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Molecular Regulation of IL-6 Activation by Asbestos in Lung Epithelial Cells

Role of Reactive Oxygen Species[1]

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IL-6 has been characterized as a pleiotropic cytokine with multiple biologic activities, but its induction and role in asbestos diseases have not been studied. Asbestos fibers were found to stimulate IL-6 expression and secretion in pulmonary type II-like epithelial A549 cells as well as in normal human bronchial epithelial cells. IL-6 induction was dependent on the intracellular redox-oxidative state, since intracellular hydroxyl scavengers and *N*-acetylcysteine, a precursor of glutathione, abrogated IL-6 secretion by asbestos or H_2O_2. IL-6 induction paralleled increased DNA binding activity to the nuclear factor-κB (NF-κB)- and NF-IL-6-recognized sites in the IL-6 promoter. The NF-κB and NF-IL-6 DNA binding proteins were immunochemically characterized as a heterodimer p65/p50 and a homodimer C/EBPβ, respectively. Stimulation of DNA binding activity to the NF-κB and NF-IL-6 binding sites of the IL-6 promoter by asbestos or H_2O_2 were inhibited by tetramethylthiourea, a hydroxyl radical scavenger. The role of local IL-6 production in the pathophysiologic processes of fiber-induced lung disorders was examined. Although less active than fibroblast growth factor, human rIL-6 also stimulated lung fibroblast growth, as evidenced by increased [^{3}H]thymidine incorporation. Furthermore, elevated IL-6 levels were found in bronchoalveolar lavage fluids from patients diagnosed with lung fibrosis and work-related histories of long term asbestos exposure. Taken together, the results suggest that asbestos-induced oxidative stress is involved in the activation of NF-κB and NF-IL-6 transcription factors, which recognize the IL-6 promoter. The resulting increase in IL-6 expression may be involved in both inflammatory and fibrotic processes in the lung. *The Journal of Immunology,* **1997, 159: 3921–3928.**

Asbestos exposure in humans is associated with inflammatory and malignant diseases in the lung (1). Cytokines and growth factors, such as TNF-α, IL-1α, IL-8, and platelet-derived growth factor, derived from alveolar macrophages, lung epithelial, and to some extent endothelial cells are strongly implicated as mediators of asbestos-induced pathophysiologic responses (2–5). The mechanisms responsible for these processes are complex (6), but largely involve the ability of asbestos fibers to generate reactive oxygen species (ROS)[2] via the interaction of iron, present as a silicate-iron complex on the fiber surface (7–9). Whereas high levels of ROS induce cytotoxicity and play a direct role in asbestos-related pulmonary damage, moderate oxidative stress can activate events in the cell signal transduction pathways leading to the production of inflammatory mediators. For example, ROS can stimulate the NF-κB nuclear transcription factor, leading to the up-regulation of a number of immune and inflammatory mediators (10, 11). Recently, NF-κB activation was found to be associated with asbestos exposure in the lung (8), and we reported that asbestos-induced oxidative stress is responsible for increased IL-8 gene expression through the activation of the NF-κB/NF-IL-6 nuclear transcription factors (9).

IL-6, a pleiotropic cytokine with multiple biologic activities, has been implicated in the pathogenesis of a variety of diseases (12). Although the induction and the role of IL-6 in asbestos exposure have not been studied, increasing evidence demonstrates that IL-6 is involved in chronic inflammatory lung diseases. For example, clinical studies have indicated elevated IL-6 levels in bronchoalveolar lavage fluid (BALF) from patients with idiopathic pulmonary fibrosis and hypersensitivity pneumonitis or from normal subjects exposed to low doses of ozone (13, 14). Furthermore, increased IL-6 expression has been reported in cells obtained from the BALFs of pneumoconiosis patients with concomitant presence of coal-mine dust particles (15). Experimental evidence has demonstrated that pulmonary cells, including macrophages, epithelial cells, fibroblasts, and endothelial cells, express and release IL-6 in response to various agents, such as TNF-α, IL-1, platelet-derived growth factor, ozone, bacterial products, viruses, or tissue injury mediators (16–21).

Like most cytokine genes, IL-6 expression is regulated by both transcriptional and post-translational mechanisms. Transcriptional regulation is mainly controlled by sequence-specific, DNA binding proteins, commonly referred to as transcription factors, that bind to the *cis*-acting elements of the IL-6 promoter and include NF-κB and NF-IL-6. Using IL-6 promoter deletion analysis, it was demonstrated recently that NF-IL-6 and NF-κB binding sites serve as obligatory elements for IL-6 induction (22, 23), and synergistic

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[2] Abbreviations used in this paper: ROS, reactive oxygen species; NF, nuclear factor; BALF, bronchoalveolar lavage fluid; TMTU, 1,1,3,3-tetramethyl-2-thiourea; DMTU, 1,3-dimethyl-2-thiourea; NAC, *N*-acetylcysteine; NHBE, normal human bronchoepithelial; LDH, lactate dehydrogenase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoresis mobility shift assay; aFGF, acidic fibroblast growth factor; AP-1, activating protein-1.

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enhancement of transcription of IL-6 promoter constructs has been reported using cotransfection with NF-κB and NF-IL-6 expression vectors (24). Most transcription factors are activated by post-translation modifications. For example, NF-κB activation requires cytosol dissociation of the inhibitory subunit IκB from the NF-κB protein complex (25), and ROS have been postulated to be involved in these modifications (10). In the present studies we examined whether IL-6 is involved in asbestos-related pulmonary damage and whether this induction is associated with NF-κB and/or NF-IL-6 activation via oxidative stress.

Materials and Methods

Reagents

Crocidolite asbestos ($Na_2Fe_3Fe_2(Si_8O_{22})(OH)_2$), identified as ML-6 (Certain-Teed Co., Ambler, PA), was obtained from a mine in South Africa located in Kuruman Hills in the Kalahari desert. This sample of crocidolite had a mean length of 19 μm, a mean width of 1 μm, and a density of 3.32 g/cm^3. Before use the mineral dust samples were sterilized and endotoxin inactivated by autoclaving for 2 h. There was no endotoxin detected in culture medium incubated with the fibers, as assessed by the *Limulus* amebocyte assay (Sigma Chemical Co., St. Louis, MO).

1,1,3,3-Tetramethyl-2-thiourea (TMTU), 1,3-dimethyl-2-thiourea (DMTU), *N*-acetylcysteine (NAC), and hypoxanthine were purchased from Sigma Chemical Co. Catalase (from bovine liver) was supplied from Calbiochem-Novabiochem Corp. (San Diego, CA). H_2O_2 was obtained from Fisher Scientific Co. (Pittsburgh, PA), and DMSO was purchased from J. T. Baker, Inc. (Phillipsburg, NJ).

Cell preparation

Pulmonary human type-II like epithelial cells (A549), originally derived from an individual with alveolar cell carcinoma, were purchased from the American Type Culture Collection (Rockville, MD). A549 epithelial cells were plated at 1×10^5 cells/well to 24-well microtiter plates (Corning Glass Works, Corning, NY) in F-12 medium (Life Technologies, Gaithersburg, MD) containing 10% FBS (HyClone, Logan, UT), 2 mM L-glutamine, 50 μg/ml gentamicin, and 50 ng/ml Fungizone (Life Technologies). These cells, although transformed, retain many of the characteristics of normal type II cells, such as surfactant production, cytoplasmic multilamellar inclusion bodies, and cuboidal appearance, and have been extensively used to assess type II pneumocyte effector cell function (26).

Cryopreserved, primary normal human bronchoepithelial (NHBE) cells were purchased from Clonetics Corp. (San Diego, CA) and grown in 25-cm^2 tissue culture flasks in bronchial epithelial cell growth medium, supplied by Clonetics. The cultures were incubated at 37°C in a humidified, 95% air/5% CO_2 atmosphere. After trypsinization, the cells were subcultured into 24-well culture plates at seeding densities of 0.5×10^5 cells/well to 24-well microtiter plates (Corning Glass Works). When the cells were 50 to 60% confluent, the medium was changed to bronchial epithelial cell growth medium without hydrocortisone, and the cells were allowed to grow for an additional 24 h.

Lung cells were incubated with asbestos fibers for 18 h at 37°C, and the supernatants were collected, filter sterilized, and stored at −70°C. Aliquots of cell culture supernatants were assayed immediately for the presence of lactic dehydrogenase (LDH), a measure of cytoplasmic leakage, as previously described (7). Triton X-100 (0.5% in PBS) was added for 30 min to control cultures to assess total LDH release. Trypan blue exclusion was used to confirm the LDH results.

TMTU, DMTU, and NAC were dissolved in culture medium and added to the cells 1 h before treatment. The pH of the NAC solution was corrected to 7.2 by 2 N NaOH. Fresh solutions of H_2O_2 were prepared in distilled water. All other chemicals were prepared fresh in PBS and added to cell cultures in 20-μl volumes. Asbestos fibers were resuspended in PBS, sonicated for 15 min and vortexed before addition to each culture well.

IL-6 ELISA

Human IL-6 concentrations were determined by ELISA using a commercially available system (R&D Systems, Minneapolis, MN). Absorbance was measured at 450 nM wavelength using a UVmax kinetic microplate reader (Molecular Devices, Corp., Menlo Park, CA) with the use of a ΔSoft Program (Molecular Devices, Corp.) for data collection. IL-6 concentrations in test samples were determined from a standard curve of human rIL-6. Data shown are representative of at least three separate experiments. Statistical significance was determined by the RS/1 multicomparison procedure using the Wilkes-Shapiro test for normality, one-way analysis of variance, and Dunnett's test for multiple comparisons with a common control group (27). When variances were nonhomogeneous, multiple comparisons using the Bonferroni adjustment of Student's *t* test were performed. Statistically significant differences were reported when $p < 0.05$.

Reverse transcription and PCR amplification

Cellular RNA was extracted using RNAzol B (Biotec Laboratories, Inc., Houston, TX) according to the manufacturer's instructions. For the synthesis of cDNA, 1 μg of RNA, quantitated spectrophotometrically (Gene Quant, Pharmacia, Cambridge, U.K.), was resuspended in a 20-μl final volume of the reaction buffer (25 mM Tris-HCl (pH 8.3), 37.5 mM KCl, 10 mM DTT, 1.5 mM $MgCl_2$, and 10 mM of each dNTP; Perkin-Elmer/Cetus, Norwalk, CT) containing 0.5 μg oligo$(dT)_{12-18}$ primer (BRL, Gaithersburg, MD). Once the reaction mixture reached 42°C, 400 U of SuperScript (Life Technologies; 200 U/μl) was added to each tube and incubated for 30 min at 42°C. The reaction was stopped by denaturing the enzyme at 99°C for 5 min, and the mixture was diluted with water to a 50-μl volume.

PCR amplification was performed using commercially available PCR primers (Clontech Laboratories, Inc., Palo Alto, CA) for human IL-6 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH), the latter an indicator of constitutive expression. The sequences of the primers used were as follows: IL-6: sense, 5′-ATGAACTCCTTCTCCACAAGCGC-3′; antisense, 5′-GAAGAGCCCTCAGGCTGGACTG-3′; and G3PDH: sense, 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′; antisense, 5′-CATGTGG GCCATGAGGTCCACCAC-3′.

Five-microliter aliquots of the synthesized cDNA, corresponding to approximately 0.1 μg of RNA, were added to 45 μl of PCR mix containing 4 μl of 10× PCR buffer, 1 μl of deoxynucleotides (1 mM each), 1 μl of 3′ and 5′ RNA-specific primers sense and antisense primers (0.15 μM), and 0.25 μl of DNA polymerase (GeneAmp PCR kit, Perkin-Elmer/Cetus). The reaction mixture was covered with wax tablets (Perkin-Elmer/Cetus). Amplification was initiated by 1 min of denaturation at 94°C for one cycle followed by 25 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min using a GeneAmp PCR 9600 DNA Thermal Cycler (Perkin-Elmer/Cetus). After the last cycle of amplification, the samples were incubated for 7 min at 72°C. For each set of primers, serial dilutions of cDNA were amplified at increasing cycles (n = 25–30) to achieve the optimal conditions for amplification. The PCR products were visualized by UV illumination following electrophoresis through 2.0% agarose (UltraPure, Sigma Chemical Co.) at 60 V for 80 min and staining in Tris borate-EDTA buffer (89 mM Tris, 89 mM boric acid, and 2.5% EDTA, pH 8.2) containing 0.5 mg/ml ethidium bromide.

Quantitative analysis of IL-6 message was conducted by means of competitive PCR (28) using stepwise dilutions of a synthetic competitor (PCR MIMICS, Clontech Laboratories, Palo Alto, CA) as a template. The competitors represent sequences complementary to the cytokine primers but with different sized PCR products. Three-microliter aliquots of cDNA were amplified for 25 cycles (G3PDH) or 30 cycles (IL-6) with the primers in the presence of the respective competitor, ranging from 10^2 to 10^{-5} attomoles. The relative amounts of mRNA transcripts were determined visually and by scanning with computerized laser densitometer (Eagle Eye II Image analysis system, Stratagene, La Jolla, CA) and the National Institutes of Health Image 1.54 analysis software package. The ratio of the target densitometry value was plotted against the reciprocal of the molar amount of the competitor. When necessary, the specificity of the PCR bands was confirmed by restriction site analysis of the amplified cDNA in which fragments were generated of the expected size (data not shown).

Nuclear extracts and electrophoresis mobility shift assay (EMSA)

Nuclear proteins were prepared from aliquots of 1×10^7 cells according to the method of Schreiber et al. (29). DNA binding reactions and EMSAs were performed as described previously (30). Briefly, the 5′ ends of the double-stranded oligonucleotides were labeled with [γ-^{32}P]ATP (New England Nuclear-DuPont, Boston, MA), using 6 to 10 U of T4 polynucleotide kinase (U.S. Biochemical Corp./Amersham, Cleveland, OH). Binding reactions (30 μl) were performed on ice for 30 min in reaction mixtures containing 10 μg of nuclear proteins, 20 mM Tris-HCl (pH 7.8), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 50 μg/ml BSA, 2 μg of poly(dI-dC)·poly(dI-dC), 10% glycerol, and approximately 0.1 ng (2×10^5 cpm) of specified probe. For detection of DNA binding activity of transcription factors, the following DNA oligonucleotides containing specific transcription factor binding sites (in italics) on the IL-6 promoter were used: NF-κB site (−73 to −64), 5′-ATGT*GGGATTTTCC*CATGAG-3′; and NF-IL-6 site (−156 to −147), 5′-GGACGTCA*CATTGCACAATC*TTAATAA-3′.

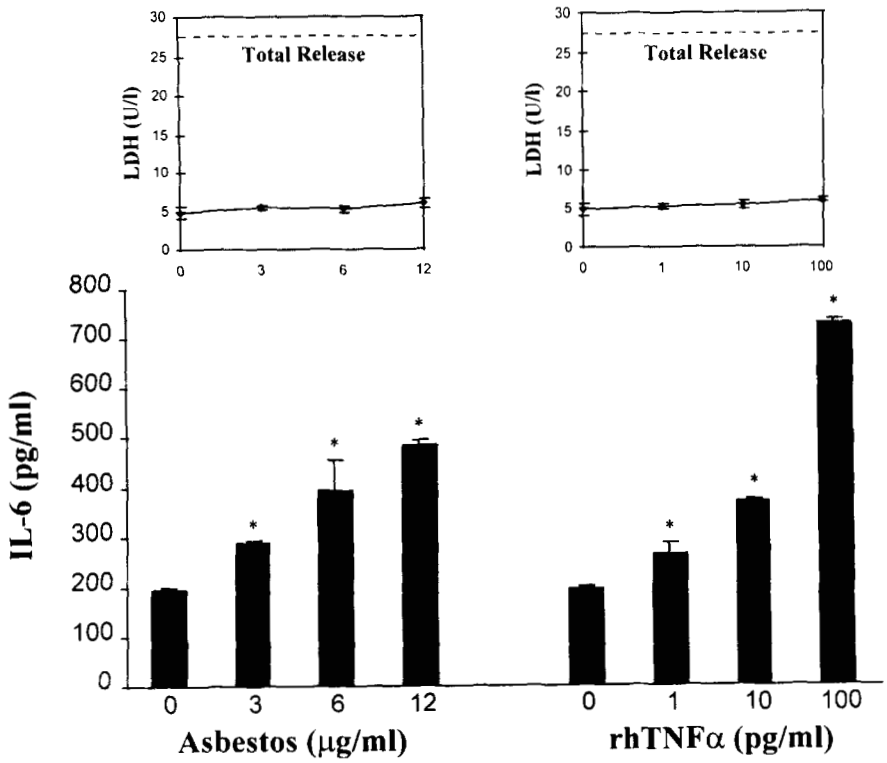

FIGURE 1. Secretion of IL-6 by asbestos fibers or human rTNF-α in normal human small bronchial epithelial cells. Cell cultures were incubated with asbestos crocidolite (0–12 μg/ml) or human rTNF-α (0–100 pg/ml) for 24 h, and IL-6 was quantified in supernatants as indicated in *Materials and Methods*. LDH release was used as a measure of cell damage. Values represent the mean ± SE of three separate experiments. The asterisk represents a significantly different ($p < 0.05$) response from that of the control group.

Protein-DNA complexes were separated on a 5% nondenaturing polyacrylamide gel. Gels were electrophoresed at 125 V in 50 mM Tris-50 mM boric acid/1 mM EDTA, dried, and autoradiographed overnight. The gels were analyzed by radioactivity image analysis (AMBIS radioisotopic imager, Scanalytics, Billerica, MA), or autoradiograms were scanned with a computerized laser densitometer (Eagle Eye II Image analysis system, Stratagene, La Jolla, CA). These results were examined using the One Dscan-gel analysis software and the National Institutes of Health Image 1.54 analysis software package. To characterize DNA binding activity, nuclear protein extracts were preincubated before addition of labeled probe with extra unlabeled oligomers for 30 min or of 2 μg/ml of Abs to the NF-κB or NF-IL-6 related proteins (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h.

Cell proliferation

Human lung fibroblasts (CCD-32Lu) were obtained from the American Type Culture Collection (Rockville, MD) and grown in MEM (Eagle's) with nonessential amino acids supplemented with 2 mM L-glutamine and 10% FBS. The cells were seeded at a concentration of 1×10^4 cells/well to 96-well flat-bottomed culture plates. After 6 h, the culture medium was replaced with MEM supplemented with insulin-selenium-transferrin (Life Technologies) and human rIL-6 (R&D Systems) or acidic fibroblast growth factor (aFGF), and the cultures were allowed to incubate for an additional 48 h. [^{3}H]Thymidine (sp. act., 6.7 Ci/mmol; DuPont-New England Nuclear) was added at a concentration of 1 μCi/well to the cultures for the last 20 h of incubation. The cells were detached from the plates by the addition of 0.1% trypsin and collected onto glass-fiber filters using an automated cell harvester (Skatron, Sterling, VA). [^{3}H]Thymidine incorporation was quantitated by liquid scintillation counting.

Bronchoalveolar lavage and subject groups

Human BALF samples were obtained from New York University Medical Center (New York, NY). The control group consisted of six men with no symptoms of respiratory diseases. All investigations, including chest radiograph and bronchoscopy, were normal, although two were former smokers. The patient group consisted of six men with biopsy-confirmed pulmonary fibrosis and a history of long term exposure to asbestos. None of these subjects was taking inhaled or systemic steroids, and none had an acute respiratory disease for at least 1 mo before the time of sampling. All individuals gave informed written consent, and the study was approved by the local human studies review board. Bronchoscopy was conducted to confirm the original diagnosis. Bronchoalveolar lavage was performed according to the method of Koren et al. (31). Following collection the samples were centrifuged (1100 rpm, 10 min, 4°C) to remove cellular components and were frozen at −20°C. Total protein was determined by a commercial kit (Bio-Rad, Hercules, CA).

Results

NHBE cells and A549 human type II pulmonary epithelial cells, cultured in the presence of asbestos crocidolite, produced a concentration-dependent increase in the secretion of immunoreactive IL-6 (Figs. 1 and 2). Asbestos-induced secretion of IL-6 was not accompanied by an increase in LDH release in NHBE cells (Fig. 1, *inset*). However, A549 cells were damaged by asbestos fibers, as demonstrated by LDH leakage, but compared with maximal LDH release, the degree of fiber-induced cell damage was minimal (Fig. 2, *inset*). RNA isolated from control and asbestos-treated A549 cells were examined for IL-6 mRNA transcripts by competitive PCR to determine whether the changes in cytokine activities observed in the culture supernatants corresponded with gene expression (Fig. 3). The density of the amplified cDNA band for IL-6 was normalized relative to the density of each corresponding band for G3PDH. Whereas vehicle-treated cells demonstrated minimal numbers of IL-6 mRNA transcripts, there was an approximately fourfold increase when cells were cultured in the presence of asbestos fibers.

As asbestos mediates many of its cellular effects through iron-mediated generation of ROS, experiments were conducted to determine whether changes in the cellular redox state are associated with the IL-6 response. As shown in Figure 4, the addition of intracellular hydroxyl radical scavengers, including DMTU, TMTU, DMSO, and *N*-acetylcysteine, to asbestos-stimulated A549 cells significantly decreased IL-6 secretion. Inhibition of

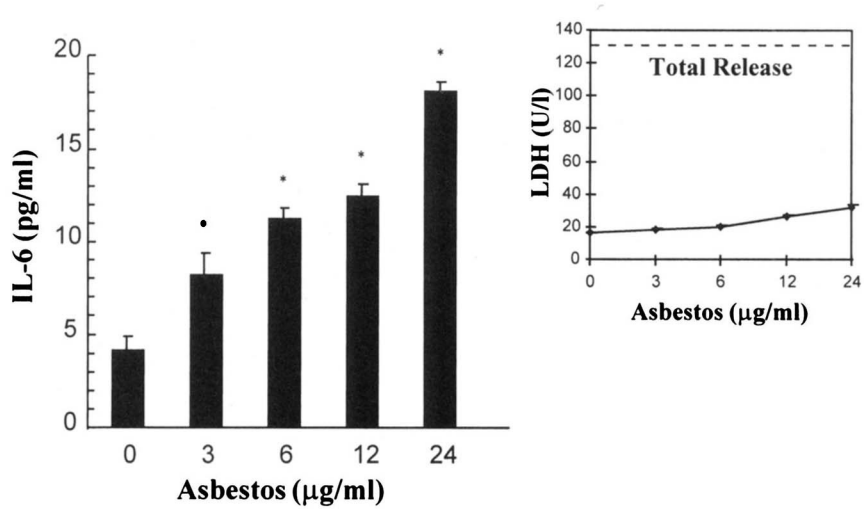

FIGURE 2. Effect of asbestos on IL-6 secretion in A549 epithelial cells. Fiber samples were added to cell cultures at concentrations ranging between 0 and 25 μg/ml. LDH release was used as a measure of cell damage. Values represent the mean ± SE of three separate experiments. The asterisk represents a significantly different ($p < 0.05$) response from that of the control group.

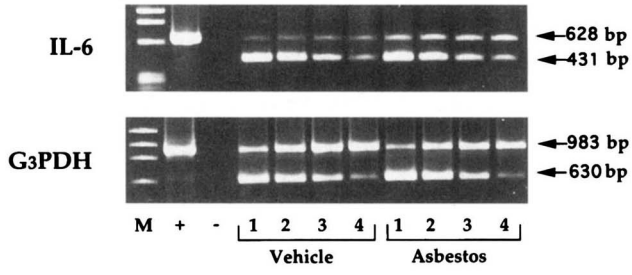

FIGURE 3. Competitive reverse transcription-PCR analysis of IL-6 and G3PDH mRNA transcripts from A549 cell cultured for 2 h in the presence of asbestos fibers (25 μg/ml). cDNA equivalents of 0.1 μg of RNA were amplified for 30 or 25 cycles in the presence of threefold dilutions of IL-6 or G3PDH competitive DNA MIMICS, respectively. For IL-6, the *top band* is the sample IL-6, and the *bottom band* is the IL-6 MIMICS. *Lane 1*, 1 amol of MIMIC; *lane 2*, 0.33 amol; *lane 3*, 0.11 amol; *lane 4*, 0.036 amol. For G3PDH, the *top band* is the G3PDH sample, and the *bottom band* is the G3PDH MIMIC. *Lane 1*, 10 amol; *lane 2*, 3.3 amol; *lane 3*, 1.1 amol; *lane 4*, 0.37 amol.

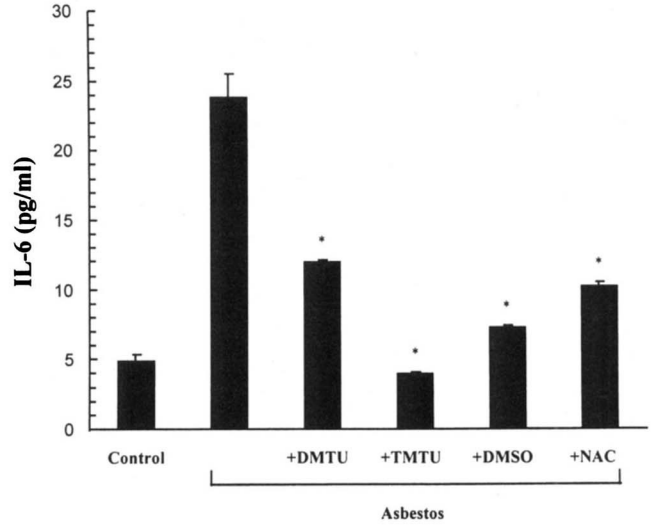

FIGURE 4. Effects of antioxidants on asbestos-induced IL-6 secretion in A549 lung epithelial cells. Cells were pretreated for 1 h with 10 mM DMTU, TMTU, or NAC or with 1% DMSO and exposed to asbestos (25 μg/ml). The ELISA values are presented in picograms per milliliter. The asterisk represents a significant ($p < 0.05$) decrease from the responses of cells treated only with asbestos fibers. Values represent the mean ± SE ($n = 3$ experiments).

IL-6 ranged from complete inhibition with TMTU to partial inhibition with DMTU. Furthermore, the direct addition of H_2O_2 to A549 cell cultures dose-dependently stimulated IL-6 secretion, while the addition of TMTU abrogated the response (Fig. 5).

Mobility shift assays were conducted to establish whether asbestos-induced IL-6 gene expression in A549 cells coincided with nuclear protein binding to regulatory elements in the IL-6 promoter. An oligonucleotide containing the NF-κB binding site in the IL-6 gene was used to probe nuclear extracts isolated from control and asbestos-treated cells. As with IL-6 gene expression, low levels of nuclear protein DNA binding were present constitutively, and the binding activity was increased following incubation with asbestos (Fig. 6). The inducible binding activity was specific, as DNA binding was prevented by the addition of excess unlabeled oligonucleotide competitor but not by an unrelated sequence containing the AP-1 binding site. The asbestos-induced DNA bound complex (bands 1 and 2) consisted of both the p50 and p65 subunits of the NF-κB transcription factor, as the complex was supershifted by addition of anti-p50 Abs and was eliminated by anti-p65 Abs. Preincubation of the nuclear extracts with nonrelated Abs did not influence the mobility of the bound complex (data not shown). Similar to asbestos, the addition of H_2O_2 to A549 cells also increased NF-κB DNA binding activity (Fig. 6). H_2O_2-inducible binding activity, although slightly weaker, demonstrated identical specificity and subunit composition as that induced by asbestos (data not shown). Nuclear extracts prepared from A549 cells cultured in the presence of TMTU revealed decreases in asbestos- and H_2O_2-stimulated DNA binding activities by 50 and 70%, respectively, as demonstrated by isotopic image analysis. Increasing preincubation with TMTU from 1 to 12 h did not increase the inhibitory effect of TMTU.

Mobility shift assays were also conducted to characterize the binding activity to the consensus binding site of the NF-IL-6 transcription factor contained in the IL-6 promoter (Fig. 7). While low

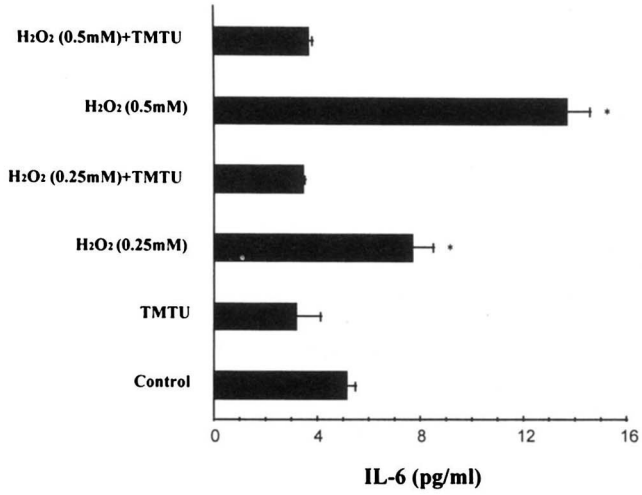

FIGURE 5. H_2O_2-induced IL-6 secretion from A549 cells and modulation of the response by TMTU. Cells were pretreated for 1 h with 10 mM TMTU and exposed to H_2O_2. The ELISA values are presented in picograms per milliliter. The asterisk represents a significant ($p < 0.05$) difference from the control value. Values represent the mean ± SE (n = 3 experiments).

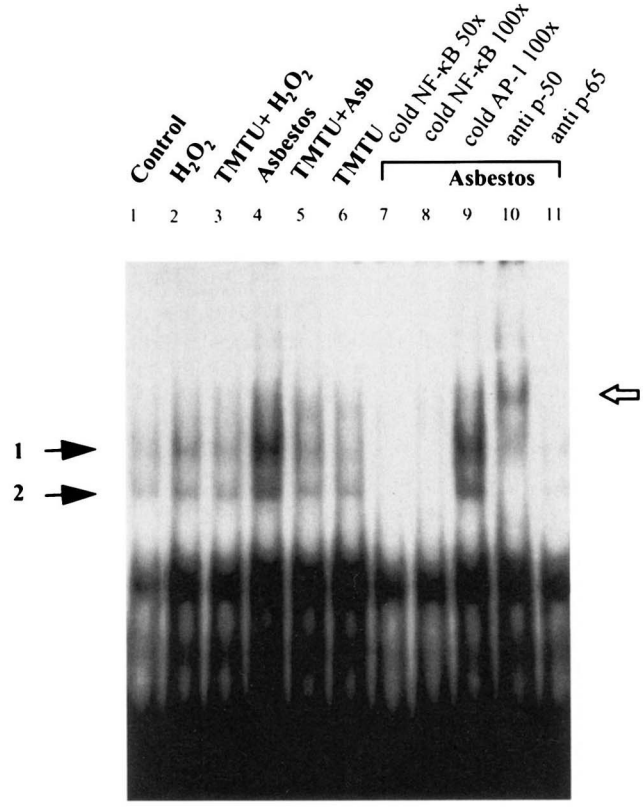

FIGURE 6. Change in DNA binding activity to the NF-κB-like binding site in the IL-6 promoter by asbestos or H_2O_2. Nuclear extracts from control, asbestos-stimulated (25 μg/ml; 2 h) or H_2O_2-stimulated (0.5 mM; 2 h) A549 cells were analyzed by EMSA. The effect of TMTU pretreatment (10 mM; 12 h) was evaluated under identical conditions. The ^{32}P-labeled double-stranded oligonucleotide containing the IL-6 binding site for NF-κB (−73/−64), was used as a probe. A 50- or 100-fold excess of unlabeled oligonucleotides containing binding sites for NF-κB or AP-1 was used as the competitor. For immunochemical evaluation, some nuclear extracts were preincubated (1 h, 4°C) with 2 μg of Abs to the p50 or p65 protein subunits. The black arrows indicate specific bands, and the open arrow indicates the supershift.

levels of constitutive DNA binding activity were present in extracts from control A549 cells, a significant increase in activity occurred from nuclear extracts of cells cultured in the presence of asbestos or H_2O_2. Both, H_2O_2-inducible (data not shown) and asbestos-inducible complexes were prevented by an excess of unlabeled NF-IL-6 probe and were unaffected by addition of the unlabeled sequence containing the NF-κB binding site of the IL-6 promoter or the AP-1 consensus binding site (Fig. 7). As NF-IL-6 is a member of the C/EBP family of transcription factors (32), the inducible DNA binding complex (bands 1–3) was characterized using Abs specific for members of the family. Abs against C/EBPβ (NF-IL-6) eliminated band 1, reduced bands 2 and 3, and formed a supershifted complex. The complex was not affected by Abs to C/EBPα or C/EBPδ subunits. To determine whether, like NF-κB regulation, ROS are associated with NF-IL-6 activation, cell cultures were pretreated with TMTU. TMTU decreased both asbestos- and H_2O_2-induced nuclear protein binding to the NF-IL-6-recognized oligonucleotide by 60 and 100%, respectively (Fig. 7).

To study whether IL-6 levels are altered during chronic fibrotic lung diseases, IL-6 was measured in BALF of controls and patients with diffuse lung interstitial fibrotic changes related to long term asbestos exposure. The results, presented as the ratio of IL-6 to the total protein of BALF, demonstrated significantly higher mean values (threefold) in patients compared with controls (Fig. 8).

To investigate whether IL-6 can serve as a mitogen for lung fibroblast, human rIL-6 was added to primary human lung fibroblast cultures, and [^{3}H]thymidine incorporation was monitored. As shown in Figure 9, IL-6 dose-dependently stimulated [^{3}H]thymidine incorporation in lung fibroblast cells, although not to the extent of aFGF, with a maximum increase of approximately 2.5-fold over the control value.

Discussion

IL-6 is a multifunctional pleiotropic cytokine produced by many cell types in the lung, including alveolar macrophages, interstitial fibroblasts, endothelial cells, and bronchial epithelial cells, following appropriate stimulation (16–21). Since the alveolar space is lined by epithelial cells that are in direct contact with the inhaled toxic agents, and hyperplasia of alveolar epithelial cells is a common finding in lung diseases such as fibrosis (33), the induction of IL-6 in these cells probably plays a role in modulating immunologic and inflammatory activities in the alveolar space. In this respect, immunoreactive IL-6 is abundant in the hyperplastic alveolar type II epithelial cells of patients with interstitial fibrosis, in contrast to that in the cells of the normal lung (17). In the current study we demonstrate that asbestos fibers, in addition to primary inflammatory cytokines such as TNF-α, directly induce IL-6 expression in NHBE cells as well as A549 cells. IL-6 expression is dependent on asbestos-induced cellular oxidative/redox changes, as intracellular antioxidants effectively inhibit the response. Asbestos treatment is also associated with elevated DNA binding to the NF-IL-6- and NF-κB-like transcription factor cognate regulatory elements located in the promoter region of the IL-6 gene. Like asbestos, H_2O_2 also induced DNA binding activity to the

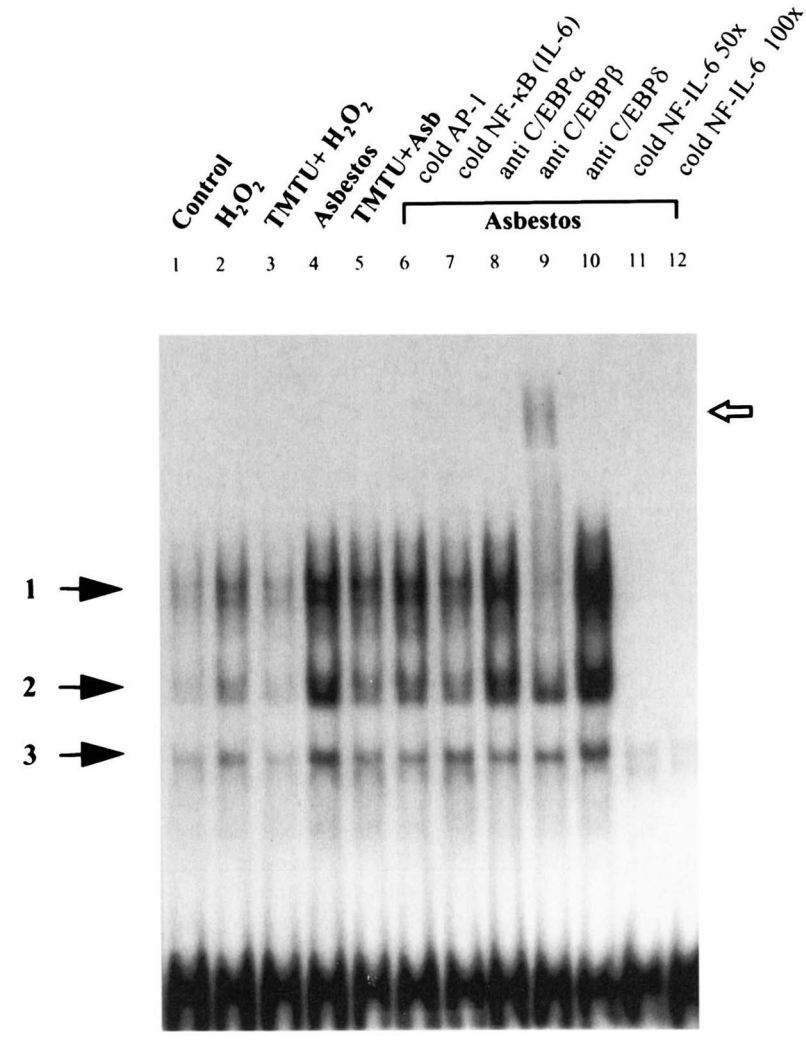

FIGURE 7. Change in DNA binding activity to the NF-IL-6 binding site in the IL-6 promoter by asbestos or H_2O_2. Nuclear extracts from control, asbestos-stimulated (25 μg/ml; 2 h), or H_2O_2-stimulated (0.5 mM; 2 h) A549 cells were analyzed by EMSA. The effect of TMTU pretreatment (10 mM; 12 h) was evaluated under identical conditions. The ^{32}P-labeled double-stranded oligonucleotide containing the IL-6 binding site for NF-IL-6 (−156/−147) was used as a probe. A 50- or 100-fold excess of unlabeled oligonucleotides containing binding sites NF-IL-6, NF-κB, or AP-1 was used as the competitor. For immunochemical evaluation, samples of nuclear extracts were preincubated (1 h, 4°C) with 2 μg of Abs to NF-IL-6-related proteins, including C/EBPα, C/EBPβ, and C/EBPδ. The black arrows indicate specific bands, and the open arrow indicates the supershift.

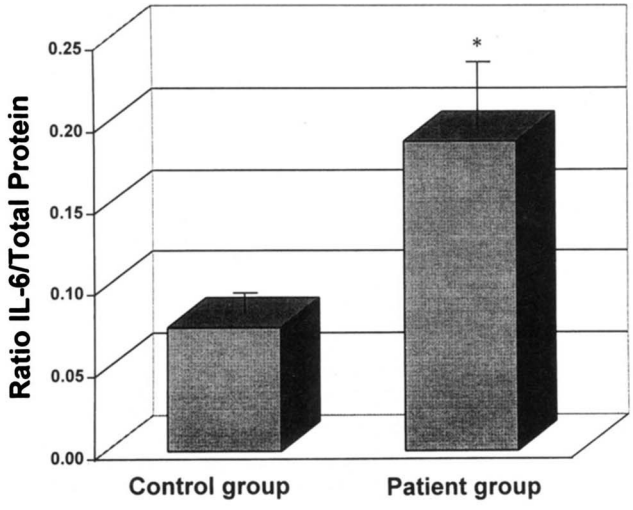

FIGURE 8. IL-6 levels in the BALF of patients with pulmonary fibrosis and a history of long term asbestos exposure. The results are presented as the ratio of IL-6 to total protein. Values represent the mean ± SE of six individuals per group. The asterisk represents a significantly different ($p < 0.05$) response from that of the control group.

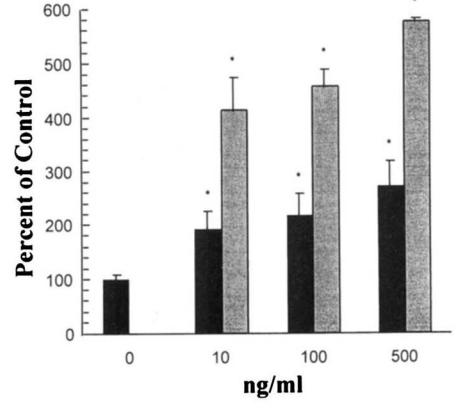

FIGURE 9. Effect of IL-6 on lung fibroblast DNA synthesis measured as [^{3}H]thymidine incorporation. Human rIL-6 or aFGF was added to the human lung fibroblast cell line (Lu35) and incubated for 48 h. [^{3}H]Thymidine was added during the last 20 h of incubation. Data for human rIL-6 (black bars) or aFGF (gray bars) treatment are expressed as a percentage of the control value using the mean ± SE of six different samples.

same binding sites in the IL-6 promoter. Changes in the intracellular redox state are a prerequisite for both H_2O_2- and asbestos-inducible binding activities, as they were blocked by TMTU.

The pulmonary epithelium is not only a physical barrier for inhaled Ags, particulates, and other potentially toxic and hazardous agents, but has the potential to synthesize and secrete inflammatory mediators such as IL-8, IL-6, and granulocyte-macrophage CSF (16, 17, 34). Recently, we demonstrated that asbestos fibers directly,

i.e., without the generation of primary response cytokines such as IL-1 and TNF-α, stimulate IL-8 expression (35), and this is mediated by up-regulation of IL-8 promoter transcriptional activity, including NF-IL-6- and NF-κB-like transcription factor activation (9). The production of IL-6 and IL-8 is often coordinated, as the promoter regions of both genes contain proximal binding sites for NF-IL-6 and NF-κB that need to be occupied for maximum expression (24). Consistent with these findings, we observed that asbestos fibers induce binding of C/EBPβ and p50/p65 regulatory proteins to the NF-IL-6- and NF-κB-like binding sites, respectively, in the IL-6 promoter. Recent studies demonstrated that NF-IL-6 (C/EBPβ) can act synergistically with homodimer p65, heterodimer p65/p50, and, to a lesser extent, homodimer p50 in IL-6 transcription activation (24).

Reactive oxygen metabolites, including hydroxyl radicals, have been implicated as important mediators in asbestos-induced lung toxicity (36). In this respect, we have previously shown that changes in the intracellular redox state generated by asbestos stimulate signaling pathways for inflammatory cytokine activation (9). The activation of NF-κB has been associated with ROS, which act as secondary messengers contributing to IκB dissociation and release of the active regulatory complex (10). H_2O_2 can also directly stimulate NF-κB DNA binding activity in cell cultures (11). We observed that intracellular antioxidants, such as TMTU, inhibit asbestos- or H_2O_2-induced DNA binding to the NF-κB-like binding site of the IL-6 gene. Consistent with these results, modulation of the glutathione level in hamster tracheal epithelial cells has been reported to suppress asbestos-stimulated DNA binding to the NF-κ consensus site (8). Additionally, we observed that oxidative stress is involved in stimulating binding to the NF-IL-6 consensus site of the IL-6 promoter. Asbestos as well as H_2O_2 triggered nuclear protein binding to the NF-IL-6 binding site in the IL-6 promoter, which was inhibited by TMTU. While post-translational modulation of C/EBP transcription factors, including NF-IL-6, has been indicated (37), the role of redox events in this process is not clear. Recently, NF-IL-6 activation has been related to IL-6 expression in endothelial cells cultured in hypoxic conditions (38). This is consistent with our observations, as the hypoxia is associated with the generation of ROS. Thus, changes in the intracellular oxidative-redox state appear to activate both NF-κB binding and NF-IL-6 binding, which coordinately regulate IL-6 expression in lung epithelial cells.

There is increasing evidence for the participation of IL-6 in inflammatory lung diseases. Transgenic mice that overexpress human IL-6 in airway epithelial cells develop peribronchial mononuclear infiltrates (39). Furthermore, it has been reported that murine lung fibroblasts express IL-6R on their surfaces, and anti-IL-6 Abs abrogate the proliferation of these cells (40). Consistent with this observation, we demonstrated that human rIL-6 stimulates lung fibroblast DNA synthesis, although less effectively than aFGF. The proliferation and activation of lung fibroblasts as well as their secretion of extracellular matrix proteins are hallmarks of lung fibrosis. Increased IL-6 levels have previously been associated with chronic pulmonary inflammatory and fibrotic diseases. For example, moderate IL-6 increases have been found in BALF of patients with idiopathic pulmonary fibrosis, hypersensitivity pneumonitis, and cystic fibrosis as well as in asymptotic pigeon breeders (13, 41). Consistent with these reports we found elevated IL-6 levels in BALF samples from patients with asbestos-related pulmonary fibrosis.

In conclusion, these studies provide evidence that IL-6 is produced locally by lung epithelial cells in response to asbestos. Asbestos mediates this response by a change in the cellular oxidative/redox state that may directly activate NF-κB and NF-IL-6 transcription factors. The local production of IL-6 in the lung may contribute in part to the pathophysiologic processes of fiber-induced lung disorders.

References

1. Mossman, B. T., and J. B. L. Gee. 1989. Medical progress: asbestos related diseases. *N. Engl. J. Med. 320:1721.*
2. Driscoll, K. E., D. G. Hassenbein, J. Carter, J. Poynter, T. N. Asquth, R. A. Grant, J. Whitten, M. P. Purdon, and R. Takigiku. 1993. Macrophage inflammatory proteins 1 and 2: expression by rat alveolar macrophages, fibroblasts, and epithelial cells and in rat lung after mineral dust exposure. *Am. J. Respir. Cell Mol. Biol. 8:311.*
3. Bonner, J. C., A. L. Goodell, P. G. Coin, and A. R. Brody. 1993. Chrysotile asbestos upregulates gene expression and production of α-receptors for platelet-derived growth factor (PDGF-AA) on rat lung fibroblasts. *J. Clin. Invest. 92:425.*
4. Li, X. Y., D. Lamb, and K. Donaldson. 1993. The production of TNF-α and IL-1-like activity by bronchoalveolar leucocytes after intratracheal instillation of crocidolite asbestos. *Int. J. Exp. Pathol. 74:403.*
5. Perkins, R. C., R. K. Scheule, R. Hamilton, G. Gomes, G. Freidman, and A. Holian. 1993. Human alveolar macrophage cytokine release in response to in vitro and in vivo asbestos exposure. *Exp. Lung Res. 19:55.*
6. Rom, W. N., W. D. Travis, and A. R. Brody. 1991. Cellular and molecular basis of the asbestos-related diseases. *Am. Rev. Respir. Dis. 143:408.*
7. Simeonova, P. P., and M. I. Luster. 1995. Iron and reactive oxygen species in the asbestos-induced tumor necrosis factor-α (TNF-α) response from alveolar macrophages. *Am. J. Respir. Cell Mol. Biol. 12:676.*
8. Janssen, Y. M. W., A. Barchowsky, M. Treadwell, K. E. Driscoll, and B. Mossman. 1995. Asbestos induces nuclear factor κB (NF-κB) DNA-binding activity and NF-κB-dependent gene expression in tracheal epithelial cells. *Proc. Natl. Acad. Sci. USA 92:8458.*
9. Simeonova, P. P., and M. I. Luster. 1996. Asbestos induction of nuclear transcription factors and IL-8 gene regulation. *Am. J. Respir. Cell Mol. Biol. 15:787.*
10. Pahl, H. L., and P. A. Baeuerle. 1994. Oxygen and the control of gene expression. *Bioessays 16:497.*
11. Meyer, M., H. L. Pahl, and P. A. Baeuerle. 1994. Regulation of the transcription factors NF-κB and AP-1 by redox changes. *Chem. Biol. Interact. 91:91.*
12. Hirano, T. 1992. Interleukin-6 and its relation to inflammation and disease. *Clin. Immunol. Immunopathol. 62:S60.*
13. Jones, K. P., S. P. Reynolds, S. J. Capper, S. Kalinka, J. H. Edwards, and B. H. Davies. 1991. Measurement of interleukin-6 in bronchoalveolar lavage fluid by radioimmunoassay: differences between patients with interstitial lung disease and control subjects. *Clin. Exp. Immunol. 83:30.*
14. Devlin, R. B., W. F. McDonnell, R. Mann, S. Becker, D. E. House, D. Schreinemachers, and H. Koren. 1991. Exposure of humans to ambient levels of ozone for 6.6 hours causes cellular and biochemical changes in the lung. *Am. J. Respir. Cell Mol. Biol. 4:72.*
15. Vanhee, D., P. Gosset, A. Boitelle, B. Wallaert, and A. B. Tonnel. 1995. Cytokines and cytokine network in silicosis and coal workers' pneumoconiosis. *Eur. Respir. J. 8:834.*
16. Cromwell, O., Q. Hamid, C. J. Corrigan, J. Barkans, Q. Meng, and P. D. Collins. 1992. Expression and generation of interleukin-8, IL-6 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells and enhancement by IL-1β and tumor necrosis factor α. *Immunology 77:330.*
17. Crestani, B., P. Cornillet, M. Dehoux, C. Rolland, M. Guenounou, and M. Aubier. 1994. Alveolar type II epithelial cells produce interleukin-6 in vitro and in vivo. *J. Clin. Invest. 94:731.*
18. Arnold, R., B. Humbert, H. Werchau, H. Gallati, and W. Konig. 1994. Interleukin-8, interleukin-6, and soluble tumour necrosis factor receptor type I release from a human pulmonary epithelial cell line (A549) exposed to respiratory syncytial virus. *Immunology 82:126.*
19. Devlin, R. B., K. P. McKinnon, T. Noah, S. Becker, and H. S. Koren. 1994. Ozone-induced release of cytokines and fibronectin by alveolar macrophages and airway epithelial cells. *Am. J. Physiol. 266:L612.*
20. Elias, J. A., V. Lentz, and P. Cummings. 1991. Transforming growth factor-β regulation of IL-6 production by unstimulated and IL-1 stimulated human fibroblasts. *J. Immunol. 146:3437.*
21. Kotloff, R. M., J. Little, and J. A. Elias. 1990. Human alveolar macrophage and blood monocyte interleukin-6 production. *Am. J. Respir. Cell Mol. Biol. 3497.*
22. Zhang, Y., M. Broser, and W. N. Rom. 1994. Activation of the interleukin 6 gene by *Mycobacterium tuberculosis* or lipopolysaccharide is mediated by nuclear factors NF-IL6 and NF-κB. *Proc. Natl. Acad. Sci. USA 91:2225.*
23. Dendorfer, U., P. Oettgen, and T. A. Libermann. 1994. Multiple regulatory elements in the interleukin-6 gene mediate induction by prostaglandins, cyclic AMP, and lipopolysaccharide. *Mol. Cell. Biol. 14:4443.*
24. Matsusaka, T., K. Fujikawa, Y. Nishio, N. Mukaida, K. Matsushima, T. Kishimoto, and S. Akira. 1993. Transcription factors NF-IL-6 and NF-κB synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proc. Natl. Acad. Sci. USA 90:10193.*
25. Kopp, E. B., and S. Ghosh. 1995. NF-κB and Rel proteins in innate immunity. *Adv. Immunol. 58:1.*
26. Lieber, M., B. Smith, A. Szakal, W. Nelson-Rees, and G. Todaro. 1976. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int. J. Cancer 17:62.*

27. Rosenthal, G. J., R. P. Stranahan, M. M. Fort, and M. I. Luster. 1989. The role of alveolar macrophage activation in the response to mineral fibers. In *Effects of Mineral Dusts on Cells*, NATO ASI Series Vol. H30. B. T. Mossman and R. O. Begin, eds. Springer-Verlag, Berlin, p. 329.
28. Wang, A. M., M. V. Doyle, and D. F. Mark. 1989. Quantitation of mRNA by the polymerase chain reaction. *Proc. Nat. Acad. Sci. USA 86:9717.*
29. Schreiber, E., P. Matthias, M. M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with "mini-extracts," prepared from a small number of cells. *Nucleic Acids Res. 17:6420.*
30. Pennypacker, K. R., L. Thai, J.-S. Hong, and M. K. McMillian. 1994. Prolonged expression of AP-1 transcription factors in the rat hippocampus after systematic kainate treatment. *J. Neurosci. 14:3998.*
31. Koren, H. S., R. B. Devlin, D. E. Graham, R. Mann, M. P. McGee, D. H. Horstman, W. J. Kozumbo, S. Becker, D. E. House, W. F. McDonnell, and P. A. Bromberg. 1989. Ozone-induced inflammation in the lower airways of human subjects. *Am. Rev. Respir. Dis. 139:407.*
32. Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto. 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J. 9:1897.*
33. Crouch, E. 1990. Pathobiology of pulmonary fibrosis. *Am. J. Physiol. 259: L159.*
34. Adler, K. B., B. M. Fischer, D. T. Wright, L. A. Cohn, and S. Becker. 1994. Interactions between respiratory epithelial cells and cytokines: relationship to lung inflammation. *Ann. NY Acad. Sci. 725:128.*
35. Rosenthal, G. J., D. R. Germolec, M. E. Blazka, E. Corsini, P. Simeonova, P. Pollock, L.-Y. Kong, J. Kwon, and M. I. Luster. 1994. Asbestos stimulated IL-8 production from human lung epithelial cells. *J. Immunol. 153:3237.*
36. Ghio, A. J., J. Zhang, and C. A. Piantadosi. 1992. Generation of hydroxyl radical by crocidolite asbestos is proportional to surface [Fe^{3+}]. *Arch. Biochem. Biophys. 298:646.*
37. Trautwein, C., C. Caelles, P. van der Geer, T. Hunter, M. Karin, and M. Chojkier. 1993. Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. *Nature 364:544.*
38. Yan, S. F., I. Tritto, D. Pinsky, H. Liao, J. Huang, G. Fuller, J. Brett, L. May, and D. Stern. Induction of interleukin 6 (IL-6) by hypoxia in vascular cells. *J. Biol. Chem. 270:11463.*
39. DiCosmo, B. F., G. P. Geba, D. Picarella, J. A. Elias, J. A. Rankin, B. R. Stripp, J. A. Whitsett, and R. A. Flavell. 1994. Airway epithelial cell expression of interleukin-6 in transgenic mice: uncoupling of airway inflammation and bronchial hyperactivity. *J. Clin. Invest. 94:2028.*
40. Fries, K. M., M. E. Felch, and R. P. Phipps. 1994. Interleukin-6 is an autocrine growth factor for murine lung fibroblast subsets. *Am. J. Respir. Cell. Biol. 11: 552.*
41. Bonfield, T. L., J. R. Panuska, M. W. Konstan, K. A. Hilliard, H. Ghnaim, and M. Berger. 1995. Inflammatory cytokines in cystic fibrosis lungs. *Am. J. Respir. Crit. Care Med. 152:2111.*