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Generation of Superoxide (O_2^-) from Alveolar Macrophages Exposed to Asbestiform and Nonfibrous Particles[1]

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ABSTRACT

Active oxygen species are implicated causally in tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate and in cell damage by asbestos, a putative tumor promoter of the respiratory tract. To determine the properties of asbestos important in generation of the oxygen free radical, superoxide (O_2^-), hamster and rat alveolar macrophages were exposed *in vitro* to nontoxic concentrations of fibrous (crocidolite, erionite, Code 100 fiberglass, sepiolite) and nonfibrous (riebeckite, mordenite, glass) particulates. The amount of O_2^- released by cells in response to dusts was determined by measuring the reduction of cytochrome *c* in the presence and absence of superoxide dismutase. Results showed that all fibrous (defined as a >3:1 length:diameter ratio) dusts caused a significant increase in both release of O_2^- from rat macrophages and enhancement of zymosan-triggered O_2^- from hamster macrophages. Nonfibrous particles were less active than fibers at comparable concentrations. These results suggest that the geometry of particulates is of critical importance in the generation of O_2^- from cells of the respiratory tract.

INTRODUCTION

Generation of oxygen free radicals has been implicated in lung injury and disease caused by a variety of perturbating agents including TPA,[3] paraquat, bleomycin, and hyperoxia (1–4). These reactive species damage cells by inducing peroxidation of lipids, depolymerization of polysaccharides, and breakage of DNA (reviewed in Ref. 5). Inflammation, cell injury, and repair and/or death of tissues then occur.

Several observations suggest a role of active oxygen metabolites in tumor promotion by TPA in the mouse skin system (reviewed in Ref. 6). For example, TPA causes marked inflammation when applied to epidermis and generation of O_2^- from fibroblasts and cells of the immune system in culture (7, 8). In this regard, the ability of various phorbol compounds to induce the production of O_2^- from polymorphonuclear leukocytes *in vitro* is related directly to their efficiency of tumor promotion *in vivo* (9). Other free radical generating compounds such as benzoyl peroxide also are effective tumor promoters when applied to mouse skin (10) or when added to two-stage mammalian cell transformation assays *in vitro* (11). Lastly, scavenger enzymes of active oxygen species and other antioxidants inhibit TPA-induced tumor promotion in mouse skin (12).

Asbestos, a family of fibrous silicates associated with the occurrence of mesothelioma and bronchogenic carcinoma in occupationally exposed individuals causes a myriad of biochemical and morphological events similar to the effects of TPA in grafts, organ cultures, and cell cultures of tracheobronchial epithelium (13–16). These include inflammatory changes (17), increases in cell proliferation (16, 18), alterations in normal cell differentiation (19, 20) and membrane perturbations such as stimulation of Na^+-K^+-dependent ATPase (21), and induction of prostaglandin synthesis (22). When injected into rat tracheal grafts exposed previously to subcarcinogenic amounts of the chemical carcinogen, dimethylbenzo(a)anthracene, asbestos also causes the development of neoplasma (14). Thus, it appears to behave as a typical tumor promoter both *in vitro* and *in vivo*.

Recent studies show that asbestos-induced toxicity to hamster tracheal epithelial cells, rat lung fibroblasts, and rat alveolar macrophages *in vitro* is ameliorated by both SOD, the enzyme scavenging O_2^-, and scavengers of the hydroxyl ($OH^\cdot$) radical (13, 23). Moreover, asbestos causes lipid peroxidation (24, 25), and chemiluminescence in rodent alveolar macrophages *in vitro* (26).

Those properties of asbestos important in cell damage and/or carcinogenesis are obscure although factors such as fibrous geometry (27), size (28) chemistry (29), and surface charge (30) are thought to be important. The goal of studies here was to determine whether geometry and/or chemical composition of asbestos and other particulates determined release of O_2^- from cells of the respiratory tract. We used lavaged hamster and rat alveolar macrophages because these cell types are effectors of mineral-induced lung disease and release detectable amounts of O_2^- after exposure to asbestos fibers and TPA *in vitro* (31).

MATERIALS AND METHODS

Minerals. A variety of fibers including crocidolite asbestos, $Na_2O \cdot Fe_2O_3 \cdot 3FeO \cdot 8SiO_2 \cdot H_2O$, (International Union Against Cancer reference sample), erionite $[Na_2 \cdot K_2 \cdot Ca \cdot Mg]_{4.5}[Al_9Si_{27}O_{72}] \cdot 27H_2O$ (Rome, Oregon; a gift from Dr. Reg Davies, MRC Pneumoconiosis Unit, Penarth, Wales), Code 100 fiberglass, SiO_2 (Manville Corp., Denver, CO), and sepiolite, $Mg_4 \cdot [Si_2O_5]_3 \cdot [OH] \cdot 6H_2O$, (Minerals Research, Clarkstown, NY), were assessed comparatively over a range of nontoxic concentrations with their nonfibrous, chemically similar analogues, *i.e.*, riebeckite $Na_2O \cdot Fe_2O_3 \cdot 3FeO \cdot 8SiO_2 \cdot H_2O$, (Wards National Science Establishment, Rochester, NY), mordenite, $[Ca \cdot Na_2 \cdot K_2]_4 \cdot Al_8Si_{40}O_{96} \cdot 28\ H_2O$, and glass beads, SiO_2, (1–4 μm diameter, Particle Information Service, Inc.), respectively. All preparations of dusts were examined for purity and size dimensions using X-ray diffraction, X-ray energy spectrometry, and scanning electron microscopy as reported previously (32). Prior to their addition to cell cultures, dusts were heat sterilized, suspended in Medium 199 (GIBCO, Grand Island, NY) containing 10% fetal calf serum, and dispersed by ultrasonication for 2 min.

Zymosan A. Zymosan A heat-killed yeast particles, (Sigma Chemical Co., St. Louis, MO) were opsonized by boiling in EBSS for 15 min, followed by incubation for 15 min at 37°C in fetal bovine serum, washing in EBSS, and resuspension in EBSS to make a 3 mg/ml stock suspension (33).

Isolation of Alveolar Macrophages. Young adult, male Syrian hamsters (EHS strain) and Fischer 344 rats (obtained from NIH) were anesthesized with phenobarbital and lung lavages performed as described previously (10). Differential cell counts of lavage constituents indicated that over 98% [98.7 ± 0.32 (SE)] of cells were macrophages. Neutrophils (0.79 ± 0.21%) and lymphocytes (0.51 ± 0.11%) constituted a very small proportion of the total cell yield. The lavage fluid was kept on ice, centrifuged for 10 min at 900 × *g*, and the cell pellet resuspended in Medium 199 supplemented with 10% fetal bovine

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[3] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; O_2^-, superoxide; EBSS, Earle's balanced salt solution.

serum. Macrophages were counted using a hemocytometer and plated in 12-well dishes at 5×10^5 cells/well in 1 ml of complete Medium 199. Cells were maintained at 37°C (95% air-5% CO_2) for 2 h at which time nonadherent cells were decanted before exposure of cultures to dusts.

Exposure to Particulates. Based on the results of preliminary studies to characterize the best protocol for optimal detection of O_2^- in each system, two different procedures were used for experiments with rat and hamster macrophages. In studies using rat cells, dusts were suspended in Medium 199 containing 10% fetal bovine serum, pelleted, and resuspended before addition to macrophages in the O_2^- reaction mixture (see below) for 1 h. In contrast, hamster macrophages were exposed to the dust for 2 h in serum-containing Medium 199 followed by removal of medium, and subsequent exposure of cells to zymosan particles in the O_2^- reaction mixture for 1 h. Nontoxic amounts of dusts were determined by exposing cells to particulates in complete medium for 24 h. At this time, numbers of viable cells excluding the vital dye nigrosin (VWR Scientific, Boston, MA) were counted using a hemocytometer.

Assay for Detection of O_2^-. Cells in 12-well dishes were incubated at 37°C in a reaction mixture consisting of 1 ml EBSS containing 40 μM cytochrome *c* (Type VI, horseheart; Sigma) (34). After 1 h, the supernatant was decanted into microfuge tubes on ice and centrifuged at $8000 \times g$ to remove debris. Reduction of cytochrome *c* was measured spectrophotometrically at 550 nm. Results were calculated as μmol reduced cytochrome *c*/5×10^5 cells/h using a 0.029 mM extinction coefficient for cytochrome *c*. All groups were run in triplicate with or without the addition of 100 μg/ml superoxide dismutase (bovine erythrocyte; Sigma). In all reaction mixtures containing superoxide dismutase, the amount of cytochrome *c* reduction observed was caused specifically by the release of O_2^- into the reaction mixture. Additional controls consisted of cell-free reaction mixtures with and without asbestos and non-dust-treated and zymosan-exposed cells. Analysis of variance by the Dunnett's procedure was used to determine statistically significant differences between groups (35).

RESULTS

Geometry and Size Dimensions of Particulates. Scanning electron microscopy of suspensions of particulates used in the present study showed the predominantly fibrous geometry of crocidolite (Fig. 1*a*), erionite (Fig. 1*c*), and Code 100 fiberglass (Fig. 1*c*). In contrast, sepiolite (Fig. 1*g*) consisted of mixed bundles of fibers and particles. Riebeckite (Fig. 1*b*), glass particles (Fig. 1*d*), and mordenite (Fig. 1*f*) were nonfibrous in nature. Fiber and particle size distributions (Fig. 2) indicated that all preparations of dusts were respirable (*i.e.*, <10 μm diameter).

Release of O_2^- from Hamster and Rat Alveolar Macrophages. Both hamster and rat cells phagocytized asbestos immediately after addition of fibers to cultures. These fibers appeared to be intracellular throughout the duration of the experiments. Fresh, unactivated hamster alveolar macrophages did not release detectable amounts of O_2^- and showed only a small increase in response to asbestos when compared to zymosan (Fig. 3*A*). Consequently, a two-step procedure for exposure to dusts and zymosan particles was adopted as described above. A significant enhancement was observed when either hamster (Fig. 3*A*) or rat (Fig. 3*B*) cells were exposed to asbestos prior to the addition of zymosan. In dose-response studies using hamster cells, the greatest induction of O_2^- release was obtained with 0.25 mg/ml medium of zymosan alone (Fig. 4*A*); thus, this concentration was used in all subsequent experiments. O_2^- release induced by either zymosan or asbestos increased with time over a 1-h period but declined thereafter in kinetic studies of O_2^- release (Fig. 5).

The results of experiments after preaddition of selected particulates and zymosan to hamster alveolar macrophages are illustrated in Fig. 6. In comparison to untreated controls,

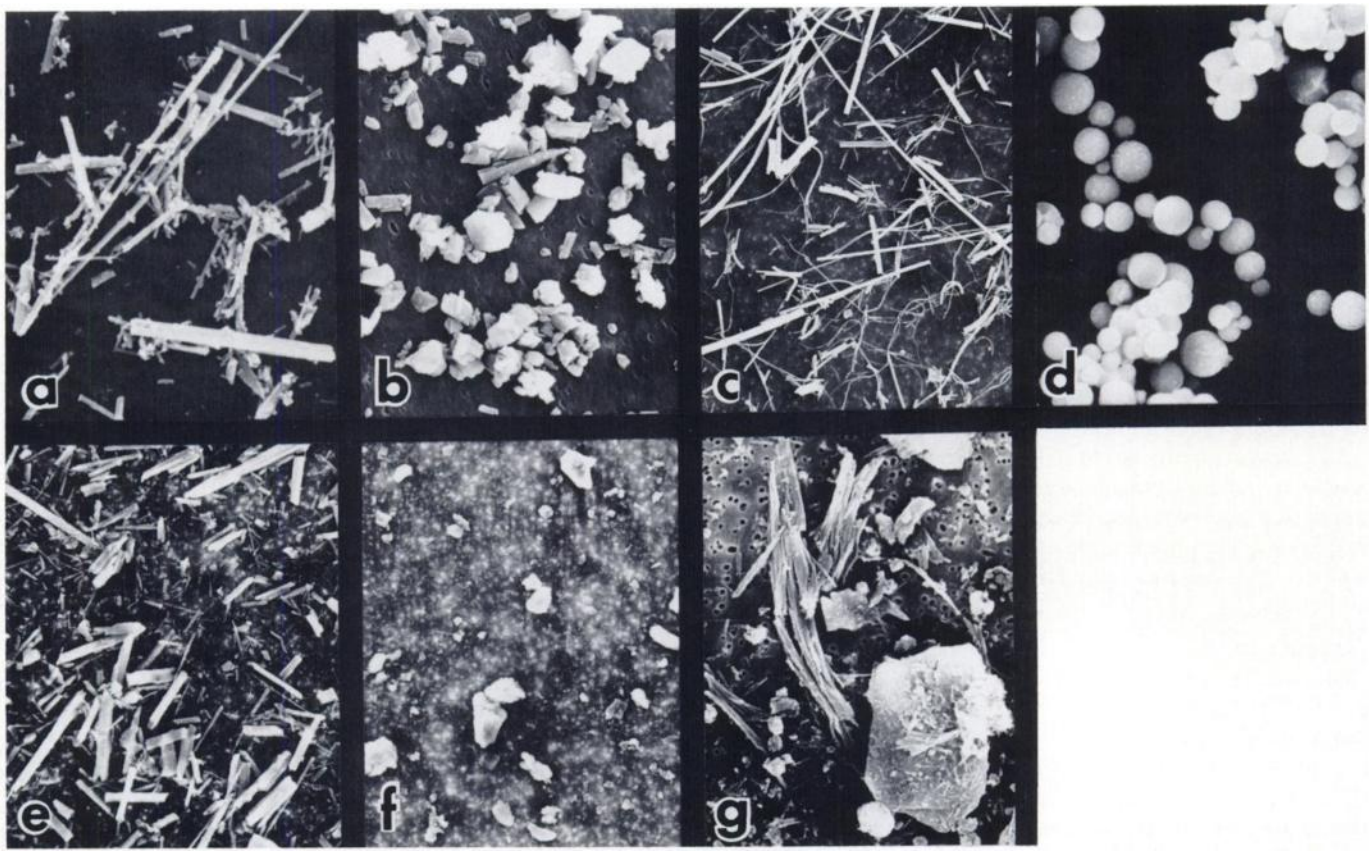

Fig. 1. Appearance of fibers and particles as documented by scanning electron microscopy. *a*, crocidolite asbestos. × 2250. *b*, Riebeckite. × 3000. *c*, Code 100 fiberglass. × 750. *d*, glass beads. × 2250. *e*, erionite. × 750. *f*, mordenite. × 750. *g*, sepolite. × 3750.

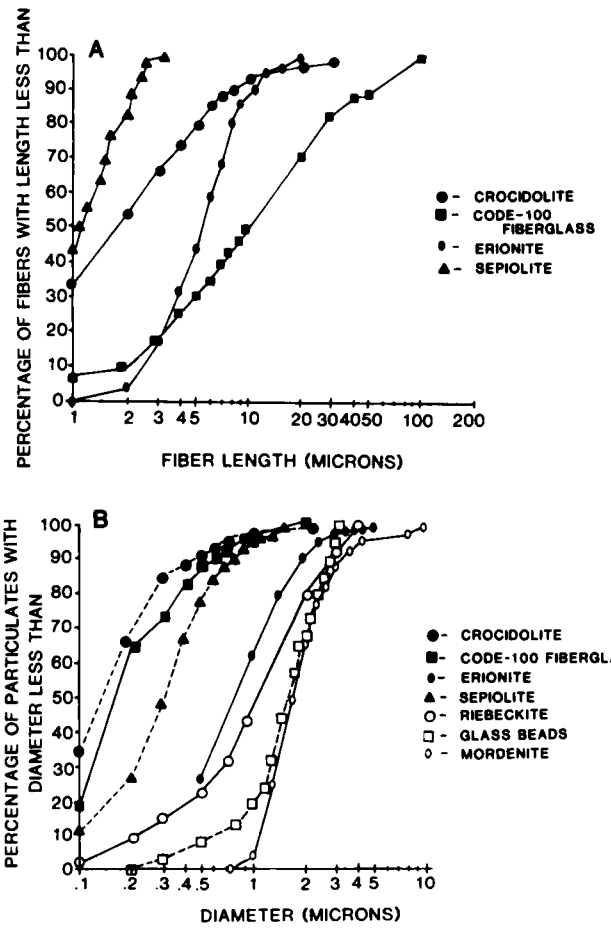

Fig. 2. Cumulative size distributions of fibers and particles as determined by scanning electron microscopy. Over 200 particulates from each preparation were measured after dispersal in medium on carbon planchets. *A*, length of fibers; *B*, diameter of fibers and particles.

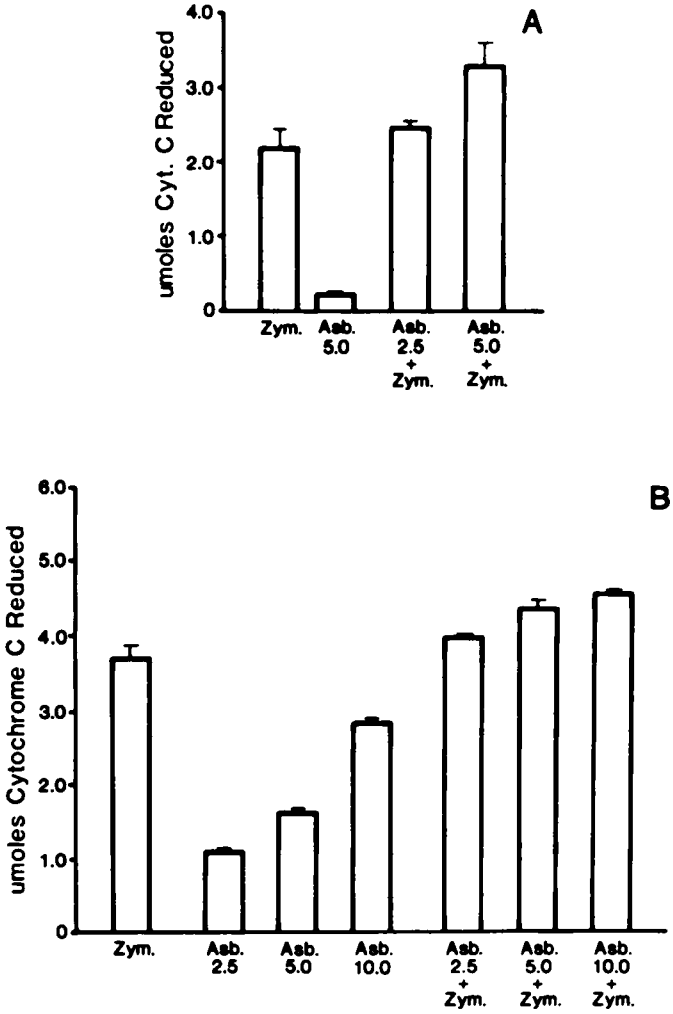

Fig. 3. Release of O_2^- [mean ± SE (*bars*); $N = 4$ to 5/group] from hamster (*A*) and rat (*B*) alveolar macrophages exposed to crocidolite asbestos (*Asb.*) and zymosan (*Zym.*) alone and in combination. Zymosan (0.25 mg/ml reaction mixture); asbestos, 2.5, 5, and 10 $\mu g/cm^2$ dish. Results are from a typical experiment. *Cyt.*, cytochrome.

exposure to crocidolite asbestos resulted in a dose-dependent increase ($P < 0.05$) in O_2^- release, whereas exposure of cells to its nonfibrous analogue, riebeckite, was ineffectual even at higher concentrations (Fig. 6*A*). Code 100 fiberglass also caused a significant dose-dependent increase ($P < 0.05$) in generation of O_2^- in contrast to glass beads at comparable and higher concentrations (Fig. 6*B*). Generation of O_2^- after exposure of cells to erionite and sepiolite also was elevated significantly ($P < 0.05$) (Fig. 6*C*).

In contrast to results with hamster macrophages, rat macrophages generated O_2^- (approximately 0.172 ± 0.071 μmol reduced cytochrome *c*/h/5 × 10^5 cells) under baseline conditions. Moreover, release of O_2^- increased significantly ($P < 0.01$ and $P < 0.05$) when cells were exposed to all particulates examined (Fig. 7). Although rat cells produced O_2^- in amounts related directly to concentrations of zymosan in medium (Fig. 3*B*), no activation by zymosan was necessary in experiments using minerals. As was observed above in the hamster system, exposure to asbestos or Code 100 fiberglass resulted in a significant increase ($P < 0.01$) in production of O_2^- by macrophages whereas the nonfibrous analogues, riebeckite and glass beads, caused less significant ($P < 0.05$) responses even at 5-fold higher concentrations (Fig. 7, *A* and *C*). Amounts of O_2^- released after exposure of rat macrophages to erionite or mordenite appeared dosage dependent although higher concentrations of mordenite were required to induce comparable responses (Fig. 7*B*). Sepiolite also caused a significant ($P < 0.01$) increase in production of O_2^- (Fig. 7*C*) as was observed in hamster macrophages.

Important controls in all experiments were cell-free reaction mixtures with fibers. Reduction of cytochrome *c* was not observed under these circumstances.

DISCUSSION

Studies here were designed to determine the properties of asbestos important in generation of O_2^- from alveolar macrophages. These cell types accumulate at sites of deposition of fibers after inhalation by animals (36) and are thought to be critical to the pathogenesis of asbestos-induced diseases (37). Observations above are consistent with the data of Donaldson *et al.* (31) who observed enhanced release of O_2^- and H_2O_2 from murine peritoneal macrophages after exposure to chrysotile asbestos. After i.p. injection of chrysotile fibers and subsequent isolation of macrophages, production of active oxygen species by cells was greater when TPA or zymosan was added to the reaction mixture. Although particulate-induced generation of O_2^- in our studies was most striking in cultures of rat alveolar macrophages, patterns of responses to dusts were remarkably consistent in both hamster and rat cells. Specifically, a number of asbestiform fibers induced a dosage-depend-

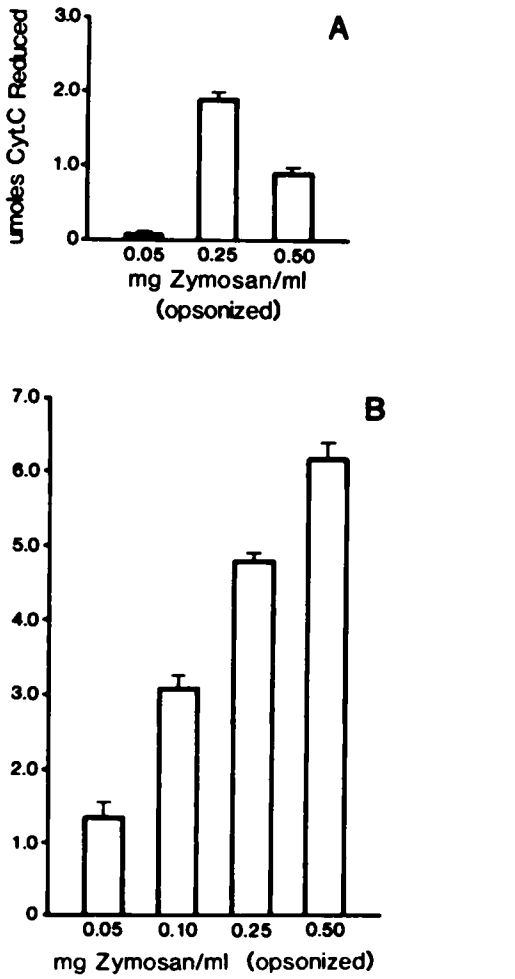

Fig. 4. Release of O_2^- [mean ± SE (*bars*); $N = 4$ to 5/group] from hamster (*A*) and rat (*B*) alveolar macrophages in response to different concentrations (0.05, 0.10, 0.25, 0.50 mg/ml reaction mixture) of zymosan. Results are the mean ± SE of duplicate experiments ($N = 4$ to 6/group). *Cyt.*, cytochrome.

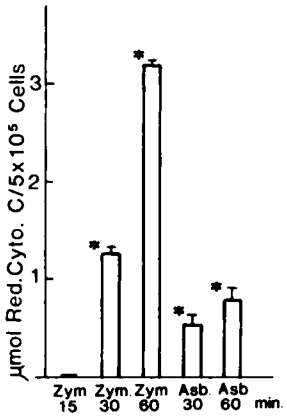

Fig. 5. Release over time [mean ± SE (*bars*); $N = 4$ to 5/group] of O_2^- from hamster alveolar macrophages in response to zymosan (*Zym.*) (0.25 mg/ml reaction mixture) and crocidolite asbestos (*Asb.*) (5.0 μg/cm² dish). Results are from a typical experiment. *, increased significantly ($P < 0.05$) in comparison to untreated controls; *Red. Cyto.*, reduced cytochrome.

ent increase in the release of O_2^- from macrophages whereas their chemically similar nonfibrous analogues caused less striking responses at comparable concentrations. Thus, results here suggest an important role of fibrous geometry in release of O_2^- by cells of the respiratory tract.

Our observations support previous work indicating a link between the fibrous nature and carcinogenicity of particulates

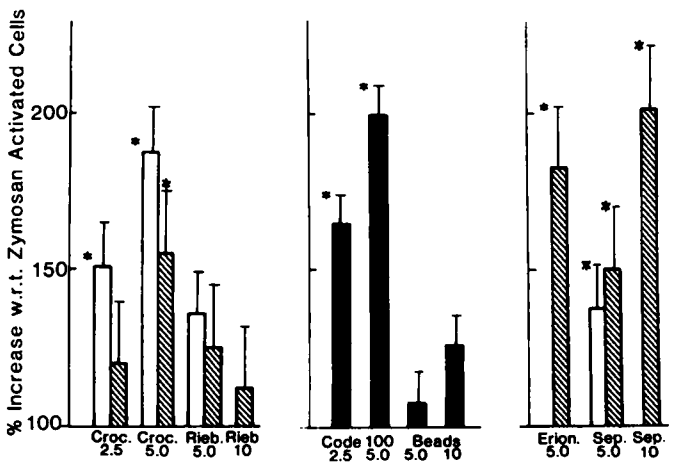

Fig. 6. Release of O_2^- from hamster alveolar macrophages exposed to particles and zymosan. Various dusts were added for 2 h in medium before addition of zymosan (0.25 mg/ml) in the reaction mixture. The amount of O_2^- release [mean ± SE (*bars*); $N = 4$ observations/group] is presented as a percentage of increase over values observed with cells exposed to zymosan alone. ■, □, results of duplicate experiments; *, increased significantly ($P < 0.05$) in comparison to zymosan controls. *w.r.t.*, with respect to; *Croc.*, crocidolite asbestos; *Rieb.*, riebeckite; *Erion.*, erionite; *Sep.*, sepiolite.

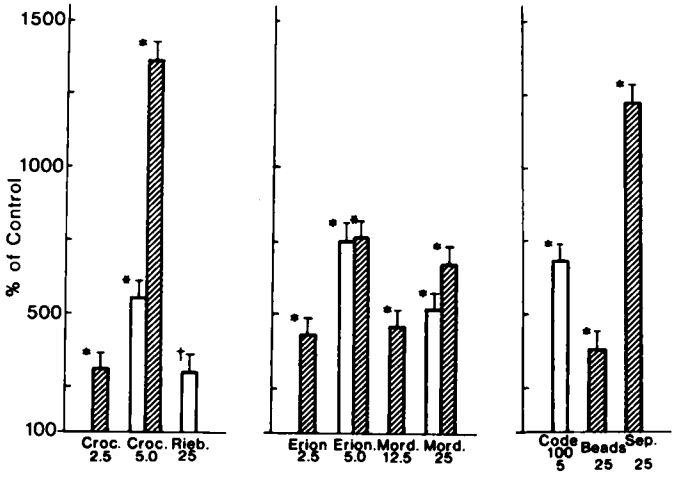

Fig. 7. Release of O_2^- from rat alveolar macrophages exposed to particulates for 1 h in the O_2^- reaction mixture. The amount of O_2^- release [mean ± SE (*bars*); $N = 3$ to 5 observations/group] is presented as a percentage of increase over values observed in unexposed controls. ■, unexposed control values = 0.172 ± 0.071 μmol reduced cytochrome c/h/5 × 10^5 cells); □, unexposed control values = 0.103 ± 0.032 μmol reduced cytochrome c/h/5 × 10^5 cells). Each represents separate experiments. *, increased significantly ($P < 0.01$) in comparison to controls; †, increased significantly ($P < 0.05$) in comparison to controls; *Croc.*, crocidolite asbestos; *Rieb.*, riebeckite; *Erion.*, crionite; *Mord.*, mordenite; *Sep.*, sepiolite.

when injected or implanted intrapleurally into rodents. In studies by Stanton *et al.* (27), Stanton and Wrench (28), Wagner (38) and Wagner *et al.* (39) a variety of fibers (defined as a >3:1 length:diameter ratio) caused the development of mesotheliomas and pleural sarcomas in rats whereas particles were ineffectual at comparable concentrations. This phenomenon might be related in part to the ability of fibers to cause proliferation of these cell types. For example, after addition to tracheal epithelial cells or organ cultures, a number of fiber types (Code 100 fiberglass, amosite, crocidolite, and chrysotile asbestos) stimulate both hyperplasia and squamous metaplasia, the conversion of normal mucociliary epithelium to keratinizing cells (32). In contrast, their respective nonfibrous analogues do not cause growth alterations. Ultrastructural observations suggest that fibers embedded on the epithelial surface serve as

supporting matrices for proliferation of epithelial cells whereas particles are cleared by or phagocytized by these cell types. We reported recently the induction of ornithine decarboxylase, an enzyme associated with cell division and tumor promotion, in tracheal epithelial cells exposed to asbestos and Code 100 fiberglass but were unable to demonstrate increases in ornithine decarboxylase activity using particulates at comparable concentrations (40).

Recent studies by Hesterberg and Barrett (41) also show selective effects of fibers in the Syrian hamster embryo transformation system. When added to hamster embryo fibroblasts, asbestos (*i.e.*, chrysotile, crocidolite) and Code 100 fiberglass cause chromosomal aberrations and morphological transformation of cells. In contrast, milled glass particles are less genotoxic. These investigators hypothesize that longer glass and asbestos fibers penetrate the nuclear membrane and interact with chromosomes during mitosis.

In addition to asbestos and fiberglass, we examined two other asbestiform fibers for their ability to trigger release of O_2^- from alveolar macrophages. Erionite, a fibrous zeolite, is endemic to the Anatolian region of Turkey and soils of the midwestern United States (42). A recent epidemic of mesothelioma and other pleural diseases in Turkey has been attributed to the presence of erionite in village dwellings (42). Like asbestos, erionite causes peritoneal mesotheliomas when injected into rodents (43). Moreover, the dust causes morphological transformation and unscheduled DNA synthesis in C3H10T1/2 and human lung cells (44). In studies here, erionite caused significant elevations in both baseline and zymosan-triggered release of O_2^- from macrophages. However, its chemically similar nonfibrous analogue, mordenite, also was effectual, albeit at five-fold higher concentrations.

Sepiolite, one of several clay minerals advocated as a commercial substitute for asbestos, consists of conglomerates of mixed fibers and particles. Clinical and radiological studies on sepiolite workers do not indicate an increased risk of pleural disease (45). Although sepiolite has not been examined in animal models of carcinogenesis, it is hemolytic and weakly toxic to macrophage-like cells *in vitro* (46). Results here indicate that this mineral is relatively potent in inducing O_2^- release from both hamster and rat macrophages.

The enhanced ability of fibrous materials to trigger release of active oxygen metabolites from cells of the respiratory tract is consistent with a theory advanced by Archer (47) to explain foreign body carcinogenesis. In brief, long thin fibers and films are thought to interact with and perturb the plasma membrane of target cells due to their inability to be phagocytized effectively (*i.e.*, "frustrated" phagocytosis). Under these circumstances, generation of active oxygen species, known clastogenic and membrane-active agents, could occur at the membrane surface or during the phagocytic process. This phenomenon could account for the documented carcinogenic and/or tumor-promoting properties of asbestos and other asbestiform fibers.

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