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Antioxidant Gene Expression in Rat Lung after Exposure to Cigarette Smoke

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To investigate the effects of cigarette smoke on the expression of genes encoding intracellular antioxidant species, we exposed rats to whole cigarette smoke or air (control) daily for 1, 2, 7, or 14 days. After sacrifice, RNA was extracted from one lung and expression of mRNA for catalase (CAT), manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (CuZnSOD), glutathione peroxidase (GPX), and metallothionein (MT) was determined by Northern blots and dot blots. The anatomical distribution of expression of these genes was determined by *in situ* hybridization studies on sections of the contralateral lung. We found that expression of both MnSOD and MT was significantly increased (to levels 70 to 400% greater than in controls) at days 1 and 2 and returned to control levels by day 7. GPX expression was slightly but significantly increased at days 7 and 14 in smoke-exposed animals. CuZnSOD and CAT expression did not change from control levels. In control lungs, MnSOD was expressed in all cell types, with the highest expression seen in bronchial epithelial cells; a notable finding was a mosaic pattern of expression in the bronchial epithelium, with contiguous areas of bronchial epithelium composed of cells expressing MnSOD at high levels (hot spots), compared with the adjacent epithelium. In smoke-exposed lungs, the hot spots became less prominent after 1 and 2 days of exposure to smoke, but after 7 and 14 days the distribution of MnSOD expression was similar in control and smoke-exposed animals. CAT, CuZnSOD, GPX, and MT also showed widespread expression in the lung by *in situ* hybridization; GPX, CuZnSOD and MT were all most highly expressed in bronchial epithelium, whereas CAT expression levels were similar in all cell types. In contrast to MnSOD, expression of CAT, CuZnSOD, GPX, and MT was uniform within the bronchial epithelium, and the distribution of expression was the same in control and smoke-exposed animals at all time points. We conclude that most of these antioxidant enzymes and scavengers show prominent bronchial expression but that MnSOD shows a unique pattern, with intense hot spots in the epithelium of the small airways. This pattern is similar to the phenomenon of clonal heterogeneity described in other tissues but not previously reported in the lung. We conclude that cigarette smoke, like other forms of oxidant attack, transiently increases expression of MnSOD, and up-regulation of MnSOD expression appears to occur particularly in bronchial epithelial cells, which normally express MnSOD at relatively low levels. MT expression is also transiently increased by smoke whereas GPX expression increases after prolonged (7 to 14 days) exposure to cigarette smoke. *(Am J Pathol 1998, 152:269–278)*

Exposure of the lung to attack by active oxygen species (AOS) is generally followed by increased production of a variety of antioxidant enzymes and substances (reviewed in Refs. 1–4). Which defensive species are induced appears to vary, in a complex and poorly understood fashion, with the nature of the attacking agent, and the mechanism of induction can be at the transcriptional or post-transcriptional level or both.[1–4]

Cigarette smoke is a highly concentrated source of AOS and other oxidizing species,[5] but surprisingly little is known of the changes in antioxidant defense produced in the lung by cigarette smoke, and the limited published data are contradictory. Drath et al[6] found no change in alveolar macrophage catalase (CAT) activity and decreased superoxide dismutase (SOD) activity in smoke-exposed rats, whereas McCusker[7] reported increased CAT and SOD activity in alveolar macrophages from both human smokers and smoke-exposed hamsters, with no change in glutathione peroxidase activity (GPX). Hilbert and Mohensin[8] found increased CAT and GPX activities in bronchoalveolar lavage cells from smokers, compared with nonsmokers, with no difference in SOD activity. York et al[9] observed a small (34%) increase in GPX activity in whole-lung extracts of smoke-exposed rats, whereas Gupta[10] reported increased glutathione levels but no change in SOD, GPX, or CAT activity in similar extracts. In all of these reports, enzyme activity was measured. The

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effects of exposure to cigarette smoke on the expression of the genes encoding these antioxidant proteins has not been investigated.

In this study we have examined changes in levels and distribution of antioxidant gene expression in the lungs of rats exposed to whole cigarette smoke for varying periods up to 2 weeks to determine whether cigarette smoke exposure affects intrapulmonary expression of genes encoding intracellular antioxidant species.

Materials and Methods

Cigarette Smoke Exposure

Male Sprague-Dawley rats weighing 250 g were exposed to whole cigarette smoke using the nose-only system we have previously described.[11] Commercial nonfiltered cigarettes were used, and the daily dose was seven cigarettes per animal. This number of cigarettes was chosen because we have previously shown that it produces pathological responses; short-term exposure rapidly induces proliferation of airway and vascular cells[12] and long-term exposure (months) produces chronic airflow obstruction.[11] This dose of cigarette smoke results in acute carboxyhemoglobin levels of approximately 15%, falling to 3 to 4% before the next day's exposure (J. L. Wright, unpublished data). Exposure times were 1, 2, 7, and 14 days, with sacrifice of the animals 24 hours after the last smoke exposure. Control animals were sham smoked. Each test or control group at each time point consisted of five animals. There was insufficient RNA isolated from one animal in the day 14 control group to analyze for expression of all of the genes, so that the control group consisted of four animals for the day 14 dot blots for GPX and metallothionein (MT) expression.

Lung RNA Levels

After animals were sacrificed by urethane overdose, the lungs were rapidly removed, and the left lung was snap frozen and stored at −80°C. Total RNA was isolated from the left lung according to the method of Chomczynski and Sacchi,[13] after the tissues were fragmented with a tissue homogenizer. The integrity of the RNA was verified by running an aliquot on a 1% agarose gel and staining with ethidium bromide; quantification was done by ultraviolet absorption spectrophotometry. Northern blots were prepared by electrophoresis of 10 μg of total RNA through a 1% agarose/2.2 mol/L formaldehyde gel whereas slot-blots were loaded with 10 μg of total RNA per sample. In each case, Nytran nylon membranes were used (Schleicher and Schuell, Keene NH) and RNA was cross-linked to the membranes by ultraviolet irradiation. ^{32}P-labeled random primer cDNA probes for MnSOD[14] (a gift from Y-S. Ho, Wayne State University, Detroit, MI), CAT[15] (a gift from S. Furuta, Shinshu University, Nagano, Japan), GPX[16] (a gift from G. Mullenbach, Chiron Corp., Emoryville, CA), CuZnSOD[17] (a gift from Y-S. Ho, Wayne State University, Detroit, MI), and MT[18] (a gift from R. Andersen, University of California at Los Angeles, Los Angeles, CA) were hybridized to the membranes with prehybridization, hybridization, and washes done according to protocols provided by the Nytran manufacturer. After exposure to preflashed X-Omat AR film (Eastman Kodak, Rochester, NY), the autoradiograms were quantified using a hand-held scanning densitometer (AAB, Fullerton, CA). To control for unequal loading, the membranes were stripped and reprobed with a β-actin cDNA probe and quantified as described above. Northern blots were used initially for all probes, and having demonstrated the specificity of the probes by their ability to detect a single band (or two bands in the case of MnSOD) of appropriate molecular weight under the hybridization conditions used, dot blots were used thereafter.

Data on mRNA levels were evaluated by taking the ratio of mRNA for the species of interest to actin, for controls and smokers. Values were log-transformed to normalize the distributions and differences between smokers and controls analyzed by analysis of variance at each time point. The graphic data are presented as nontransformed means and standard deviations as these provide a good visual sense of the data.

In Situ *Hybridization*

The right lower lobe was inflated and fixed with 10% neutral buffered formalin, at a constant pressure of 25 cm H_2O, for 48 to 72 hours. Pilot experiments showed that this fixative and duration of fixation gave the best signal to background ratio and preservation of morphological detail with the probes used. The *in situ* hybridization was done according to published protocols. Briefly, after embedding midsagittal sections of the lobe in paraffin, 3-μm sections were cut, deparaffinized in xylene, rehydrated in graded ethanols, and digested in 1 μg/ml proteinase K, in 10 mmol/L Tris/5 mmol/L EDTA/0.5% SDS at 37°C for 30 minutes. After washing in 0.1 mol/L triethanolamine (pH 8.0), the slides were incubated in 0.1 mol/L triethanolamine (pH 8.0)/0.25% acetic anhydride for 10 minutes at 20°C. After washing in 2X SSC, the slides were dehydrated through graded ethanols, and 50 μl of hybridization buffer (50% formamide, 1X Denhardt's reagent, 1 mmol/L EDTA, 2X SSC, 20 mmol/L Tris, 10% dextran sulfate, 100 mmol/L dithiothreitol (DTT), and 500 μg/ml yeast tRNA) was applied to each slide and prehybridization done at 58°C for 1 to 3 hours. After prehybridization, the slides were drained and blotted, hybridization buffer with 2×10^6 cpm of ^{35}S-labeled probe was applied, and the slides were coverslipped, sealed with rubber cement, and incubated overnight at 58°C. Sense and antisense riboprobes were prepared according to standard protocols. Washes after hybridization were as follows: 4X SSC/10 mmol/L DTT for 10 minutes at 20°C (twice), 0.5X SSC/10 mmol/L DTT for 15 minutes at 20°C, 0.1X SSC/10 mmol/L DTT for 15 minutes at 60°c, RNAse buffer (0.5 mol/L NaCl/10 mmol/L Tris/1 mmol/L EDTA) for 1 minute at 37°C, RNAse buffer plus 20 μg/ml RNAse A for 30 minutes at 37°C, RNAse buffer for 30 minutes at 37°C, 2X SSC for 30 minutes at 20°C, 0.1X SSC for 15

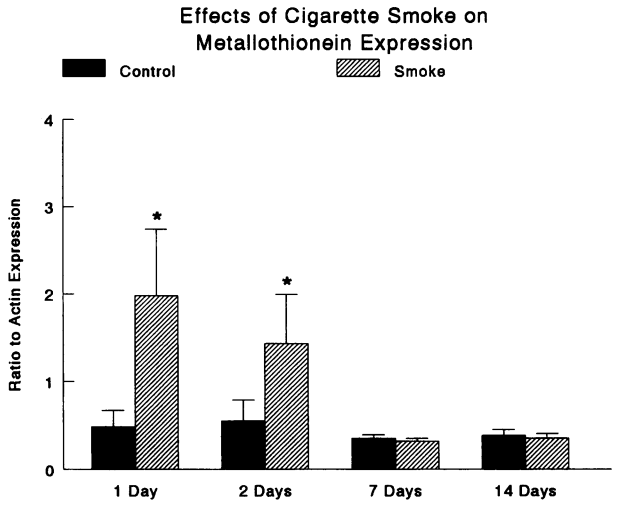

Figure 1. MT expression in controls *versus* smokers. There is a marked increase in expression in smokers at days 1 and 2, which this disappears by day 7.

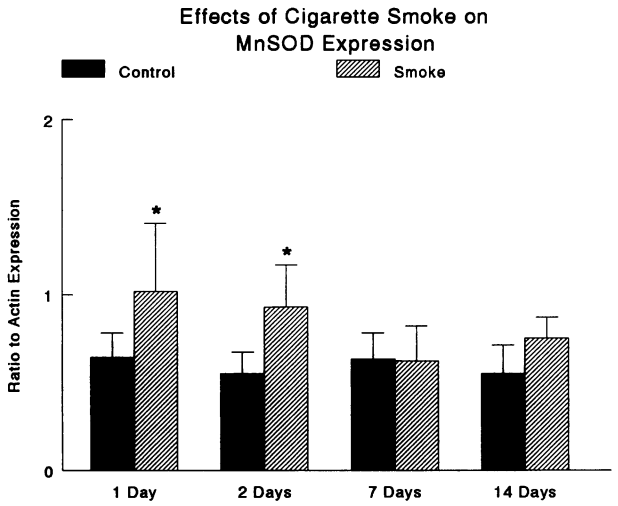

Figure 2. MnSOD expression in controls *versus* smokers. There is a significant increase in MnSOD expression in smokers at day 1 and day 2. No significant differences are seen between smoke-exposed and control animals at days 7 and 14.

minutes at 60°C, 0.1X SSC for 30 minutes at 20°C. The slides were then dehydrated through graded ethanols, air dried, and dipped in Kodak NTB-2 emulsion, diluted 1:1 with sterile distilled H_2O, at 42°C to 45°C. After drying, the slides were stored at 4°C with dessicant. After 1 to 8 weeks of exposure, the slides were developed in Kodak D-19 developer for 4.5 minutes, washed in H_2O for 30 seconds, and fixed with Ilford paper fixer for 5 minutes. Sense probes showed only a light scattered background signal for all of the genes studied (a representative sense probe is shown in Figure 8C). Slides from three control and three smoke-exposed animals were used for *in situ* hybridization for each time point.

Morphometric Analysis of MnSOD Hot Spots

A Bioquant image analysis system was used to measure both the internal bronchiolar diameter and the circumference of all conducting (noncartilagenous) airways in each section of the right lower lobe prepared for visualization of MnSOD expression. For each airway, the length of the epithelium with markedly increased MnSOD expression, as assessed visually, was measured and expressed as a percentage of the circumference. Because of the highly skewed distribution, comparisons of the percentage of epithelial length expressing MnSOD at high levels (hot spots), between controls and smokers, were done by the Kruskal-Wallis analysis of variance. For all figures, data are presented as mean ± SD, and asterisks indicate statistical significance at $P < 0.05$.

Results

Figures 1 to 5 show expression of MT, MnSOD, GPX, CAT, and CuZnSOD in smoke-exposed animals (smokers) compared with controls over the course of the experiment. In smokers, there was a significant increase of both MT (nearly 4-fold) and MnSOD (approximately 1.7-fold) expression at day 1; at day 2, both MnSOD and MT were again significantly elevated (approximately 2.5- and 1.7-fold) in smokers; these differences disappeared by day 7. There was a small but significant increase in GPX expression at days 7 and 14. Expression of CAT and CuZnSOD did not change, compared with controls. Figure 6 shows a representative set of dot blots for a single time point (14 days of exposure to cigarette smoke).

In situ hybridization for MnSOD showed that expression was most prominent in the epithelium of the small airways. A unique feature of MnSOD expression was marked variability of MnSOD RNA content in bronchiolar epithelial cells, with contiguous areas of high expression (hot spots) present, consisting of from 5 to greater than 100 cells (Figure 7, A–D). These areas of high expression were observed consistently in serial sections (Figure 8, A–C) and were not seen with the probes for the other antioxidant enzyme genes, indicating they were not an

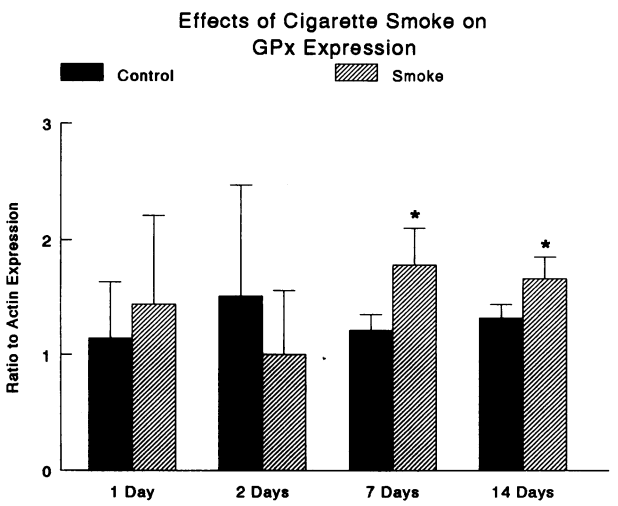

Figure 3. GPX expression in controls *versus* smokers. There is a small but significant increase in expression in smokers at days 7 and 14.

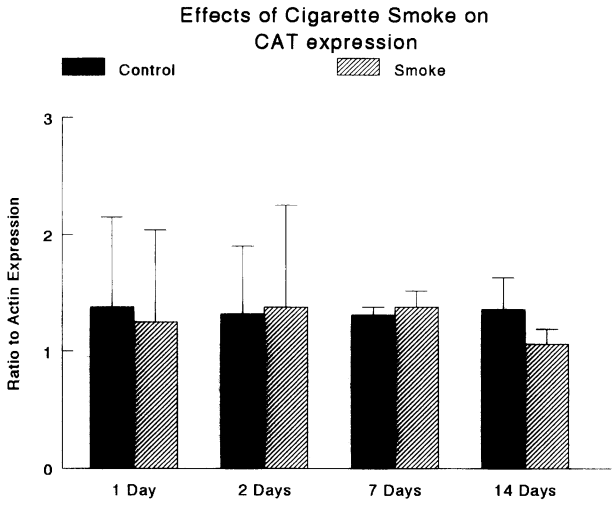

Figure 4. Catalase expression in controls vs. smokers. No differences are seen between smokers and controls.

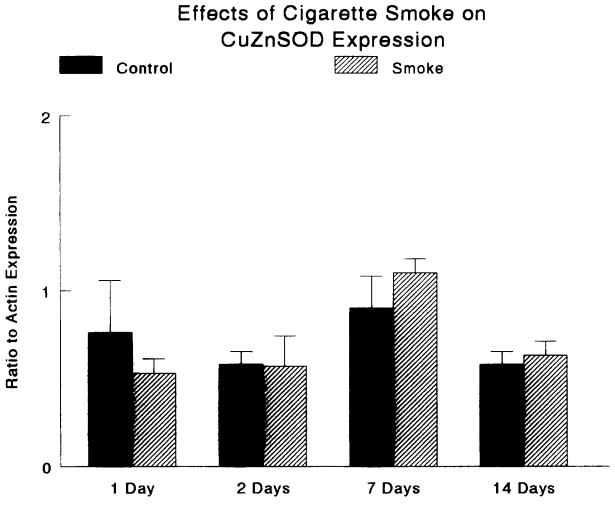

Figure 5. CuZnSOD expression in controls *versus* smokers. No differences are seen between smokers and controls.

artifact due to fixation or the *in situ* hybridization protocol used. The areas of high MnSOD expression showed no predilection for specific locations within the airways (eg, airway bifurcations) but were more common in the larger airways. MnSOD expression was also present in the alveolar parenchyma, and within the obtainable resolution, signal was seen over type II cells, type I cells, and interstitium; the intensity of expression in the ordinary (not hot spot) bronchiolar epithelium was greater than in the parenchyma (Figure 9).

Because the distributions of percentage of bronchiolar epithelium that appeared as hot spots was highly skewed (with many airways having no hot spots), the data on hot spots are presented in Table 1 as the number of airways having various percentages of the epithelial length occupied by hot spots. In the animals exposed to cigarette smoke, hot spots were present but, after 1 and 2 days of exposure to cigarette smoke, constituted significantly less of the bronchiolar epithelium, compared with controls (Table 1). After 14 days of exposure, the relative length of the hot spots was greater in smokers, although this difference was not statistically significant (Table 1).

CuZnSOD was expressed in all cells in the lung, including stromal cells and pneumocytes, but with higher levels of expression seen in bronchiolar epithelial cells (Figure 10, A and B). GPX was also expressed in all cells, with higher expression in bronchiolar epithelial cells compared with pneumocytes (Figure 10, C and D). CAT expression was very uniform within the pulmonary epithelial cells, and in contrast to the other genes studied, bronchiolar epithelial cells did not express CAT at levels visually greater than was observed in pneumocytes (Figure 10, E and F). MT was expressed in all cell types, with visually higher levels in bronchiolar epithelial cells compared with other cells (Figure 10, G and H). For GPX, CuZnSOD, CAT, and MT, no difference in the distribution of expression was seen in smoke-exposed animals compared with controls.

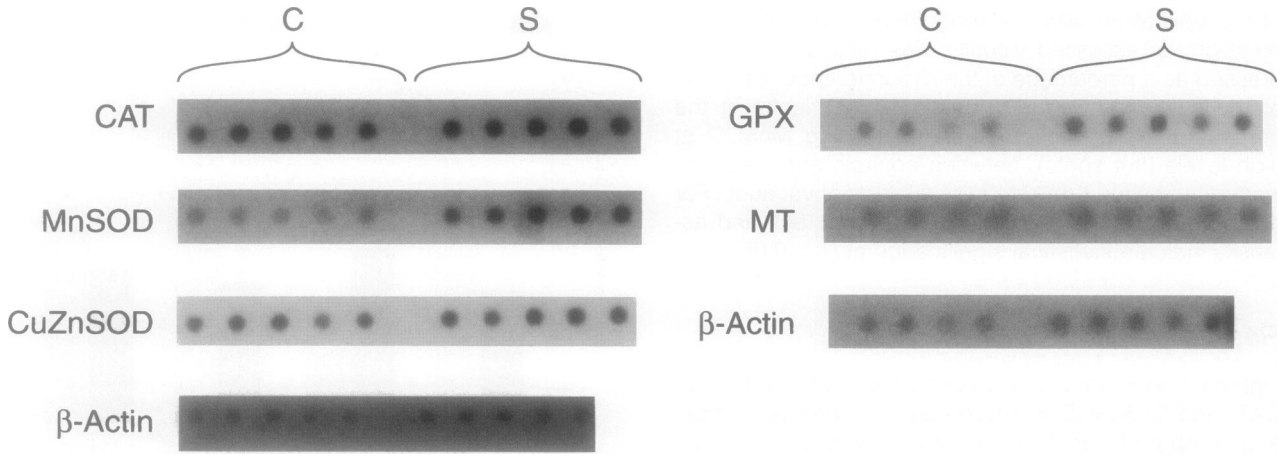

Figure 6. Dot blots for CAT, MnSOD, CuZnSOD, GPX, MT, and β-actin expression after 14 days exposure to cigarette smoke (C, control; S, smoke). Significant differences are seen only for GPX, with a slight increase in GPX expression detected in smokers, compared with controls.

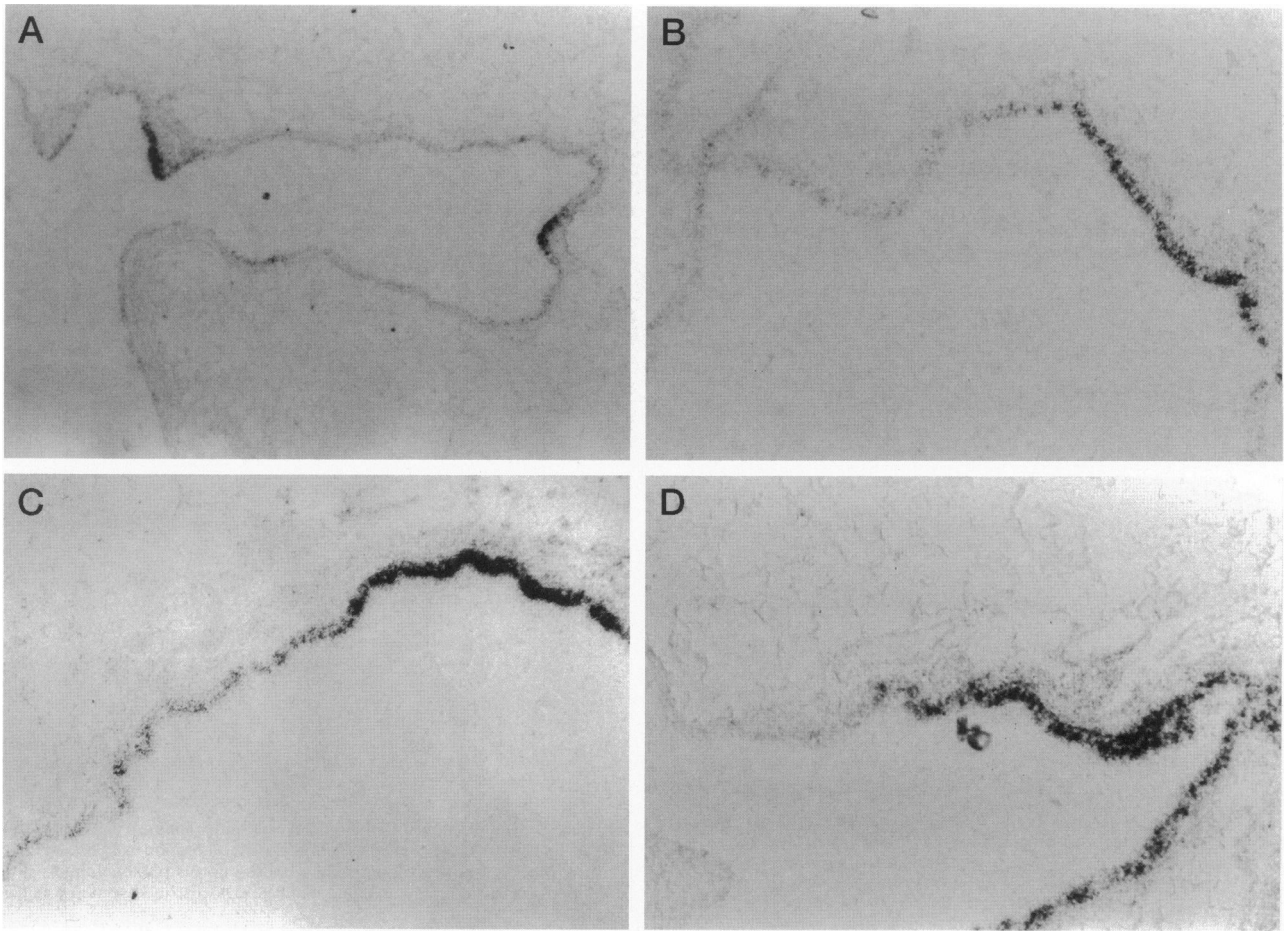

Figure 7. Distribution of expression of MnSOD in bronchiolar epithelial cells, with contiguous areas of high expressing cells (hot spots) present in some airways. Unstained sections; magnification, ×20 (**A** and **B**) and ×50 (**C** and **D**).

Discussion

The lung is particularly subject to oxidant attack, and oxidant damage has been implicated in the pathogenesis of numerous lung diseases including various pneumoconioses, adult respiratory distress syndrome, chronic obstructive pulmonary disease, and lung cancer.[19] The most commonly encountered oxidants are active oxygen species (AOS), products of the progressive reduction of molecular oxygen to water,[1,2] and include the highly reactive moieties superoxide anion, hydrogen peroxide, and hydroxyl radical. AOS are also produced in the lung, as in all organs, as a result of normal oxidative metabolism; in addition, preformed AOS are frequently inhaled, and cigarette smoke contains AOS in high concentrations.[3]

AOS are potentially harmful to cells because of their highly reactive nature and ability to modify a spectrum of biomolecules, and mammalian cells have evolved a complex set of defenses to deal with AOS.[20] Superoxide anion is destroyed by dismutation to hydrogen peroxide by superoxide dismutases (SODs). There are two forms of intracellular SOD; the cytoplasmic form is a copper- and zinc-binding protein (CuZnSOD), and the mitochondrial form of the enzyme, which is present at the site of generation of most intracellular active oxygen species, is a manganese-binding protein (MnSOD). Catalase (CAT) catalyzes conversion of hydrogen peroxide to water and oxygen. A fourth intracellular antioxidant enzyme, glutathione peroxidase (GPX), scavenges hydroperoxides but also can metabolize hydrogen peroxide to water and oxygen. Metallothioneins (MTs) are a group of small, cysteine-rich intracellular proteins that bind avidly to copper, zinc, and cadmium.[21] Their exact function in normal cells is unknown, although it has been proposed that by binding zinc they have a role in regulation of DNA replication and repair.[1] They also appear to function as scavengers of AOS, particularly hydroxyl radical,[21] and may be important in protecting DNA from oxidant attack.[22] In this study we have shown that cigarette smoke rapidly but transiently increases whole-lung expression of Mn-SOD and MT, with increased expression of GPX appearing later and persisting, whereas CAT and CuZnSOD expression appear not to be affected by cigarette smoke.

As noted in the introduction, different forms of AOS injury lead to different patterns of antioxidant response, although increased expression of MnSOD appears to be one response that is common to most if not all types of oxidant attack.[1–4] Such increases have been seen with hyperoxia,[23] and with exposure to crocidolite asbestos and silica (cristobalite),[24] although with some variation.

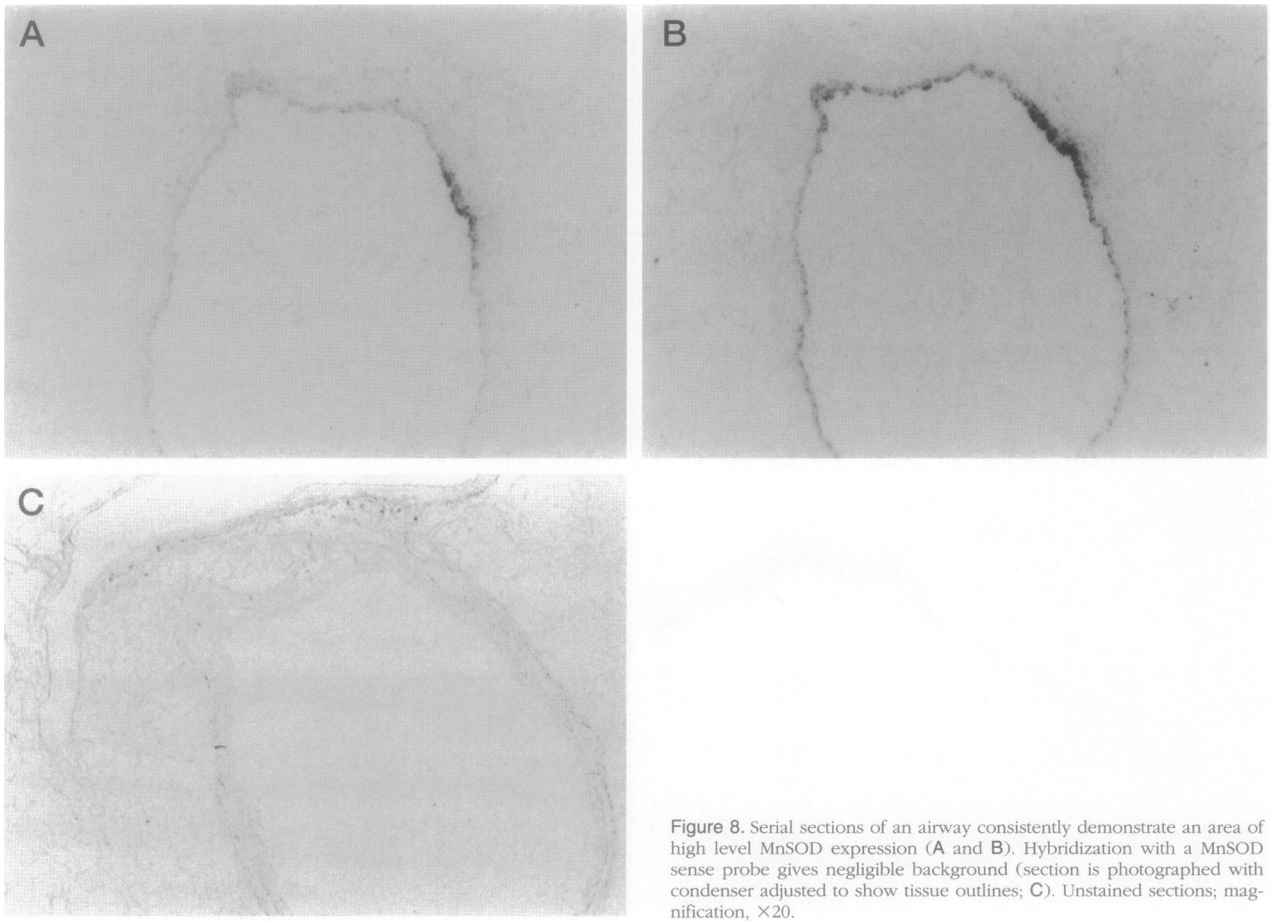

Figure 8. Serial sections of an airway consistently demonstrate an area of high level MnSOD expression (A and B). Hybridization with a MnSOD sense probe gives negligible background (section is photographed with condenser adjusted to show tissue outlines; C). Unstained sections; magnification, ×20.

Crocidolite, like smoke, induces an early transient increase, whereas cristobalite causes a sustained induction.[24] Whether increased levels of MnSOD expression are a direct reflection of oxidant exposure is unclear, and it has been suggested that for most or all of the agents just listed, increased MnSOD expression may be a response to the inflammatory reaction and, specifically, the inflammation-derived cytokines tumor necrosis factor-α and interleukin-1, which are known to induce rapid increases in MnSOD expression.[23] Also, AOS can directly regulate gene expression; for example, DNA binding by the transcription factors NFκB and AP-1 appears to be regulated by the intracellular redox state.[25] The relative contributions of the inflammatory response *versus* direct effects of AOS in the cellular response to oxidant stress is unknown, and either possibility could apply to cigarette smoke, as smoke contains very high levels of oxidants[5] and also evokes a polymorphonuclear and chronic inflammatory reaction.[26] Regardless of the mechanism, our observations do support the idea that increases in MnSOD expression are a consistent response of the lung to oxidant attack. It should be noted that, although we are

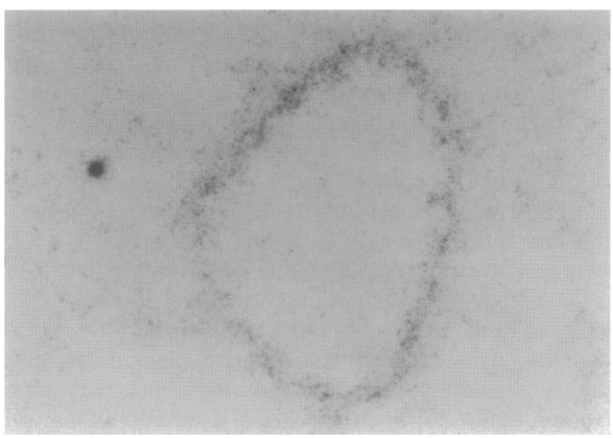

Figure 9. Expression of MnSOD in ordinary (not hot spot) bronchiolar epithelium and adjacent parenchyma. Unstained section; magnification, ×20.

Table 1. Number of Airways with Indicated Percentage of Bronchiolar Epithelium Occupied by Hot Spots

Treatment	0–4.9%	5.0–9.9%	10.0–19.9%	>20.0%	*P**
Control, 1 day	23	2	3	3	<0.02
Smoke, 1 day	61	3	2	0	
Control, 2 days	26	5	4	0	<0.01
Smoke, 2 days	54	3	3	0	
Control, 7 days	23	3	4	0	NS
Smoke, 7 days	63	2	4	1	
Control, 14 days	31	1	6	2	NS
Smoke, 14 days	41	4	8	13	

*By Kruskal-Wallis test. NS, not significant.

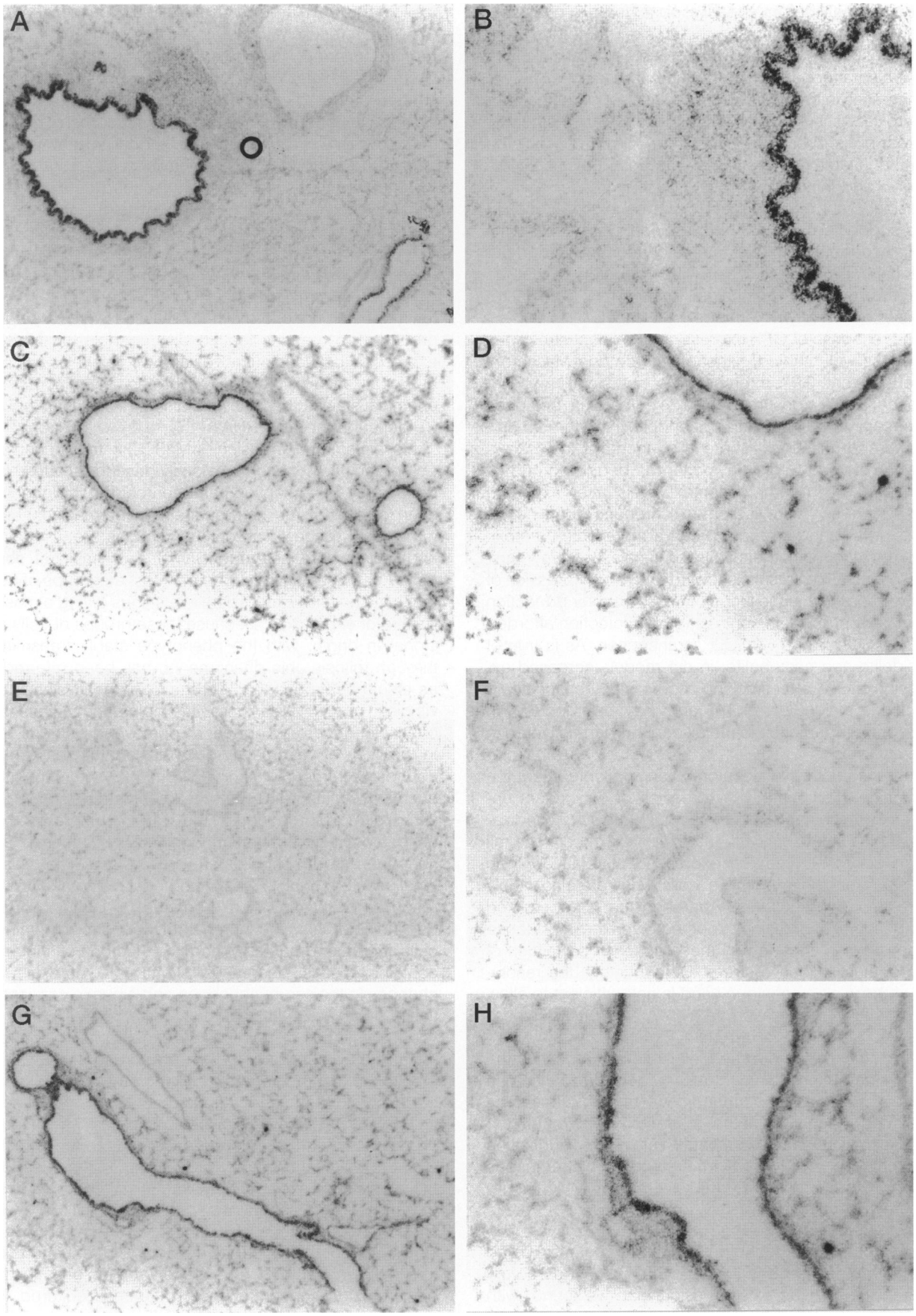

Figure 10. Distribution of expression of CuZnSOD (**A** and **B**), GPX (**C** and **D**), CAT (**E** and **F**), and MT (**G** and **H**). All except catalase show higher expression levels in small airways, compared with parenchyma. Unstained sections; magnification, ×20 (**A**, **C**, **E**, and **G**) and ×50 (**B**, **D**, **F**, and **H**).

assuming that the changes we observed are related to the presence of oxidants in the smoke, numerous other toxic substances are also present in cigarette smoke that might also affect antioxidant gene expression.

Increases in MT expression[21] have been reported after hyperoxia. As is true of MnSOD, it is unclear whether MT expression is directly induced by AOS or is associated with the inflammatory response, as both tumor necrosis factor-α and interleukin-6 can induce increased MT expression,[27] and Piedbouf et al,[21] using *in situ* hybridization, suggested that MT expression in hyperoxia was spatially correlated with inflammatory cells. Although our data cannot resolve this issue, they do suggest that MT expression may be another fairly nonspecific acute response to a variety of forms of AOS attack.

The response of GPX expression to oxidant stress has been less well studied, although Clerch and Massaro[28] observed that GPX expression is not significantly increased after exposure of rats to hyperoxia but does increase after administration of endotoxin. With both crocidolite and cristobalite there is an increase in GPX expression starting 6 to 9 days after the beginning of dust exposure, with return to control levels after exposure ceases.[24] Also, there is some discrepancy between *in vitro* studies showing that GPX is a more potent antoxidant enzyme than the SODs or catalase on a molar basis[20] and *in vivo* results showing failure of expression of a GPX transgene in the lungs of mice to confer protection against hyperoxia[29] in contrast to the protection afforded by CuZnSOD[30] and MnSOD[31] transgenes. As is true of crocidolite and cristobalite, in our model, increased expression of GPX after smoking occurs later than the acute elevations of MnSOD or MT and may be important in longer-term adaptation to the oxidant burden delivered in cigarette smoke.

There is relatively little information available about the anatomical distribution of expression of antioxidant enzymes and substances in the lung. Our *in situ* hybridization studies show that, with the exception of catalase, all of the genes studied are expressed at relatively high levels in bronchiolar epithelial cells. They are also widely expressed in the pulmonary parenchyma, a generalized distribution of expression expected given the importance of the proteins encoded by these genes in protection of cells against oxidative stress. Unfortunately, the resolution of the *in situ* technique used is not sufficient to allow more detailed localization, ie, type I cells *versus* type II cells *versus* endothelial cells, etc. Except for MnSOD (see below), smoke did not produce visible differences in the expression pattern of any of these agents. The increase in MT expression that we found on Northern blot analysis presumably could be either quite diffuse through the lung, so that the anatomical pattern of *in situ* hybridization does not appear different in the smokers, or perhaps is buried in the bronchiolar epithelial signal, which is intense even in nonsmokers.

The distribution of MnSOD mRNA by *in situ* hybridization has previously been studied by Clyde et al.[32] Using the same probe used in this study, they reported a very different distribution of expression than we found, with localization to arterioles, alveolar duct septal tips, and mesothelium. We are at a loss to explain these discordant results; our results with the MnSOD probe have been very reproducible, and the same results were obtained when we followed exactly the *in situ* hybridization protocol described by Clyde et al. The only difference between the studies we can identify is the tissue fixation: 4% paraformaldehyde *versus* 10% buffered formalin in the present study. The decision to use formalin was based on pilot experiments using the probes described herein as well as a procollagen cDNA probe, which gives a cell-type-specific signal rather than the more generalized expression seen with the antioxidant genes, which are expressed in all cells. Although results with paraformaldehyde and formalin were similar, the signal was consistently greater in the formalin-fixed tissues with less background. The reasons for the discrepancies in MnSOD expression between ourselves and Clyde et al is unclear, but localization of the MnSOD protein by immunohistochemical staining has shown a distribution similar to what we describe for the mRNA.[33–35]

Mosaicism of expression of MnSOD in the bronchiolar epithelium has not been previously described, and in fact remarkably little attention has been paid to the phenomenon of phenotypic heterogeneity in normal nonhematolymphoid tissues. The only exceptions are in the skin and liver, where heterogeneity of expression of some genes has been shown to be manifest as groups of cells that have uniform expression levels, thus giving a mosaic pattern of expression. Because these groups of cells are clonal in origin, with the phenotype stably transmitted through mitosis, this phenomenon has been referred to as clonal heterogeneity.[36] In the skin, these clones are visible in many mouse lines with single gene mutations in coat color genes and appear as dorsoventral stripes.[37,38] In the liver, approximately 0.1% of hepatocytes contain abundant intracytoplasmic albumin by immunohistochemical staining and are presumably the source of the serum albumin produced by the liver.[39] A different subset of hepatocytes produce fibrinogen, and the albumin- and fibrinogen-producing cells are present as single cells or small clusters of cells within the hepatic parenchyma. In response to inflammation produced by injection of turpentine into rats, the number of fibrinogen-containing cells increases markedly and the number of albumin-containing cells decreases slightly, in parallel with changes in the serum fibrinogen and albumin concentrations secondary to inflammation.

The possibility of clonal heterogeneity playing a role in antioxidant defenses of the lung was recognized during previous experiments from our laboratory on hydrogen-peroxide-induced oxidant injury to tracheal epithelial cells in rats.[40] In that study, the comet assay was used to allow detection of hydrogen-peroxide-induced DNA strand breaks in single-cell suspensions of tracheal epithelial cells. We showed that, although there was a dose-dependent increase in the number of cells showing strand breaks after exposure to hydrogen peroxide, a significant number of cells were very resistant and failed to show evidence of damage, even at the highest hydrogen peroxide concentrations. In contrast, cultured V79 lung fibroblasts showed a very uniform response to dif-

ferent doses of hydrogen peroxide, with little cell to cell variability. This suggested the possibility of heterogeneity in expression of antioxidant enzymes as a mechanism that might account for this heterogeneity in response to an oxidant stress.

Clonal heterogeneity of antioxidant enzyme expression within the lung could provide improved adaptability through the presence of clones that are differentially resistant to oxidant stresses, with the ability of the resistant clones to survive and proliferate in response to an insult that could irreparably damage nonresistant clones. Although the observed mosaicism of MnSOD expression may be an example of clonal heterogeneity, proof of this will require demonstration that the phenotype (ie, high *versus* low expresser) is transmitted through mitotic cell division to daughter cells.

It was interesting to note that, in this study, MnSOD showed an anatomically changed pattern of expression after exposure to cigarette smoke, with apparent disappearance of many of the discrete hot spots after 1 and 2 days of smoke exposure (Table 1), so that the bronchiolar epithelium became more homogeneously hot, with reappearance of the discrete areas of high expression by the end of the experiment. This finding, together with the increased total MnSOD mRNA present in the lungs of these animals, suggests that there is initial up-regulation and then rapid down-regulation of MnSOD expression in the initially low-expressing bronchiolar epithelial cells in response to cigarette smoke; this could represent an acute protective mechanism against oxidant damage. The transient nature of these these changes, despite continuing cigarette smoke exposure, indicates that they are unlikely to contribute to long-term adaptive tolerance.

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