrey Mine in Quebec)	0 (+49.4 ± 2.1)
Short" fibers		
ntigorite[d]	Wards Scientific Est. (sample from Arizona)	0
berglass		
iber	Owens Corning Fiberglas, Toledo, Ohio	NA[e]
ode-100 fiber	Manville Corp.	0 (−48.6 ± 4.1)
ass particles	Prepared from samples of code-100 fiberglass	0
tapulgite	Clay Mineral Society, Columbia, Mo. (sample from Nevada)	0

ctrophoretic mobility (22) of particles which were suspended in MEM. umbers in parentheses, zeta potential (22) determinations of fibers in distilled ± S.E.

ample of riebeckite contained <1% fibers (aspect ratio >3).

ntigorite contained <3% fibers and small amounts of the minerals picrolite zardite. No contamination was detected in samples of the other minerals.

A, not applicable. β Fiberglass rapidly settled from suspension due to the iameter of fibers. This precluded accurate determinations of zeta potential.

After experimental treatments, 4 explants were transferred to 30- x 15-mm plastic Petri dishes (Costar, Cambridge, Mass.) the surface of which had been scored to facilitate attachment. We added 0.5 ml of MEM containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (Sigma Chemical Co., St. Louis, Mo.), gentamicin (100 μg/ml), and nystatin (25 units/ml). This volume of medium was sufficient to wet but not submerge the mucosal surface of the explant. Cultures were maintained at 35–36° in an humidified atmosphere containing 5% CO_2, and the medium was changed every 3 days. Explants maintain normal mucociliary differentiation for at least 4 weeks under these conditions (16). Representative explants were examined at intervals of 2, 4, and 6 weeks after exposure to particles.

Assessment of Metaplasia. The extent of squamous metaplasia was evaluated by SEM. Explants were rinsed twice in HBSS to remove adherent mucin and were placed in modified Karnovsky's fixative for 12 hr. The specimens were dehydrated in ethanol, critical-point dried, sputter-coated with gold-palladium, and examined in a JEOL JSM 350 SEM.

The method used to grade the extent of the epithelium on the explants exhibiting squamous metaplasia has been described previously (32). An image of the entire mucosal surface was centered on the viewing screen of the SEM at a 0° tilt. A rectangular area (0.138sq mm, which comprised approximately 5% of the mucosal surface) in the center of each specimen then was defined after increasing the magnification 10-fold. The central region of the explant was analyzed since artifactual metaplastic changes usually develop at the cut margin (presumably due to traumatization of the tissue during preparation).

The percentage of mucosal surface within this area which showed either squamous differentiation or cytotoxic changes (cell necrosis and sloughing) was measured by placing a sheet of clear plastic over the viewing screen and outlining the distribution of lesions. The areas demarcated in this way were then converted to numerical values using a graphics tablet (Apple Computer, Inc., Cupertino, Calif.) and image analysis software (Optomax, Inc., Hollis, N. H.). Squamous cells were distinguished by their polygonal configuration and large diameter (>15

Table 2

Cumulative frequency distribution of fiber length

	% of particles at following fiber length[a]									
Test material	≤1 μm	≤5 μm	≤10 μm	≤20 μm	≤30 μm	≤40 μm	≤50 μm	≤100 μm	≤200 μm	≤300 μm	≤500 μm
ulgite	94	100									
t" chrysotile	59	100									
dolite	21	69	87	95	98	99	100				
-100 fiberglass	2	31	54	78	87	92	94	100			
" chrysotile	0	0	31	43	50	56	60	84	98	100	
rglass	0	0	1	5	10	16	20	43	77	90	100

lues indicate cumulative percentage of particles equal to or less than a given size.

Table 3

Cumulative frequency distribution of fiber/particle diameter

	% of particles at following fiber/particle diameter[a]								
Test material	≤0.2 μm	≤0.4 μm	≤0.6 μm	≤0.8 μm	≤1.0 μm	≤2.0 μm	≤3.0 μm	≤4.0 μm	≤5.0 μm
"Short" chrysotile	89	99	100						
Attapulgite	1	4	25	60	100				
"Long" chrysotile	65	82	90	93	96	98	99	100	
Antigorite	47	71	85	93	99	99	100		
Crocidolite	64	83	89		94	98	100		
Code-100	50	73	83	89	94	98	100		
Glass powder	7	20	33		51	91	98	99	100
Riebeckite	14	27		47	57	88	95	97	100
β-fiberglass	0	0	0	0	0	0	3	97	100

[a] Values indicate the cumulative percentage of particles equal to or less than a given size.

μm). Stratified squamous metaplasia was conspicuous because the superficial cells "heaped up" to form mounds. However, we did not attempt to distinguish simple squamous metaplasia from stratified squamous metaplasia by SEM.

Autoradiography. Explants were labeled for 5 hr with [^{3}H]thymidine (20 μCi/ml; specific activity, 49 Ci/mmol; Amersham-Searle Corp., Arlington Heights, Ill.) and then rinsed twice in HBSS at 37° before fixation. Using this protocol, approximately 0.5 to 2.0% of the tracheal epithelial cells are labeled in MEM after 2 weeks in culture (16). After examination by SEM, selected specimens were processed for light microscopy (2). Tissues were rehydrated and embedded in Paraplast (American Scientific Products, Bedford, Mass.), and sections of 5-μm thickness were cut. Sections mounted on glass slides were dipped in Kodak NTB immulsion (Eastman Kodak Co., Rochester, N. Y.) and then exposed at 4° for 1 week. Autoradiographs were developed in Kodak D-19 and stained with Harris hematoxylin.

One hundred epithelial cells (basal and suprabasal) on each side of the approximate center of the explant in 4 sections (a total of 800 epithelial cells per specimen) were counted to determine the labeling index (18).

RESULTS

Untreated explants exhibit normal mucociliary differentiation for 4 weeks *in vitro* although small foci of metaplasia occasionally develop over this period. In contrast, explants exposed to asbestos and other fibrous materials underwent both proliferative and metaplastic alterations. Analysis of variance and Fisher's least significant difference procedures were used to compare experimental groups (15). Crocidolite and fiberglass induced significant increases ($p < 0.05$) in the proportion of the mucosa involved by squamous metaplasia after both 2 and 4 weeks in culture (Chart 1). Metaplastic changes were most pronounced after 4 weeks whereas degeneration of the epithelium, characterized by cell sloughing and/or a spindly epithelium of one cell thickness, often was evident after 6 weeks. Both the "long" and "short" fibers of chrysotile asbestos induced a significant increase in metaplasia at low dosages (1.0 and 4.0 mg/ml, respectively). Exposure to attapulgite resulted in similar effect although the increase was not statistically significant. High concentrations (16.0 mg/ml) of "long" chrysotile were markedly cytotoxic, as determined by desquamation and necrotic alterations in epithelial cells.

The labeling index of epithelial cells was increased significantly in cultures exposed to both crocidolite asbestos and fiberglass for 2 weeks (Chart 2). Attapulgite and "long" chrysotile also caused increases in the labeling index, although the changes were not statistically significant. No change was observed after exposure for 2 weeks to "short" chrysotile. After 4 weeks in culture, labeling indices were low, and experimental groups did not differ from controls. The decrease in labeling of epithelial cells after extended periods *in vitro* has been observed previously in this system (16).

Nonfibrous analogues (riebeckite, antigorite, and glass particles) failed to induce significant increases in both the labeling index and extent of squamous metaplasia over a range of dosages and durations of exposure.

The pattern of deposition of materials on the mucosal surface was studied by SEM to determine the association of fibers with metaplastic lesions. Most fibers aggregated at the margins of the explant, although small numbers of individual fibers also were distributed randomly on the mucosal surface. These fibers either rested on nonciliated cells (Fig. 1) or protruded into the mucosal surface where they often were encompassed by accumulations of epithelial cells (Figs. 2 to 5). Metaplastic foci were usually

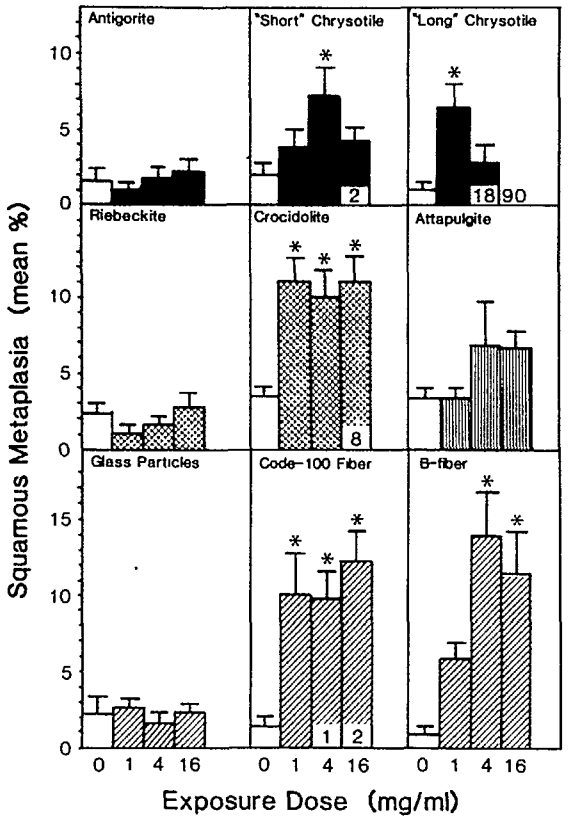

Chart 1. Development of squamous metaplasia 4 weeks after exposure of tracheal organ cultures to fibrous and nonfibrous materials. The percentage of squamous metaplasia represents the area of the mucosa showing squamous change divided by the total area examined. *, responses which are statistically different ($p < 0.05$) than the corresponding nonexposed controls (□); *bars*, S.E.; the number of observations varied between 19 and 26. *numbers* within *columns*, percentage of mucosal surface with cytotoxic alterations (*i.e.*, loss of epithelium).

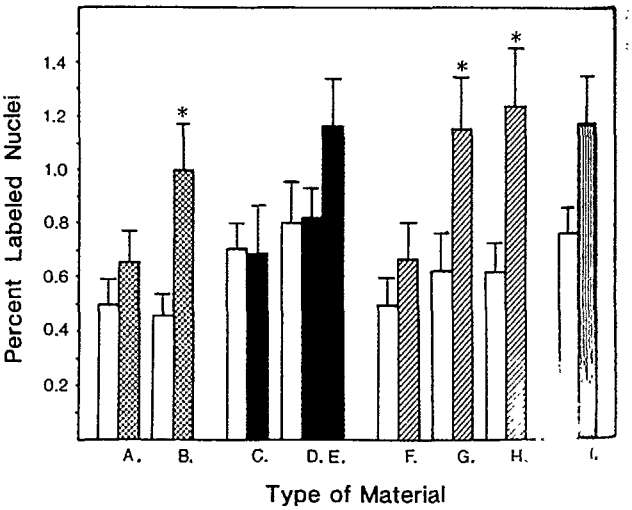

Chart 2. Percentage of epithelial cells with labeled nuclei on explants of hamster trachea 2 weeks after exposure to fibrous and nonfibrous materials. The explants were exposed to (*A*) nebeckite, (*B*) crocidolite, (*C*) antigorite, (*D*) "short" chrysotile, (*E*) "long" chrysotile, (*F*) glass particles, (*G*) β fiberglass, (*H*) code-100 fiberglass, and (*I*) attapulgite at 4.0 mg/ml medium. *, responses which were significantly different than the corresponding nonexposed controls (□); *bars*, S.E.; number of observations varied between 19 and 26.

(<50 μm in diameter), but occasionally larger expanses of mucosa were affected (approximate diameter > 300 μm; Fig. Although many metaplastic lesions developed in association fibers, foci also were located at sites where deposits of rs could not be found.

s noted above, "long" fibers of chrysotile (in contrast to the er minerals) induced cell distortion and sloughing. These fibers n formed aggregates which were associated with clusters of rotic cells (Fig. 6). Many fibers became coated with material ng a smooth, bead-like appearance, suggesting deposition mucin or proteinaceous material (Fig. 7). Cytotoxicity of nat- y occurring asbestos has been described by others in a iety of cell types (11, 19).

The surface of explants treated with nonfibrous materials was atively free of particles. When present, this material accumu- d into aggregations which were scattered sparingly over the cosal surface.

CUSSION

Asbestos and cigarette smoke act synergistically in the devel- ment of bronchogenic carcinoma (9, 24). The stimulation of amous metaplasia by asbestos might predispose epithelial s to transformation by carcinogens in cigarette smoke. The dies recorded here were designed to investigate the role of ticle geometry in the induction of squamous metaplasia by ous materials. Fibers of differing physicochemical composi- induced hyperplasia and metaplasia in cultured tracheal ants. Their nonfibrous analogues do not.

Hyperplastic and metaplastic lesions are found in the airways ats after the inhalation of asbestos (4, 31). Therefore, our ervations using cultured tissues are consistent with findings nimals.

The importance of fiber morphology in the induction of bron- genic carcinoma is unclear, but fibrous shape is critical in the erimental production of both mesotheliomas (26) and pul- nary fibrosis (33). Intrapleural innoculation of materials com- d of long, thin fibers results in mesotheliomas, whereas rt, blocky fibers are less tumorigenic (26). In the studies of nton *et al.* (26), maximum carcinogenic activity was observed fibers <0.25 μm in diameter and >8 μm in length; however, documented the metaplastic capability of β fiberglass, a paration containing fibers >3 μm in diameter. Thus, fiber th in comparison to diameter seems more important in ing squamous metaplasia. Phagocytosis and subsequent nation of short fibers might explain biological inactivity (13, 33), but there has been no satisfactory explanation for the inogenicity of long fibers in the pleural and peritoneal cavity. ther or not long fibers of types other than asbestos are inogenic in tracheobronchial epithelium is unproven in ani- s because of the difficulty in generating sized preparations of rs for inhalation studies.

Our morphological observations suggest a hypothetical model which long fibers stimulate squamous metaplasia (Chart 3). have found that fibers occur between cells of the mucociliary thelium where they appear to interact with the underlying al cells (19, 32). This process is accompanied by necrosis of perficial epithelial cells (19). Although no definitive evidence sts to explain how fibers stimulate squamous metaplasia, the ocess probably involves more than compensatory changes

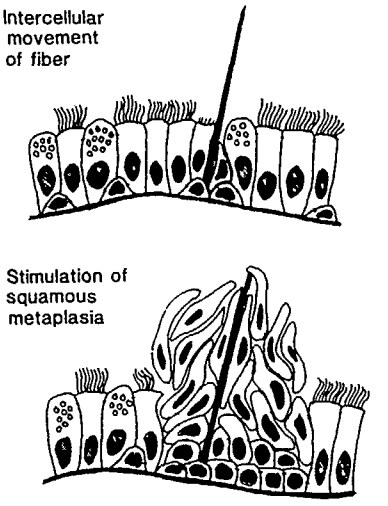

Chart 3. Hypothetical mechanism by which long fibers stimulate squamous metaplasia. Fibers occurred between cells of the mucociliary epithelium and appeared to interact with the underlying basal cells. This process causes injury and compensatory hyperplasia of adjacent basal cells (19). Alternatively, the long fibers which interact with the mucosal surface might serve as an artificial substrate which stimulates the outward migration of epithelial cells. Subsequently, these cells divide and differentiate into squamous cells.

resulting from cell death. For example, "long" chrysotile was the most cytotoxic material, but both crocidolite asbestos and fiberglass induced more extensive squamous metaplasia.

The nature of the substrate upon which cells rest influences their type of differentiation (8). Long fibers of glass provide a unique substrate for attachment of cultured fibroblasts and also act as a stimulus to promote cell division (13). Epithelial cells which interact with fibers might respond similarly. The failure of nonfibrous materials to stimulate metaplasia could reflect their rapid elimination from the mucosa by either phagocytosis or mucociliary clearance.

Squamous metaplasia in the human respiratory tract is topographically associated with squamous cell carcinoma (29). It might predispose the respiratory epithelial cells to malignant transformation by carcinogens in cigarette smoke. The rapid proliferation of cells which accompanies metaplasia (12) can enhance DNA damage by chemical carcinogens (14). Alternatively, chronic hyperplasia might promote neoplastic progression by previously initiated cells. Squamous differentiation also results in loss of protective mucociliary function, possibly leading to both the accumulation and prolonged interaction of carcinogens with epithelial cells (32). Cigarette smoking also causes squamous metaplasia (1) and modifies the deposition and clearance of inhaled particles (23). Hence, both substances could interact to increase retention of chemical carcinogens in the respiratory tract (6).

Because occupational exposure to asbestos is a recognized health hazard, a variety of nonasbestiform fibers recently have been promoted as substitutes (3, 21). These include both naturally occurring fibrous minerals (*i.e.*, attapulgite) and a number of man-made mineral fibers (*i.e.*, fiberglass). Although these materials are used widely, their biological effects are incompletely understood (7, 10, 20, 26, 30). Our results suggest that the ability of inorganic particulates to induce squamous metaplasia depends upon their fibrous geometry and not on any particular chemical or structural property unique to asbestos. Thus, other fibrous materials have the potential to induce hyperplasia and

metaplastic alterations in respiratory epithelium. Our results should be useful in predicting the potential of specific inorganic particulates to induce proliferative and metaplastic changes in epithelial cells.

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