Environmental Health Perspectives
Vol. 51, pp. 27-33, 1983

Interaction of Asbestos with Metaplastic Squamous Epithelium Developing in Organ Cultures of Hamster Trachea

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The normal mucociliary epithelium of the respiratory tract in chronic cigarette smokers often is replaced focally by a metaplastic squamous epithelium. Because asbestos workers who smoke have a substantially greater risk of bronchogenic carcinoma than nonsmokers, we hypothesized that interaction of asbestos with squamous epithelium might be a contributing factor. To address this question, an *in vitro* model was developed to study the interaction of asbestos with both mucociliary and squamous epithelium. Explants of tracheas from hamsters were cultured in either a chemically defined minimal essential medium, which maintains a differentiated epithelium, or a nutritionally complex medium, which encourages the development of squamous metaplasia. Scanning electron microscopy (SEM) was used to measure quantitatively the development of a squamous epithelial surface on the explants. The interaction of chrysotile and crocidolite asbestos with cells of the mucociliary and squamous epithelium was studied using both SEM and transmission electron microscopy (TEM). Long fibers of asbestos were cleared, whereas shorter fibers were phagocytized by cells of the mucociliary epithelium. In contrast, asbestos was phagocytized by superficial squamous cells regardless of fiber length, and fibers penetrated between intercellular junctions in the metaplastic epithelium. The relevance of these interactions to the induction of bronchogenic carcinoma is discussed.

Introduction

The synergistic action of asbestos and cigarette smoke in the development of bronchogenic carcinoma by asbestos workers has been documented by Selikoff and colleagues (*1*). However, the underlying mechanisms of synergism are not understood. During studies undertaken to explore the cellular basis of this synergism, we became interested in squamous metaplasia, a pathological process in which the normal epithelium is converted to a flattened, squamous type. This reversible change occurs commonly in the respiratory tract of cigarette smokers (*2*) and those exposed to other noxious agents. We hypothesized that fibrous minerals might accumulate preferentially in areas of squamous epithelium due to the loss of normal mucociliary clearance. Accordingly, a study was undertaken to determine how asbestos interacts with the metaplastic squamous epithelium of the respiratory tract *in vitro*.

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Materials and Methods

Tracheal Organ Cultures

Organ cultures of hamster trachea were prepared from 6- to 8-week-old male Syrian hamsters by methods described previously (*3*). The trachea was removed from the animal and divided into 14-16 explants of approximately 2 × 3 mm. These were maintained in either Eagle's Minimal Essential Medium (MEM, GIBCO, Grand Island, NY) to preserve the normal differentiation of the epithelium, or in the more complex medium, Waymouth's MAB 87/3 (W-87/3, GIBCO) to stimulate the development of squamous metaplasia (*3*). Both media contained 25 *mM* HEPES buffer, 100 μg/mL Gentamicin and 25 units/mL nystatin.

Evaluation of Squamous Metaplasia

The percentage of epithelial surface of the tracheal culture which exhibited squamous differentiation was measured quantitatively by using SEM. Each explant was bisected manually with a scalpel,

and an image of the surface of each half was obtained with the SEM. Two representative sites on each half of the explant were chosen using a clear plastic grid superimposed on the viewing screen (Fig. 1*a*). The grid defined two areas which were adjacent to, but did not include, the band of squamous epithelium, which customarily is found at the edges of the explant. The magnification was increased to 600×, and each area was assessed for the percentage of squamous and mucociliary differentiation

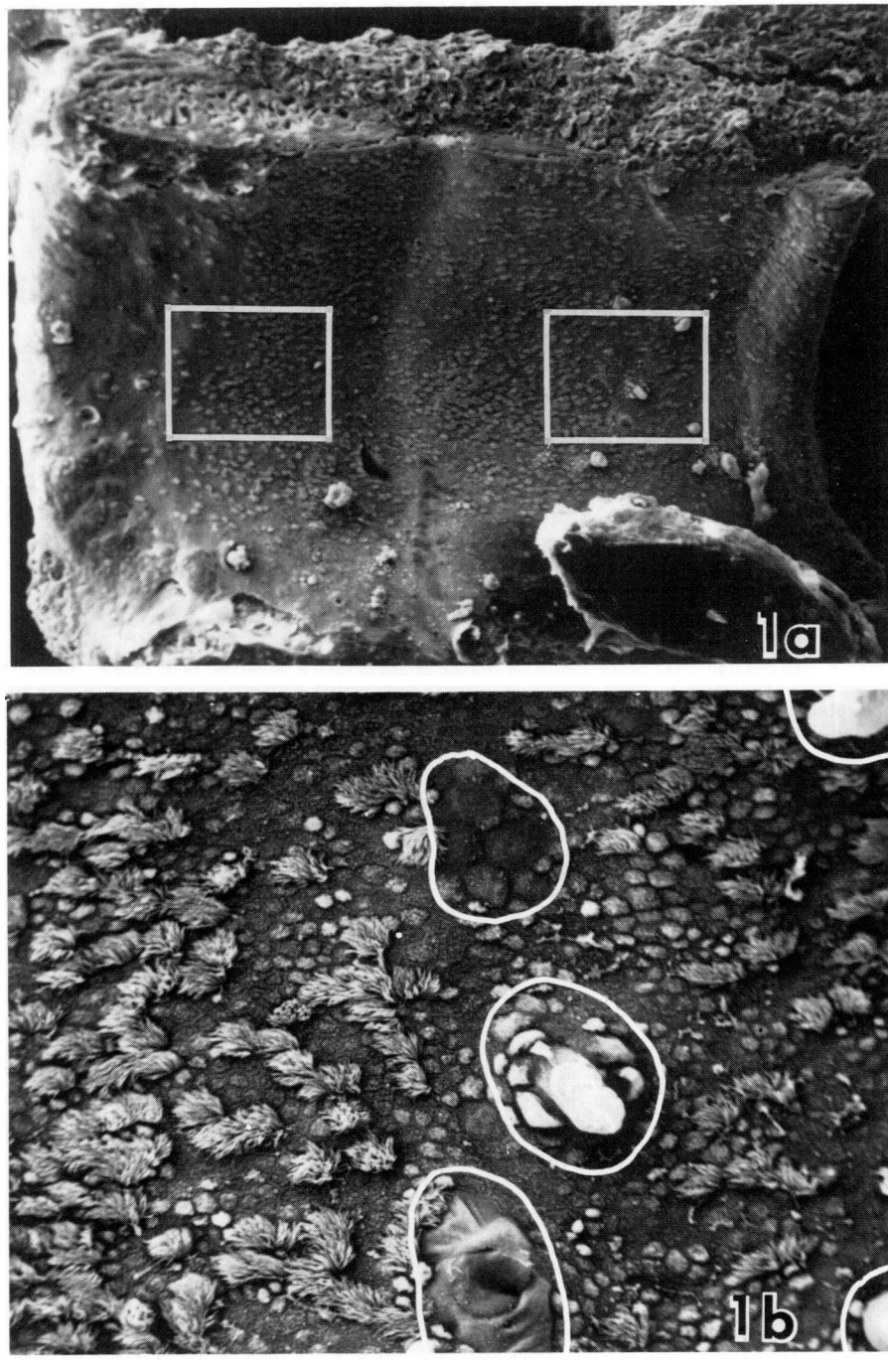


FIGURE 1. An SEM (*a*) of low magnification depicting half of an explant. The white rectangles were the areas analyzed (× 120). (*b*) SEM of the previously defined area (on right). The areas within the white lines represent regions which were graded as metaplastic (× 600).

(Fig. 1b) with the aid of an Optomax image analysis system (Optomax Inc., Hollis, NH). Squamous cells were identified by their relatively large diameter (> 20 μm) and polygonal configuration. The cells also tended to "heap up" to form mounds. In this manner four surface areas/explant were measured. This represented approximately 120,000 μm^2 or 5% of the total surface area.

Exposure to Fibrous Materials

Crocidolite and Canadian Chrysotile (UICC reference samples) were used in these studies. Samples of fibers were suspended in Hank's balanced salt solution (HBSS) and dispersed by gentle repetitive pipetting. In replicate experiments, fibers were added in 2% heated (56°C for 30 min) calf serum to ameliorate fiber-induced cytotoxicity (4). Suspensions of fibers (0.3, 1.0, 3.0 and 10.0 mg/mL) were added to cultures of explants exhibiting either squamous or mucociliary differentiation (i.e., cultures maintained previously for 2 weeks in W-87/3 or freshly prepared explants, respectively). The explants remained submerged for 1 hr to allow the precipitation of fibers on the mucosal surface. The dust-containing medium then was replaced by either MEM or W-87/3, and the explants incubated in a humidified 95% air-5% CO_2 environment at 35-36°C. The tissues were prepared for morphological examination immediately following exposure and after incubation for 3, 8 and 24 hr, and 3 and 7 days.

Electron Microscopy

Explants were rinsed in HBSS at 35°C and placed in modified Karnovsky's fixative for 24 hr. For SEM the tissues were dehydrated, critical point-dried, sputter-coated with gold-palladium, and examined in a JEOL JSM-35 scanning electron microscope. For transmission electron microscopy (TEM), the explants were postfixed in 2% osmium tetroxide and embedded in Araldite 502 epoxy resin. Sections 700 Å thick were cut by using a diamond knife, post-stained with uranyl acetate and lead citrate and examined with a Phillips 201 transmission microscope at 80 kV.

Results

Development of Squamous Metaplasia

The normal pattern of mucociliary differentiation was maintained for as long as 4 weeks when explants were cultured in MEM (Fig. 2). In contrast, "hillocks" of squamous epithelium developed rapidly when organ cultures were maintained in the nutritionally complex medium, W-87/3 (Fig. 3). These lesions originated as small foci and expanded subse-

FIGURE 2. Normal mucociliary epithelium from hamster trachea maintained for 3 weeks in MEM (× 720).

FIGURE 3. Development of focal squamous metaplasia in an explant after culture for 3 weeks in W-87/3. The mucociliary epithelium has been replaced in 3 regions by large, flattened squamous cells. (× 480).

quently to form large patches and ridges. Keratinization, judged by the loose attachment and sloughing of superficial squamous cells, was observed frequently. Squamous metaplasia occurred preferentially over cartilage rings, either as isolated foci or as ingrowths from the extensive areas of metaplastic epithelium covering the edges of the explant.

The progression of these changes was measured quantitatively using SEM (Fig. 4). Although focal metaplasia occurred as early as 1 week, extensive conversion to squamous epithelium appeared after 2- weeks. Little progression of these lesions was observed thereafter.

Interaction of Fibers with Mucociliary Epithelium

When added to cultures with mucociliary epithelium, the longer fibers (>5 μm) became entrapped in the cilia and appeared to be transported to the periphery of the explant. An undetermined number of short fibers of crocidolite or chrysotile deposited on the surface of nonciliated cells with microvilli. In some instances these fibers were phagocytized and incorporated into lysosomes as observed by TEM. The exposure to high concentrations of crocidolite (10.0 mg/mL) or chrysotile (3.0 mg/mL) resulted in the sloughing of ciliated and goblet cells. These results are consistent with previous findings of Mossman et al. (*5, 6*).

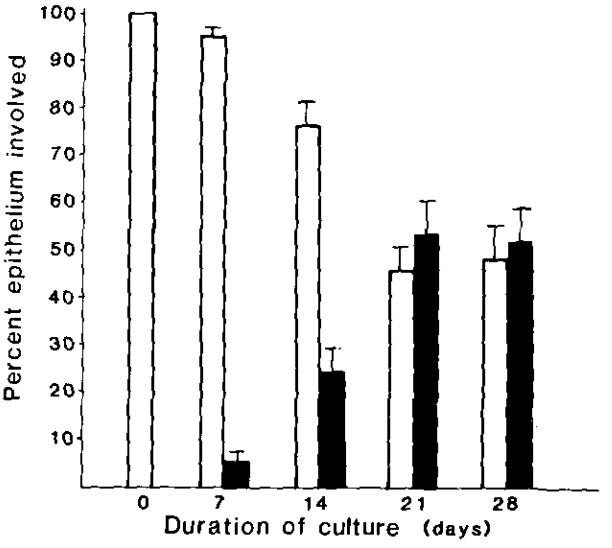
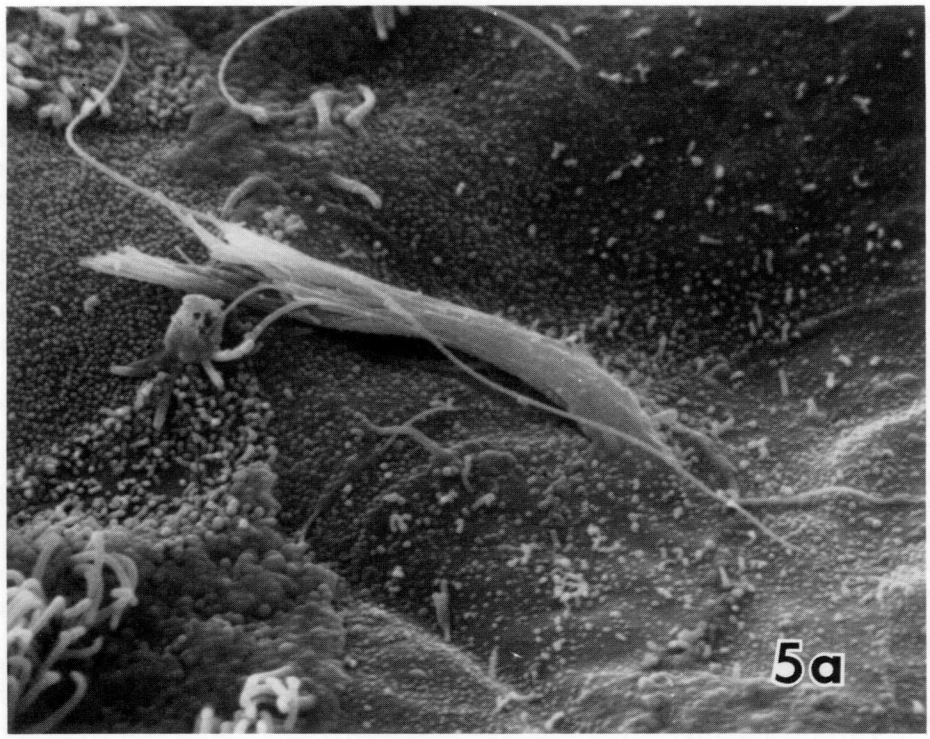


FIGURE 4. Development of squamous metaplasia (■) and loss of mucociliary epithelium (□) were most pronounced after culture for 2-3 weeks in W-87/3. Surface changes were evaluated with SEM in conjunction with image analysis. Histologic sections from the same explants were examined to confirm these results (*14*).



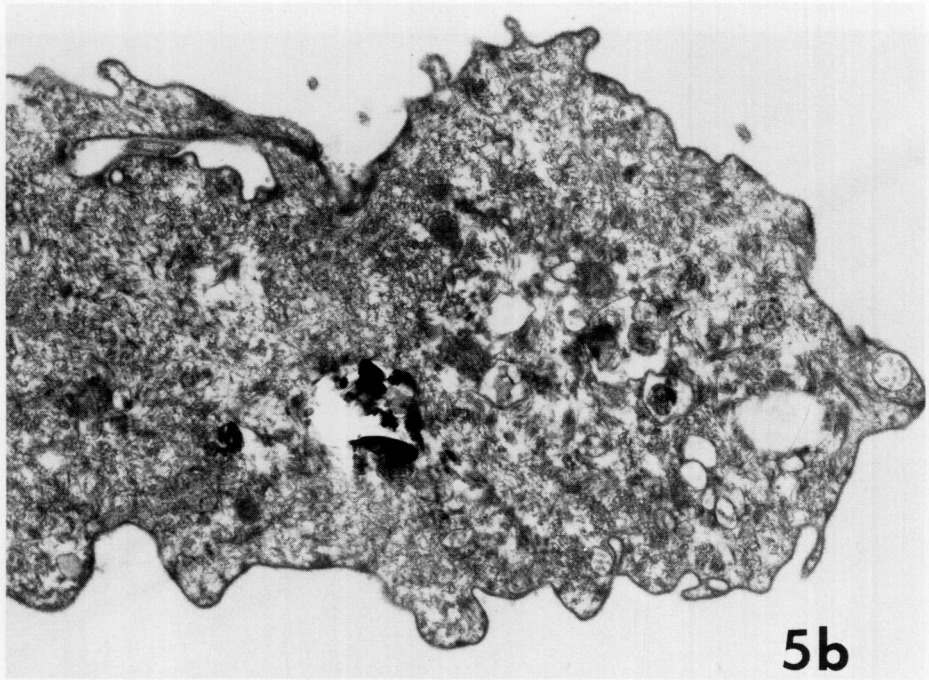


FIGURE 5. Micrographs showing (*a*) a superficial squamous cell phagocytizing a long fiber of chrysotile (3.0 mg/mL, 3 days post exposure, × 4400); (*b*) TEM illustrating an accumulation of chrysotile (arrow) within a superficial squamous epithelial cell (10.0 mg/mL, 1 day post exposure, × 22,000).

Interaction with Squamous Epithelium

In contrast, when asbestos was added to cultures exhibiting squamous differentiation, fibers of various lengths were deposited on the squamous epithelium and did not appear to be cleared. Scanning electron microscopy proved to be a valuable tool for analyzing the entire epithelial surface (six explants/time interval were examined). The superficial squamous cells phagocytized both short and also long fibers of crocidolite and chrysotile (Fig. 5*a*) within 8 hr, either in the presence or absence of serum. Representative thin sections from replicate organ cultures were examined by TEM (6-12 explants at 0, 1, and 3 days). Asbestos was observed occasionally within superficial squamous epithelial cells (Fig. 5*b*). In addition, both long and short fibers were observed between the intercellular junctions of squamous epithelial cells (Fig. 6). The occurrence of both the rodlike crocidolite and flexible chrysotile fibers between cells was observed 24 hr after treatment.

Discussion

Our studies demonstrate that long and short fibers of asbestos are phagocytized by metaplastic squamous cells of the respiratory tract. These fibers also penetrate between junctions of squamous cells. Previous workers have shown that epidermal keratinocytes phagocytize a variety of particulates *in vivo*, including Thorotrast (*7*) and melanin granules (*8*). Asbestos also is phagocytized by type I cells of pulmonary epithelium *in vivo* (*9*).

The cellular mechanisms involved in the pathogenesis of asbestos-associated bronchogenic carcinoma are complex (*10*). Asbestos might act as a co-carcinogen, due in part to its ability to adsorb and transport chemical carcinogens to susceptible cells. In addition, crocidolite asbestos induces chronic hyperplasia and DNA synthesis, a necessary prerequisite for cellular transformation by chemical carcinogens (*11*).

Our observations suggest another mechanism whereby inhaled asbestos and components of cigarette smoke could act synergistically in the induction of bronchogenic carcinoma. Squamous metaplasia occurs commonly at bronchial bifurcations where appreciable amounts of inhaled materials are deposited by impaction (*12*). Metaplastic areas might interrupt the normal mucociliary clearance of materials resulting in their interception and accumulation (*13*). This could result in a prolonged interaction between inhaled carcinogens and the respiratory epithelium. An interaction of asbestos with the underlying layer of basal cells might occur by several mechanisms including the intercellular penetration of long fibers or by intracellular transport of short

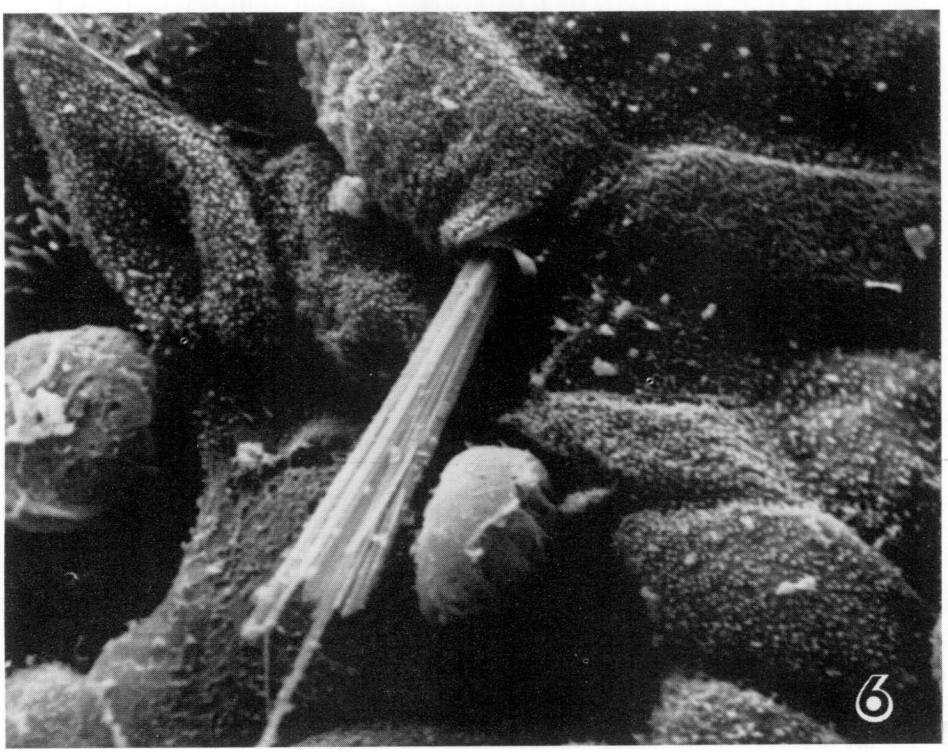


FIGURE 6. A bundle of crocidolite which has penetrated between the junctions of adjacent squamous cells (3.0 mg/mL, 1 day post exposure, × 3000).

fibers by squamous cells. Alternatively, in the normal epithelium, exposure to asbestos results in cytotoxicity and sloughing of ciliated and goblet cells, exposing the underlying basal cells to the fibers (5). Since basal cells are presumably the progenitors of bronchogenic carcinoma, this scheme appears relevant to the causation of this disease.

The authors would like to thank Ms. Joan Carrassi for her excellent secretarial assistance. This research was supported by Grant 00888 from NIOSH.

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