Experimental and Molecular Pathology **66,** 201–210 (1999)
Article ID exmp.1999.2268, available online at http://www.idealibrary.com on IDEAL®

Role of Transcription Factor NF-κB in Asbestos-Induced TNFα Response from Macrophages

Ningli Cheng,* Xianglin Shi,† Jianping Ye,† Vincent Castranova,† Fei Chen,‡ Stephen S. Leonard,† Val Vallyathan,† and Yon Rojanasakul*,[1]

**Department of Basic Pharmaceutical Sciences, West Virginia University, Morgantown, West Virginia 26506; †Pathology and Physiology Research Branch, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505; and ‡Department of Pathology, Pennsylvania State University, Milton S. Hershey Medical Center, Hershey, Pennsylvania 17033*

Received January 25, 1999, and in revised form May 20, 1999

Asbestos exposure in humans is associated with inflammatory, fibrotic, and malignant diseases in the lung. Increasing evidence supports the hypothesis that the production of proinflammatory cytokines such as tumor necrosis factor-α (TNFα) is an important mediator of the pathologic responses of asbestosis. In this study, we examine the role of nuclear transcription factor-κB (NF-κB) and free oxygen radicals in asbestos-induced TNFα gene and protein expression in lung macrophages. Exposure of the cells to crocidolite asbestos caused a parallel increase in TNFα production and NF-κB activation, as analyzed by enzyme-linked immunosorbent assay and electrophoretic mobility shift assay. Inhibition of NF-κB by SN50, an inhibitor of NF-κB nuclear translocation, or by sequence-specific oligonucleotides directed against the NF-κB binding site of TNFα promoter attenuated the asbestos effect on TNFα production. Gene transfection assays using an expression plasmid containing a luciferase reporter gene and a TNFα-derived NF-κB gene promoter further indicated the dependence of NF-κB activation on asbestos-induced gene expression. The effects of asbestos on NF-κB and TNFα activation were inhibited by oxygen radical scavengers and were enhanced by antioxidant enzyme inhibitors. These results indicate that asbestos-induced TNFα gene expression is mediated through a process that involves NF-κB activation and free radical reactions.

[1]To whom correspondence should be addressed at Department of Basic Pharmaceutical Sciences, West Virginia University, Health Sciences Center, P.O. Box 9530, Morgantown, WV 26506. Fax: (304) 293-5483. E-mail: yrojanasakul@hsc.wvu.edu.

INTRODUCTION

Clinical and experimental studies show that asbestos exposure causes lung diseases such as inflammation, fibrosis, and cancer (Mossman and Gee 1989; Zhang *et al.* 1993; Janssen *et al.* 1994). Although the mechanisms of asbestos-induced lung diseases remain unclear, an elaboration of macrophage cytokines and activation of reactive oxygen species (ROS) are thought to play a key role in the disease process. Tumor necrosis factor-α (TNFα) is an important cytokine that mediates inflammatory and fibrotic reactions (Zhang *et al.* 1993; Perkins *et al.* 1993). It can initiate a cascade of events including the secretion of chemotactic cytokines by immune and nonimmune cells, expression of adhesion molecules on endothelial cells, and responses that contribute to lung toxicities (Emgelmann *et al.* 1990; Li *et al.* 1993; Perkins *et al.* 1993; Ljungman *et al.* 1994).

The expression of TNFα is regulated at different levels, transcriptional and posttranscriptional (Beutler 1992). At the transcriptional level, TNFα is regulated by sequence-specific transcription factors, which interact with the gene's promoter or enhancer regions (Collart *et al.* 1990). Because the TNFα gene promoter contains NF-κB binding sites (Lenardo and

0014-4800/99 $30.00

Baltimore 1989; Collart *et al.* 1990), we hypothesize that asbestos-induced TNFα production is mediated through NF-κB activation. The possible role of NF-κB in asbestos-induced lung disease has also been recently suggested. Janssen *et al.* (1995) reported that asbestos was able to induce NF-κB DNA binding activity in hamster tracheal epithelial cells, and Simeonova and Luster (1996) showed that asbestos activated NF-κB-like transcription factors in the human pulmonary epithelial cell line A549. Although these studies suggest that asbestos is able to activate NF-κB, whether or not this activation is responsible for TNFα production remains to be established. Furthermore, asbestos-induced NF-κB activation has not been demonstrated in pulmonary alveolar macrophages. This is important since alveolar macrophages are the primary source of TNFα production in the lung and are the principal target for inhaled toxicants (Kelly 1990). Thus, in this study we focus on the role of NF-κB in asbestos-induced TNFα activation in macrophages.

Asbestos-induced lung toxicity has also been associated with the generation of free radicals and other ROS by macrophages and other inflammatory cells. ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot$OH) have been reported to cause lung injury after asbestos exposure (Kamp *et al.* 1992; Schapira *et al.* 1994). Recent studies have also indicated that ROS can activate a number of cytokine genes including TNFα (Gossart *et al.* 1996; Simeonova and Luster 1995). These findings suggest that ROS may serve as mediators of TNFα and other cytokine-related pathologic responses in asbestosis. As ROS has been implicated in the activation of numerous transcription factors, including NF-κB (Schreck *et al.* 1991), we hypothesize that asbestos induces TNFα gene expression via ROS-dependent NF-κB activation. Here, we demonstrate that asbestos activates TNFα promoter-mediated transcription and stimulates binding activity to the NF-κB regulatory elements in the TNFα gene. The contribution of ROS and the role of specific oxidative species were evaluated in the regulation of these responses.

MATERIALS AND METHODS

Reagents

Crocidolite asbestos [$(Na_2(Fe^{III})_2(Fe^{II})_3Si_8O_{22}(OH)_2$] was obtained from the National Institute of Occupational Safety and Health (Morgantown, WV). This sample, originally obtained from a mine in South Africa, had a median fiber length of 11.5 μm and a surface area of 17.1 m^2/g. Titanium dioxide (Sigma Chemical Co., St. Louis, MO) was spherical in shape and had a diameter between 0.1 and 0.55 μm. Prior to use, the samples were heat-sterilized for 2 h at 200°C and dispersed in incubating medium by ultrasonication. The presence of endotoxin in the samples was analyzed by the Limulus amebocyte assay (Sigma) and was undetectable. Double-stranded (ds) oligodeoxynucleotide with a sequence specific to the NF-κB binding site of the TNFα promoter (NF-ON) (5′-GGTCCGTGAATTCCCAGGGC-3′) (Collart *et al.* 1990) and a nonspecific control sequence (C-ON) (5′-GTGAAGGTCCAGGGCTTCCC-3′) were synthesized on an automated solid-phase synthesizer using standard phosphoramidite chemistry. The antisense strands of these ds oligomers were the reverse complements. The oligonucleotides were purified by high-performance liquid chromatography and were >98% pure. SN50 peptide was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). All other test agents including deferoxamine mesylate, sodium formate, mercaptosuccinic acid, and diethyldithiocarbamic acid were purchased from Sigma Chemical Co.

Cell Preparation and Culture

Alveolar macrophages were harvested from male Sprague–Dawley rats (200–250 mg) by bronchoalveolar lavage as previously described (Rojanasakul *et al.* 1997). Aliquots of 200 μl containing 2×10^5 cells in DMEM medium with 10% fetal bovine serum were added onto a 96-well plate and incubated at 37°C in a humidified atmosphere at 5% CO_2. The macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (ATCC, Rockville, MD) and was similarly maintained.

Cell Treatment

In a typical experiment, cells were treated with crocidolite asbestos in DMEM medium at a subcytotoxic concentration of 50 μg/ml. In studies designed to evaluate the effect of concentration of asbestos on TNFα production and NF-κB activation, 25–100 μg/ml of asbestos was used. All other test agents, including deferoxamine, sodium formate, PBN, mercaptosuccinic acid, and diethyldithiocarbamic acid, at indicated concentrations, were added to the cells 2 h prior to asbestos stimulation.

Nuclear Extracts

Nuclear extracts were prepared as follows: 5×10^7 cells were treated with 500 μl lysis buffer (50 mM KCl, 0.5%

NP-40, 25 mM Hepes, 1 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μM DTT) on ice for 4 min. Nuclei were pelleted by centrifugation at 14,000 rpm for 1 min and were resuspended in 300 μl extraction buffer (500 mM KCl, 10% glycerol, 25 mM Hepes, 1 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μM DTT). After centrifugation at 14,000 rpm for 5 min, the supernatant was harvested and stored at -70°C. The protein concentration of the resulting nuclear protein extract was determined by BCA protein assay reagent (Pierce, Rockford, IL).

Electrophoretic Mobility Shift Assay

The DNA–protein binding reaction was conducted in a 24-μl reaction mixture including 3 μg nuclear protein extract, 1 μg poly(dI·dC) (sigma), 3 μg BSA, 4×10^4 cpm of ^{32}P-labeled oligonucleotide probe (see below), and 12 μl of 2× Y buffer (Ye *et al.* 1996). In some cases, nonlabeled oligomer (100-fold excess) was also added as a cold competitor. The mixture was incubated on ice for 10 min with or without antibody specific to NF-κB p50 in the absence of radiolabeled probe and then for 20 min at room temperature in the presence of radiolabeled probe. The mixture was resolved on a 5% polyacrylamide gel that had been prerun at 170 V for 30 min with 0.5× TBE buffer. The loaded gel was run at 200 V for 90 min, dried, and placed on Kodak X-OMAT film (Eastman Kodak, Rochester, NY). This film was developed after overnight exposure at -70°C. The preparation of radiolabeled oligonucleotide probe was performed as previously described (Isshiki *et al.* 1990). An NF-κB binding sequence (5′TGGGATTTTCCCATGAGTCT-3′) was used to synthesize the probe. The synthesized oligonucleotide probe was denatured at 80°C for 5 min and annealed with its complementary sequence at room temperature. An AP1 binding oligonucleotide was also used as a nonspecific competitor or as a probe to examine the AP1 binding activity (Ye *et al.* 1996). The ds probe was labeled with [^{32}P]ATP (Amersham, Arlington Heights, IL) using T4 kinase (BRL, Gaithersburgh, MD).

Transient Transfection and Luciferase Assay

The reporter gene vector used in this study was generously provided by Dr. S. T. Fan at the Scripps Research Institute (La Jolla, CA) (Yao *et al.* 1997). The luciferase vector contains NF-κB binding sites derived from the $-615/+15$ promoter fragment of the TNFα gene. RAW 264.7 cells (1×10^6/well) were plated in 6-well plates for 16 h, after which they were transfected with the reporter DNA (5 μg) using the DEAE–dextran method (Yao *et al.* 1997). After transfection, the cells were washed and added to normal culture medium containing 10% fetal bovine serum. After a 24-h incubation at 37°C, the cells were washed and used for asbestos stimulation studies. Luciferase activity was measured 6 h after asbestos stimulation, with or without other test agents, using the Promaga Luciferase Assay kit (Promega, Madison, WI). To account for the potential cytotoxic effect caused by the transfecting agent, asbestos, or other test agents during the experiments, total cell protein was determined and used to normalize the measured luciferase activity.

Enzyme-Linked Immunoadsorbent Assay

Analysis of TNFα protein level was performed using the Genzyme TNFα enzyme-linked immunosorbent assay (ELISA) kit (Genzyme Corp., Cambridge, MA) according to the manufacturer's instructions. Absorbance measurement of the enzyme product was carried out at a wavelength of 450 nm using the Bio-Rad 500 microplate reader.

RESULTS

Asbestos Induces TNFα Production

To study the effect of asbestos on TNFα production, ELISA experiments were conducted using primary alveolar macrophages and macrophage RAW 264.7 cells. The cells were treated with varying concentrations of crocidolite asbestos (25–100 μg/ml) for up to 24 h, and the cell supernatants were collected and analyzed for TNFα. Figures 1 and 2 show that asbestos stimulated TNFα production in a dose- and time-dependent manner. The saturation level was reached in about 8 h at a crocidolite concentration of approximately 50 μg/ml. The nonfibrogenic control dust, titanium dioxide, had no stimulatory effect on TNFα secretion (Fig. 1). Under the optimal stimulatory condition (50 μg/ml, 8 h), asbestos did not cause a significant cytotoxic effect over control, as analyzed by trypan blue dye exclusion assay, i.e., $7 \pm 3\%$ vs $5 \pm 4\%$ ($P < 0.05$, $n = 4$). These results are in good agreement with previous studies that indicated that asbestos was able to induce TNFα secretion both *in vivo* and *in vitro* (Perkins *et al.* 1993; Driscoll *et al.* 1990; Simeonova and Luster 1995).

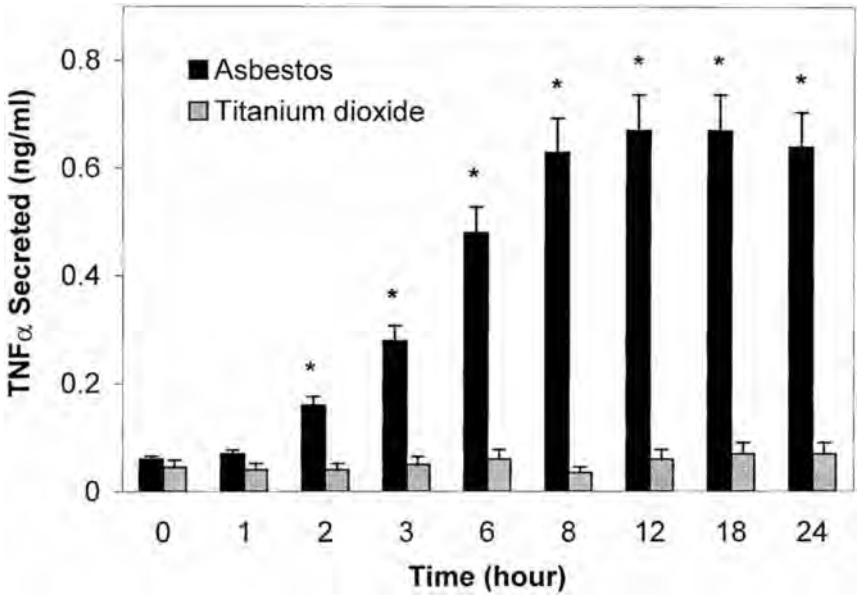

FIG. 1. Time course of TNFα production in asbestos-treated alveolar macrophages. Lavage cells (2×10^5/well) were incubated with heat-sterilized crocidolite asbestos (50 μg/ml) or titanium dioxide (50 μg/ml) at 37°C in culture medium. At the indicated times, the culture media were collected and analyzed for TNFα by ELISA. The values represent mean ± SE of three measurements obtained from different cell preparations. *Significant difference from untreated control ($P < 0.05$).

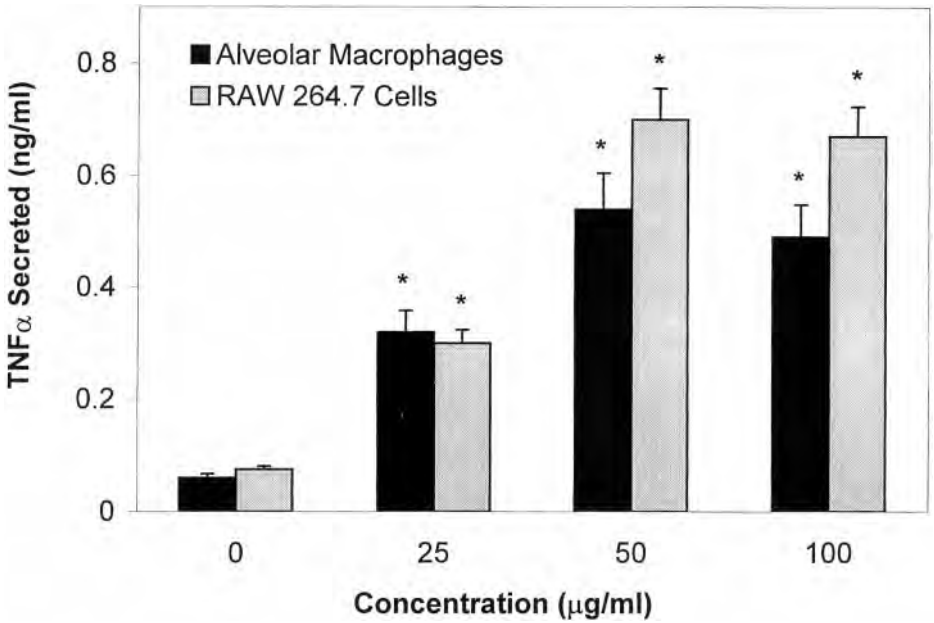

FIG. 2. Dose effect of crocidolite asbestos on TNFα production in alveolar macrophages and RAW 264.7 cells. Cells were treated with varying concentrations of crocidolite asbestos (0–100 μg/ml) for 6 h at 37°C. The values represent mean ± SE, $n = 3$. *Significant difference from untreated control ($P < 0.05$).

Inhibition of Asbestos-Induced TNFα Production by NF-κB Inhibitors

Since NF-κB binding sites have been found in the promoter region of the TNFα gene (Collart *et al.* 1990; Lenardo and Baltimore 1989), NF-κB may be involved in the regulation of asbestos-induced TNFα expression. To test this possibility, cells were treated with asbestos in the presence of SN50, a cell-permeable inhibitory peptide of NF-κB. This peptide carries a functional domain that inhibits the translocation of activated NF-κB complexes from the cytoplasm to the nucleus (Lin *et al.* 1995). As shown in Fig. 3, treatment of the cells with SN50 resulted in a complete inhibition of TNFα, suggesting the regulatory role of NF-κB in asbestos-induced TNFα activation.

To confirm the above observation, oligonucleotide inhibition assays were conducted. Double-stranded oligonucleotide containing the sequence specific to the NF-κB binding site of TNFα promoter was added to the cells prior to asbestos stimulation. As a control, nonspecific oligonucleotide sequence having the same base composition and length was used. Figure 3 shows that the NF-ON was able to inhibit TNFα production, whereas the control C-ON had no effect. These results suggest the binding specificity of NF-ON to its NF-κB target and the involvement of NF-κB in asbestos-induced TNFα activation.

EMSA and Gene Transfection Studies

The observation that NF-κB inhibitors attenuated TNFα production indicates that the activation of NF-κB by asbestos may be required for the induction of TNFα. The possible activation of NF-κB by crocidolite asbestos was further examined by electrophoretic mobility shift assay (EMSA) and luciferase gene transfection assays. In the EMSA study, alveolar macrophages were exposed to crocidolite asbestos for 6 h, and the NF-κB was analyzed in the nuclear extracts. As shown in Fig. 4A, the cells exhibited a dose-dependent increase in NF-κB binding activity. The specificity of NF-κB binding was verified by competitive inhibition and antibody supershift assays (Fig. 4B). The results of this study showed that the NF-κB binding activity of the nuclear extract could be competed by a nonlabeled NF-κB DNA probe but not by a nonspecific AP-1 DNA probe, and the antibody specific to NF-κB caused a bandshift of the NF-κB complexes. Pretreatment of the cells with SN50 or NF-ON effectively inhibited asbestos-induced NF-κB activation, whereas the nonspecific inhibitor C-ON had no effect (Fig. 4C). In the second set of experiments, a more quantitative gene transfection assay was used to probe the promoter activity of the TNFα gene under asbestos stimulation. The TNFα promoter-containing vector that carries NF-κB binding sites upstream of

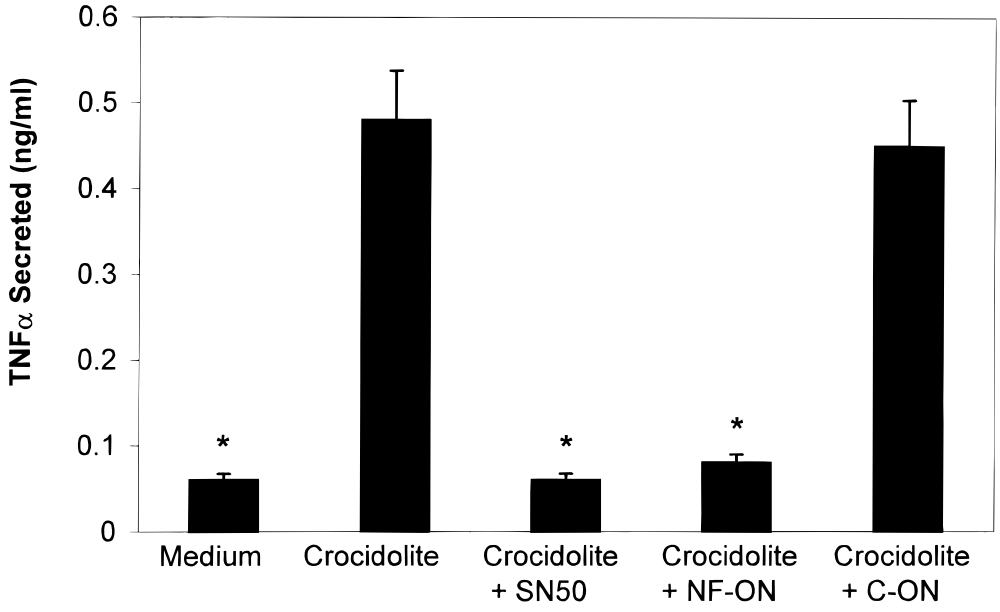

FIG. 3. Effect of NF-κB inhibitors on crocidolite-induced TNFα production. Alveolar macrophages were pretreated with SN50 (100 μg/ml), NF-ON (25 μg/ml), or C-ON (25 μg/ml) for 2 h, followed by asbestos stimulation (50 μg/ml) for another 6 h. The values represent mean $\pm$ SE, $n = 3$. *Significant difference from crocidolite-treated control ($P < 0.05$).

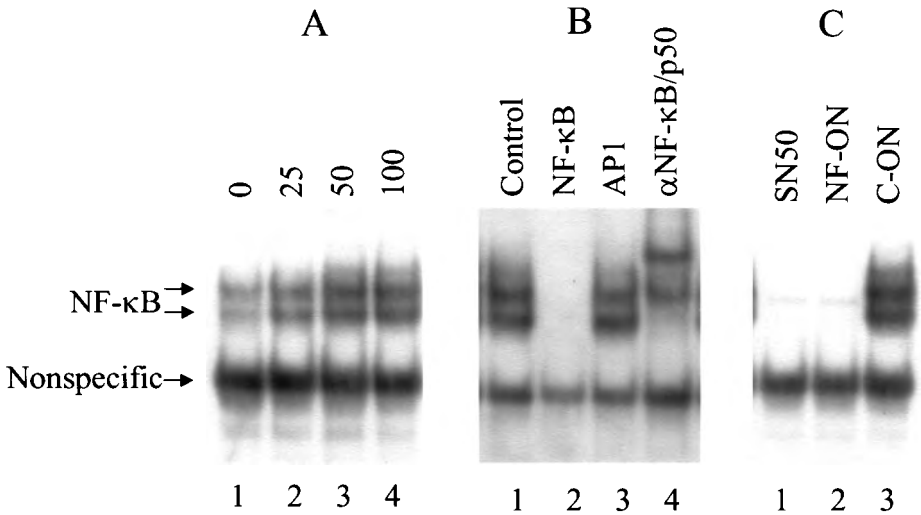

FIG. 4. NF-κB activation by asbestos and its inhibition by NF-κB inhibitors. (A) Dose-dependence activation of NF-κB by asbestos. Alveolar macrophages were stimulated by crocidolite asbestos at varying concentrations (0, 25, 50, and 100 μg/ml) (lane 1–4) for 6 h at 37°C, and then nuclear extracts were prepared. (B) Competition and antibody supershift assays of NF-κB binding activity for nuclear extract from cells treated with asbestos (50 μg/ml) for 6 h. The competitors and antibody used in each lane are as indicated. (C) Effect of NF-κB inhibitors on asbestos-induced NF-κB activation. Cells were pretreated with SN50 (100 μg/ml) (lane 1), NF-ON (25 μg/ml) (lane 2), or C-ON (25 μg/ml) (lane 3), for 2 h, followed by crocidolite stimulation (50 μg/ml) for an additional 6 h.

the luciferase reporter gene was introduced into the macrophages. In this study, the macrophage RAW 264.7 cells were used instead of the alveolar macrophage cells since the latter were found to be refractory to gene transfection. The transfected RAW 264.7 cells were treated with varying concentrations of asbestos in the presence or in the absence of SN50. Figure 5 shows that such treatment caused a dose-dependent increase in luciferase activity, and the SN50 effectively inhibited this activation. These results demonstrate that asbestos is able to activate the TNFα gene promoter and that the NF-κB binding site is required for such activation.

Effects of Oxygen Radical Scavengers

The role of free radical reactions in asbestos-induced NF-κB and TNFα activation was examined using free radical scavengers and antioxidant enzyme inhibitors. It has been reported that asbestos is capable of generating free radicals from hydrogen peroxide (Kamp *et al.* 1992). Free radical scavenger (sodium formate) and metal chelator (deferoxamine) were used to examine the effects of asbestos on NF-κB activation and TNFα production. As shown in Fig. 6, cells treated with these agents prior to asbestos stimulation exhibited a dose-dependent decrease in NF-κB activity. Similarly, treatment of the cells with these agents also caused a parallel decrease in TNFα production (Fig. 7).

Most cells are endowed with a sophisticated network of antioxidant defense mechanisms. To test whether endogenous antioxidant enzymes are involved in asbestos-induced TNFα activation, two specific antioxidant enzyme inhibitors, mercaptosuccinate and diethyldithiocarbamic acid, were used. As shown in Fig. 7, mercaptosuccinate, an inhibitor of glutathione peroxidase that scavenges H_2O_2, enhanced macrophage secretion of TNFα, whereas diethyldithiocarbamic acid, an inhibitor of superoxide dismutase that scavenges superoxide anion to generate H_2O_2, inhibited it. These results indicate the role of endogenous antioxidant enzymes in the regulation of asbestos-induced ROS generation and TNFα production.

DISCUSSION

It is well documented that asbestos exposure induces pulmonary diseases (Craighead and Mossman 1982; Zhang *et al.* 1993). The pathologic responses in the lung are characterized by the accumulation of macrophages at the site of fiber deposition and the release of inflammatory mediators, including proinflammatory cytokines, chemotactic peptides, and growth factors (Zhang *et al.* 1993; Perkins *et al.* 1993;

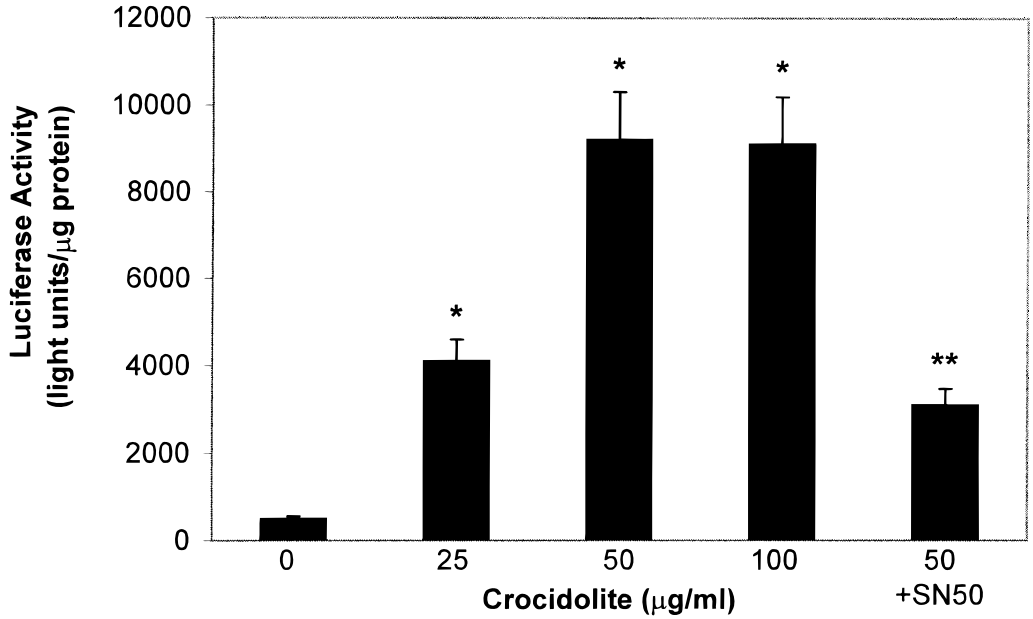

FIG. 5. Effect of crocidolite and SN50 on NF-κB-dependent luciferase gene expression. RAW 264.7 cells were transiently transfected with an expression plasmid containing luciferase gene transcribed from the NF-κB-dependent promoter. The transfected cells were treated with varying concentrations of crocidolite (0–100 μg/ml) in the absence or in the presence of SN50 (100 μg/ml) for 6 h at 37°C. The values represent mean $\pm$ SE, $n = 3$. *Significant difference from non-crocidolite-treated control A ($P < 0.05$). **Significant difference from crocidolite-treated control C ($P < 0.05$).

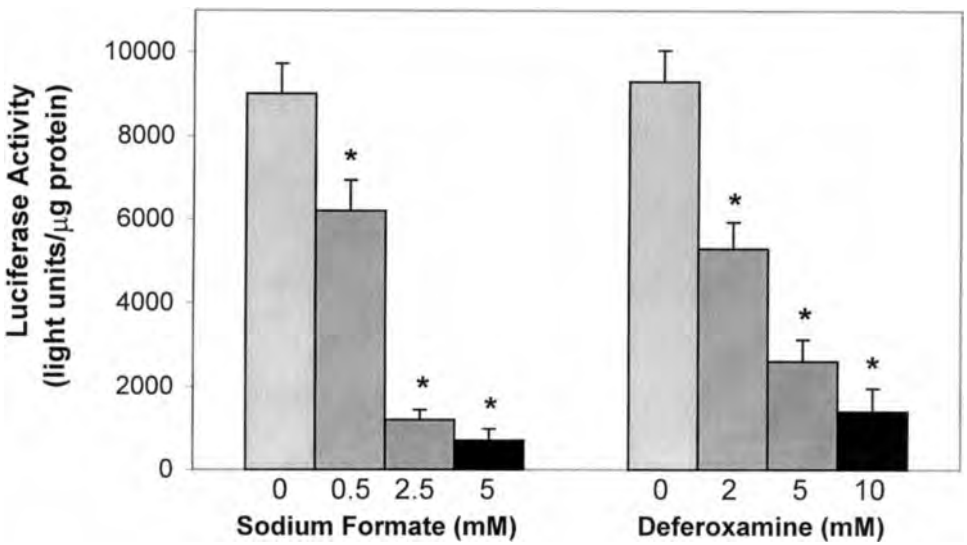

FIG. 6. Effect of oxygen radical scavengers on NF-κB-dependent luciferase activity in crocidolite-treated cells. Transfected RAW 264.7 cells were pretreated with various concentrations of sodium formate or deferoxamine for 2 h at 37°C and then treated with crocidolite for 6 h at 37°C. The values represent mean $\pm$ SE, $n = 3$. *Significant difference from crocidolite-treated control B ($P < 0.05$).

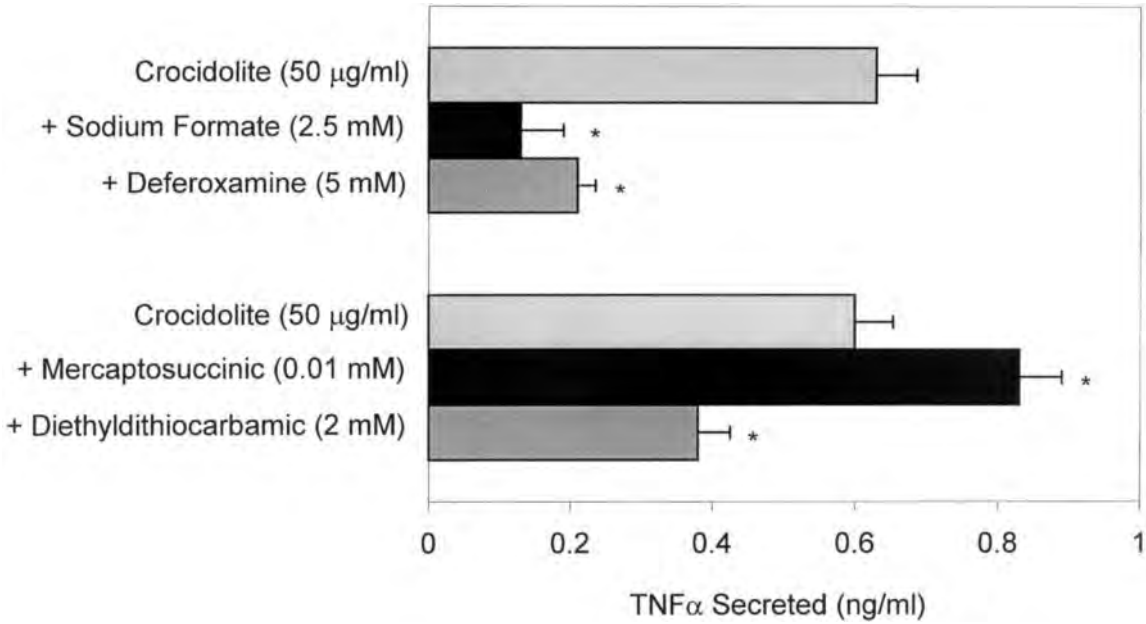

FIG. 7. Effect of oxygen radical scavengers and antioxidant enzyme inhibitors on TNFα production in crocidolite-treated cells. RAW 264.7 cells were pretreated with various radical scavengers and enzyme inhibitors for 2 h at 37°C and then treated with crocidolite for 6 h at 37°C. The values represent mean ± SE, $n = 3$. *Significant difference from crocidolite-treated control B ($P < 0.05$).

Li *et al.* 1993). Among the proinflammatory cytokines, TNFα has received the most attention and several lines of evidence suggest that it plays a crucial role in asbestos-induced lung diseases, including (1) alveolar macrophages from asbestosis patients (Zhang *et al.* 1993) or from patients with a history of chronic asbestos exposure (Perkins *et al.* 1993) who produce an increased amount of TNFα; (2) *in vivo* administration of TNFα causes many of the pathologic responses associated with asbestosis (Piguet *et al.* 1990a; Fiers 1991); and (3) administration of antibodies to TNFα prevents the pathologic effects caused by related fibrogenic material (Piguet *et al.* 1990b). Despite these findings, relatively little is known about the molecular mechanism by which asbestos stimulates TNFα production. Recent studies suggest that ROS produced by macrophages are involved in TNFα activation and antioxidants inhibit this activation (Gossart *et al.* 1996; Simeonova and Luster 1995). In the present study, we further demonstrate that ROS-dependent TNFα activation by asbestos is mediated through NF-κB activation.

The expression of TNFα is regulated at different levels, transcriptional and posttranscriptional (Beutler 1992). It is well known that transcriptional regulation is a key step in the control of TNFα gene expression (Collart *et al.* 1990). The activation of the TNFα gene is controlled by transcription factors that bind to the gene's promoter or enhancer regions. NF-κB is one such factor that is involved in the transcriptional regulation of several cytokines, including interleukins and TNFα (Baeuerle and Henkel 1994; Siebenlist *et al.* 1994). This study demonstrated that crocidolite asbestos induced NF-κB activation and stimulated TNFα production in alveolar macrophages and RAW 264.7 cells. The inhibitory effects were dose-dependent and inhibited by the NF-κB inhibitor SN50. The SN50 is a cell-permeable peptide that carries a functional domain consisting of the nuclear localization sequence of the transcription factor NF-κB p50 (Lin *et al.* 1995). It inhibits subcellular traffic of NF-κB/Rel complexes from the cytoplasm to the nucleus. This peptide has been reported to inhibit LPS-induced NF-κB activation in a dose-dependent manner. At 100 μg/ml, this peptide inhibits 85% of the NF-κB activation (Lin *et al.* 1995). Thus, it appears from this study that asbestos-induced TNFα expression is regulated by NF-κB activation.

NF-κB is known to activate gene transcription by interacting with a specific DNA sequence in the promoter region of a target gene. Since this interaction is sequence specific, we rationalize that specific oligonucleotides having the same base sequence as that of the NF-κB recognition site may be

used to inhibit transcriptional activation. Using a ds oligonucleotide targeted to the TNFα binding site of NF-κB, we have shown that specific inhibition of TNFα gene expression can be achieved. The relative inability of the nonspecific control sequence to inhibit TNFα activation under identical stimulation conditions indicates the target binding specificity of NF-ON. These results are consistent with the SN50 finding and indicate that NF-κB is involved in asbestos-induced TNFα activation.

The dependence of ROS in asbestos-induced NF-κB activation and TNFα production is further demonstrated by the following observations: (1) sodium formate, a hydroxyl radical scavenger, inhibited asbestos-induced NF-κB activation and TNFα production; (2) mercaptosuccinic acid, an inhibitor of endogenous glutathione peroxidase, enhanced the TNFα induction, whereas diethyldithiocarbamic acid, an inhibitor of superoxide dismutase, decreased the effect. The major function of glutathione peroxidase is to protect the cell against oxidative damage by scavenging H_2O_2. The inhibition of this enzyme increases the cellular level of H_2O_2. The major function of superoxide dismutase is to scavenge superoxide radicals to generate H_2O_2. Thus, a decrease in superoxide dismutase activity reduces the H_2O_2 level. It should be noted that H_2O_2 is a major source of hydroxyl radicals, i.e., via the metal-catalyzed Fenton reaction (Halliwell and Gutterridge 1986). (3) The metal chelator deferoxamine reduced TNFα induction. Deferoxamine chelates metal ions, such as Fe(II) and Fe(III), making them less reactive toward H_2O_2 and thus decreasing their ability to generate hydroxyl radicals. Previous studies have shown that the treatment of asbestos with deferoxamine effectively removes Fe from the fiber surface and decreases its ability to generate hydroxyl radicals (Ghio *et al.* 1992a). Based on the above information, the following scheme is proposed for asbestos-induced TNFα production:

$$\text{Asbestos} \rightarrow \text{Macrophage} \rightarrow H_2O_2 \xrightarrow{\text{Fe(II)/Fe(III)}} \cdot OH \rightarrow \text{NF-}\kappa\text{B} \rightarrow \text{TNF}\alpha.$$

The dependence of iron on fiber reactivity suggests that fibers with high iron content (e.g., crocidolite) should be more biologically active than those with lower iron content (e.g., chrysotile). Indeed, previous studies have shown that crocidolite is a stronger inducer of TNFα (Simeonova and Luster 1995) and is also more toxic to the pulmonary system than chrysotile (Craighead and Mossman 1982). Titanium dioxide, a mineral oxide with minimal iron binding capacity (Ghio *et al.* 1992b), was unable to stimulate TNFα in the present study.

In conclusion, the present study demonstrates that crocidolite asbestos is able to activate TNFα production in macrophages and that this activation can be inhibited by NF-κB inhibitors and free radical scavengers. NF-κB activation via metal-mediated free radical reactions is an important step in asbestos-induced TNFα production.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant HL54291 and by the National Institute of Occupational Safety and Health.

REFERENCES

Baeuerle, P. A., and Henkel, T. (1994). Function and activation of NF-κB in the immune system. *Annual Review of Immunology* **12,** 141–179.

Beutler, B. (1992). Application of transcriptional and posttranscriptional reporter constructs to the analysis of tumor necrosis factor gene regulation. *American Journal of Medical Sciences* **303,** 129–133.

Collart, M. A., Baeuerle, P. A., and Vassal, P. (1990). Regulation of tumor necrosis factor alpha transcription in macrophages: Involvement of four κB-like motifs and of constitutive and inducible forms of NF-κB. *Molecular and Cellular Biology* **10,** 1498–506.

Craighead, J. E., and Mossman, B. T. (1982). The pathogenesis of asbestos-associated disease. *New England Journal of Medicine* **306,** 1446–1455.

Driscoll, K. E., Higgins, J. M. Leytart, M. J., and Crosby, L. L. (1990). Differential effects of mineral dusts on the in vitro activation of alveolar macrophage eicosanoid and cytokine release. *Toxicology In Vitro* **4,** 284–288.

Emgelmann, H., Holtmann, H., Brakebusch, C., Avni, Y. S., Sarov, I., Nophar, Y., Hadas, E., Leitmer, O., and Wallach, D. (1990). Antibodies to a soluble form of a tumor necrosis factor (TNF) receptor have TNF-like activity. *Journal of Biological Chemistry* **265,** 14497–14504.

Fiers, W. (1991). Tumor necrosis factor. Characterization at the molecular, cellular, and *in vivo* level. *FEBS Letter* **285,** 199–212.

Ghio, A. J., Zhang, J., and Piantadosi, C. A. (1992a). Generation of hydroxyl radical by crocidolite asbestos is proportional to surface [Fe^{3+}]. *Archives Biochemistry and Biophysics* **298,** 646–650.

Ghio, A. J., Kennedy, T. P., Whorton, A. R., Crumbliss, A. L., Hatch, G. E., and Hoidal, J. R. (1992b). Role of surface complexed iron in oxidant generation and lung inflammation induced by silicates. *American Journal of Physiology* **263,** 511–518.

Gossart, S., Cambon, C., Orfila, C., Seguelas, M. H., Lepert, J. C., Rami, J., Carre, P., and Pipy, B. (1996). Reactive oxygen intermediates as

regulators of TNF-α production in rat lung inflammation induced by silica. *Journal of Immunology* **156,** 1540–1548.

Halliwell, B., and Gutterridge, J. M. C. (1986). Oxygen free radicals and iron in relation to biology and medicine: Some problems and concepts. *Archives of Biochemistry and Biophysics* **246,** 501–514.

Isshiki, H., Akira, S., Tanabe, O., Nakajima, I., Shimamoto, T., Hirano, T., and Kishimoto, T. (1990). Constitutive and interleukin-1 (IL-1)-induced factors interact with the IL-1-responsive element in the IL-6 gene. *Molecular and Cellular Biology* **10,** 2757–2764.

Janssen, Y. M. W., Heintz, N. H., Marsh, J. B., Born, P. D. J., and Mossman, B. T. (1994). Induction of c-*fos* and c-*jun* pro-oncogenes in target cells of lung and pleura by carcinogenic fibers. *American Journal of Respiratory Cell and Molecular Biology* **11,** 522–530.

Janssen, Y. M. W., Barchowsky, A., Treadwell, M., Driscoll, K. E., and Mossman, B. T. (1995). Asbestos induces nuclear factor κB (NF-κB) DNA-binding activity and NF-κB-dependent gene expression in tracheal epithelial cells. *Proceedings of the National Academy of Sciences of the USA* **92,** 8458–8462.

Kamp, B. T., Graceffa, P., Pryor, W. A., and Weitzman, S. A. (1992). The role of free radicals in asbestos-induced diseases. *Free Radical Biology and Medicine* **12,** 293–315.

Kelly, J. (1990). Cytokines of the lung. *American Review of Respiratory Disease* **141,** 765–788.

Lenardo, M. J., and Baltimore, D. (1989). NF-κB: A pleiotropic mediator of inducible and tissue-specific gene control. *Cell* **58,** 227–229.

Li, X. Y., Lamb, D., and Donaldson, K. (1993). The production of TNF-α and IL-1 like activity by bronchoalveolar leukocytes after intratracheal instillation of crocidolite asbestos. *International Journal of Experimental Pathology* **74,** 403–410.

Lin, Y. Z., Yao, S. Y., Veach, R. A., Torgerson, T. R., and Hawiger, J. (1995). Inhibition of nuclear translocation of transcription factor NF-κB by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. *Journal of Biological Chemistry* **270,** 14255–14258.

Ljungman, A. G., Lindahl, M., and Tagesson, C. (1994). Asbestos fibers and manmade mineral fibers: Induction and release of tumor necrosis factor-α from rat alveolar macrophages. *Occupational and Environmental Medicine* **51,** 777–783.

Mossman, B. T., and Gee, J. B. L. (1989). Medical progress: Asbestos-related diseases. *New England Journal of Medicine* **320,** 1721–1730.

Perkins, R. C., Scheule, R. K., Hamilton, R., Gomes, G., Freidman, G., and Holian, A. (1993). Human alveolar macrophages asbestos exposure. *Experimental Lung Research* **19,** 55–65.

Piguet, P. F., Grau, G. E., and Vassalli, P. (1990a). Subcutaneous perfusion of tumor necrosis factor induces local proliferation of fibroblasts, capillaries, and epidermal cells, or massive tissue necrosis. *American Journal of Pathology* **136,** 103–110.

Piguet, P. F., Collart, M. A., Grau, G. E., Sappino, A. P., and Vassalli, P. (1990b). Requirement of tumor necrosis factor for development of silica-induced pulmonary fibrosis. *Nature* **344,** 245–247.

Rojanasakul, Y., Weissman, D. N., Shi, X., Castranova, V., Ma, J. K. H., and Liang, W. (1997). Antisense inhibition of silica-induced tumor necrosis factor in alveolar macrophages. *Journal of Biological Chemistry* **272,** 3910–3914.

Schapira, R. M., Ghio, A. J., Effros, R. M., Morrisey, J., Dawson, C. A., and Hacker, A. D. (1994). Hydroxyl radicals are formed in the rat lung following asbestos instillation *in vivo*. *American Journal of Respiratory Cell and Molecular Biology* **10,** 573–579.

Schreck, R., Rieber, P., and Baeuerle, P. A. (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *EMBO Journal* **10,** 2247–2258.

Siebenlist, U., Franzoso, G., and Brown, K. (1994). Structure, regulation, and function of NF-κB. *Annual Review of Cell Biology* **10,** 405–455.

Simeonova, P. P., and Luster, M. I. (1995). Iron and reactive oxygen species in the asbestos-induced tumor necrosis factor-α response from alveolar macrophages. *American Journal of Respiratory Cell and Molecular Biology* **12,** 676–683.

Simeonova, P. P., and Luster, M. I. (1996). Asbestos induction of nuclear transcription factors and interleukin 8 gene expression. *American Journal of Respiratory Cell and Molecular Biology* **15,** 787–795.

Yao, J., Mackman, N., Edgington, T. S., and Fan, S. T. (1997). Lipopolysaccharide induction of the tumor necrosis factor-alpha promoter in human monocytic cells. Regulation by Egr-1, c-jun, and NF-κB transcription factors. *Journal of Biological Chemistry* **272,** 17795–17801.

Ye, J., Zhang, X., and Dong, Z. (1996). Characterization of the human granulocytemacrophage colony-stimulating factor gene promoter: An AP1 complex and an Sp1-related complex transactivate the promoter activity that is suppressed by a YY1 complex. *Molecular and Cellular Biology* **16,** 157–167.

Zhang, Y., Lee, T. C., Guillemin, B., Yu, M. C., and Rom, W. N. (1993). Enhanced IL-1β and tumor necrosis factor-α release and messenger RNA expression in macrophages from idiopathic pulmonary fibrosis after asbestos exposure. *Journal of Immunology* **150,** 4188–4196.