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0023-6837/86/5402-0204$02.00/0
LABORATORY INVESTIGATION
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Vol. 54, No. 2, p. 204, 1986
Printed in U.S.A.

Alteration of Superoxide Dismutase Activity in Tracheal Epithelial Cells by Asbestos and Inhibition of Cytotoxicity by Antioxidants

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We report here the inhibition of asbestos-induced cytotoxicity in a hamster tracheal epithelial cell line by superoxide dismutase, a scavenger of superoxide (O_2^-), and by mannitol and dimethylthiourea, scavengers of the hydroxyl radical ($OH^\cdot$). By using these agents, cell damage was ameliorated in cultures exposed to long (> 10 μm in length) fibers of chrysotile and crocidolite asbestos. In contrast, injury to epithelial cells by short (≤2μm) chrysotile or glass fibers was not prevented by scavengers of O_2^-, $OH^\cdot$, H_2O_2 or $^1O_2$ (singlet oxygen). These results implicate active oxygen species as mediators of injury by long asbestos fibers to cells of the respiratory tract.

By using immunocytochemical and biochemical techniques, we detected appreciable amounts of copper-zinc superoxide dismutase in hamster tracheobronchial epithelial cells and alveolar macrophages *in vitro* and in histologic sections of rat and human respiratory tract. Activity of total endogenous superoxide dismutase (copper-zinc and manganese forms) increased in tracheal epithelial cells exposed for several days *in vitro* to either crocidolite or chrysotile asbestos but was unchanged in untreated cells and those exposed to comparable amounts of glass fibers. After inhalation of asbestos by rats, or exposure of cells in culture to asbestos, long fibers were observed protruding from both epithelial cells and alveolar macrophages. The unsuccessful phagocytosis of long fibers of asbestos coupled with generation of oxygen free radicals might explain the increased pathogenic potential of long fibers in asbestos-associated diseases of the respiratory tract.

Additional key words: Crocidolite, Chrysotile, Fibrous glass, Hydroxyl radical, phagocytosis, oxygen free radicals.

Occupational exposure to asbestos is associated with an increased risk in man of bronchogenic carcinoma, pleural and peritoneal mesothelioma, and asbestosis, a progressive and often fatal disease (6). In an effort to understand the pathogenesis of asbestos-related diseases, investigators have focused on the elucidation of possible cellular mechanisms of injury by fibers (reviewed in references 3, 6, 40, 43). In this regard, experiments have been complicated by the fact that asbestos is not one, but a family of minerals, each with individual physicochemical features. Chrysotile ($3\ MgO \cdot 2SiO_2 \cdot 2H_2O$), a serpentine fiber accounting for approximately 90% of the world production, and crocidolite ($Na_2O \cdot Fe_2O_3 \cdot 3FeO \cdot 8SiO_2$), a fiber generally more pathogenic than chrysotile in man (6), have been studied extensively in bioassays both *in vitro* and *in vivo*.

Cytotoxicity by chrysotile and crocidolite is attributed to various features of these minerals including fibrous geometry (14), size (4, 24), surface area (42) and charge (28). With the red blood cell as a model, Harington, Miller, and Macnab (18) suggest that chrysotile injures cells by an ionic attraction of its positively charged magnesium to negative sialic acids on the plasma membrane. These investigators speculate on the subsequent creation of intermembranous protein channels, resulting in eventual osmotic imbalance and hemolysis. In related studies, Jaurand *et al.* (22) show extraction of red blood cell lipids and proteins by chrysotile and their adsorption to fibers (22); thus hemolysis and cytotoxicity to mammalian cells in culture are ameliorated when fibers are precoated with phospholipids and lung fluids (8, 9, 21).

Although experiments with chrysotile suggest that interaction of positively-charged fibers with membranes is intrinsic to cell injury, the mechanisms of cell damage by crocidolite, a type of asbestos with a net negative charge (28), are more obscure. In comparison to chrysotile, crocidolite is weakly hemolytic and less cytotoxic *in vitro* (3, 6, 40, 43).

We showed previously the inhibition of chrysotile-induced hypersecretion of mucin in tracheal explants by using prior addition of lectins, proteins binding to selected carbohydrates on the cell surface (34, 37). This interference indicates interaction of fibers with specific moieties on the plasma membrane, possibly receptors triggering subsequent biological responses. As oxygen free radicals are generated from membranes in response to a number of perturbations (15, 32), we tested the hypothesis that asbestos-induced injury might involve

production of free radicals. Consequently, asbestos-associated damage could be prevented by addition of antioxidants to cells of the respiratory tract in culture. To determine if asbestos altered normal amounts of scavengers of active oxygen species in cells, endogenous levels of superoxide dismutase (SOD), the enzyme converting superoxide (O_2^-) to H_2O_2, were measured in tracheal epithelial cells exposed to minerals *in vitro*.

MATERIALS AND METHODS

Determination of Mineral-Induced Epithelial Cell Damage in Vitro

A [^{75}Se]selenomethionine labeling technique (27, 39) was used as a sensitive assay for quantitation of mineral-induced cell injury in a cloned diploid line of tracheal epithelial cells (devoid of phagocytes) derived from a neonatal hamster and characterized by this laboratory (36). Tracheal epithelial cells were incubated in Ham's F12 medium (10% calf serum, 50 μg/ml of gentamycin, 25 units/ml of nystatin) containing 2.5 μCi/ml of L-[^{75}Se]selenomethionine (specific activity 0.6 to 4 Ci/mM, Amersham Corporation, Arlington Heights, Illinois). After 16 to 18 hours, the [^{75}Se]-labeled medium was removed and cells were rinsed 3 times in phosphate-buffered saline, pH 7.4, before plating in 12-well dishes (5×10^4 cells/well). At this juncture, asbestos containing a diversity of fiber lengths (UICC crocidolite and chrysotile) and preparations of sized chrysotile (Manville Corporation, Denver, Colorado; >10 and ≤ 2μm in length) were added in medium (0.36 to 5.8 μg/cm^2 surface area of dish). Code 100 fibrous glass (Manville Corporation) of comparable dimension to UICC asbestos preparations was used as an example of a nonasbestos fiber. The mineralogical purity of particulates was characterized by x-ray diffraction and x-ray energy-dispersive spectrometry (55). Use of the latter technique showed that the Mg/Si ratio of all chrysotile preparations ranged from 1.4/2 to 1.5/2. All fiber size distributions were confirmed by scanning electron microscopy (SEM) and reported previously (55). At 24 hours after addition of fibers, the supernatant medium was removed, and 100-μl aliquots were counted in a gamma counter. Adherent cells were washed 3 times in phosphate buffered saline, dissolved in 1.0 N NaOH and the radioactivity was determined in 100-μl aliquots as described above. The release index for individual wells was calculated by the equation:

$$\frac{\text{cpm of medium}}{\text{Total counts (cpm of medium + cpm of cells)}}$$

and expressed as a percentage of the respective control (untreated) culture with or without respective scavenger. The Student's *t*-test adjusting for multiple comparisons between groups (Dunnett's procedure) was used to determine statistical significance (11).

Addition of Scavengers of Oxygen Free Radicals to Hamster Tracheal Epithelial Cells

An approximately equivalent, sublethal concentration of each mineral was used as a positive control in studies to evaluate the effects of specific scavengers of oxygen free radicals on asbestos and fibrous glass induced injury. Controls included cultures without addition of minerals and cultures with addition of scavengers alone. To determine whether damage to tracheal epithelial cells by various types of fibers could be prevented, bovine erythrocyte SOD (280 units/ml of medium), catalase (105 units/ml of medium) (Sigma Chemical Co, St. Louis, Missouri), the enzyme converting H_2O_2 to H_2O and O_2, and 1,4-diazobicyclo[2.2.2]octane (DABCO, 0.1 and 0.5 mM, Aldrich Chemical Company, Milwaukee, Wisconsin), a scavenger of singlet oxygen ($^1O_2$), were added simultaneously with the minerals in culture medium. In additional experiments, scavengers of the OH$^\cdot$ radical including dimethylthiourea (20 and 40 mM, Aldrich Chemical Company), mannitol (10 and 20 mM, Sigma Chemical Company) and sodium benzoate (10 and 20 mM, Fisher Scientific, Pittsburgh, Pennsylvania) were evaluated with an identical protocol. The concentrations of the antioxidants used here were based on preliminary screening and effective *in vitro* amounts reported by others (15, 32). Initially, all scavengers were evaluated using the ^{75}Se labeling technique for toxicity at three concentrations in cell cultures without addition of minerals. Catalase, in comparison to other antioxidants, was toxic at 210 units/ml of medium, thus a lower, nontoxic concentration of enzyme was selected for evaluation in studies concerning effects on mineral-induced cell damage.

Preparation of Antisera to Copper-Zinc SOD

Antisera to SOD was raised in New Zealand white rabbits (Fred Weston, Underhill, Vermont) following conventional methods for the preparation of polyclonal antibodies. In brief, each rabbit received four SC injections (0.4 ml/injection site) of a stock solution consisting of 1 mg of bovine copper-zinc SOD (Orgotein Diagnostic Data Inc., Mountain View, California), 2 ml of phosphate buffered saline and 2 ml of Freund's complete adjuvant (GIBCO, Grand Island, New York). This was followed 4 weeks later by another series of injections of SOD in Freund's incomplete adjuvant. After an additional 4 weeks the rabbits were bled, the serum was isolated, and antisera to SOD was verified by the immunoblot technique (Fig. 1). Activity of antibody to copper-zinc SOD was ascertained by using an enzyme-linked immunoadsorbance assay. Using a standard peroxidase-antiperoxidase sandwich technique, this antibody was used to localize SOD in tissue sections from rat lung and human bronchus obtained at autopsy. For localization of SOD in cell cultures, hamster tracheal epithelial cells were fixed *in situ* in methanol before staining by using a fluorescein-labeled goat anti-rabbit IgG as the secondary antibody. Cells were examined with a Zeiss MC 68 fluorescence microscope.

Assay for Intracellular SOD Activity

Tracheal epithelial cells (5×10^5 cells/100 mm per Petri dish) were plated in F12 medium (GIBCO) supplemented with 10% calf serum and 50 units/ml of gentamycin with and without equivalent minimally toxic amounts of UICC chrysotile (0.36 μg/cm^2), UICC crocidolite (1.8 μg/cm^2) or Code 100 glass fibers (1.8 μg/cm^2). The activity of total endogenous SOD (both copper-zinc

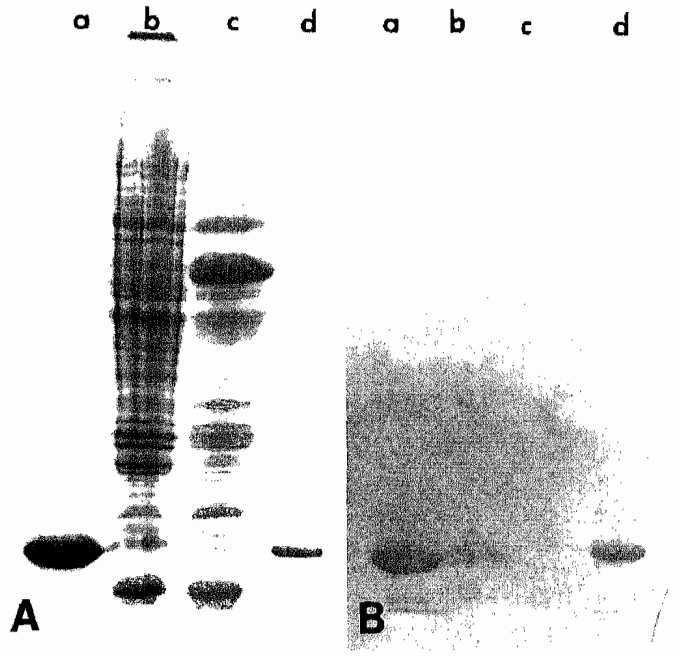

FIG. 1. Characterization of the antibody to copper-zinc SOD. *A*) Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis gels containing: (a) bovine copper-zinc SOD (25 μg of protein); (b) rat liver cell homogenate (150 μg of protein); (c) rat pancreatic cell homogenate (150 μg of protein); (d) bovine copper-zinc SOD (2 μg of protein). *B*) Corresponding immunoblot showing selective reaction of SOD antibody with bands of copper-zinc SOD. Diaminobenzidine is used as an indicator.

and manganese-containing forms) then was determined in cell-free extracts prepared by sonication in potassium phosphate buffer (pH 7.8) at 24, 48, 72, and 96 hours after plating. The assay employed a xanthine-xanthine oxidase O_2^- generating system with nitro-blue tetrazolium as the detector, and was modified slightly from procedures described by McCord, Crapo, and Fridovich (30) and Beauchamp and Fridovich (2) by adding 0.33 mg of gelatin per milliliter of assay medium to prevent the precipitation of the formazan. The sensitivity of this assay exceeded that using cytochrome C as a detector of O_2^- (30). The Student's *t*-test adjusting for multiple group comparisons (Dunnett's procedure) was used to determine statistical significance (11).

INHALATION STUDIES

Pathogen-free adult, male Fischer 344 rats (Taconic Farms Inc, Germantown, New York) were exposed by inhalation to National Institute of Environmental Health Science crocidolite (10.36 ± 6.08 mg/m^3 of air; 6 hours per day for 6 days of exposure) using a Timbrell generator. The fiber-size dimensions of aerosolized crocidolite indicated that these fibers were respirable (Shatos *et al*, unpublished data). After perfusion of the lungs through the vasculature with Karnovsky's fixative, the tracheas from asbestos-exposed and untreated control rats were removed, and the left lobe of the lung was sliced in sagittal section in preparation for immunocytochemistry or SEM as described below. The objective of these studies was to determine if asbestos interacted with tracheobronchial epithelial cells *in vivo*. We were interested specifically in how cells responded to longer (ca. >10 μm length) fibers.

ELECTRON MICROSCOPY

To determine whether epithelial cells in monolayer interacted with various fibers in a fashion analogous to that observed after inhalation, cells were fixed *in situ* with 2.5% glutaraldehyde in 0.1 N sodium cacodylate buffer for transmission electron microscopy or plated on Thermonox coverslips (Miles Labs Inc., Naperville, Illinois) before fixation and preparation for SEM. For transmission electron microscopy, cells were postfixed with 1% buffered osmium tetroxide, embedded in Epon as described previously (36), and examined using a Philips 201 electron microscope. Cells on coverslips for SEM were washed with PBS, fixed in Karnovsky's fixative, dehydrated in a graded ethyl alcohol-freon series, critical-point dried, and sputter-coated with gold-palladium in a vacuum evaporator. Cells were examined with a JEOL JSM-35C by SEM, and asbestos was identified by

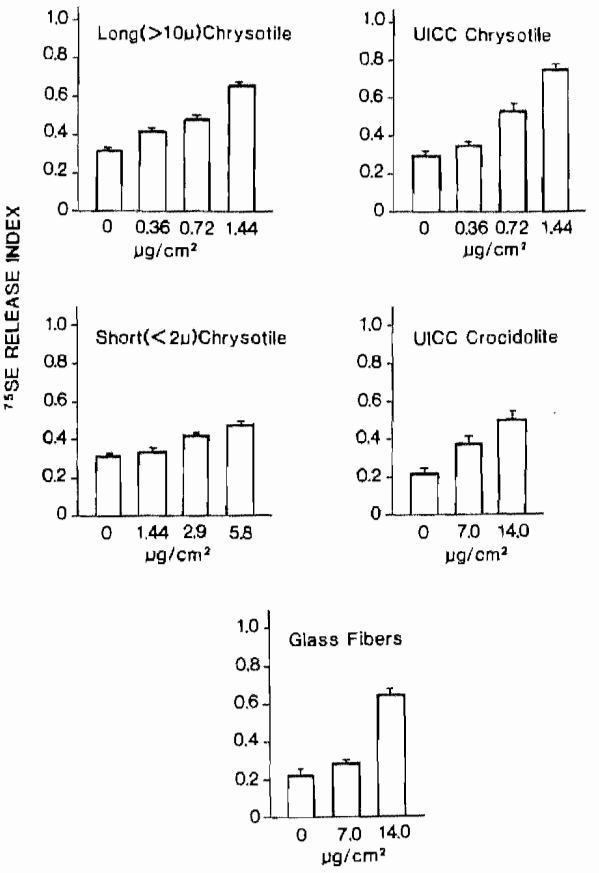

FIG. 2. Release of ^{75}Se from tracheal epithelial cells in culture after exposure for 24 hours to selected fibers. The release index for individual wells (*N*, 4 to 6 per concentration of dust, all experiments in duplicate) was calculated by the equation:

$$\frac{\text{cpm of medium}}{\text{total counts (cpm of medium + cpm of cells)}}.$$

Long (> 10 μm) and UICC chrysotile caused the most release of ^{75}Se followed by short (≤ 2 μm) chrysotile, UICC crocidolite, and glass fibers.

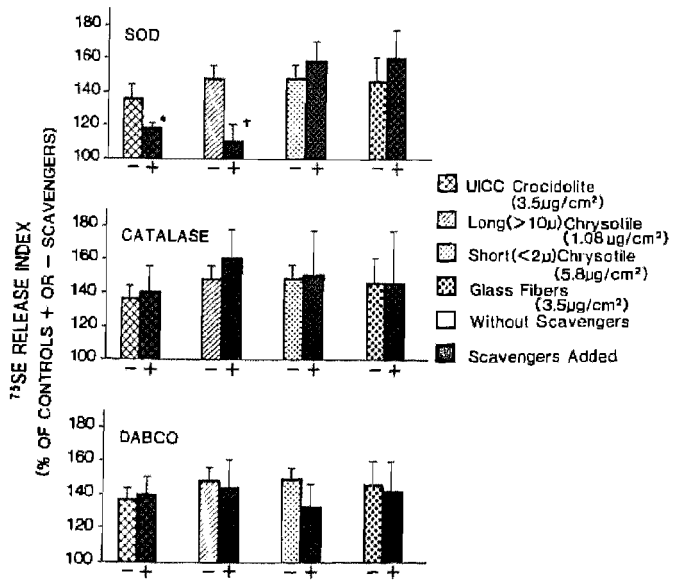

FIG. 3. Effects of SOD (280 units/ml of medium), catalase (105 units/ml of medium) and DABCO (0.5 µM) on release of ^{75}Se from tracheal epithelial cells exposed to equitoxic amounts of UICC crocidolite (3.5 µg/cm² per dish); long (> 10µm) chrysotile (0.72 µg/cm² per dish), short (≤ 2 µm) chrysotile (2.9 µg/cm² per dish), and glass fibers (3.5 µg/cm² per dish). SOD, the scavenger of O_2^-, inhibited release of ^{75}Se induced by crocidolite and long chrysotile but not that induced by short chrysotile or glass fibers. In contrast, catalase, a scavenger of H_2O_2, and DABCO, a scavenger of $^1O_2$, have no effect on ameliorating fiber-associated cytotoxicity. The Students *t*-test adjusting for multiple group comparisons was used to determine statistical significance (11). *, $p < 0.005$; +, $p < 0.001$.

X-ray energy-dispersive spectrometry (Kevex Corporation, Burlingame, California).

RESULTS

Effect of Scavengers of Active Oxygen Species on Release of ^{75}Selenium (^{75}Se) after Exposure to Minerals

After exposure to equivalent amounts of test fibers, the order of toxicity was long chrysotile = UICC chrysotile > short chrysotile > UICC crocidolite = Code 100 glass fibers (Fig. 2). In comparison to untreated controls, amounts of ^{75}Se released by cells exposed to fibers were elevated and were related directly to increasing concentrations of minerals. The release of ^{75}Se was inversely proportional to cell viability as determined by cell counting using exclusion of trypan blue. On the basis of these findings, an approximately equivalent cytotoxic concentration of each preparation was used to evaluate the effects of specific scavengers of oxygen free radicals.

The addition of SOD, but not catalase or DABCO, inhibited release of ^{75}Se induced by UICC crocidolite and long chrysotile (Fig. 3). In contrast, SOD (and other scavengers) did not prevent release of ^{75}Se caused by short chrysotile or glass fibers. To eliminate the possibility that concentrations of SOD here might have peroxidase-like activity, SOD inactivated by incubation of the enzyme with H_2O_2 under alkaline conditions (20) was tested in additional experiments. Under these circumstances, the SOD activity, but not the peroxidase activity, of the enzyme is lost upon inactivation of critical catalytic residues. Results indicated that chemically inactivated SOD did not prevent asbestos-associated release of ^{75}Se (Table 1).

In the presence of iron, O_2^- supplies an electron for the reduction of H_2O_2 to $OH^\cdot$ by a modified Haber-Weiss (Fenton-type) reaction ($O_2^- + Fe^{3+} \rightarrow O_2^- + Fe^{2+}$; $H_2O_2 + Fe^{2+} \rightarrow OH^- + Fe^{3+} + OH^\cdot$) (15, 32). Thus, scavengers of $OH^\cdot$ were evaluated in comparative studies (Fig. 4). Both DMTU and mannitol inhibited ^{75}Se release caused by UICC crocidolite and long chrysotile fibers, whereas sodium benzoate diminished release of ^{75}Se in response to crocidolite only. None of the $OH^\cdot$ scavengers affected release of ^{75}Se by short (≤ 2 µM) chrysotile or glass fibers.

Immunocytochemistry

To confirm the presence of copper-zinc SOD in tracheobronchial epithelial cells, hamster tracheal epithelial cells fixed *in vitro* on glass coverslips and 5 µm paraffin sections from normal human bronchi and perfused rat lungs were examined by light microscopy using an immunocytochemical technique for localization of the enzyme. Cultured hamster tracheal epithelial cells incubated with antiserum to copper-zinc SOD exhibited intense cytoplasmic stain (Fig. 5). In slices of whole rat lung and human bronchus, the reaction was most prominent in macrophages and in bronchiolar epithelial cells that appeared in many cases to be basal cells (Fig. 6). Lung fibroblasts stained less intensely than these cell types in both rat and human sections. This comparative immunocytochemistry indicates the cross reactivity of the antibody to SOD in hamster, rat, and human.

Assay for SOD

In comparison to untreated cultures or those with addition of Code 100 glass fibers, activity of endogenous SOD (total Cu-Zn and Mn forms) decreased at 48 hours

TABLE 1. COMPARATIVE EFFECTS OF SUPEROXIDE DISMUTASE (SOD) AND CHEMICALLY INACTIVATED SOD ON ASBESTOS-INDUCED RELEASE OF [^{75}]SELENIUM IN TRACHEAL EPITHELIAL CELLS

Agents	Amount of asbestos (µg/cm² dish)	Release index (mean ± SE)	% Decrease in comparison to controls
No dust		0.22 ± 0.014[a]	
No dust + SOD[b]		0.21 ± 0.009	NS[c]
No dust + inactivated[d] SOD[b]		0.23 ± 0.004	NS
UICC crocidolite	3.5	0.39 ± 0.020	
UICC crocidolite + SOD	3.5	0.30 ± 0.117	$p < 0.01$
UICC crocidolite + inactivated SOD	3.5	0.41 ± 0.018	NS
Long chrysotile	1.08	0.47 ± 0.013	
Long chrysotile + SOD	1.08	0.33 ± 0.000	$p < 0.01$
Long chrysotile + inactivated SOD	1.08	0.46 ± 0.020	NS

[a] *N*, four dishes per group.
[b] 280 Units/ml of medium.
[c] NS, not significant (11).
[d] Inactivated by incubation with H_2O_2 under alkaline conditions (20).

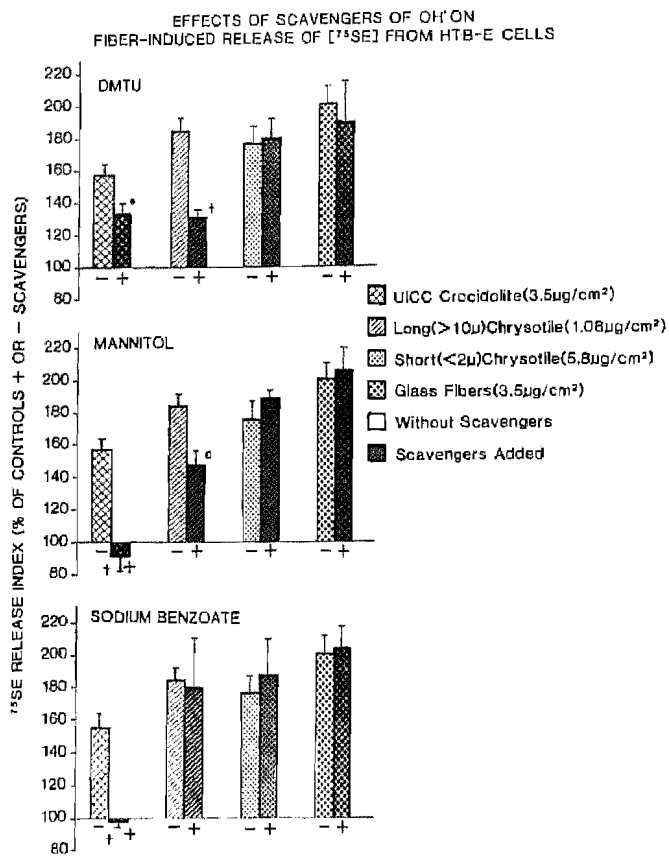

FIG. 4. Effects of scavengers of OH˙ on fiber-induced release of ^{75}Se from tracheal epithelial cells. DMTU (40 mM) and mannitol (10 mM) inhibited release of ^{75}Se associated with both crocidolite and long (> 10 µm) chrysotile, whereas sodium benzoate (10 mM) was effective with use of crocidolite alone. ^{75}Se release by short (≤ 2 µm) chrysotile and glass fibers was unaffected by addition of scavengers of OH˙. Approximately equitoxic amounts of fibers were used and data were analyzed as described in the legend of Figure 3. *, $p < 0.005$; +, $p < 0.001$; ˙, $p < 0.01$.

after addition of chrysotile ($p < .005$). However, activity of the enzyme increased two- to three-fold at 72 and 96 hours after addition of both types of asbestos ($p < 0.001$) (Fig. 7).

ELECTRON MICROSCOPY

Epithelial cells responded similarly to asbestos both *in vitro* and *in vivo*. Whereas shorter fibers were phagocytized (38), longer fibers were taken up incompletely by tracheal epithelium in cultures (Fig. 8*A*) and bronchiolar epithelial cells after exposure to fibers by inhalation (Fig. 8*B*). These fibers protruded from the plasma membrane.

GENERATION OF OXYGEN FREE RADICALS BY ASBESTOS

Observations above indicate a common mechanism of cell damage, *i.e.*, generation of oxygen free radicals, by crocidolite and chrysotile asbestos. We show here the ability to prevent asbestos-associated cell damage with scavengers of O_2^- and OH˙, reactive species that cause lipid peroxidation, depolymerization of polysaccharides, and damage to macromolecules (15, 32). Cytotoxicity is increased in epithelial cells after unsuccessful phagocytosis of long asbestos, presumably due to prolonged or increased generation of active oxygen species (39). In this regard, production of O_2^- has been documented in tracheal epithelial cells after exposure to mineral fibers (41).

Administration of SOD or scavengers of OH˙ does not prevent release of ^{75}Se caused by glass or short chrysotile indicating alternative mechanisms of damage by these types of fibers. Conceivably, short chrysotile and glass fibers might injure lysosomal membranes after phagocytosis, causing autodigestion of cells. Under these circumstances, production of oxygen free radicals could occur also within the lysosome, but scavengers might be ineffectual due to either their inability to penetrate lysosomal membranes or their degradation by lysosomal enzymes. On the other hand, amorphous glass fibers might injure membranes directly by other mechanisms.

H_2O_2 is required to generate OH˙ by the modified Haber-Weiss reaction (15, 32). Thus, it is surprising in our studies that no protection of chrysotile and crocidolite-induced cell damage was achieved from the addition

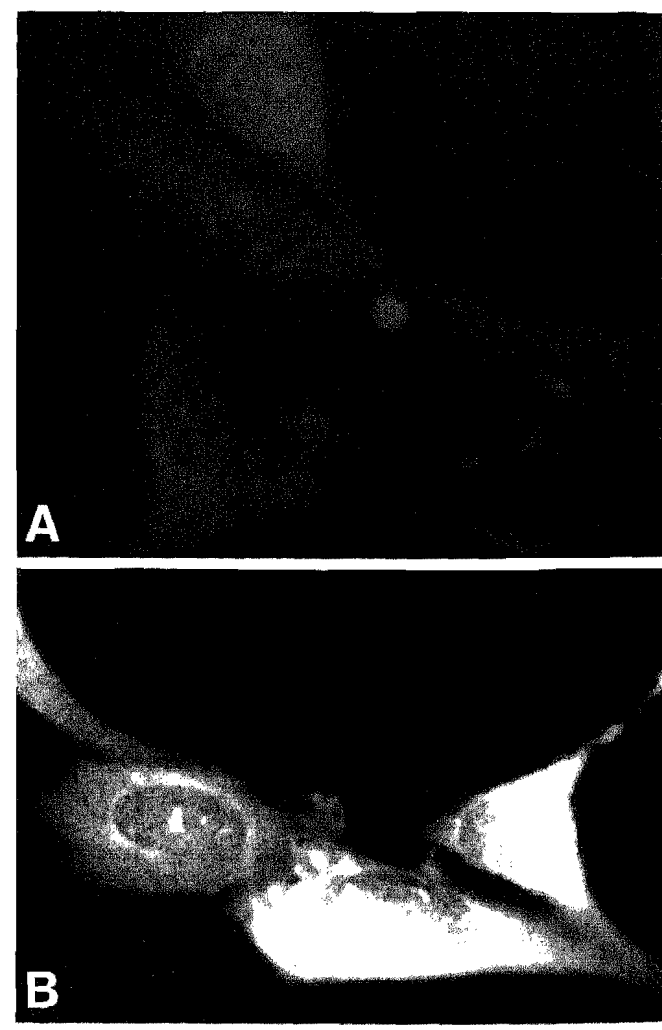

FIG. 5. Immunocytochemical localization of copper-zinc SOD in cultures of hamster tracheal epithelial cells using fluorescence. *A*) Preimmune serum; *B*) antiserum to copper-zinc SOD. Figure 5, *A* and *B*, ×350.

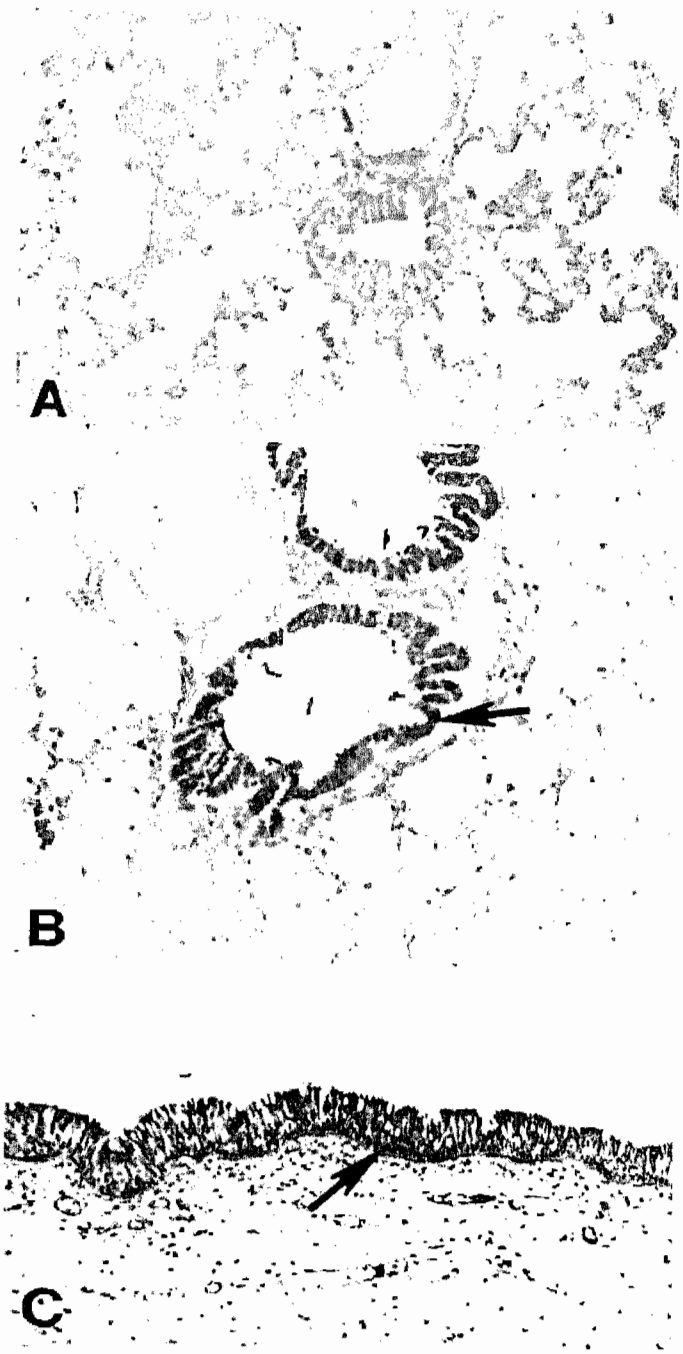

FIG. 6. Immunocytochemical localization of copper-zinc SOD in normal, perfused Fischer 344 rat lung (*A,B*) and human bronchus (*C*). *A*) Stained with peroxidase coupled to preimmune sera. *B*) and *C*) Stained with peroxidase coupled to antiserum to copper-zinc SOD. Note the intense peroxidase staining of basilar bronchiolar epithelial cells in *B* and *C* (*arrows*). Figure 6, *A* to *C*, ×75.

of catalase. Like SOD, this scavenger does not penetrate the cell unlike the situation observed with the low molecular weight scavengers of $OH^{\cdot}$. The inability of catalase to protect cells here does not exclude the possible importance of intracellular H_2O_2 which is inaccessible to exogenously administered catalase.

Several mechanisms could explain why asbestos elicits production of oxygen free radicals in the lung. First, metals and other chemical moieties on the surface of the fibers might cause reduction of oxygen directly. For example, iron (II) salts can react with oxygen in an aqueous environment to yield O_2^- and $OH^{\cdot}$ (*i.e.*, $Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^-$) (54). Secondly, asbestos might cause the generation of active oxygen species from a product of the cell. In support of this view, crocidolite, amosite, and chrysotile asbestos catalyze the formation of $OH^{\cdot}$ and O_2^- from H_2O_2 in a cell-free system (53). Based on the inhibition of the reaction with use of the iron chelator, desferroxamine, the authors propose that iron on the asbestos fiber drives the reactions: H_2O_2 + asbestos-$Fe^{2+} \rightarrow OH^{\cdot} + OH^- +$ asbestos-Fe^{3+}. O_2^- then might be generated by the reaction: $OH^{\cdot} + H_2O_2 \rightarrow H_2O + H^+ + O_2^-$ and/or: asbestos-$Fe^{3+} + H_2O_2 \rightarrow$ asbestos-$Fe^{2+} + 2H^+ + O_2^-$. Lastly, asbestos could stimulate a membrane-associated or soluble enzyme catalyzing the production of active oxygen species. Stimulation of a reduced nicotinamide-adenine dinucleotide (NADH) or reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase (15, 32) and/or conversion of xanthine dehydrogenase to xanthine oxidase in the cytoplasm (31) are both possibilities although untested at the present time with asbestos. In this regard, conversion of xanthine dehydrogenase to xanthine oxidase requires an increase in cytosolic calcium concentrations, and asbestos-induced cytotoxicity appears to be calcium-dependent, an event probably representing an influx of Ca^{2+} across the damaged plasma membrane (44). The interaction of asbestos with enzyme systems on the plasmalemma or in the cytosol also is supported by morphologic studies

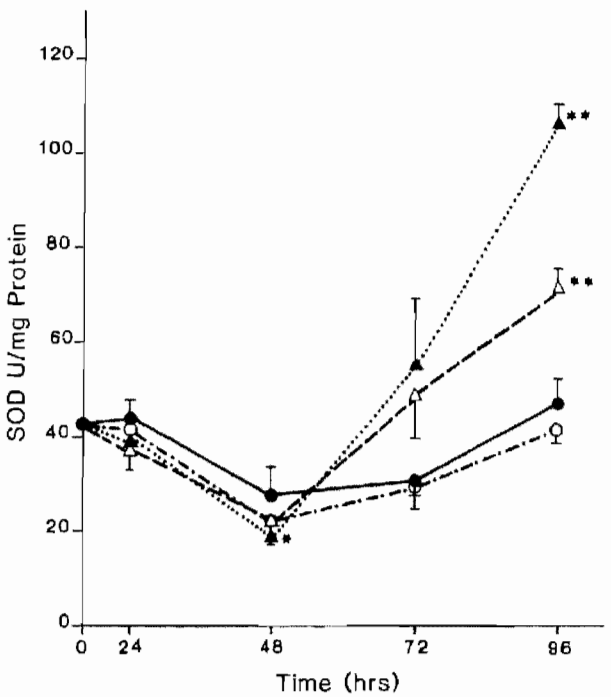

FIG. 7. Total endogenous SOD (units/ml of protein) in tracheal epithelial cells after addition of asbestos or glass fibers at plating. In comparison to untreated cultures (●) and those exposed to glass fibers (○; 1.8 $\mu g/cm^2$ per dish) cultures with addition of UICC crocidolite (△; 1.8 $\mu g/cm^2$ per dish) or chrysotile (▲; 0.36 $\mu g/cm^2$ dish) exhibited increased activity of endogenous SOD at 72 and 96 hours. **, $p < 0.001$; *, $p < 0.005$ (Student's *t*-test adjusting for multiple group comparisons).

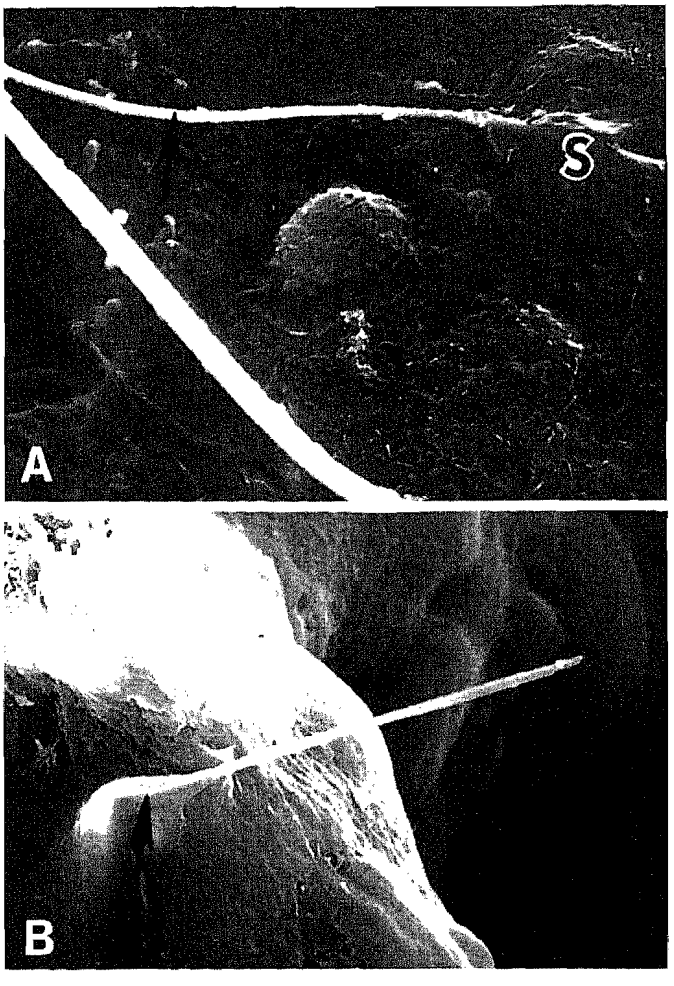

FIG. 8. Scanning electron micrographs illustrating similar interactions of long crocidolite fibers with epithelial cells *in vitro* and *in vivo* after inhalation by Fischer 344 rats. *A*, Crocidolite fibers (*arrows*) protruding from the tracheal epithelial cells *in vitro*, gold-palladium; *B*, interaction of a fiber (*arrow*) with a bronchiolar epithelial cell after inhalation, gold-palladium. Figure 8, *A* and *B*, ×4,000.

documenting the presence of fibers on the surface of cells, in phagolysosomes and free in the cytoplasm. (3, 40, 43). A recent report documenting lipid peroxidation by crocidolite asbestos in microsomal preparations from liver and lung suggests that binding of fibers to membrane is necessary to permit reaction of active oxygen species with the unsaturated bonds of phospholipids (17).

ALTERATION OF SOD ACTIVITY BY ASBESTOS

The stimulation of total endogenous SOD at 72 and 96 hours after addition of asbestos to tracheal epithelial cells suggests that the enxyme is inducible in cells after a protracted period of exposure to the mineral *in vitro*. In contrast, glass fibers of similar dimension (39) do not alter endogenous SOD activity. Biochemical and cytochemical studies here demonstrate that epithelial cells, particularly basal cells, of the respiratory tract are endowed with relatively large amounts of scavengers of oxygen free radicals, an important observation in view of the fact that these cell types are, in general, more resistant than differentiated epithelial cells to a variety of chemical and physical insults (33, 38, 45).

IMPORTANCE OF FIBER LENGTH AND PATHOGENIC RESPONSE

Studies here indicate a relationship between fiber dimension and damage to cells by oxygen free radicals. They also appear to shed some light on the question, "Why are longer asbestos fibers more pathogenic that shorter fibers in man and laboratory animals?". In the classical studies of Stanton *et al.* (48, 49) and Wagner, Berry, and Timbrell (51) mesotheliomas occur in rodents after intrapleural injection of long, thin fibers (*i.e.*, those > 8 μm in length and <2.5 μm in diameter) of a variety of types. Alternatively, few tumors are observed after injection of short fibers or particles. In support of these experimental results, mesotheliomas frequently are found in asbestos workers in the Northwest Cape of Africa, a region where long, fine crocidolite is mined, whereas few tumors are observed in the Transvaal, a region containing a harsher, more blocklike type of crocidolite (52).

A direct correlation between fiber length and consequent fibrosis (*i.e.*, "asbestosis") also exists. After inhalation by or intratracheal instillation into animals, longer in comparison to shorter asbestos fibers are associated with a greater risk of fibrotic lung disease (16, 25, 50). The influence of longer fibers in the development of bronchogenic carcinoma has not been explored in depth, but Davis (7) attributes the increased numbers of tumors observed in rats after inhalation of chrysotile to the presence of longer fibers (>20 μm) in dust clouds. In these studies, clouds of crocidolite and amosite asbestos containing equal numbers of shorter fibers were less carcinogenic in animals. Our data lend credence to a theory advanced by Archer (1) to explain the carcinogenesis of fibers and films (*i.e.*, "foreign bodies"). He hypothesizes that exocytosis of long thin fibers and their flexion permit the escape of cellular superoxide radicals which then induce somatic mutations. Although the ability of asbestos to act as a mutagen is questionable (5, 23, 29), human mesothelial cells (26) and Syrian hamster embryo cells (19) exhibit chromosomal aberrations after exposure to asbestos *in vitro*. Conceivably, breakage of DNA under these circumstances could occur via the generation of active oxygen species although the ability of these short-lived species to diffuse long enough distances in order to exert nuclear effects is questionable. More stable, secondary products of generation (lipid oxidation products, H_2O_2) are more probable candidates. On the other hand, we and other investigators have been unable to demonstrate single-strand breaks by alkaline elution in hamster (35) and human (13) tracheobronchial epithelial cell DNA after exposure to UICC crocidolite or chrysotile asbestos. Since these cell types are rich in SOD, DNA-damaging radicals might be scavenged more effectively than by mesothelial cells and fibroblasts, the cell types affected in mesothelioma and foreign body carcinogenesis, respectively.

Epithelial cells of the airways are the cells giving rise to bronchogenic carcinoma, a tumor whose incidence is increased substantially in asbestos workers who smoke (46, 47). To address possible mechanisms of synergism between asbestos and polycyclic aromatic hydrocarbons in cigarette smoke, we measured uptake of radiolabeled

benzo(a)pyrene by tracheal epithelial cells after addition of the hydrocarbon to medium alone in comparison to its adsorption and introduction on asbestos fibers (12, 35). Under the latter circumstances, cellular uptake of BaP was increased and alkylation of BaP to DNA, an event related intrinsically to carcinogenesis, persisted for extended periods of time. These observations and a recent report showing that oxidizing agents generated during lipid peroxidation initiate the epoxidation of inactive BaP to active carcinogenic forms (10) suggest that asbestos not only affects the capacity of the cell to remove polycyclic aromatic hydrocarbons but also favors the conversion of these chemicals to species capable of interacting with DNA (via production of oxygen free radicals).

The ability to ameliorate asbestos-associated injury at the cellular level using scavengers of O_2^- and OH· is exciting in view of the possible extension of preventive approaches to asbestosis, bronchogenic carcinoma, and mesothelioma in man. A common, early feature of all these asbestos-associated diseases is inflammation, a pathologic change characterized by the influx of macrophages and polymorphonuclear leukocytes, cells associated with production of oxygen free radicals (15, 32). It will be important to determine whether inflammation, acute cell injury and/or consequent mineral lung disease can be prevented by administration of antioxidants to animals.

Acknowledgments: We thank Joan Carrassi, Lucy Jean, and Judith Kessler for excellent technical assistance. Dr. John E. Craighead and Dr. Kenneth B. Adler provided helpful editorial advice. Dr. Janet Mitchell, Anne Burkhardt, and Thomas Orfeo assisted in the preparation and verification of antibody to superoxide dismutase.

Date of acceptance: September 18, 1985. An abstract describing some aspects of this work was presented at the 25th Aspen Lung Conference in Aspen, Colorado, 1983.

This work was supported by Grant BC-415 from the American Cancer Society, Public Health Service Grant RO1 33501 from the National Cancer Institute and Specialized Center of Organized Research Public Health Service 14212 from the National Heart, Lung and Blood Institute. Dr. Shatos is a Parker B. Francis Fellow.

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