American Journal of Pathology, Vol. 139, No. 3, September 1991

Histochemical Evidence for Generation of Active Oxygen Species on the Apical Surface of Cigarette-smoke–exposed Tracheal Explants

Jane Hobson, Joanne Wright, and Andrew Churg

From the Department of Pathology and University Hospital, University of British Columbia, Vancouver, British Columbia, Canada

Cigarette smoke is known to contain many types of free radicals, and solutions of smoke tar have been shown to liberate hydrogen peroxide as well as superoxide radical. To further investigate the relationship of smoke exposure and generation of active oxygen species, the authors exposed rat tracheal explants to varying amounts of smoke for 10 minutes in a humidified chamber. After smoke exposure was completed, tracheal segments were incubated in a modification of the ultrastructural cerium chloride technique that was devised by Briggs et al. to demonstrate hydrogen peroxide production. Smoke dose-dependent deposition of cerium-containing reaction product was found on the cilia and the apical membranes; with low-dose smoke, the reaction product appeared as individual dots along the apical surface, but with greater amounts of smoke, heavy linear deposits of reaction product were found along the apical membranes. Smoke produced focal dose-related cell damage with blebbing of the apical membranes, loss of cilia, and focal cell necrosis. Catalase prevented both the positive histochemical reaction and the cell damage; if the catalase was first boiled, its protective effect was destroyed. Similarly, after smoke exposure was completed, tracheal segments were covered with a solution of nitroblue tetrazolium to demonstrate production of superoxide anion. A positive reaction was observed by light microscopy on the surface of tracheas that was exposed to smoke but not that exposed to air; the reaction could be prevented by addition of superoxide dismutase. The authors conclude that exposure of tracheal explants to cigarette smoke* in vitro *is associated with histochemical evidence of continuing production of both hydrogen peroxide and superoxide anion at the apical cell membrane. (Am J Pathol 1991, 139:573–580)

Cigarette smoke is known to contain many types of free radicals, and it is believed that free radicals and reactive chemical species generated by free radicals are important in the pathogenesis of smoke-induced lung disease.[1–3] Smoke produces a variety of radicals in both the gas and tar phases,[1–3] and aqueous solutions of smoke tar generate both superoxide anion and hydrogen peroxide.[1–5] Previous data from our laboratory on the uptake of asbestos fibers by tracheal epithelial cells in organ culture have indicated that smoke augments fiber uptake, and that this effect can be entirely prevented by scavengers of active oxygen species.[6–9] The fact that the asbestos is added *after* the completion of smoke exposure in these experiments suggested that there is ongoing generation of active oxygen species on the tracheal epithelial surface after smoke exposure, probably as a result of tar deposition during smoke exposure (see Discussion).

We used histochemical techniques to detect hydrogen peroxide and superoxide anion and showed that the generation of these two active oxygen species can be observed on the apical tracheal cell surfaces after cigarette smoke exposure.

Materials and Methods

Tracheal explants were prepared from 200 g female Sprague-Dawley rats using a modification of the method of Mossman[10] as previously described.[6–9] The segments were placed, serosal side down, on Millipore filters

Supported by grants MA8051 and MA7820 from the Medical Research Council of Canada.

Accepted for publication April 30, 1991.

Address reprint requests to Dr. Andrew Churg, Department of Pathology, University of British Columbia, 2211 Wesbrook Mall, Vancouver, BC Canada V6T 2B5.

that were moistened with a small amount of culture medium (Dulbecco's minimal essential medium, Gibco Products), and then exposed to various amounts of whole smoke from a commercial nonfilter cigarette for 10 minutes in a humidified chamber at 37°C. Control explants were exposed to air in a similar chamber.

For demonstration of hydrogen peroxide production, the tracheal explants were exposed to 1, 3, or 6 puffs of smoke; after 10 minutes, they were removed from the smoke chamber, held for 1 minute in air, and transferred to a modification of the cerium chloride medium described by Briggs et al.[11] Briggs et al. were originally interested in showing production of hydrogen peroxide by the NADH oxidase located in the cell membranes of polymorphonuclear leukocytes, and hence, included NADH as substrate in the medium. Since we were only interested in exogenous hydrogen peroxide production, we omitted NADH from the medium.

Explants were incubated in the cerium chloride medium for 30 minutes; they were then rinsed 4 times with tris-maleate buffer to remove unreacted cerium, fixed overnight in 2.5% glutaraldehyde in 0.1M cacodylate buffer, postfixed in osmium tetroxide, dehydrated in ethanol, and embedded in epoxy resin. In some instances, osmium or lead and uranium counterstains were omitted to provide better contrast between tissue and reaction product. Sections were examined in an electron microscope that was equipped with energy dispersive x-ray

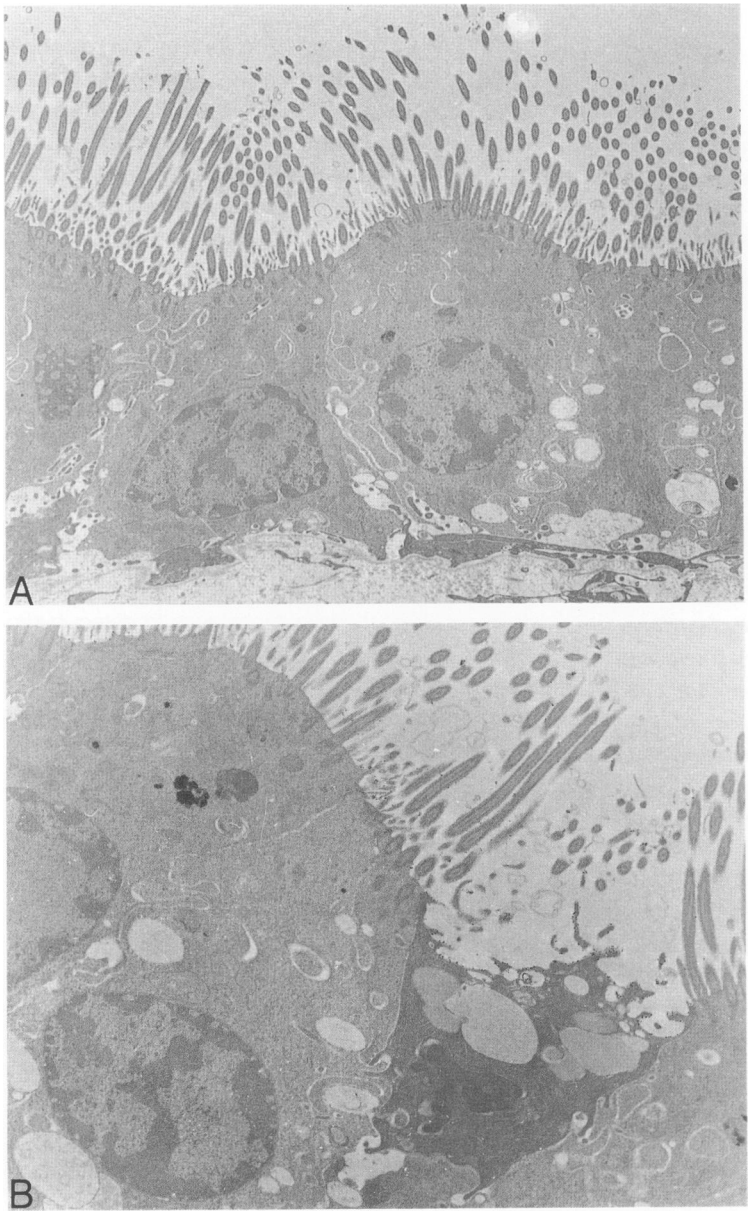

Figure 1. *Electron micrographs of control tracheas exposed to air and then incubated for 30 minutes in the cerium chloride medium of Briggs et al.*[11] **A**: *Low-power view. Note overall good morphologic preservation but tendency toward separation of cells along their lateral edges, particularly toward the basal pole.* **B**: *Higher-power view of another area showing apical surfaces of ciliated and mucous cells. No cerium reaction product is present (Glutaraldehyde and osmium fixation, counterstained with lead citrate and uranyl acetate;* **A**: *×5200;* **B**: *×8100).*

spectrometer. The latter was used to confirm the presence of cerium in the precipitate.

As a negative control, tracheal segments were exposed to air instead of smoke and incubated in the reaction mixture. To demonstrate the specificity of the reaction, sets of tracheas were briefly immersed before smoking in culture medium containing 1300U/ml catalase (Boehringer-Mannheim); alternately, the catalase was added after smoke exposure to the cerium chloride medium. Additional explants were incubated with 1300U/ml of catalase that had been boiled for 10 minutes to inactivate the enzyme.

To show the production of superoxide anion,[12–14] the tracheal segments were exposed to six puffs of smoke as described earlier, removed from the smoke, and held in air for 1 minute. A drop of a 3 mg/ml solution of nitroblue tetrazolium (NBT) (Sigma Chemicals) in Hank's balanced salt solution (Gibco) pH 7.3 was placed on the apical surface of each segment for 10 minutes. Controls were run by exposing the segments to air instead of smoke. To

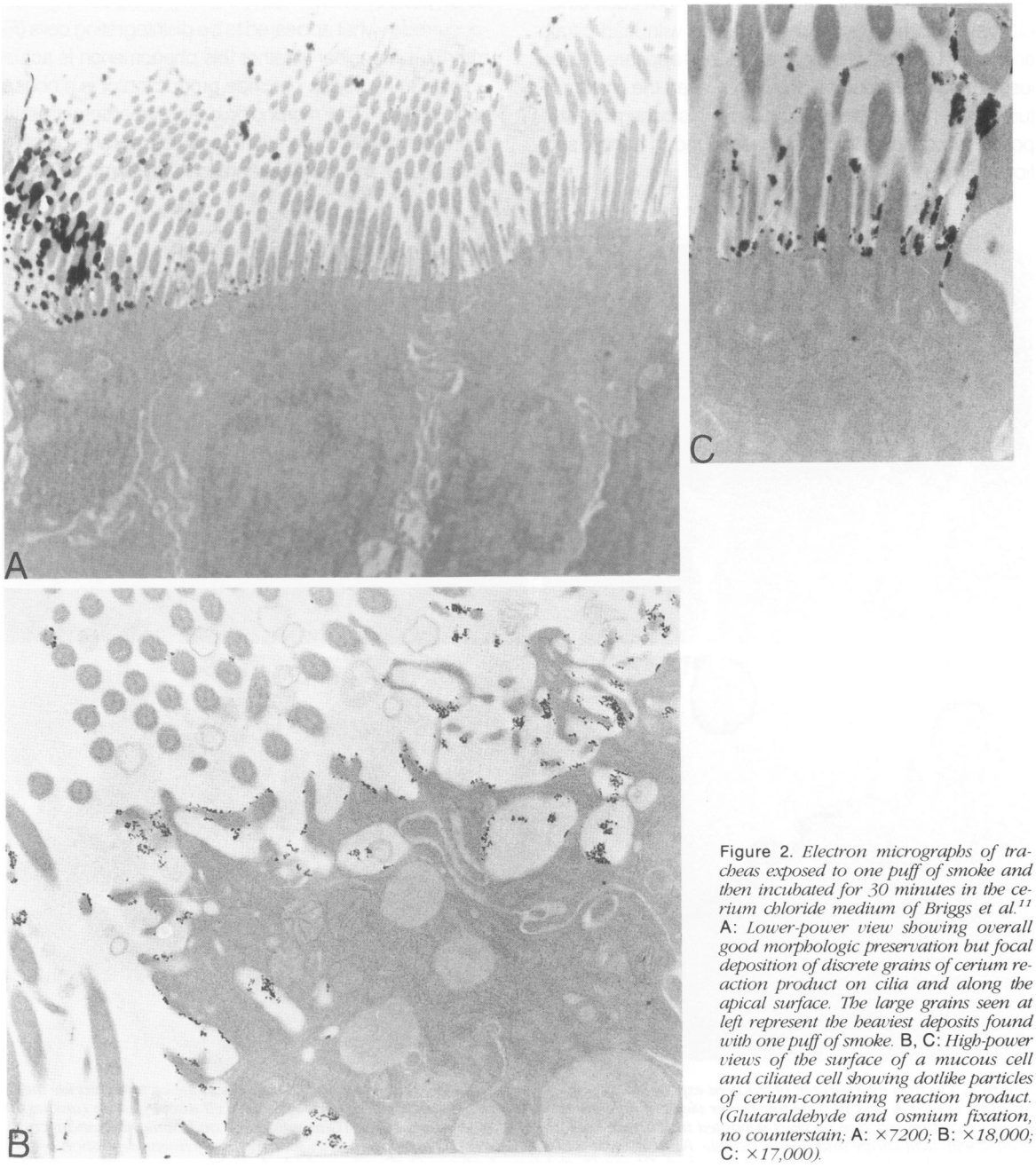

Figure 2. *Electron micrographs of tracheas exposed to one puff of smoke and then incubated for 30 minutes in the cerium chloride medium of Briggs et al.*[11] A: *Lower-power view showing overall good morphologic preservation but focal deposition of discrete grains of cerium reaction product on cilia and along the apical surface. The large grains seen at left represent the heaviest deposits found with one puff of smoke.* B, C: *High-power views of the surface of a mucous cell and ciliated cell showing dotlike particles of cerium-containing reaction product. (Glutaraldehyde and osmium fixation, no counterstain;* A: *×7200;* B: *×18,000;* C: *×17,000).*

demonstrate specificity, the NBT was added mixed with 600U/ml superoxide dismutase (SOD) (Sigma Chemicals).

Results

Ultrastructural Changes and Hydrogen Peroxide Localization

Control (Air-exposed) Explants

Use of the cerium chloride medium resulted in some degradation of ultrastructure, in particular, separation of the basal portions of the epithelial cells from each other. Contrast was also generally low, even with lead citrate and uranyl acetate staining, and there appeared to be loss of fine ultrastructural detail. However, the ultrastructural preservation was adequate for histochemical purposes. No cerium precipitate was found in control sections (Figure 1).

Smoke-exposed Explants

Smoke-exposed explants showed clearly dose-dependent deposition of cerium containing precipitate and dose-dependent damage to the cells. Cerium-containing precipitate was seen on apical cell surfaces in amounts that paralleled amount of smoke exposure. With low doses of smoke, individual grains of precipitate could be found on and between cilia and on the surface of mucous cells (Figure 2). With high doses of smoke, the precipitate, although still focal in overall distribution, appeared as a dense linear band along the cell membranes and cilia (Figure 3). In some segments that were exposed to six puffs of smoke, heavy staining of the cell organelles occurred in what appeared to be disintegrating cells (Figure 5); it is unclear whether this phenomenon is actually related to hydrogen peroxide production or is a nonspecific artifact. The morphologic abnormalities consisted primarily of cell separation, focal membrane blebbing, loss of cilia, and cell disintegration. Cell damage was rare

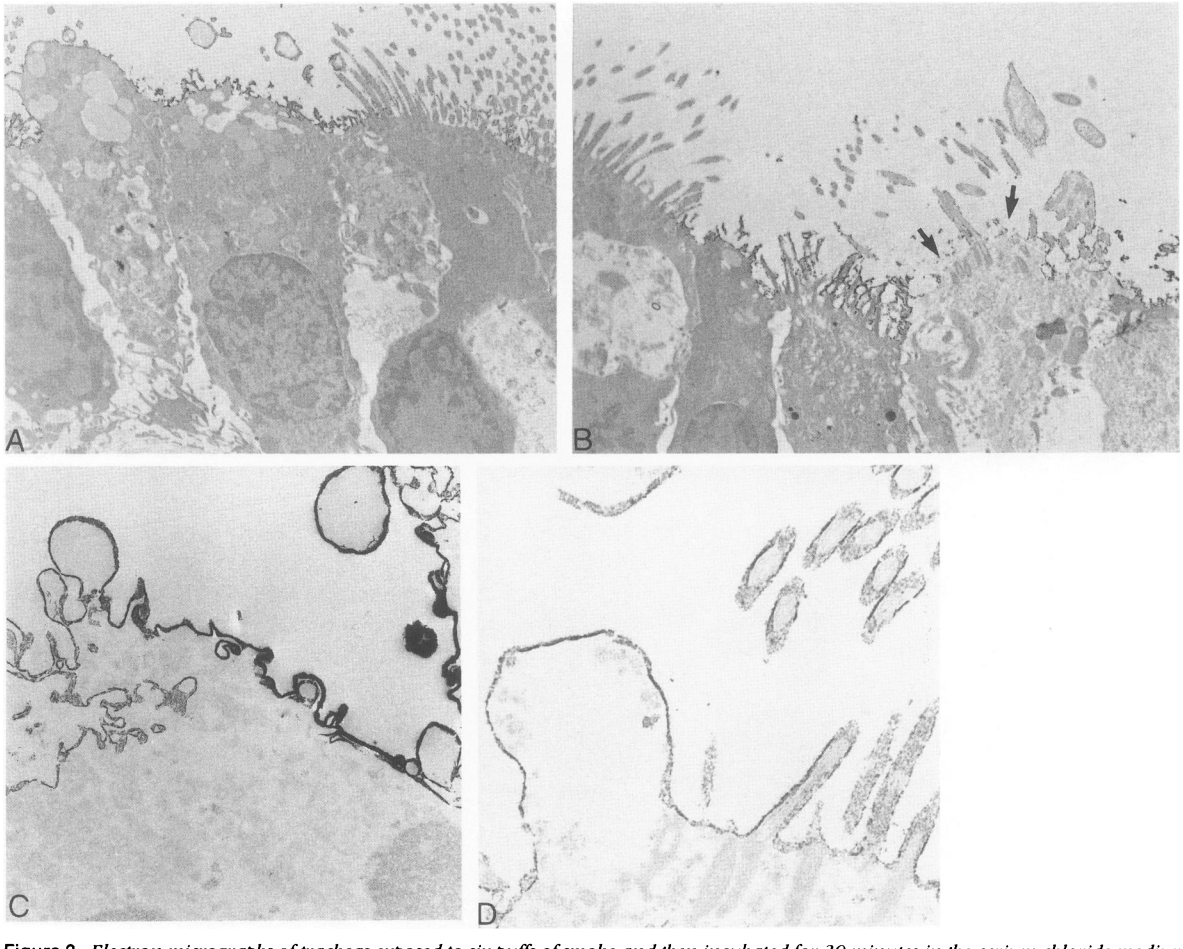

Figure 3. *Electron micrographs of tracheas exposed to six puffs of smoke and then incubated for 30 minutes in the cerium chloride medium of Briggs et al.*[11] **A** *and* **B**: *Two low-power views of different areas showing marked cell separation, focal cell disintegration (arrows), and extensive cerium reaction product deposited in a linear fashion along the apical surfaces (Glutaraldehyde and osmium fixation, counterstained with lead citrate and uranyl acetate;* **A**, *×6600;* **B**, *×6000).* **C** and **D**: *High-power views showing heavy linear deposition of cerium reaction product. Note membrane blebs in both* **C** *and* **D** *and loss of cilia in* **D** (**C**: *Glutaraldehyde fixation only, no counterstain;* **D**: *Glutaraldehyde and osmium fixation, no counterstain;* **C**, *×15,000;* **D**, *×20,000).*

in tracheas exposed to one puff of smoke and was more severe and more widespread with increasing smoke exposure (Figure 3); however, even with six puffs of smoke, most of the epithelium was not obviously damaged.

Catalase-treated Explants

Addition of catalase to the incubation medium abolished both the evidence of cell damage and all but minimal and focal deposits of cerium precipitate (Figure 4). Better protection was obtained if the explants were treated with catalase before smoke exposure rather than after exposure (i.e., in the latter circumstance the catalase was included in the cerium incubation medium), but the differences were not marked. If the catalase was first boiled, both cell damage and cerium precipitate were seen in a pattern identical to that found with cigarette smoke alone (Figure 5).

Demonstration of Superoxide Anion

Tracheal segments exposed to smoke and then to the NBT solution demonstrated deposition of discrete, apparently randomly distributed, blue formazan grains (Figure 6). Controls exposed to air and segments exposed to smoke plus superoxide dismutase showed only faint reactions (Figure 6).

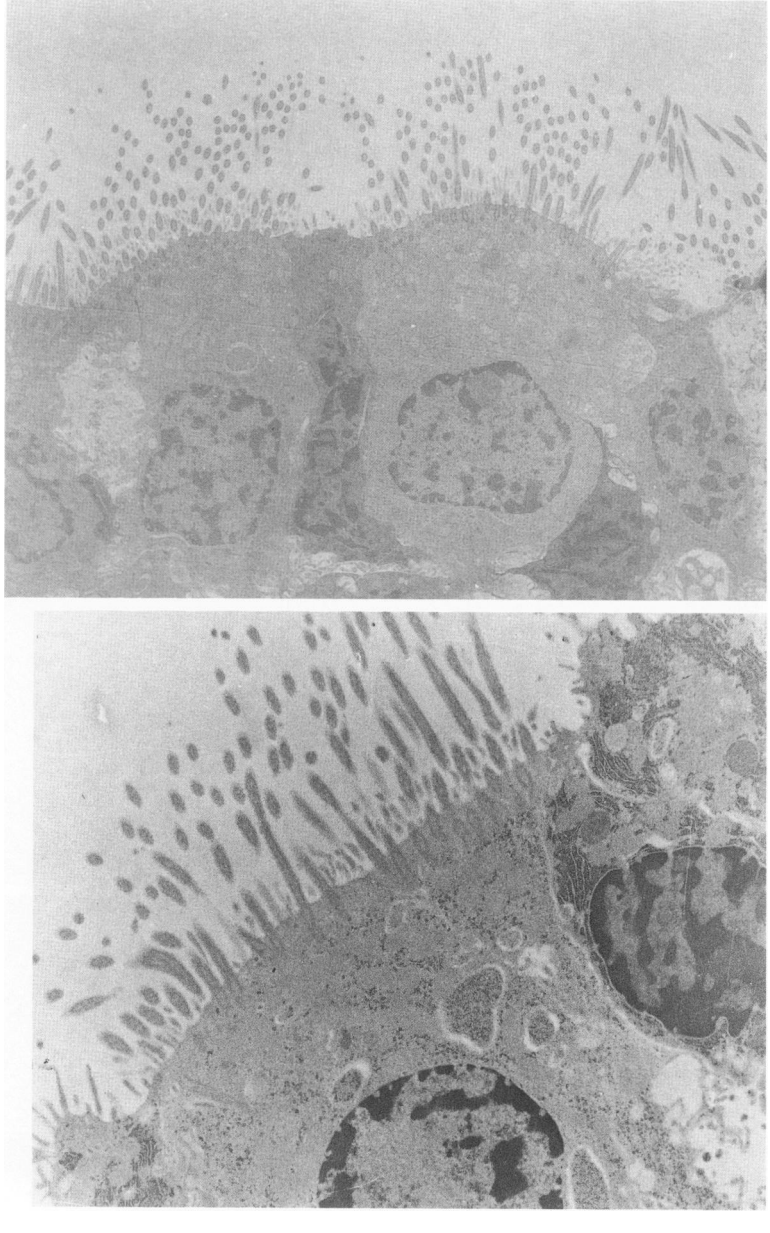

Figure 4. *Electron micrographs of tracheas exposed to six puffs of cigarette smoke and treated with catalase/cerium chloride medium as described in text.* **A** *and* **B**: *Low- and high-power views showing cell separation but no obvious cell damage and no cerium reaction product (Glutaraldehyde and osmium fixation, counterstained with lead citrate and uranyl acetate;* **A**, *×5500;* **B**, *×11,000).*

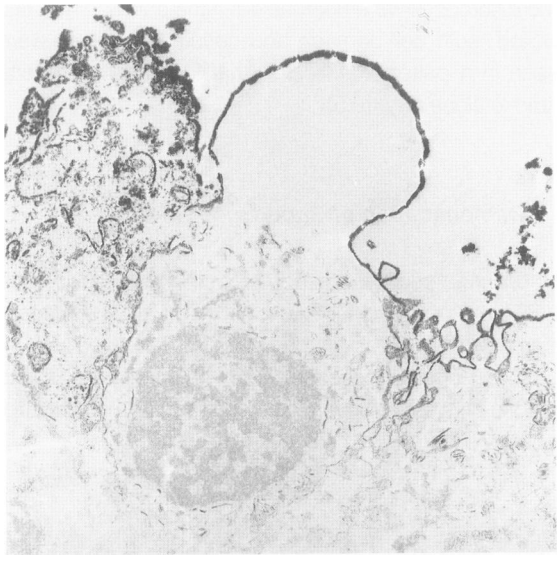

Figure 5. *Electron micrograph of trachea exposed to six puffs of cigarette smoke and treated with catalase inactivated by boiling/cerium chloride medium as described in text. There is membrane blebbing and loss of cytologic detail along with linear heavy linear deposition of reaction product along apical membrane. The cell to the left is disintegrating and shows extensive staining that may not be specific. The overall appearance is essentially identical to that seen with six puffs of smoke and no catalase (Glutaraldehyde fixation only, no counterstain, ×8000).*

Discussion

As noted in the Introduction, cigarette smoke contains and produces a variety of radicals.[1–5] The gas-phase radicals are believed to be both carbon- and oxygen-centered species maintained by oxidation of nitric oxide; compared with the tar phase, gas-phase radicals are present in lower concentration and are short lived.[1,2] The tar-phase radicals appear to be quinones held in the tar matrix; the quinones reduce dioxygen to superoxide anion with subsequent dismutation to hydrogen peroxide.[1–3] If transitional metal ions are present, the reactive hydroxyl radical can also be formed.[15] Tar-phase radicals are present in high concentrations and are long lived;[1–3] because of the long lifetime (characterized by Pryor[2] as "essentially infinite") of these radicals, the production of active oxygen species can continue for remarkably long periods. Nakayama et al.[5] showed that hydrogen peroxide production could be detected as much as 24 hours (the longest period they examined) after an aqueous extract of fresh smoke tar was prepared. In fact their graph shows not only continuing production, but slowly increasing concentration of hydrogen peroxide/ml of solution with increasing time, and their data imply that hydrogen peroxide production would continue for much longer periods.

In previous experiments that used a tracheal explant system, we found that brief exposure of explants to smoke *followed by* exposure to amosite asbestos increased fiber uptake compared with exposure to air followed by asbestos.[6,7,9] In this system, enhanced fiber uptake was still seen with delays between smoke and asbestos exposure of 1 minute to 48 hours. Catalase, superoxide dismutase, and deferoxamine all abolished the smoke effect in a dose-related fashion. These findings lead us to propose that smoke-enhanced fiber uptake is mediated by cell damage caused by hydroxyl radical formed from the superoxide anion and hydrogen peroxide generated in smoke tar; in this situation the iron on the surface of the asbestos fiber is believed to serve

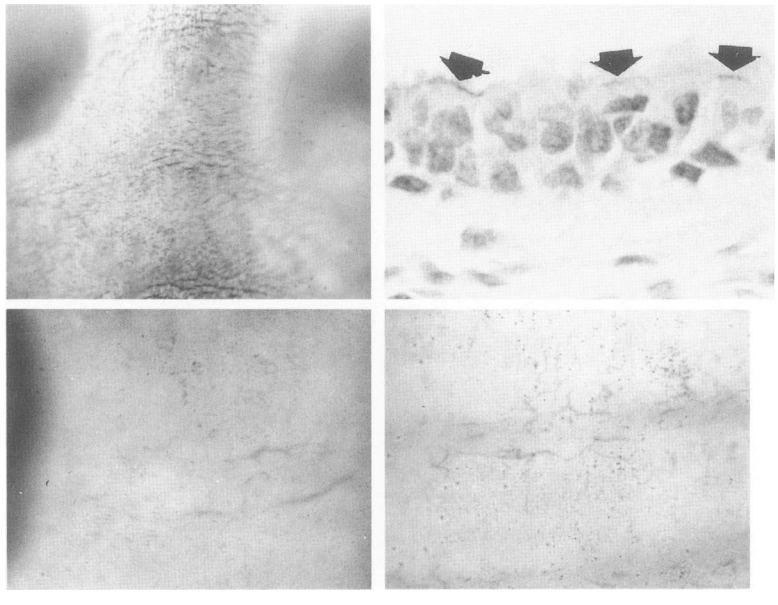

Figure 6. *Light micrographs of tracheal segments exposed to smoke for 10 minutes followed by air for 1 minute, and then covered with NBT solution as described in text. Photographs are taken from above the tracheal surface by transmitted light and show epithelium and underlying blood vessels (except* **B**, *which is a histologic cross-section); shadows at edges of field are overhanging curve of the tracheal wall. A diffuse positive reaction (blue dots of formazan) indicating the presence of superoxide anion is seen with smoke exposure* (**A**); *the surface location of the reaction is confirmed (arrows) in the histologic cross section* (**B**). *If the NBT is mixed with superoxide dismutase the positive reaction is abolished* (**C**). *Little reaction is seen in a control* (**D**) *in which air exposure is substituted for smoke exposure.* **A**, **C**, **D**, ×50; **B**, ×400.

as a Fenton catalyst to promote formation of hydroxyl radical (see[16] for a recent review).

One interpretation of the findings just described with asbestos and smoke is that tar is deposited during smoke exposure on the apical surface (mucous blanket) of the tracheal cells, in effect creating a long-term active oxygen species generator on the cell membrane. The current experiments were designed to test this latter hypothesis using the much simpler system of smoke without asbestos by directly visualizing the appearance of both hydrogen peroxide and superoxide anion on the tracheal cells after smoke exposure had ceased.

A few comments about the histochemical methods are in order. The hydrogen peroxide method of Briggs et al.[11] makes use of the fact that cerous ions in the presence of peroxide form cerium perhydroxide, an insoluble and electron-dense precipitate. The method is not ideal from the point of view of morphologic preservation. However, the specificity of the procedure was extensively investigated by Briggs et al., and Briggs et al. examined in detail the effect of catalase in this system and carefully ruled out artificial effects from complexing of catalase with cerium ions. Thus the abolition of precipitate on smoke-exposed tracheas by active but not inactive catalase reinforces our belief that the method is visualizing smoke-related hydrogen peroxide present at the surface of the tracheal epithelial cells. The clear smoke-dose dependent increases in cerium reaction product also argue for the specificity of the technique.

The NBT reaction depends on reduction of NBT to formazan, an insoluble blue precipitate. NBT reduction has been used as an indicator of the presence of superoxide anion, although the reaction is not entirely specific.[12–14] Cigarette smoke (tar) has other reducing substances[1–3] and thus other species might be causing the reactions we observe; however, the fact that the reaction is almost completely abolished in the presence of superoxide dismutase suggests that most of the formazan is produced by effects of superoxide anion.

We are not attempting a detailed morphologic study of the effects of smoke on rat tracheal epithelial cells in this article, and the histochemical technique interferes with structural detail enough to make such a study difficult; however, the morphologic findings in the smoke-exposed tracheas are consistent with those known to be caused by active oxygen species, particularly hydrogen peroxide. Ciliary damage is not in and of itself distinctive, but hydrogen peroxide and ozone, as well as cigarette smoke, have been shown to damage cilia.[17,18] Membrane blebbing, although hardly specific, is a remarkably constant finding in many different types of cells exposed to hydrogen peroxide.[19] It has been proposed that, in this circumstance, blebbing reflects oxidant-induced damage to the cytoskeleton and dissociation of the cytoskeleton from the cell membrane.[19,20] Cell necrosis is similarly nonspecific but is seen with exposure to both ozone and hydrogen peroxide.[17,19,20]

Therefore, in our system, exposure to cigarette smoke leads to the random deposition of smoke tar on the surface of the tracheal epithelium cells. The tar probably partially dissolves in or at least is covered by the mucous blanket, and hence, the situation is functionally equivalent to that reported after bubbling smoke through water.[4,5] We suggest that, effectively, a microscopic reaction chamber is formed on the apical cell surface, and from this site, the tar generates active oxygen species that damage the underlying cells.

References

1. Church DF, Pryor WA: The oxidative stress placed on the lung by cigarette smoke. The Lung: Scientific Foundations. Edited by RG Crystal, JB West. New York, Raven Press, 1991, 1973–1979.
2. Pryor WA: The free radical chemistry of cigarette smoke and inactivation of alpha-1-proteinase inhibitor. Pulmonary Emphysema and Proteolysis. Edited by JC Taylor, C Mittman. New York, Academic Press, 1987, pp 369–392
3. Church DF, Pryor WA: Free radical chemistry of cigarette smoke and its toxicological implications. Environ Health Perspect 1985, 64:111–126
4. Nakayama T, Kodama M, Nagata C: Generation of hydrogen peroxide and superoxide anion radical from cigarette smoke. Gann 1984, 75:95–98
5. Nakayama T, Church DF, Pryor WA: Quantitative analysis of the hydrogen peroxide formed in aqueous cigarette tar extracts. Free Rad Biol Med 1989, 7:9–15
6. Hobson J, Gilks B, Wright J, Churg A: Direct enhancement by cigarette smoke of asbestos fiber penetration and asbestos-induced epithelial proliferation in rat tracheal explants. J Natl Cancer Inst 1988, 80:518–521
7. Churg A, Hobson J, Berean K, Wright J: Scavengers of active oxygen species prevent cigarette smoke-induced asbestos fiber penetration in rat tracheal explants. Am J Pathol 1989, 135:599–603
8. Hobson J, Wright JL, Churg A: Active oxygen species mediate asbestos fiber uptake by tracheal epithelial cells. FASEB J 1990, 4:3135–3139
9. Churg A, Hobson J, Wright JL: Effects of cigarette smoke dose and time after smoke exposure on uptake of asbestos fibers by rat tracheal epithelial cells. Am J Resp Cell Molec Biol 1990, 3:265–269
10. Mossman B, Craighead JE: Use of hamster tracheal organ cultures for assessing the cocarcinogenic effects of inorganic particulates on the respiratory epithelium. Prog Exp Tumor Res 1979, 24:37–47
11. Briggs RT, Drath DB, Karnovsky ML, Karnovsky MJ: Local-

ization of NADH oxidase on the surface of hyman polymorphonuclear leukocytes by a new cytochemical method. J Cell Biol 1975, 67:566–586
12. Goodglick L, Kane AB: Cytotoxicity of long and short crocidolite asbestos fibers *in vitro* and *in vivo*. Cancer Res 1990, 50:5153–5163
13. Goodglick LA, Pietras LA, Kane AB: Evaluation of the causal relationship between crocidolite asbestos-induced lipid peroxidation and toxicity to macrophages. Am Rev Respir Dis 1989, 139:1265–1273
14. Goodglick L, Kane AB: Role of reactive oxygen metabolites in crocidolite asbestos toxicity to mouse macrophages. Cancer Res 1986, 46:5558–5566
15. Cosgrove JP, Borish ET, Church DF, Pryor WA: The metal—mediated formation of hydroxyl radical by aqueous extracts of cigarette tar. Biochem Biophys Res Comm 1985, 132:390–396
16. Mossman BT, Marsh JP: Evidence supporting a role for active oxygen species in asbestos-induced toxicity and lung disease. Environ Health Perspect 1989, 81:91–94
17. Nikula KJ, Wilson DW, Giri SN, Plopper CG, Dungworth DL: The response of the rat tracheal epithelium to ozone exposure. Am J Pathol 1988, 131:373–384
18. Burman WJ, Martin WJ: Oxidant-mediated ciliary dysfunction. Possible role in airway disease. Chest 1986, 89:410–413
19. Hinshaw DB, Sklar LA, Bohl B, Schrauffstatter IU, Hyslop PA, Rossi MW, Spragg RG, Cochrane CG: Cytoskeletal and morphologic impact of cellular oxidant injury. Am J Pathol 1986, 123:454–464
20. Raghu G, Striker L, Harlan J, Gown A, Striker G: Cytoskeletal changes as an early event in hydrogen peroxide induced cell injury: A study in A549 cells. Br J Exptl Pathol 1986, 67:105–112