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# Asbestos Causes Translocation of p65 Protein and Increases NF-κB DNA Binding Activity in Rat Lung Epithelial and Pleural Mesothelial Cells

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**The mechanisms of cell signaling and altered gene expression by asbestos, a potent inflammatory, fibrogenic, and carcinogenic agent, are unclear. Activation of the transcription factor, nuclear factor (NF)-κB, is critical in up-regulating the expression of many genes linked to inflammation and proliferation. Inhalation models of crocidolite- and chrysotile-induced inflammation and asbestosis were used to study the localization of p65, a protein subunit of the NF-κB transcription factor, in sham control rats and those exposed to asbestos. In addition, we investigated, using electrophoretic mobility shift analysis, whether *in vitro* exposure of rat lung epithelial cells and rat pleural mesothelial cells to asbestos increased binding of nuclear proteins, including p65, to the NF-κB DNA response element. Furthermore, translocation of p65 into the nucleus was determined by confocal microscopy. In comparison with sham animals, striking increases in p65 immunofluorescence were observed in airway epithelial cells of rats at 5 days after inhalation of asbestos. These increases were diminished by 20 days, the time period necessary for development of fibrotic lesions. In contrast, although inter-animal variability was observed, immunoreactivity for p65 was more dramatic in the interstitial compartment of asbestos-exposed rat lungs at both 5 and 20 days. Changes in p65 expression in pleural mesothelial cells exposed to asbestos in inhalation experiments were unremarkable. Exposure to asbestos also caused significant increases in nuclear protein complexes that bind the NF-κB consensus DNA sequence in both rat lung epithelial and rat pleural mesothelial cells. Using confocal microscopy, we observed partial nuclear translocation of p65 in rat pleural mesothelial cells exposed to asbestos. This partial response contrasted with the effects of lipopolysaccharide, which caused rapid and complete translocation of p65 from cytoplasm to nucleus. Our studies are the first to show the presence of the NF-κB system in lung tissue and evidence of activation *in vitro* and *in vivo* after exposure to a potent inflammatory, fibrinogenic, and carcinogenic environmental agent. (*Am J Pathol 1997, 151:389–401*)**

Asbestos fibers are a family of hydrated silicate fibers associated with the development of lung cancers, mesothelioma, and asbestosis.[1–3] The molecular events that precede development of lung disease and are triggered in pulmonary target cells by asbestos are unclear. Work to date suggests that asbestos may act through a number of mechanisms that include generation of active oxygen species (AOS; reviewed in Ref. 3) and inflammatory cytokines.[4–6] For instance, in human mesothelial cells and in lungs of rodents exposed by inhalation, asbestos causes an oxidant stress response that is characterized by increases in gene expression of the antioxidant proteins manganese-containing superoxide dismutase[7] and heme oxygenase.[8] Rodent alveolar and peritoneal macrophages generate increased amounts of oxidants after phagocytosis of asbestos fibers,[9,10] and fibers also catalyze formation of the hydroxyl radical ($OH^{\cdot}$) from hydrogen peroxide ($H_2O_2$).[11] Furthermore, exposure of rat pleural mesothelial (RPM) cells to asbestos causes a depletion of cellular glutathione levels, indicating that asbestos also may alter the redox state of the cells.[12] Stimulation of an oxidant burst in phagocytic cells contacting asbestos fibers or inhalation of asbestos also causes release of a spectrum of cytokines including tumor necrosis factor (TNF)[5,6] and interleukin (IL)-1.[4,13] These inflammatory cytokines and AOS may act in concert to cause alterations in cell signaling pathways and gene expression that are critical to the development of pulmonary fibrosis and tumorigenesis.[14]

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AOS and inflammatory cytokines both cause activation of the transcription factor nuclear factor (NF)-κB in a number of cell types.[15–17] Recently, we have demonstrated that asbestos causes transcriptional activation of NF-κB-dependent genes in transient transfection assays using hamster tracheal epithelial cells.[18] Therefore, activation of NF-κB may be integral in the development of asbestos-associated lung diseases, as this transcription factor modulates expression of a number of genes important in inflammation,[19–21] proliferation,[18,22,23] and apoptosis.[24–26] Activation of NF-κB has been demonstrated in a number of *in vitro* models that involve oxidant stress by radiation[27,28] and chemical generating systems of oxidants.[15–17] The process of NF-κB activation involves cytoplasmic phosphorylation of inhibitory protein complexes (IκBα, etc), which then are ubiquitinated and targeted for rapid degradation through the proteasome pathway.[29–31] Active transcription factor complexes, which include p65 protein subunits, then translocate to the nucleus where they up-regulate expression of a variety of genes with diverse functions, including nitric oxide synthase,[32] c-*myc*,[33] the cytokines IL-6 and IL-8, and monocyte chemoattractant protein-1.[21,22,34] Activation of NF-κB-dependent genes may contribute to lung disease associated with exposure to asbestos. For example, we have recently demonstrated increased mRNA levels of c-*myc*, a proto-oncogene with NF-κB regulatory sequences in its promoter region in tracheal epithelial cells after exposure to asbestos.[18]

The goals of the studies here, using well defined rodent models of inflammation and asbestosis,[35–38] were to characterize p65 (RelA) protein localization, the subunit responsible for the transcriptional activation potential of NF-κB[39] in lung. Moreover, we examined binding of nuclear proteins to the NF-κB consensus sequence and nuclear translocation of p65 in RPM cells and rat lung epithelial (RLE) cells exposed to asbestos or nonpathogenic particles (eg, glass or riebeckite) *in vitro*. In parallel studies, we demonstrate prominent localization of p65 protein in bronchial epithelium and alveolar epithelial cells *in vivo* and striking increases in reactivity after brief inhalation of either crocidolite or chrysotile asbestos. Both RPM and RLE cells *in vitro* exhibited dose-dependent increases in p65/p50 nuclear protein complexes in response to crocidolite asbestos fibers but not nonfibrous particulates. Asbestos-associated elevations in NF-κB binding activity were less in magnitude in comparison with responses obtained with lipopolysaccharide (LPS) and may reflect partial as opposed to complete nuclear translocation of p65 as observed by confocal microscopy. Our findings *in vivo* and *in vitro* support a correlative relationship between exposure to an important environmental toxicant, NF-κB activation, and the development of lung disease.

## Materials and Methods

### Inhalation Models of Lung Injury

Previously characterized rodent inhalation models of asbestos-induced inflammation and fibrosis[35–38] were used here to relate patterns of p65 immunoreactivity to the development of disease. The chemical and physical characteristics of National Institute of Environmental Health Sciences reference samples of chrysotile and crocidolite asbestos used here (Thermal Insulation Manufacturers Association Fiber Repository, Littleton, CO) have been reported previously.[40] Male Fischer 344 rats weighing 200 to 250 g were exposed to room air and to either chrysotile or crocidolite asbestos. Rats were exposed for 6 hours/day, 5 days/week for a total of 5 or 20 days. Asbestos fibers were aerosolized using a modified Timbrell generator.[41] Gravimetric concentrations in chambers were measured and recorded on a daily basis. The respirability of the two fiber types was determined by analysis of aerodynamic fiber distributions using a Sierra cascade impactor (Sierra Instruments, Carmel, CA). The median average fiber diameter of chrysotile and crocidolite was 0.34 and 0.40 μm with average daily gravimetric chamber concentrations of 8.25 ± 0.23 and 7.20 ± 0.27 mg/m$^3$ of air. Sham control rats were transferred to chambers with clean air and treated identically.

### Cell Cultures and in Vitro Studies

A spontaneously immortalized, differentiated line of rat type II epithelial cells (RLE-6TN)[42] was propagated in DMEM/F12 medium containing penicillin and streptomycin (GIBCO-BRL, Grand Island, NY) and 10% newborn bovine serum (GIBCO-BRL). RPM cells were isolated from Fischer 344 rats[43] and propagated in DMEM/F12 medium containing 50 U/ml penicillin, 50 μg/ml streptomycin, 10% fetal bovine serum, 100 ng/ml hydrocortisone, 2.5 μg/ml insulin, 2.5 μg/ml transferrin, and 2.5 ng/ml selenium (Sigma Chemical Co., St. Louis, MO). Cells were grown to confluency, and 24 hours before addition of test agents, the growth medium was replaced with medium containing 2% serum.

For *in vitro* studies, we used National Institute of Environmental Health Sciences processed crocidolite asbestos, the most potent asbestos type in the causation of human mesothelioma[1–3] and asbestosis in our inhalation models.[35,36] In comparative studies to determine the specificity of fiber-induced effects, the nonfibrous particulate analogue of crocidolite, riebeckite, was prepared according to procedures described previously.[44] In addition, glass beads (1 to 4 μm in size) were obtained from Particle Information Services, Kingston, WA. Particulates were sterilized at 225°F for 12 to 15 hours and suspended in Hanks' balanced salt solution (GIBCO-BRL). Asbestos fibers were triturated eight times through a 22-gauge needle to obtain a homogeneous suspension, as described elsewhere.[44] Minerals were added directly to the medium at noncytolytic concentrations ranging from 1.25 to 20 μg/cm$^2$ area of dish.[44] LPS (*Escherichia coli* 026:B6, Sigma) was used as a positive control for NF-κB activation.

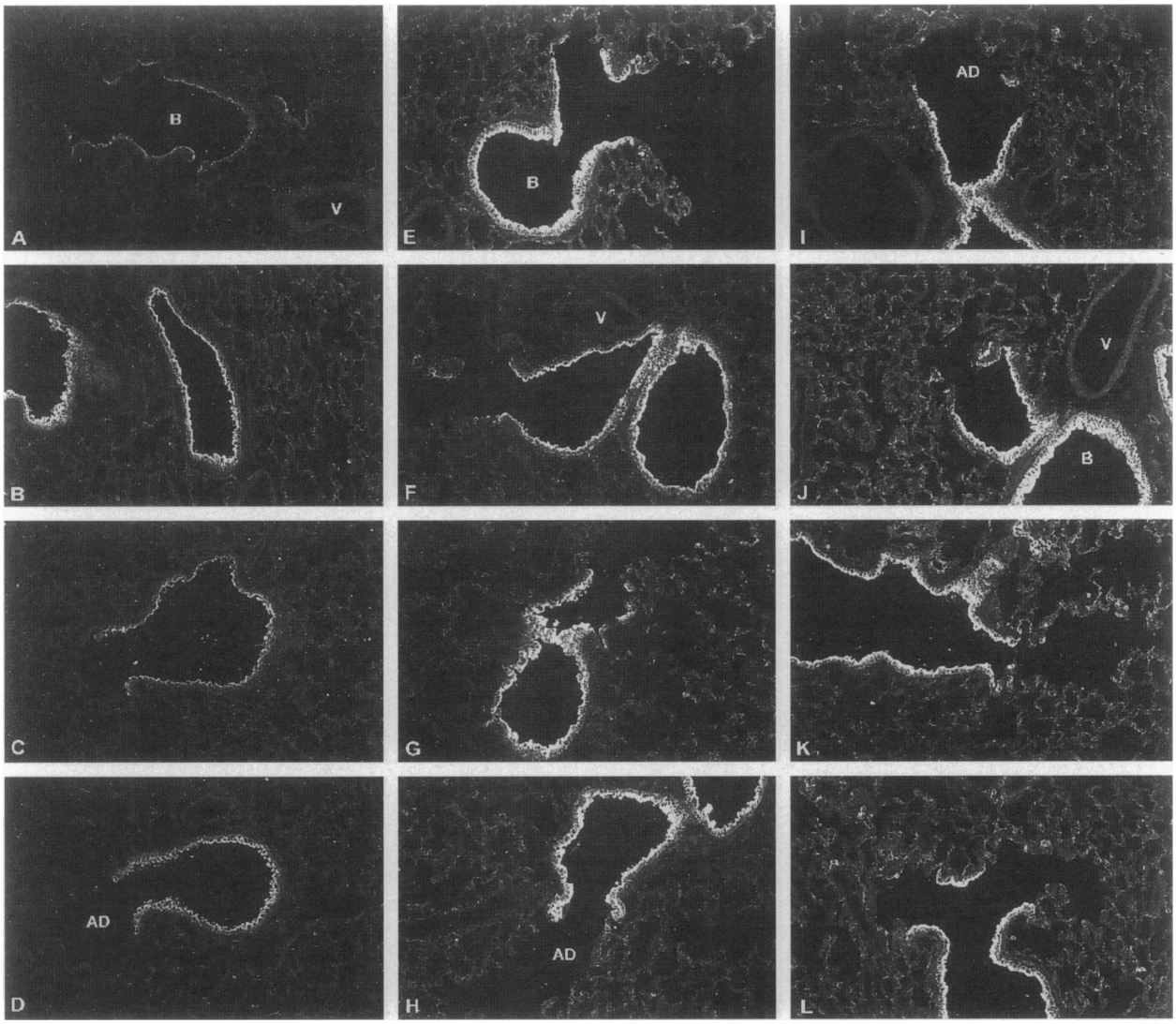


**Figure 1.** Confocal microscopy showing immunoreactivity of p65 protein in bronchiolar epithelial cells of sham (**A** to **D**), chrysotile-exposed (**E** to **H**), and crocidolite-exposed (**I** to **L**) rat lungs at 5 days. Note the striking epithelial cell fluorescence in discrete bronchioles (B) and alveolar duct epithelium (AD) and lack of fluorescence in vessels (V). n = 4/group; magnification, ×200.

## *Immunofluorescence Technique and Confocal Microscopy*

To determine patterns of p65 (RelA) immunofluorescence in lung and their relationship to the development of pulmonary fibrosis, Fischer 344 rats were exposed to chrysotile or crocidolite asbestos for 5 or 20 days.[35–38] At the former time point, increased proliferation in bronchial epithelial cells, interstitial cells, and mesothelial cells is observed in both models of asbestosis. In asbestos-exposed rat lungs, focal lesions of inflammation also are observed in the alveolar duct region, which are accompanied by increased neutrophil infiltration in bronchoalveolar lavage fluids.[35–37] At 20 days, focal areas of pulmonary fibrosis occur that are more striking in severity and extent in crocidolite-exposed rats.[35–38] At each time point, rats (n = 3 to 4/group) were sacrificed, and lungs were perfused through the vasculature with heparinized phosphate-buffered saline (PBS). Lungs then were removed, fixed in 70% ethanol, and paraffin embedded. For immunofluorescence, 5-μm-thick lung sections were deparaffinized with xylene and rehydrated in a graded series of ethanol according to standard procedures. All subsequent incubations were done at room temperature. Permeabilization was performed with PBS containing 0.1% Triton X-100 for 30 minutes, followed by blocking of sections in 1% bovine serum albumin (BSA) containing PBS (twice for 30 minutes each). Slides were incubated with a polyclonal rabbit anti-p65 antibody (SC-372, Santa Cruz Biotechnology, Santa Cruz, CA) at 2.5 μg/ml for 1 hour. This antibody recognizes both active and inactive NF-κB complexes. Hence, p65 immunostaining is expected both in cytoplasmic and nuclear compartments. After three 20-minute incubations in PBS/BSA, lung sec-

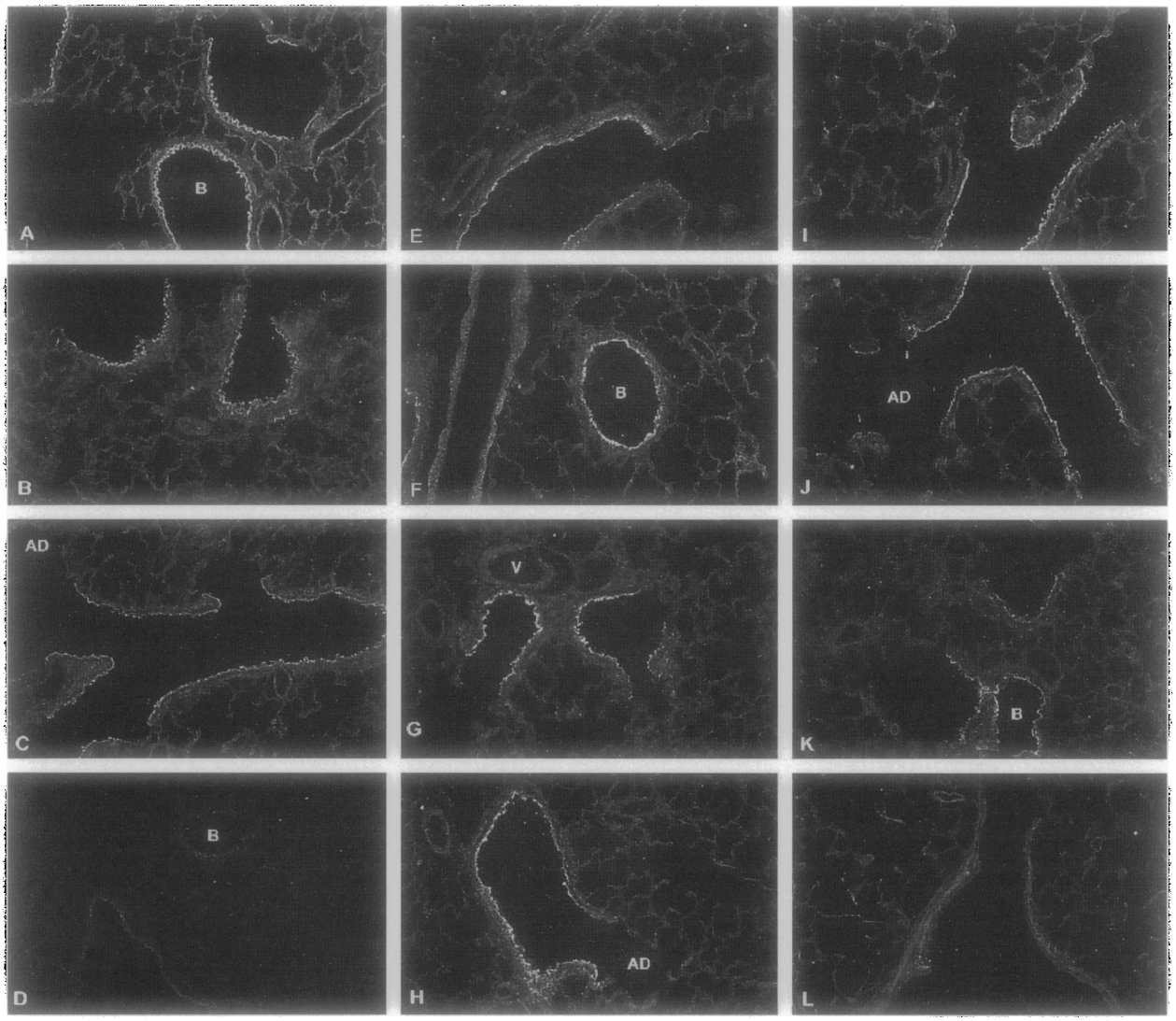


**Figure 2.** Confocal microscopy showing immunoreactivity of p65 protein in bronchiolar epithelial cells of sham (**A** to **C**), chrysotile-exposed (**E** to **H**), and crocidolite-exposed (**I** to **K**) rat lungs at 20 days. B, bronchiole; AD, alveolar duct; V, vessel. **D**: Secondary antibody control. **L**: Peptide neutralization control. Magnification, ×200.

tions were incubated with a Cy5-conjugated secondary antibody (20 μg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour, washed in PBS, and mounted in Vectashield (Vector Laboratories, Burlingame, CA). To determine the specificity of p65 immunofluorescence in lung tissue or cells *in vitro* (see below), p65 antibody was incubated with a 10-fold excess of antigen overnight, according to the instructions of the manufacturer (Santa Cruz Biotechnology). This procedure and sections treated only with secondary antibody resulted in a diminution or lack of visible fluorescence (see Figures 2, D and L, and 4L). Sections were then examined on a Bio-Rad confocal microscope (Hercules, CA). To scan p65 immunofluorescence, identical instrument settings were used for all samples. The instrument gain was at 1000 V, the iris at 3.0 mm, and the laser power at 30%. Subsequently, images were averaged using five Kalman collections.

## *Confocal Microscopy to Determine Nuclear Translocation of p65*

Activation of the transcription factor NF-κB involves cytoplasmic dissociation of the inhibitor protein IκB and translocation of the active NF-κB complex into the nucleus. To determine whether nuclear translocation of p65 occurred after exposure of cells to asbestos *in vitro*, immunofluorescence studies were performed in RPM cells exposed to crocidolite asbestos or glass beads for 8 hours or LPS for 2 hours. Sham control cells were left untreated and manipulated identically. Cells were harvested and rinsed in PBS followed by fixation in methanol for 20 minutes at room temperature. Sections then were permeabilized with 0.1% Triton X-100 and 4% fetal bovine serum in PBS for 20 minutes and subsequently blocked in PBS/BSA for 15 minutes. Coverslips were then incubated with anti-p65

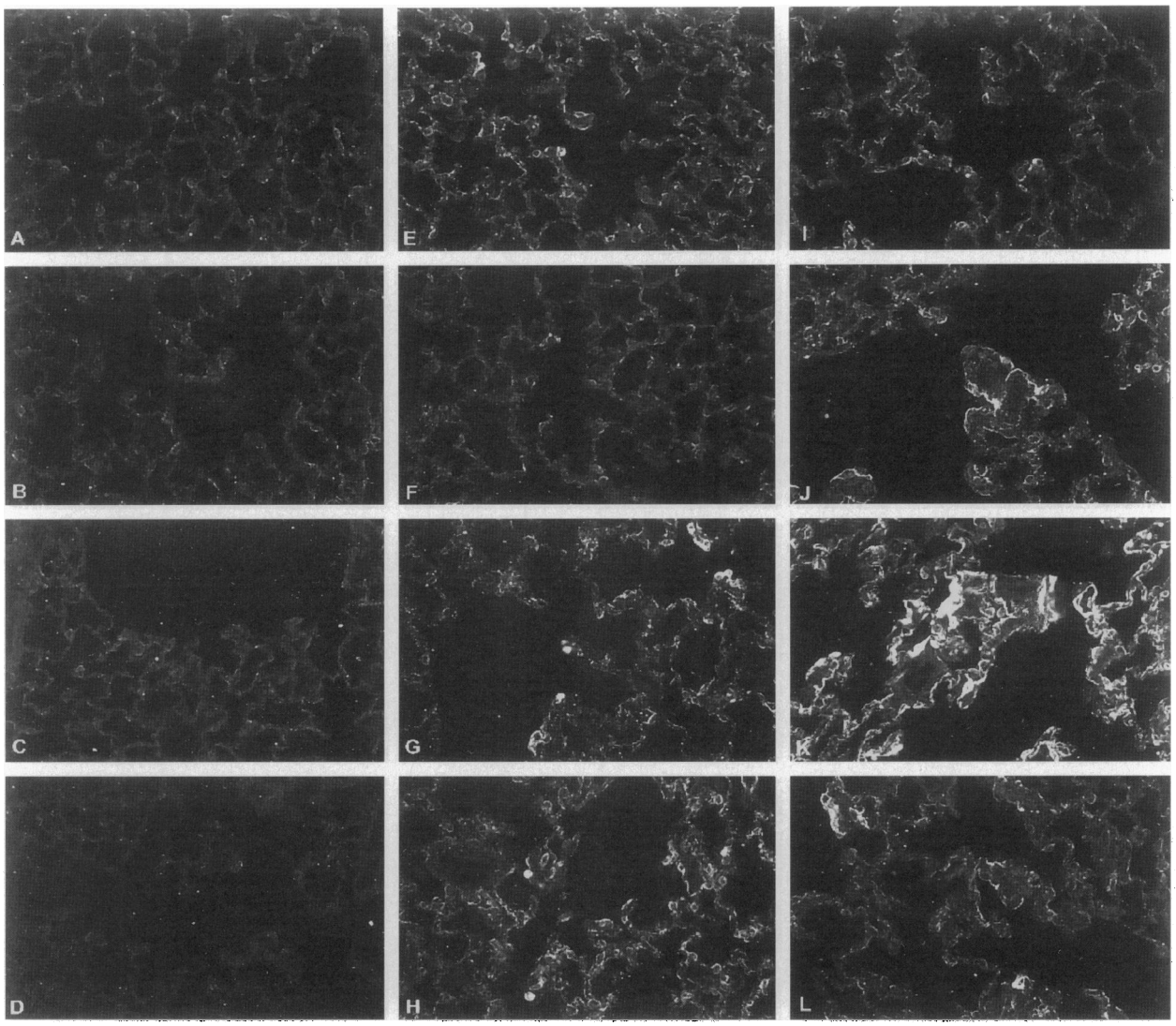


**Figure 3.** Confocal microscopy showing immunoreactivity of p65 protein in interstitial compartments of the lung in sham (**A** to **D**), chrysotile-exposed (**E** to **H**), and crocidolite-exposed (**I** to **L**) rats at 5 days. Note increases in immunofluorescence in asbestos-exposed lungs. Magnification, ×400.

antibody as described above. Sections were then washed with PBS (three times for 5 minutes each) and incubated with a rhodamine/lissamine-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) at 20 μg/ml for 1 hour in the presence of 500 nmol/L YOYO-1 iodide (YOYO, Molecular Probes, Eugene, OR) to stain nuclei. Coverslips were washed in PBS and mounted in Vectashield mounting medium (Vector). Immunofluorescence of p65 or YOYO was examined using a confocal microscope (Bio-Rad). From 100 to 300 random cells on two coverslips per treatment (n = 2 dishes/group) were examined for p65 fluorescence, and results were expressed as a percentage of cells staining positive/total cells examined. Merged images of p65 and YOYO were analyzed to confirm nuclear localization of p65.

## *Electrophoretic Mobility Shift Analyses (EMSAs)*

After various time periods of exposure to particulates or LPS, nuclear extracts were isolated as described by Staal et al.[45] Cells were lysed in hypotonic buffer and incubated on ice for 15 minutes, and 0.6% Nonidet P-40 was added and lysates were vortexed for 15 seconds. Nuclei were pelleted by centrifugation at 14,000 rpm for 30 seconds at 4°C. Nuclear proteins were then extracted and stored at −80°C.[45] EMSAs were performed using procedures as described previously.[18] Briefly, 4 μg of nuclear protein extract was incubated for 20 minutes at room temperature in DNA-binding buffer containing 40 mmol/L Hepes buffer, 4% Ficoll 400, 200 ng of poly(dI)·(dC) per μl, 1 mmol/L $MgCl_2$, 0.1 mmol/L dithiothreitol, and 0.175 pmol of a $^{32}$P-end-labeled double-stranded oligonucleotide containing a consensus NF-κB site (Promega, Madison, WI). Samples were loaded onto a 5% polyacrylamide gel and electrophoresed in 0.25X Tris borate-EDTA buffer for 2 hours at 120 V. Gels were dried and visualized by exposure to Kodak X-Omat film. Subsequently, radioactivity in retarded binding complexes was quantitated on a phosphoimager (Bio-Rad) or by densitometric analysis of autoradiographs (Technolo-

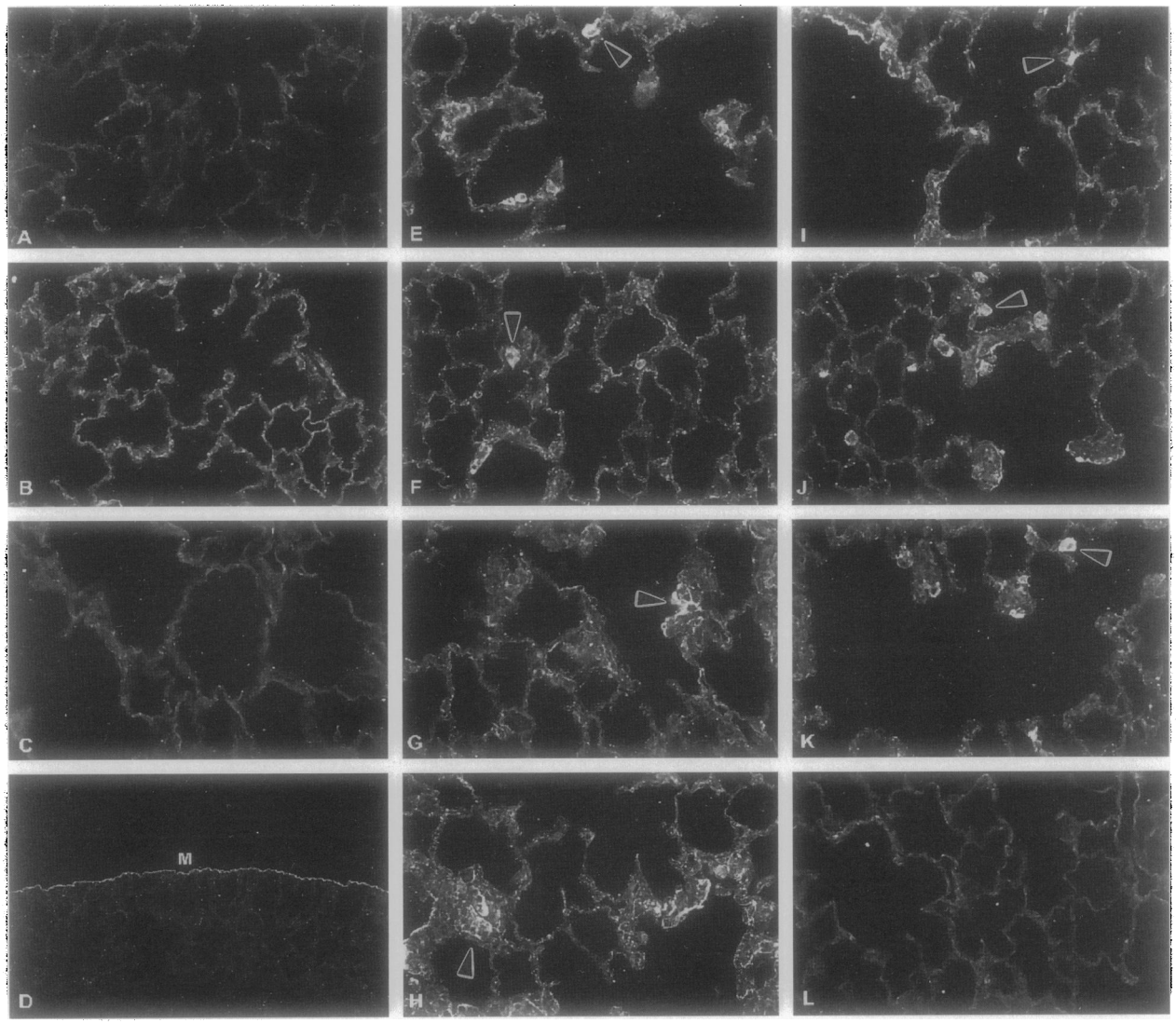


**Figure 4.** Confocal microscopy illustrating immunoreactivity of p65 protein in the interstitial compartments in sham (**A** to **C**), chrysotile-exposed (**E** to **H**), and crocidolite-exposed (**I** to **K**) lungs at 20 days. **D:** Pleural mesothelial cells (M) at 5 days. **L:** Peptide neutralization control section from a chrysotile-exposed animal. Magnification, ×400.

gy Resources, Nashville, TN). The specificity of DNA-binding patterns was characterized using antibodies recognizing p65 and p50 proteins SC-109 (or SC-372) and SC-114, respectively (Santa Cruz Biotechnology). The p65 antibody supershifts the upper binding complex (see Figure 5A) whereas the p50 antibody affects both complexes, demonstrating the presence of these proteins in two binding complexes. An excess of unlabeled NF-κB oligonucleotide competed away all binding complexes, whereas excess of AP-1 oligonucleotide did not modify gel shift complexes, demonstrating the specificity of NF-κB gel shift patterns.[18]

### *Statistical Analyses*

Data were analyzed by analysis of variance corrected for multiple comparisons according to the Student-Newman-Keuls procedures. Trend analyses also were performed to examine linear trends over time and dose.

## *Results*

### *Immunofluorescence Studies in Lung*

Rodent inhalation models were used to determine which cell types in lung exhibited localization of p65 (RelA) protein and whether inhalation of asbestos caused alterations in the immunoreactivity or distribution of p65 protein. Confocal microscopy showed that localization of p65 was most striking in bronchial epithelial cells of both sham and asbestos-exposed rats at both 5 (Figure 1) and 20 days (Figure 2). Inhalation of asbestos for 5 days resulted in more pronounced increases in p65 immunofluorescence in bronchial epithelial cells (Figure 1, E–L). At 20 days, immunoreactivity of p65 was comparable in bronchiolar epithelium of sham animals (Figure 2, A–C) and those exposed to either chrysotile (Figure 2, E–H) or crocidolite (Figure 2, I–K). Immunofluorescence was less dramatic in interstitial compartments of the lung in sham

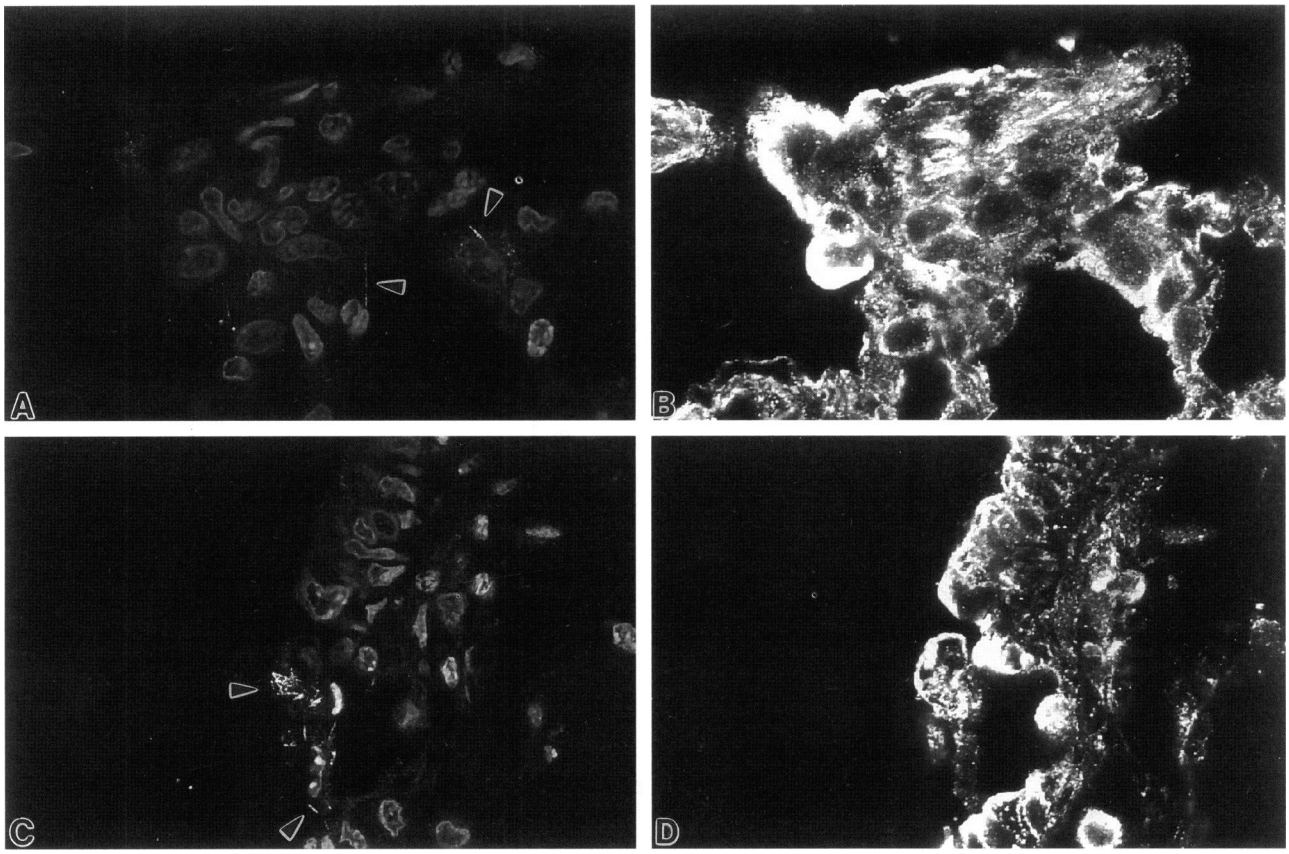


**Figure 5.** Airway bifurcation (**A** and **B**) and alveolar duct region (**C** and **D**) of an animal exposed to crocidolite asbestos for 20 days. **B** and **D** show the widespread occurrence of p65 immunofluorescence. The same regions were also scanned using the reflecting mode (**A** and **C**) to visualize asbestos fibers (indicated by **arrowheads**). Note that p65 occurs in regions where no asbestos can be detected. Magnification, ×1500.

controls (Figures 3 and 4), but focal increases in p65 localization were apparent in rats exposed to both fiber types at 5 and 20 days. At 20 days, lung sections from both chrysotile-exposed (Figure 4, E–H) and crocidolite-exposed (Figure 4, I–K) rats showed marked fluorescence of p65 in discrete cells lining both the alveolar ducts and within the interstitium (arrowheads). The patterns of localization of these cells and morphology suggested that they were alveolar macrophages or type II alveolar epithelial cells. Figure 5 further illustrates a higher magnification of p65 immunofluorescence in airway bifurcations (B) and alveolar ducts (D) after 20 days of inhalation of crocidolite asbestos. Using the reflection mode on the confocal microscope to visualize reflecting substances such as asbestos, asbestos fibers were apparent in airway bifurcations (Figure 5A) and alveolar ducts (Figure 5C). These findings illustrate the generalized patterns of p65 immunofluorescence in the absence of asbestos fibers. Immunoreactivity of p65 was apparent in the mesothelium of sham or asbestos-exposed animals at both time points (Figure 4D). However, no clear increases in immunofluorescence were observed at any time point after asbestos exposure.

## *EMSA Studies* in Vitro

We next determined by EMSA whether crocidolite asbestos caused increases in nuclear proteins that bind to the NF-κB consensus DNA sequence in RLE and RPM cells *in vitro*. In both cell types, after exposure to crocidolite asbestos (Figures 6 to 8), increases in DNA binding activity to the NF-κB sequence were observed. Nuclear extracts from asbestos-exposed RPM and RLE cells showed increased binding of multiple complexes to the NF-κB consensus sequence. Using antibodies recognizing p65 and p50 proteins, we showed that the predominant upper binding complex was composed of both p65 and p50 protein, whereas the lower complex contained p50 (Figure 6A).[18] Unlabeled NF-κB oligonucleotide competed away all of the binding, whereas unlabeled AP-1 oligonucleotide did not modify binding patterns (not shown).

To demonstrate the specificity of asbestos-mediated increases in protein binding to the NF-κB DNA sequence, we exposed RPM cells to nonpathogenic particulates for 8 hours, the time point of maximal increases by asbestos as observed above (Figure 6B). As shown in Figure 7, exposure of RPM cells to the nonfibrous, chemically similar analogue of crocidolite asbestos, riebeckite, at 5 $\mu g/cm^2$, caused insignificant increases in nuclear protein complexes binding the NF-κB sequence. Exposure of RPM cells to 10 $\mu g/cm^2$ of glass beads also did not alter nuclear protein complexes. In contrast, a 2-hour exposure to the inflammatory mediator LPS caused striking increases in binding activity to the NF-κB sequence.

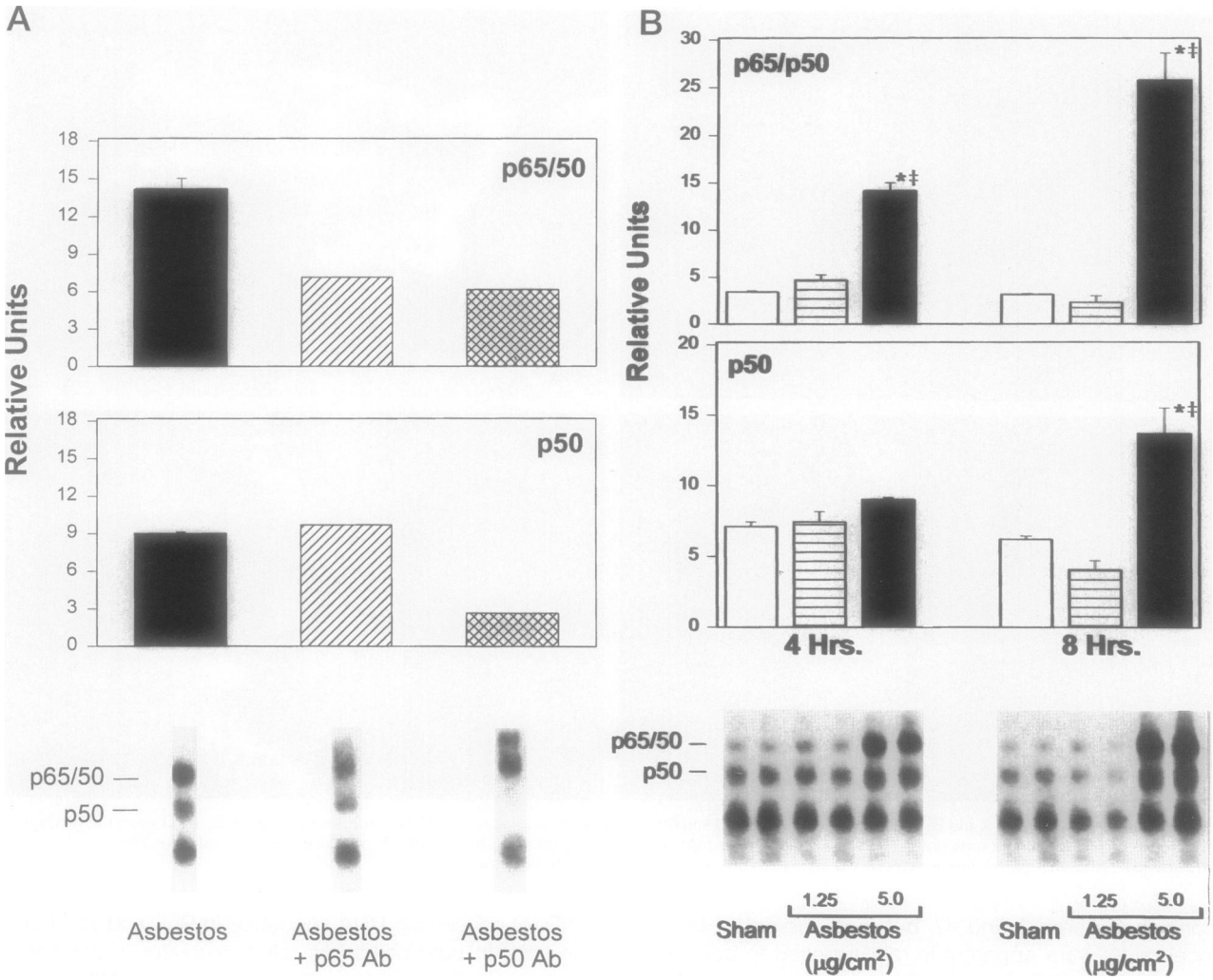


**Figure 6. A:** Specificity of nuclear proteins that bind to the NF-κB consensus sequence as shown by EMSA in RPM cells exposed to asbestos. Nuclear extracts from asbestos-exposed cells were incubated for 20 minutes at room temperature at which time 2 μl of antibodies recognizing p65 or p50 were added for an additional 30 minutes. **Left lane**, DNA binding pattern from nuclear extract from asbestos-exposed RPM cells; **middle lane**, DNA binding in the presence of p65 antibody; **right lane**, DNA binding in the presence of p50 antibody. **B:** EMSA showing dosage-dependent increases of binding of nuclear proteins to the NF-κB consensus DNA sequence in RPM cells exposed to crocidolite asbestos (1.25 or 5 μg/cm²) for 4 or 8 hours. *Significantly increased ($P < 0.05$ compared with sham controls at the same time point. ‡Significantly increased ($P < 0.05$ compared with the group exposed to 1.25 μg/cm² asbestos at the same time point. Error bars show mean ± SEM of duplicate samples as determined by phosphoimage analysis.

We next determined, using RLE cells, whether asbestos- and LPS-induced binding to the NF-κB sequence were comparable to patterns observed in RPM cells. Similar to results in RPM cells, RLE cells exhibited multiple distinct bands of nuclear NF-κB binding activity. Dose-related increases in both p65/p50 complexes, observed in this cell type after 6 hours of exposure to asbestos, are depicted in Figure 8A. Trend analysis confirmed a significant linear trend in binding of the p65/p50 complex with increasing doses of asbestos ($P < 0.05$). As was seen in RPM cells, LPS (50 ng/ml) caused more dramatic increases in nuclear proteins that bound to the NF-κB sequence (Figure 8B). This time course study in RLE cells employing 10 μg/cm² crocidolite asbestos also revealed that increases in nuclear proteins recognizing the NF-κB consensus sequence became more striking over time (Figure 8B).

p65 (RelA) is a major component of the NF-κB family that is associated with transcriptional activation of target genes.[39] As this protein complex increased most dramatically in EMSA studies evaluating asbestos-treated RPM and RLE cells, subsequent experiments focused on nuclear translocation of this protein in RPM cells in response to asbestos, LPS, and glass beads (negative control). Figure 9 shows p65 immunofluorescence in RPM cells exposed to 5 μg/cm² crocidolite asbestos for 8 hours or 100 ng/ml LPS for 2 hours, the time points of maximal increases of p65/p50 complexes observed by EMSA. In control RPM cells, p65 occurred almost exclusively in the cytoplasm, and nuclear staining was virtually absent. However, the majority of RPM cells exposed to LPS for 2 hours exhibited complete nuclear translocation of p65 and an absence of cytoplasmic fluorescence. In RPM cells exposed to asbestos, a large percentage of cells

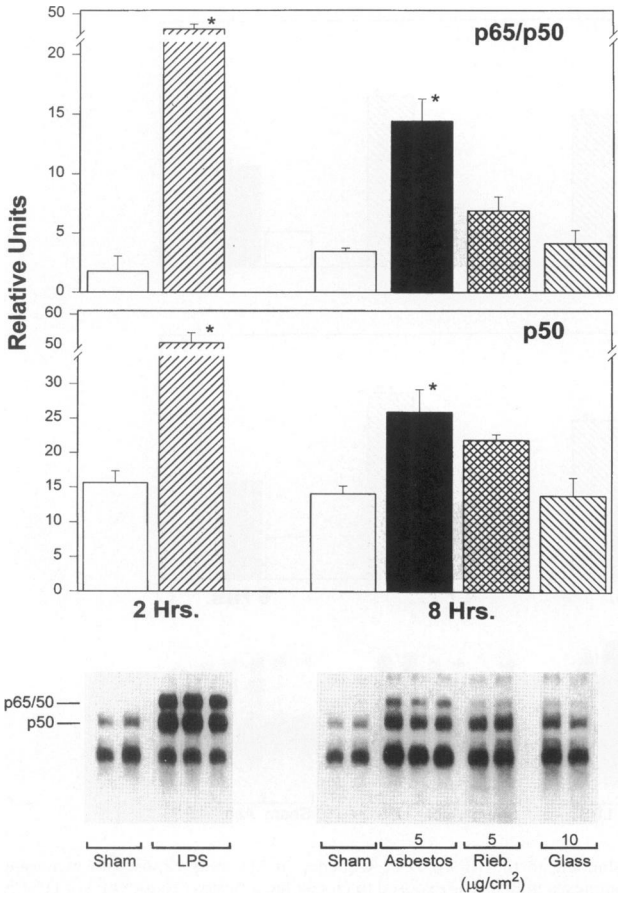


**Figure 7.** EMSA in RPM cells showing stimulatory effects of LPS (100 ng/ml for 2 hours) and crocidolite asbestos (8-hour exposure) on binding of nuclear protein complexes to the NF-κB consensus DNA sequence (*$P <$ 0.05 compared with sham controls at the same time point). Note the lack of significant increases in DNA binding with nuclear extracts obtained from RPM cells exposed to the particles, riebeckite, or glass for 8 hours. Error bars show mean ± SEM of duplicate samples as determined by phosphoimage analysis.

exhibited p65 fluorescence in both the cytoplasm and nucleus (Figure 9; Table 1). Table 1 summarizes the data from quantitative experiments in which RPM cells were examined for nuclear p65 immunofluorescence after exposure to test agents. Whereas approximately 1 to 3% of total cells showed nuclear localization of p65 in cultures after addition of medium alone (sham) or glass beads, approximately 50% of total cells were labeled after exposure to crocidolite asbestos. In contrast, exposure to LPS resulted in 90% of cells showing immunofluorescence of p65 in the nucleus. To further illustrate this phenomenon, we performed dual-labeling confocal microscopy to confirm nuclear p65 using the DNA stain YOYO. Nuclear p65 immunofluorescence merged with YOYO results in an orange color. Results in Figure 9 confirm the absence of nuclear p65 in untreated controls and complete translocation of p65 into the nucleus of cells exposed to LPS. However, merged images of p65 and YOYO demonstrate that p65 immunofluorescence occurs in both the cytoplasm and nucleus of RPM cells exposed to crocidolite asbestos, indicating partial translocation of the p65 subunit of NF-κB.

## *Discussion*

In studies here, we used rodent inhalation models of asbestosis and differentiated cultures of pleural mesothelial and alveolar type II epithelial cells to demonstrate activation of the NF-κB system in response to asbestos fibers. These *in vivo* and *in vitro* approaches allowed us to study the patterns of activation of this transcription factor in relationship to the development of lung disease and the specificity of asbestos-induced responses in comparison with the inflammatory agent LPS or the noninflammatory inert particles riebeckite and glass beads.

Results presented here show that exposure to crocidolite asbestos *in vitro* increases nuclear protein complexes binding the NF-κB sequence in RLE and RPM cells, progenitor cells of the asbestos-induced diseases, lung cancer, and mesothelioma. In contrast to asbestos, the inflammatory mediator LPS caused more dramatic increases in nuclear complexes binding to the NF-κB sequence. This may be due to the soluble nature and receptor affinity of LPS, enabling its rapid association with cells, as opposed to asbestos fibers that make focal contact with and precipitate onto cells *in vitro* over several hours.[44] Our results also demonstrate that the nonpathogenic particles, glass and riebeckite, do not enhance nuclear NF-κB complexes. As riebeckite is a chemically similar analogue of crocidolite, differing only in its geometry as it is defined as a particle (<3:1 length to diameter ratio) as opposed to a fiber (>3:1 length to diameter ratio), results indicate that the fibrous nature of asbestos is essential for activation of NF-κB. AOS may be mediators of the NF-κB response to asbestos, based on several observations. First, nonfibrous particles are phagocytized effectively, whereas incomplete phagocytosis of long, thin crocidolite asbestos fibers generates elevated amounts of AOS,[9] documented activators of the NF-κB pathway in other cell types.[15–17] Addition of *N*-acetyl-L-cysteine to tracheal epithelial cells also ameliorates asbestos-induced activation of NF-κB, an observation suggesting that oxidant generation triggered by asbestos fibers may be critical in activation of the NF-κB system.[18]

We show here for the first time the localization of p65 protein, an active component important in the transactivation of NF-κB target genes[39] in lung and apparent increases in immunoreactivity as early as 5 days after exposure to either crocidolite or chrysotile asbestos. At high airborne concentrations used here, both types of asbestos cause rapid inflammatory and proliferative changes in lung and the development of focal asbestosis at 20 days.[35–38] In comparison with other cell types in normal rat lungs, the extent of p65 immunofluorescence was most remarkable in bronchiolar epithelial cells, including those occurring at the alveolar duct region where asbestos fibers are initially deposited after inhalation.[46] Higher basal levels of p65 protein in airway epithelial cells may be important in responses to a number of inhaled oxidative stresses. Thus, the early increases in p65 immunofluorescence that occur in these areas in response to asbestos are consistent with other findings showing increased expression of antioxidant enzymes[7,47] and cytokines[6,48] and reversible increases in

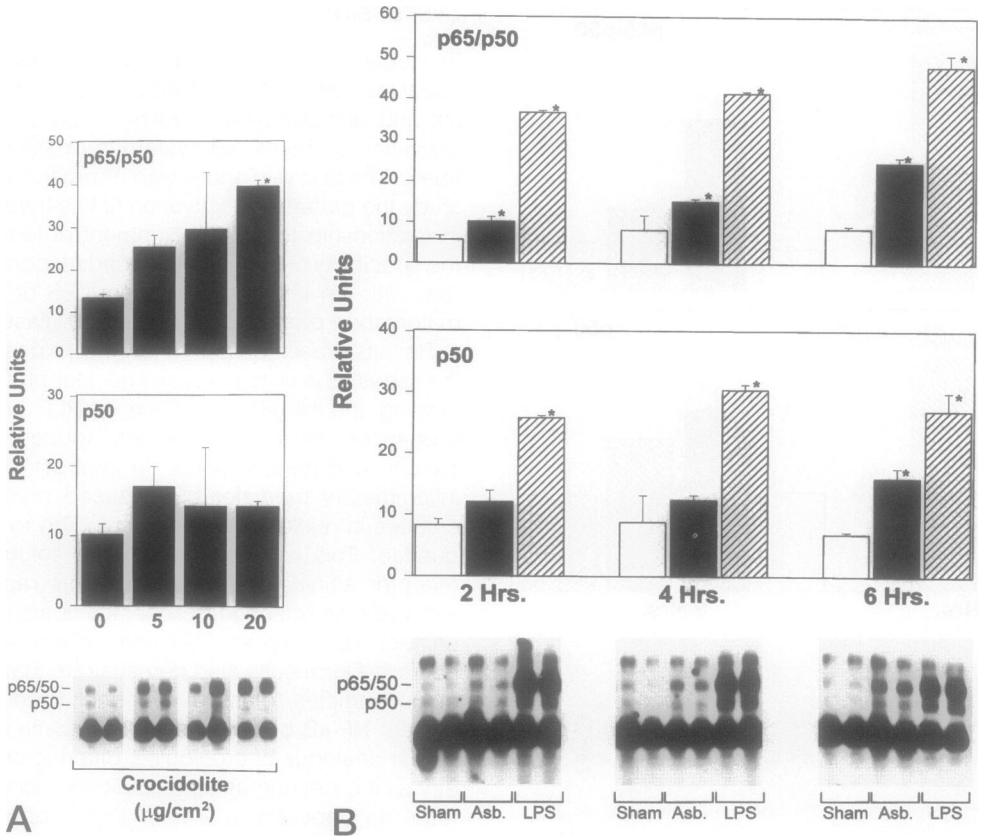


**Figure 8. A:** Dose-dependent increases in p65/p50 and p50 protein complexes binding to the NF-κB DNA sequence in RLE cells exposed to increasing concentrations of asbestos for 6 hours ($P < 0.05$, linear trend analysis). **B:** EMSA complexes in RLE cells exposed to crocidolite asbestos (10 μg/cm²) or LPS (50 ng/ml) for 2, 4, or 6 hours. Nuclear extracts were examined for binding of p65/p50 or p50 protein complexes to the NF-κB consensus DNA sequence (*$P < 0.05$). Error bars represent mean ± SEM of duplicate samples as measured by densitometry.

cell proliferation[38,49] after short exposures to mineral dusts. Coincidentally, immunoreactivity of p65 in bronchiolar epithelial cells decreased at 20 days as does cell proliferation in this compartment of the lung after exposure to asbestos. In contrast, inflammation is striking at both 5 and 20 days in asbestos-exposed lungs. The increases of p65 immunofluorescence in airways after inhalation of asbestos are generalized and do not appear related to sites of fiber deposition (Figure 5). Release of inflammatory cytokines at these time points may account for the generalized increases of p65 immunofluorescence observed here. Despite immunolocalization of p65 in mesothelium, no clear increases were observed in this compartment after inhalation of asbestos. The lack of effect may be due to the lack of translocation of asbestos to the mesothelium at these time points of investigation. In addition, inflammation may be more pronounced in the airways or the interstitium, accounting for increases of p65 in these compartments *versus* the mesothelium.

The mechanisms intrinsic to the development of asbestos-induced pulmonary disease are unclear to date. However, an increasing body of evidence suggests that both AOS and inflammatory mediators are important factors in the induction of asbestos-associated pulmonary disease (reviewed in Ref. 3). For example, several studies have demonstrated that asbestos-mediated cell damage and inflammation is prevented by antioxidants or iron chelators.[50–52] More importantly, asbestos-induced cell injury, inflammation, and asbestosis in rats is ameliorated by systemic administration of the antioxidant enzyme catalase.[35] Inflammatory cytokines such as TNF are released from different cell types of the lung in response to asbestos fibers *in vitro* and *in vivo* and may also contribute to the development of disease.[4–6,48,53] The ramifications of NF-κB activation in lung by asbestos are undoubtedly complex. A number of laboratories have shown increased expression of genes with NF-κB binding sequences in their regulatory regions in lungs and cells *in vitro* after exposure to asbestos. For example, inhalation of crocidolite asbestos (8 mg/m³ of air) for 3 or 6 hours leads to increases in lung of steady-state mRNA levels of macrophage inflammatory protein-2 and cytokine-induced neutrophil chemoattractant, chemokines involved in the recruitment of inflammatory cells to the site of injury.[6,48] Nitric oxide synthase (NOS) is another protein under transcriptional control of NF-κB that can be induced by cytokines and LPS[54] and contributes to lung inflammation.[55] Recent work by our laboratory (T. R. Quinlan, K. A. Berube, M. P. Hacker, D. J. Taatjes, C. R. Timblin, J. Goldberg, P. Kimberley, P. O'Shaughnessy, D.

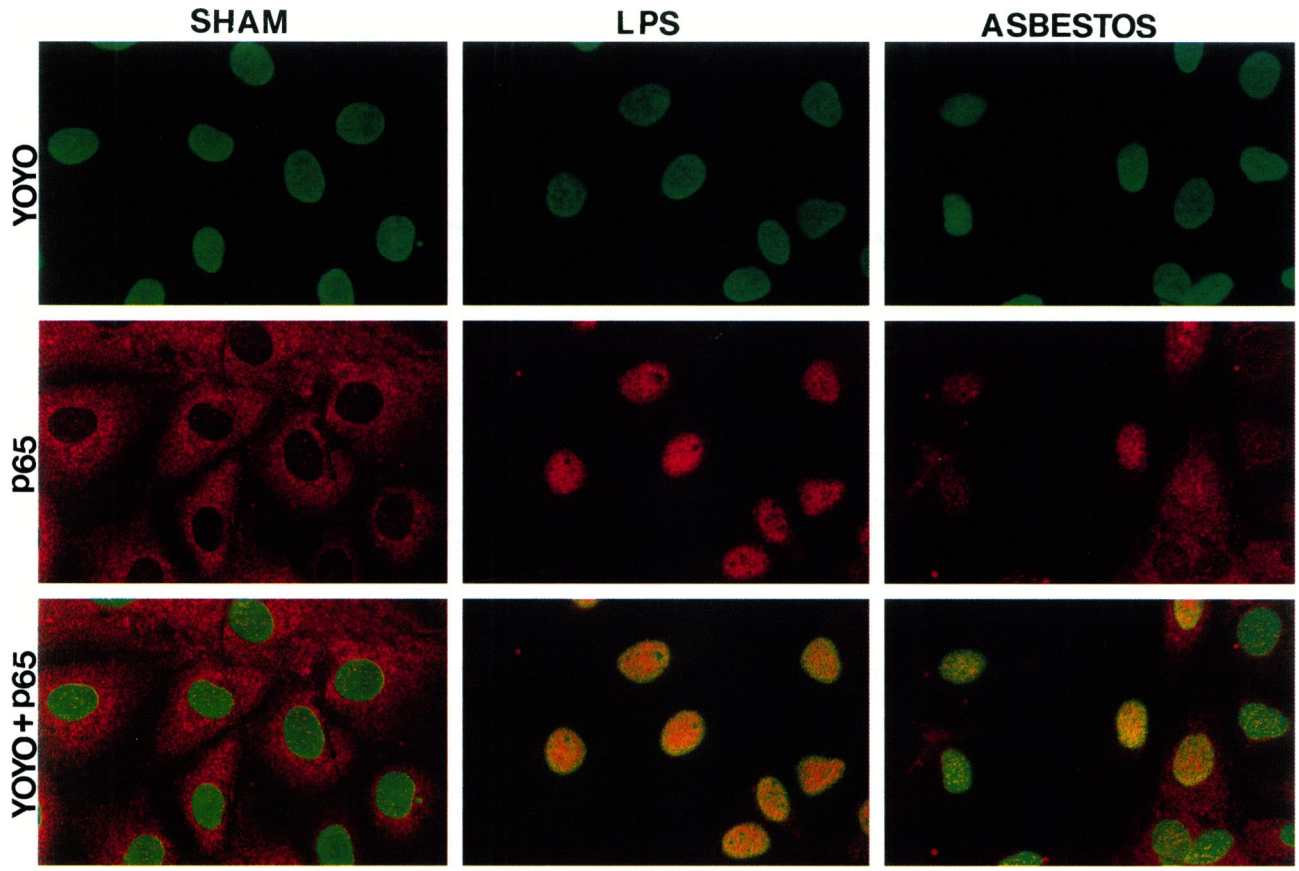


**Figure 9.** Confocal microscopy showing YOYO DNA stain (green) and cellular localization of p65 protein in RPM cells (red). Sham control RPM cells (sham) or RPM exposed to LPS for 2 hours (LPS) or crocidolite asbestos (5 $\mu g/cm^2$) for 8 hours (asbestos) were examined for p65 immunofluorescence and co-localization with YOYO. Cells were incubated with an antibody recognizing p65 protein and subsequently with a lissamine-rhodamine-conjugated secondary antibody in the presence of 500 nmol/L YOYO-1 iodide. YOYO and p65 images were collected individually and images were then merged (YOYO + p65) to confirm nuclear localization of p65 in response to LPS or asbestos (orange). Magnification, ×1080.

Hemenway, J. Torino, L. A. Jimenez, and B. T. Mossman, submitted for publication) and others[56] documents increased levels of nitrates and nitrites in alveolar macrophages exposed to asbestos and in bronchoalveolar lavage fluids from rats after inhalation of asbestos. These increases appear to be indicative of up-regulation of inducible NOS (iNOS) as increases in iNOS mRNA are observed in alveolar macrophages exposed to asbestos *in vitro* (T. R. Quinlan, K. A. Berube, M. P. Hacker, D. J. Taatjes, C. R. Timblin, J. Goldberg, P. Kimberley, P. O'Shaughnessy, D. Hemenway, J. Torino, L. A. Jimenez, and B. T. Mossman, submitted for publication).

**Table 1.** Nuclear Fluorescence of p65 in RPM Cells

| Treatment | Total cell number | Cells with NF | % NF |
|---|---|---|---|
| Sham | 290 | 3 | 1 |
| LPS (100 ng/ml, 2 hours) | 280 | 250 | 89 |
| Crocidolite (5 $\mu g/cm^2$, 8 hours) | 127 | 66 | 52 |
| Glass (10 $\mu g/cm^2$, 8 hours) | 278 | 7 | 3 |

Cells were exposed to test agents and examined for p65 immunofluorescence as described in text. Cells were counted in random fields in duplicate cultures using a blind code system, and the percentage of those exhibiting nuclear fluorescence was determined. NF, nuclear fluorescence.

Unlike LPS, which induces a rapid nuclear translocation of p65, asbestos causes only a partial nuclear translocation of p65 in RPM and RLE cells. This response, as quantitated using confocal microscopy, may explain why EMSA changes are less striking in magnitude in asbestos-exposed cells compared with LPS. In contrast to LPS, a soluble agent, target cells of asbestos-induced disease interact with asbestos fibers either directly by contact or indirectly via oxidant stress. Thus, partial nuclear translocation of p65 may reflect localized responses to fibers or the limited diffusion capacity of AOS released during phagocytosis of fibers. Our studies suggest that sustained activation of NF-κB by asbestos and transactivation of NF-κB-dependent genes may contribute to the chronic inflammatory process occurring in the lungs of experimental animals[35–38] and workers after occupational exposures to asbestos.[57]

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## References

1. Mossman BT, Bignon J, Corn M, Seaton A, Gee JBL: Asbestos: scientific developments and implications for public policy. Science 1990, 247:294–301
2. Mossman BT, Gee JBL: Medical progress: asbestos-related diseases. N Engl J Med 1989, 320:1721–1730
3. Mossman B, Kamp D, Weitzman S: Mechanisms of carcinogenesis and clinical features of asbestos-associated cancers. Cancer Invest 1996, 14:466–480
4. Griffith DE, Miller EJ, Gray LD, Idell S, Johnson AR: Interleukin-1-mediated release of interleukin-8 by asbestos-stimulated human pleural mesothelial cells. Am J Respir Cell Mol Biol 1994, 10:245–252
5. Zhang Y, Lee TC, Guillemin B, Yu MC, Rom WN: Enhanced IL-1 $\beta$ and tumor necrosis factor-$\alpha$ release and messenger RNA expression in macrophages from idiopathic pulmonary fibrosis or after asbestos exposure. J Immunol 1993, 150:4188–4196
6. Driscoll KE, DG Hassenbein, JM Carter, SL Kunkel, T. R: Quinlan, Mossman BT: 1995, TNF-$\alpha$ and increased chemokine expression in rat lung after particle exposure. Toxicol. Lett:82/83:483–489
7. Janssen YMW, Marsh JP, Absher MP, Hemenway D, Vacek PM, Leslie KO, Borm PJA, Mossman BT: Expression of antioxidant enzymes in rat lungs after inhalation of asbestos or silica. J Biol Chem 1992, 267:10625–10630
8. Janssen YMW, Marsh JP, Absher MP, Gabrielson E, Borm PJA, Driscoll K, Mossman BT: Oxidant stress responses in human pleural mesothelial cells exposed to asbestos. Am J Respir Crit Care Med 1994, 149:795–802
9. Hansen K, Mossman BT: Generation of superoxide ($O_2^-$) from alveolar macrophages exposed to asbestiform and nonfibrous particles. Cancer Res 1987, 47:1681–1686
10. Goodglick LA, Kane AB: Role of reactive oxygen metabolites in crocidolite asbestos toxicity to mouse macrophages. Cancer Res 1986, 46:5558–5566
11. Weitzman SA, Graceffa P: Asbestos catalyzes hydroxyl and superoxide radical generation from hydrogen peroxide. Arch Biochem Biophys 1984, 228:373–376
12. Janssen Y, Heintz N, Mossman B: Induction of c-*fos* and c-*jun* protooncogene by asbestos is ameliorated by *N*-acetyl-L-cysteine in mesothelial cells. Cancer Res 1995, 55:2085–2089
13. Perkins RC, Scheule RK, Hamilton R, Gomes G, Freidman G, Holian A: Human alveolar macrophage cytokine release in response to in vitro and in vivo asbestos exposure. Exp Lung Res 1993, 19:55–65
14. Mossman BT, Faux S, Janssen Y, Jiminez LA, Timblin C, Zanella C, Goldberg J, Walsh E, Barchowsky A, Driscoll K: Cell signalling pathways elicited by asbestos. Environ Health Perspect 1997 (in press)
15. Anderson MT, Staal FJT, Gitler C, Herzenberg LA, Herzenberg LA: Separation of oxidant-initiated and redox-regulated steps in the NF-$\kappa$B signal transduction pathway. Proc Natl Acad Sci USA 1994, 91:11527–11531
16. Barchowsky A, Munro SR, Morana SJ, Vincenti MP, Treadwell M: Oxidant-sensitive and phosphorylation-dependent activation of NF-$\kappa$B and AP-1 in endothelial cells. Am J Physiol 1995, 269:L829–L836
17. Meyer M, Schreck R, Baeuerle PA: $H_2O_2$ and antioxidants have opposite effects on activation of NF-$\kappa$B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. EMBO J 1993, 12:2005–2015
18. Janssen YMW, Barchowsky A, Treadwell M, Driscoll KE, Mossman BT: Asbestos induces nuclear factor $\kappa$B(NF-$\kappa$B)DNA-binding activity and NF-$\kappa$B-dependent gene expression in tracheal epithelial cells. Proc Natl Acad Sci USA 1995, 92:8458–8462
19. Read MA, Whitley MZ, Williams AJ, Collins T: NF-$\kappa$B and I$\kappa$B$\alpha$: an inducible regulatory system in endothelial activation. J Exp Med 1994, 179:503–512
20. Kunsch C, Rosen CA: NF-$\kappa$B subunit-specific regulation of the interleukin-8 promoter. Mol Cell Biol 1993, 13:6137–6146
21. Matsusaka T, Fujikawa K, Nishio Y, Mukaida N, Matsushima K, Kishimoto T, Akira S: Transcription factors NF-IL6 and NF-$\kappa$B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. Proc Natl Acad Sci USA 1993, 90:10193–10197
22. O'Connell MA, Cleere R, Long A, O'Neill LA, Kelleher D: Cellular proliferation and activation of NF kappa B are induced by autocrine production of tumor necrosis factor $\alpha$ in the human T lymphoma line HuT 78. J Biol Chem 1995, 270:7399–7404
23. Autieri MW, Yue TL, Ferstein GZ, Ohlstein E: Antisense oligonucleotides to the p65 subunit of NF-$\kappa$B inhibit human vascular smooth muscle cell adherence and proliferation and prevent neointima formation in rat carotid arteries. Biochem Biophys Res Commun 1995, 213:827–836
24. Beg AA, Baltimore D: An essential role for NF-$\kappa$B in preventing TNF-$\alpha$-induced cell death. Science 1996, 274:782–784
25. Wang C-Y, Mayo MW, Baldwin AS Jr: TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-$\kappa$B. Science 1996, 274:784–787
26. Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM: Suppression of TNF-$\alpha$-induced apoptosis by NF-$\kappa$B. Science 1996, 274:787–789
27. Devary Y, Rosette C, DiDonato JA, Karin M: NF-$\kappa$B activation by ultraviolet light not dependent on a nuclear signal. Science 1993, 261:1442–1445
28. Mohan N, Meltz ML: Induction of nuclear factor $\kappa$B after low-dose ionizing radiation involves a reactive oxygen intermediate signaling pathway. Radiat Res 1994, 140:97–104
29. Israel A: A role for phosphorylation and degradation in the control of NF-$\kappa$B activity. Trends Genet 1995, 11:203–205
30. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T: The ubiquitin-proteosome pathway is required for processing the NF-$\kappa$B1 precursor protein and the activation of NF-$\kappa$B. Cell 1994, 78:773–785
31. Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T: Transcriptional regulation of endothelial cell adhesion molecules: NF-$\kappa$B and cytokine-inducible enhancers. FASEB J 1995, 9:899–909
32. Xie Q-W, Kashiwabara Y, Nathan C: Role of transcription factor NF-$\kappa$B/Rel in induction of nitric oxide synthase. J Biol Chem 1994, 269: 4705–4708
33. LaRosa FA, Pierce JW, Sonenshein GE: Differential regulation of the c-*myc* oncogene promoter by the NF-$\kappa$B rel family of transcription factors. Mol Cell Biol 1994, 14:1039–1044
34. Ueda A, Okuda K, Ohno S, Shirai A, Igarashi T, Matsunaga K, Fukushima J, Kawamoto S, Ishigatsubo Y, Okubo T: NF-kappa B and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. J Immunol 1994, 153:2052–2063
35. Mossman BT, Marsh JP, Sesko A, Hill S, Shatos MA, Doherty J, Petruska J, Adler KB, Hemenway D, Mickey R, Vacek P, Kagan E: Inhibition of lung injury, inflammation and interstitial pulmonary fibrosis by polyethylene glycol-conjugated catalase in a rapid inhalation model of asbestosis. Am Rev Respir Dis 1990, 141:1266–1271
36. Quinlan TR, Marsh JP, Janssen YMW, Leslie KO, Hemenway D, Vacek P, Mossman BT: Dose responsive increases in pulmonary fibrosis after inhalation of asbestos. Am J Respir Crit Care Med 1994, 150:200–206
37. Quinlan TR, BéruBé KA, Marsh JP, Janssen YMW, Taishi P, Leslie KO, Hemenway D, O'Shaughnessy PT, Vacek P, Mossman BT: Patterns of inflammation, cell proliferation, and related gene expression in lung after inhalation of chrysotile asbestos. Am J Pathol 1995, 147:728–739
38. BéruBé KA, Quinlan TR, Moulton G, Hemenway D, O'Shaughnessy P, Vacek P, Mossman BT: Comparative proliferative and histopathologic changes in rat lungs after inhalation of chrysotile and crocidolite asbestos. Toxicol Appl Pharmacol 1996, 137:67–74
39. Schmitz R, Baeuerle P: The p65 subunit is responsible for the strong transcription activating potential of NF-$\kappa$B. EMBO J 1991, 10:3805–3817
40. Campbell WJ, Huggins CW, Wylie AG: Chemical and physical characterization of amosite, chrysotile, crocidolite, and nonfibrous tremolite for oral ingestion studies by the National Institute of Environmental Health Sciences. US Bureau of Mines Report of Investigations, No 8452, 1980
41. Hemenway DR, MacAskill SM: Design, development and test results of a horizontal flow facility. Am Ind Hyg Assoc 1982, 43:874–879
42. Driscoll KE, Carter JM, Iype PT, Kumari HL, Crosby LL, Aardema MJ,

Isfort RJ, Cody D, Chestnut MH, Burns JL, LeBoeuf RA: Establishment of immortalized alveolar type II epithelial cell lines from adult rats. In Vitro Cell Dev Biol Animal 1995, 31:516–527
43. Jaurand MC, Renier A, van der Meeren A, Magne J, Pinchon L, Bignon J: Rat pleural mesothelial cell in culture. In Vitro 1981, 17:98–108
44. Janssen YMW, Heintz NH, Marsh JP, Borm PJA, Mossman BT: Induction of c-*fos* and c-*jun* proto-oncogenes in target cells of the lung and pleura by carcinogenic fibers. Am J Respir Cell Mol Biol 1994, 11:522–530
45. Staal FJ, Roederer M, Herzenberg LA, Herzenberg LA: Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. Proc Natl Acad Sci USA 1994, 87:9943–9947
46. Brody AR, Hill LH: Interstitial accumulation of inhaled chrysotile asbestos fibers and consequent formation of microcalcifications. Am J Pathol 1982, 109:107–114
47. Holley JA, Janssen YMW, Mossman BT, Taatjes DJ: Increased manganese superoxide dismutase protein in type II epithelial cells of rat lungs after inhalation of crocidolite asbestos or cristobalite silica. Am J Pathol 1992, 141:475–485
48. Driscoll KE, Maurer JK, Higgins J, Poynter J: Alveolar macrophage and growth factor production in a rat model of crocidolite-induced pulmonary inflammation and fibrosis. J Toxicol Environ Health 1995, 46:155–169
49. Brody AR, Overby LH: Incorporation of tritiated thymidine by epithelial and interstitial cells in bronchiolar-alveolar regions of asbestos-exposed rats. Am J Pathol 1989, 134:133–140
50. Shatos MA, Doherty JM, Marsh JP, Mossman BT: Prevention of asbestos-induced cell death in rat lung fibroblasts and alveolar macrophages by scavengers of active oxygen species. Environ Res 1987, 48:709–714
51. Goodglick LA, Pietras LA, Kane A: Evaluation of the causal relationship between crocidolite asbestos-induced lipid peroxidation and toxicity to macrophages. Am Rev Respir Dis 1989, 139:1265–1273
52. Lund LG, Aust AE: Iron mobilization from crocidolite asbestos greatly enhances crocidolite-dependent formation of DNA single-strand breaks in phi X174 RFI DNA. Carcinogenesis 1992, 13:637–642
53. Simeonova PP, Luster MI: Iron and reactive oxygen species in the asbestos-induced tumor necrosis factor-α response from alveolar macrophages. Am J Respir Cell Mol Biol 1995, 12:676–683
54. Owens MW, Grisham MB: Nitric oxide synthesis by rat pleural mesothelial cells: induction by cytokines and lipopolysaccharide. Am J Physiol 1993, 265:110–116
55. Flak TA, Goldman WE: Autotoxicity of nitric oxide in airway disease. Am J Respir Crit Care Med 1996, 154:S202–S206
56. Thomas G, Ando T, Verma K, Kagan E: Asbestos fibers and interferon-γ upregulate nitric oxide production in rat alveolar macrophages. Am J Respir Cell Mol Biol 1994, 11:707–715
57. Craighead JE, Abraham JL, Churg A, Green FHY, Kleinerman J, Pratt PC, Seemayer TA, Vallyathan V, Weill H: The pathology of asbestos-associated diseases of the lung and pleural cavities: diagnostic criteria and proposed grading schema. Arch Pathol Lab Med 1982, 106:544–597