Asbestos exposure upregulates the adhesion of pleural leukocytes to pleural mesothelial cells via VCAM-1

NONGHOON CHOE,1 JUN ZHANG,1 AKITAKA IWAGAKI,2 SHOGO TANAKA,1 DAVID R. HEMENWAY,3 AND ELLIOTT KAGAN1

1Department of Pathology and 2Department of Medicine, F. Edward Hébert School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799; and 3Department of Civil and Environmental Engineering, University of Vermont, Burlington, Vermont 05405

Asbestos exposure upregulates the adhesion of pleural leukocytes to pleural mesothelial cells via VCAM-1. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L292–L300, 1999.—This study was designed to assess the effects of in vitro and in vivo asbestos exposure on the adhesion of rat pleural leukocytes (RPLs) labeled with the fluorochrome calcine AM to rat pleural mesothelial cells (RPMCs). Exposure of RPMCs for 24 h to either crocidolite or chrysotile fibers (1.25–10 µg/cm²) increased the adhesion of RPLs to RPMCs in a dose-dependent fashion, an effect that was potentiated by interleukin-1β. These findings were not observed with nonfibrogenic carbonyl iron particles. Crocidolite and chrysotile plus interleukin-1β also upregulated vascular cell adhesion molecule-1 (VCAM-1) mRNA and protein expression in RPMCs, and the binding of RPL to asbestos-treated RPMCs was abrogated by anti-VCAM-1 antibody. PRLs exposed by intermittent inhalation to crocidolite for 2 wk manifested significantly greater binding to RPMCs than did RPLs from sham-exposed animals. The ability of asbestos fibers to upregulate RPL adhesion to RPMCs may play a role in the induction and/or potentiation of asbestos-induced pleural injury.

crocidolite; chrysotile; intercellular adhesion molecule-1; vascular cell adhesion molecule-1
that both crocidolite and chrysotile exposure in vitro upregulated the adhesion of unexposed rat pleural leukocytes (RPLs) to RPMCs that were derived from unexposed animals. This effect was potentiated by interleukin (IL)-1β but was abrogated by anti-VCAM-1 antibody. Furthermore, both types of asbestos in combination with IL-1β enhanced VCAM-1 mRNA and protein expression in RPMCs. We also showed that RPLs obtained from crocidolite-exposed rats demonstrated increased adhesiveness to both unexposed and asbestos-exposed RPMCs.

**METHODS**

Mineral samples. For in vitro exposures, crocidolite and chrysotile samples were obtained from the National Institute of Environmental Health Sciences (Research Triangle Park, NC). These samples were previously characterized (3) and were shown to be fibrogenic to rats in vivo (28) and to induce pleural inflammation in a rat inhalation model (5). Union Internationale Contre le Cancer (UICC) crocidolite samples were utilized for in vivo exposures and were a generous gift from Dr. David Rees (National Centre for Occupational Health, Johannesburg, South Africa). These also were previously characterized (33) and shown to induce interstitial pulmonary fibrosis in rats (12). Carbonyl iron particles (size range 1–10 μm; average particle size 4.5–5.2 μm) purchased from Sigma (St. Louis, MO) were used as a control particulate. These particles were shown previously to be nonfibrogenic in a rat inhalation model (25). All of the mineral samples comprised a significant respirable fraction.

Reagents. DMEM, RPMI 1640 medium, Hanks' balanced salt solution, PBS, L-glutamine, fetal calf serum, penicillin, streptomycin, and Fungizone were supplied by Biofluids (Rockville, MD). Cell culture freezing medium-DMSO, trypsin-EDTA, Moloney murine leukemia virus reverse transcriptase, oligo(dT) primer, deoxyribonucleotide triphosphate (dNTP), dithiothreitol, and Trizol were obtained from Gibco BRL (Life Technologies, Gaithersburg, MD). Mouse monoclonal IgG1 anti-rat ICAM-1 antibody, recombinant human IL-1β, and recombinant mouse tumor necrosis factor (TNF)-α were supplied by Genzyme Diagnostics (Cambridge, MA). Normal mouse IgG1, κ (control) was obtained from Southern Biotechnology Associates (Birmingham, AL). Calcium AM was obtained from Molecular Probes (Eugene, OR), and mouse monoclonal IgG2a, κ anti-rat VCAM-1 was supplied by BabCO (Richmond, CA). Aminoguanidine and BSA were purchased from Sigma. Peroxidase-labeled goat antirabbit IgG antibody (rat serum absorbed), 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate, and TMB stop solution were purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Taq polymerase was obtained from Perkin-Elmer (Norwalk, CT). Diff-Quik stain was purchased from Baxter Healthcare (McGaw Park, IL).

Animals and inhalation exposure regimen. For the inhalation exposures, two groups of male Fischer 344 rats were placed in whole-body inhalation chambers and exposed to either UICC crocidolite asbestos fibers or filtered room air (sham-exposed group). The rats in both groups were comparably matched for age (41 days old) and size (mean weight for each group 116 g) before inhalation exposure. With an established inhalation exposure protocol (5, 28), both groups were exposed for 6 h/day on 5 days/wk over 2 wk. The rats were killed 10 days after the cessation of exposure by the intraperitoneal administration of pentobarbital sodium (50 mg/kg) followed by exsanguination via the abdominal aorta.

Total fiber mass aerosol concentrations were measured on membrane filters with 25-mm conductive cowl filter holders (Nudapore, Carmel, CA) equipped with 0.8-μm-pore filters (DM-800, Gelman Sciences, Ann Arbor, MI) by standard gravimetric analysis. All total mass measurements were done on a daily basis at the nose level of the exposed animals. The asbestos aerosols were generated with a modified Timbrell dust generator (BGI, Waltham, MA). Aerodynamic particle-size distributions were measured with a Sierra eight-stage model 210 cascade impactor (Andersen Instruments, Atlanta, GA) throughout the exposure period. The time-weighted exposure concentration for crocidolite was 10.52 ± 2.31 (SE) mg/m³. These exposure levels were comparable with historic asbestos dust concentrations recorded in the workplace environment of asbestos mines and mills (34). Analysis of all impactor data for crocidolite indicated the following: geometric mean diameter = 0.71 μm and geometric SD = 2.38. The aerosols were very similar in aerodynamic size distributions and were highly respirable.

RPMC cultures and treatment protocols. RPMCs were obtained in primary culture from unexposed Fischer 344 rats as previously described (22). The cells were seeded into 75-cm² tissue culture flasks (Corning, Wexford, PA). The cultures then were maintained in a humidified environment containing 5% CO₂ at 37°C in DMEM supplemented with 10% fetal bovine serum, 100 µg/ml of streptomycin, 100 U/ml of penicillin, 2.5 µg/ml of Fungizone, and 2 mM L-glutamine (“supplemented DMEM”). The present study utilized cultures from passages 13 to 16. The cultured cells displayed the typical characteristics of mesothelial cells: a polygonal, “cobblestone” morphological appearance, delicate surface microvilli, and junctional complexes on transmission electron microscopy, and positive immunocytochemical reactivity for 40- to 55-kDa cytokeratins and vimentin (22). The cells were cultured until confluent in DMEM plus 10% fetal bovine serum in 96-well culture plates (Costar) at 37°C.

For assessment of the effects of particulate challenge on RPMCs, parallel experiments were performed on unstimulated cultures as well as on RPMCs stimulated with either cytokines alone, particulates alone, or cytokines plus particulates. Accordingly, RPMCs were cultured for 2–24 h in 5% CO₂ at 37°C in supplemented DMEM. Cytokine stimulation comprised the addition of either recombinant human IL-1β (20 ng/ml) or recombinant mouse TNF-α (10 ng/ml) to cultured RPMCs. For some experiments, nonfibrogenic carbonyl iron particles were used as a control particulate. Before use, all particulate samples (carbonyl iron particles, crocidolite fibers, and chrysotile fibers) were autoclaved, suspended in supplemented DMEM, and dispersed by repeated passage through a 22-gauge needle. Additionally, asbestos fibers were sonicated three times for 15 s. Thereafter, particulates were added to cultures at concentrations ranging from 1.25 to 10.0 µg/cm². Cell viability was measured by lactate dehydrogenase (LDH) activity in conditioned medium with a commercial LDH kit (Sigma).

Assessment of the role of nitric oxide formation. To evaluate the effects of nitric oxide (·NO) on the adhesion of RPLs to RPMCs and on adhesion molecule expression, aminoguanidine, a specific inhibitor of the inducible form of ·NO synthase (iNOS), was added to cultured RPMCs (300 µM) in the presence and absence of particulates or cytokines. Conditioned medium was obtained from 24-h mesothelial cell cultures, and ·NO formation was analyzed after nitrate reductase treatment of conditioned medium by measuring the ·NO oxidation product nitrite (NO₂⁻) via the Griess reaction (4).
Collection of RPLs. RPLs were obtained by pleural lavage from either unexposed or asbestos-exposed Fischer 344 rats. Before lavage, the rats were exsanguinated after intraperitoneal anesthesia with pentobarbital sodium (50 mg/kg). The technique of pleural lavage was performed as previously described (5). Differential cell counts of RPL populations were performed on cytospin preparations stained with Diff-Quik.

Cell adhesion assays. Cell adhesion assays were performed with a modification of a previously published procedure (2) with RPLs (from either unexposed or asbestos-exposed rats) labeled with the AM form of the cytoplasmic fluorescent dye calcein (calcein AM). Cells labeled with calcein AM demonstrated a high level of intracellular retention of the dye, and its low-level release after cytoplasmic incorporation excludes possible labeling of the underlying monolayer, thereby enabling its use in cell-cell adhesion assays (2).

Briefly, freshly isolated RPLs (2 × 10⁶ cells/ml) were resuspended in 1% BSA-PBS and incubated with 20 µM calcein AM for 30 min at 37°C on a rocking platform. Unincorporated dye was removed after three washes with PBS, and the leukocytes were resuspended at a concentration of 2 × 10⁶ cells/ml in warm RPMI 1640 medium. Thereafter, unstimulated or stimulated RPMCs were washed three times with warm DMEM, and 100 µl of labeled leukocytes were added to each well. In some experiments, to evaluate the role of ICAM-1 and VCAM-1 in this adhesion process, calcein AM-labeled RPLs were added to the culture wells at the same time as 10 µg/ml of either anti-rat ICAM-1, anti-rat VCAM-1, or normal mouse IgG1, κ (which was employed as a nonspecific antibody control). In another set of experiments, the RPMCs were fixed in 2% paraformaldehyde for 20 min before addition of the RPLs. In all instances, after the RPLs were coincubated with the RPMCs for 30 min at 37°C, the plates were carefully washed three times with warm PBS (150 µl/well) to remove nonadherent leukocytes. The remaining fluorescence in the well was measured and is expressed as the relative fluorescence intensity as determined with a luminescence microplate reader (Perkin-Elmer model LS50B) at 485-nm excitation and 530-nm emission, with a sensitivity setting of 3. Adhesion assays utilizing increasing numbers of calcein AM-labeled RPLs deposited on unstimulated RPMCs demonstrated a linear relationship between the relative fluorescence intensity and the number of calcein AM-labeled RPLs (results not shown).

Measurement of ICAM-1 and VCAM-1 gene expression. For RT-PCR studies, confluent parietal pleural mesothelial cell cultures in 25-cm² flasks (Costar) were incubated in the presence (5 µg/cm²) and absence of crocidolite or chrysotile asbestos fibers and in the presence (20 ng/ml) and absence of IL-1β for 2, 4, 8, or 12 h at 37°C in DMEM in 5% CO₂. Total cellular RNA was extracted by the guanidinium thiocyanate method (6). RNA yield and integrity were assessed by ultraviolet spectrophotometry and ethidium bromide staining. One microgram of RNA from each sample was heat denatured at 65°C for 15 min. After being cooled on ice, mRNA transcripts were reverse transcribed into first-strand cDNA in a 20-µl RT mixture containing 400 U of Moloney murine leukemia virus reverse transcriptase, 0.5 µg of oligo(dT) primer, 10 nmol of each dNTP, and 10 mmol of dithiothreitol. The transcription reaction was allowed to proceed at 37°C for 60 min and was then terminated by heating the sample to 70°C for 10 min. Expression of the gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was evaluated concurrently with ICAM-1 and VCAM-1 mRNAs as an internal control gene. PCR primers were designed from published sequences for rat ICAM-1 (20) and rat VCAM-1 (40) deposited in the GenBank database (accession no. D00913 for ICAM-1 and accession no. X63722 for VCAM-1) and were found by using a primer analysis software program (Primer 3, Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA).

RESULTS

Cellular composition of RPLs. As shown in Table 1, RPLs comprised a heterogeneous cell population that was composed predominantly of macrophages. No significant differences were observed between the composition of RPLs obtained from unexposed rats and those obtained from crocidolite-exposed rats.

Effects of in vitro asbestos exposure on the adhesion of RPLs to RPMCs. When RPLs from unexposed rats were deposited on 24-h cultures from either unstimulated or asbestos-exposed RPMCs, distinct differences were noted (Fig. 1). Notably, the adherence of RPLs to RPMCs was 209 bp, whereas that of VCAM-1 was 235 bp and that of GAPDH was 529 bp as confirmed by the patterns of fragmentation cutting with restriction enzymes. The amplification mixture (50 µl) contained 5 µl of cDNA, 2.5 U of Taq polymerase, 100 pmol of sense and antisense primers, 10 nmol of each dNTP, and 2.5 mmol of MgCl₂. PCR amplification was carried out by denaturation at 94°C for 1 min, oligonucleotide annealing at 60°C for 2 min, and primary extension at 72°C for 2 min. Amplification for GAPDH was performed over 18–20 cycles, whereas 27 cycles of amplification were used for ICAM-1 and VCAM-1. Reactions were electrophoresed in 2% agarose gels containing 1 µg/ml of ethidium bromide in Tris-acetate-EDTA buffer to visualize the PCR products. Amplified cDNA bands were visualized under ultraviolet illumination and evaluated by densitometry with an Eagle Eye II still video system (Stratagene, La Jolla, CA). The yield of the amplified product was tested and found to be linear for the amount of input RNA and PCR cycle number.

Whole cell ELISA for ICAM-1 and VCAM-1 expression. Surface ICAM-1 and VCAM-1 expression on RPMCs was quantified by an adaptation of a published ELISA method (32). RPMCs were cultured with supplemented DMEM in 96-well plates at 37°C in 5% CO₂ until confluent, after which the cells were stimulated with and without particulates and in the presence and absence of cytokines. After 24 h, the cells were washed with PBS and fixed in 2% glutaraldehyde for 5 min. The plates were washed three times in PBS, after which a routine ELISA assay was performed with 10 µg/ml of monoclonal anti-rat ICAM-1 or anti-rat VCAM-1 primary antibody, peroxidase-labeled mouse IgG as the secondary antibody, and TMB as a substrate. Color development was measured with a microplate reader at 650 nm for ICAM-1 and after color development was stopped with 0.18 M H₂SO₄ at 450 nm for VCAM-1.

Statistics. The data in Table 1 are means ± SE of 3–10 rats, whereas the data in Figs. 1–6 are expressed as means ± SE of 3–4 independent experiments. Each experiment employed 5–6 well replicates. To determine whether there were any differences between exposure groups, data were analyzed by analysis of variance with Bartlett’s test. Individual comparisons between different exposure groups were made with Student’s unpaired t-test. Values of P < 0.05 were considered significant.
RPMCs was significantly increased after both crocidolite and chrysotile asbestos exposure compared with the adhesion of RPLs to unstimulated mesothelial cell cultures. Moreover, asbestos exposure upregulated the binding of RPLs to RPMCs in a dose-dependent fashion, with maximal adhesiveness evident at fiber concentrations of 10 µg/cm². To determine whether the ability to upregulate the adhesiveness of RPLs to RPMCs was peculiar to asbestos fibers or whether it was a nonspecific effect of particulate challenge, parallel adhesion studies were performed on cultured RPMCs treated with nonfibrogenic carbonyl iron particles at concentrations of 1.25–10 µg/cm² (Fig. 1). Although carbonyl iron treatment of mesothelial cells did augment the binding of RPLs to RPMCs, no dose-response relationship was noted. Furthermore, both crocidolite and chrysotile fibers at doses of 5–10 µg/cm² induced significantly greater pleural leukocyte adhesion than did carbonyl iron. When adhesion assays were performed on RPMCs fixed in 2% paraformaldehyde before the addition of RPLs, leukocyte adhesion was largely abrogated (results not shown), indicating that the adhesion process required the presence of intact, viable mesothelial cells.

To assess whether the effects of asbestos exposure might be augmented by cytokine stimulation, adhesion assays were performed on RPMCs cultured with and without added asbestos fibers in the presence and absence of TNF-α (10 ng/ml) or IL-1β (20 ng/ml). Because both crocidolite and chrysotile fibers induced unacceptable cytotoxicity (as determined by measurement of LDH) at a dose of 10 µg/cm² in cytokine-containing cultures, an asbestos fiber concentration of 5 µg/cm² was employed in all subsequent experiments. As illustrated in Fig. 2, cultures stimulated with TNF-α in the absence of asbestos fibers increased the binding of RPLs to RPMCs by ~44%. However, the effects of asbestos and TNF-α were additive because the adhesion of RPLs to RPMCs stimulated with TNF-α was increased by ~70 and 103% after crocidolite and chrysotile exposure, respectively (Fig. 2). The addition of IL-1β to asbestos-containing cultures induced even greater leukocyte attachment to mesothelial cells because adhesion was upregulated by ~118 and 134% after crocidolite and chrysotile challenge, respectively (Fig. 2). Accordingly, all subsequent experiments assessing the effects of cytokine stimulation on RPMCs were performed with IL-1β exclusively.

Effects of NO formation on the adhesion of RPLs to RPMCs. Choe et al. (4) have previously shown that asbestos exposure upregulates the formation of NO by RPMCs. Because NO is known to be anti-inflammatory and to decrease leukocyte adhesiveness to endothelium (21, 27), additional studies were performed to assess the effects of NO synthesis on the adhesion of RPLs to RPMCs. When aminoguanidine (300 µM) was added to either unstimulated mesothelial cultures or RPMCs stimulated with asbestos fibers, it induced a significant increase in the adhesion of RPLs to RPMCs (Fig. 3). In contrast, when added to RPMCs stimulated with IL-1β (20 ng/ml) and either crocidolite or chrysotile fibers (5 µg/cm²), aminoguanidine had no noticeable effect on

Table 1. Cellular composition of RPLs obtained from unexposed and crocidolite-exposed rats

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Proportion of Total Population of RPLs, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unexposed</td>
</tr>
<tr>
<td>Macrophages</td>
<td>74.8 ± 0.6</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>10.7 ± 0.5</td>
</tr>
<tr>
<td>Mast cells</td>
<td>10.8 ± 0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE of 10 rats/group for unexposed rats and 3 rats/group for crocidolite-exposed rats. RPMCs, rat pleural leukocytes.
the adhesion of RPLs to RPMCs despite the fact that NO₂ formation was abolished by the presence of the iNOS inhibitor (Fig. 4).

ICAM-1 and VCAM-1 studies. Additional studies were performed to determine the effects of anti-rat VCAM-1 and anti-rat ICAM-1 antibodies on the adhesion of RPLs to RPMCs. As seen in Fig. 5, the presence of anti-VCAM-1 significantly inhibited pleural leukocyte attachment in cultures challenged with either crocidolite or chrysotile fibers (5 µg/cm²) as well as in cultures stimulated with IL-1β (20 ng/ml). However, anti-VCAM-1 had no appreciable effect on the adhesion of RPLs to unstimulated RPMCs. In contrast, anti-ICAM-1 antibody did not suppress the attachment of RPLs to RPMCs stimulated with either IL-1β or asbestos fibers (results not shown). The specificity of the anti-VCAM-1 antibody further was confirmed by the fact that normal mouse IgG1 had no effect on pleural leukocyte adhesiveness to RPMCs (Fig. 5).

When RPMCs were cultured for 2–12 h in the absence of either asbestos fibers or IL-1β, some constitutive expression of both ICAM-1 and VCAM-1 mRNAs was detected that did not vary with the period of culture. However, when the results were normalized to GAPDH mRNA expression, the addition of either asbestos fibers (5 µg/cm²) or IL-1β (20 ng/ml) to mesothelial cultures induced an early increase in VCAM-1 mRNA expression at 2 h, an effect that peaked at 8 h and then declined to near baseline levels by 12 h. The addition of either chrysotile or crocidolite fibers appeared to enhance the capacity of IL-1β to upregulate VCAM-1 mRNA expression (Fig. 6). Although IL-1β also induced a modest increase in ICAM-1 mRNA expression, this effect was not enhanced by the action of either crocidolite or chrysotile fibers (Fig. 6).
Unstimulated RPMCs demonstrated strong constitutive expression of ICAM-1 and weaker constitutive expression of VCAM-1 proteins in 24-h cultures, findings that were not significantly altered by challenge with either chrysotile or crocidolite fibers (5 µg/cm²). When, however, the cultures were stimulated with IL-1β (20 ng/ml), VCAM-1 protein expression was upregulated by ~357%, an effect that was potentiated by the addition of either chrysotile or crocidolite fibers (Fig. 7). In contrast, IL-1β induced only a modest (~33%) increase in ICAM-1 protein expression that was not potentiated by asbestos fibers (Fig. 7). Carbonyl iron particles (5 µg/cm²) had no significant effect on either VCAM-1 or ICAM-1 protein expression by RPMCs (results not shown).

Effect of crocidolite inhalation on the adhesion of RPLs to RPMCs. As was noted with RPLs from unexposed rats (Figs. 1 and 2), the adhesion of RPLs from sham-exposed rats to cultured RPMCs was upregulated when the mesothelial cells were challenged with either 5 µg/cm² of asbestos fibers or 20 ng/ml of IL-1β (Fig. 7). However, when compared with RPLs from sham-exposed rats, the pleural leukocytes from crocidolite-exposed rats demonstrated significantly greater

Fig. 6. IL-1β and asbestos fibers upregulated VCAM-1 mRNA expression by RPMCs. RPMCs were cultured for 8 h without asbestos or IL-1β or with IL-1β alone (20 ng/ml), asbestos fibers alone (5 µg/cm²), or IL-1β plus asbestos fibers. A: RT-PCR products for VCAM-1, intercellular adhesion molecule (ICAM)-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on ethidium bromide-stained gel. Lane 1, unstimulated RPMCs; lane 2, IL-1β; lane 3, crocidolite; lane 4, chrysotile; lane 5, IL-1β plus crocidolite; lane 6, IL-1β plus chrysotile. These results are representative of 3 separate experiments. B and C: VCAM-1 and ICAM-1 mRNA expression, respectively, normalized to GAPDH mRNA expression in RPMCs cultured for 8 h. These results are representative of 3 separate experiments.

Fig. 7. Potentiating effect of crocidolite and chrysotile asbestos fibers on IL-1β-induced VCAM-1 protein expression by RPMCs (A). No such effect was seen on ICAM-1 protein expression (B). RPMCs were cultured for 24 h without asbestos or IL-1β (dashed line) or with IL-1β alone (20 ng/ml), asbestos fibers alone (5 µg/cm²), or IL-1β plus asbestos fibers before whole cell ELISA assays for VCAM-1 and ICAM-1 were performed. Values are means ± SE; n = 3 rats/group. *P < 0.0001 compared with unstimulated RPMCs and with crocidolite or chrysotile alone. †P < 0.05 compared with IL-1β alone. §P < 0.01 compared with unstimulated RPMCs.
attachment to both unstimulated RPMCs and RPMCs stimulated with either crocidolite asbestos fibers or IL-1β (Fig. 8).

DISCUSSION

Asbestos fibers are known to translocate to the pleural space and to be phagocytosed by pleural mesothelial cells after the inhalation or intrapulmonary instillation of asbestos (5, 8, 39). Because in vivo asbestos challenge has also been shown to induce a protracted inflammatory response (composed predominantly of macrophages) within the pleural space (5, 31, 37), the present study was undertaken to determine whether asbestos exposure might stimulate the adhesion of pleural leukocytes to pleural mesothelial cells. We found that the addition of either crocidolite or chrysotile fibers to cultured RPMCs enhanced the attachment of RPLs to the mesothelial cells in a dose-dependent fashion. Although we observed that the effect of chrysotile was greater than that of crocidolite when based on mass measurements, our findings were inconclusive as to whether there truly were differences between fiber types because mass measurements do not indicate actual fiber numbers. In this regard, it should be noted that chrysotile has many more fibers than crocidolite per given mass. Nevertheless, when comparisons were made between the effects of both types of asbestos and those of carbonyl iron particles (which are nonfibrogenic and noncarcinogenic), distinct differences were noted. Although carbonyl iron treatment of RPMCs, no dose-response relationship was noted. Furthermore, both crocidolite and chrysotile fibers at doses of 5–10 µg/cm² induced significantly greater pleural leukocyte adhesion than did carbonyl iron. It is of interest in this regard that another study (38) demonstrated that the attachment of human neutrophils to cultured porcine endothelial cells was increased after asbestos exposure.

Because asbestos inhalation has been shown to up-regulate pleural macrophage cytokine secretion (5), we evaluated the effects of cytokine stimulation of RPMCs on pleural leukocyte adhesion and noted that both IL-1β and TNF-α stimulated the attachment of RPLs to RPMCs. These observations were consistent with those of other investigators (16, 23, 43) who demonstrated that leukocyte adhesiveness to human peritoneal mesothelial cells could be modulated by cytokine administration. The present study has also shown that the ability of IL-1β to stimulate the attachment of RPLs to RPMCs was significantly enhanced by amphibole as well as by serpentine asbestos fibers. Additionally, we observed that pleural leukocyte adhesiveness was upregulated as a consequence of in vivo asbestos exposure because pleural leukocytes from crocidolite-exposed rats demonstrated significantly greater attachment to RPMCs than RPLs from sham-exposed animals. Although there is no obvious explanation for this finding, it may relate to excessive and persistent cytokine secretion into the pleural space by pleural macrophages after asbestos inhalation (5).

Studies were performed to determine which adhesion molecule(s) was mediating the attachment process. It is known that cultured peritoneal mesothelial cells express ICAM-1 and VCAM-1 constitutively and that the expression of these adhesion molecules can be enhanced by IL-1β or TNF-α (16, 23, 43). We observed that whereas both chrysotile and crocidolite fibers potentiated the ability of IL-1β to upregulate VCAM-1 mRNA and protein expression by RPMCs, this effect was not induced by carbonyl iron particles. Furthermore, when mesothelial cell cultures were costimulated with IL-1β and asbestos fibers, the adhesion of RPLs to RPMCs was significantly abrogated by the addition of anti-rat VCAM-1 antibody to the cultures. In contrast, asbestos fibers did not upregulate ICAM-1 expression nor did anti-rat ICAM-1 antibody decrease the attachment of RPLs to RPMCs. Collectively, these observations underscore the importance of VCAM-1, but not of ICAM-1, in the binding of RPLs to RPMCs.

It is well recognized that NO formation can inhibit leukocyte adhesion to vascular endothelium via an effect on adhesion molecule expression (1, 27). Because Choe and colleagues (4, 5) and Tanaka et al. (37) have previously shown that both in vitro and in vivo asbestos exposure can induce the production of NO and other reactive nitrogen species in pleural cells, in the present study, we also assessed the effect of iNOS inhibition on the adhesion of RPLs to RPMCs in the context of asbestos and cytokine stimulation. Mesothelial cell cultures challenged with IL-1β plus either crocidolite or chrysotile fibers generated significant quantities of NO (expressed as its oxidation product NO₂⁻), and when 300 µM aminoguanidine was added to those cultures, NO production was largely abolished. Although aminoguanidine administration significantly enhanced the attachment of RPLs to RPMCs stimu-
lated with IL-1β alone, it did not noticeably affect pleural leukocyte adhesion to RPMCs stimulated with IL-1β plus either chrysotile or crocidolite fibers. The latter finding was unexpected and suggested that NO formation appeared to play only a minor role in modulating the adhesiveness of RPLs to RPMCs costimulated with asbestos and IL-1β. Although this study did not address the mechanisms whereby asbestos fibers stimulated VCAM-1 expression, it is likely that complex transduction pathways may be involved, especially the protein kinase (PK) C and nuclear factor (NF)-κB signaling cascades. Prior studies (26, 41) have demonstrated that TNF-α-induced upregulation of VCAM-1 expression in endothelial and renal tubular epithelial cells was linked to PKC activation. In this regard, it is significant that PKC has been shown to play a critical role in asbestos-induced signaling pathways because PKC inhibitors have been shown to suppress the ability of asbestos fibers to upregulate reactive oxygen species formation in rat alveolar macrophages and c-fos mRNA expression in RPMCs (9, 24). There is also evidence that asbestos exposure can induce NF-κB activation as evidenced by the fact that in vitro crocidolite asbestos exposure has been demonstrated to cause significant increases in nuclear protein complexes binding the NF-κB consensus DNA sequence in RPMCs, rat lung epithelial cells, and A549 cells (15, 35). Furthermore, both chrysotile and crocidolite inhalation have been shown to enhance immunoreactive expression of the p65 subunit of NF-κB within rat airway epithelial cells (15). These findings may have relevance to our observations because the induction of VCAM-1 expression by cytokines in rat cardiac myocytes (13) and murine glomerular mesangial cells (19) has been shown to involve NF-κB activation and the interaction of NF-κB with the proximal VCAM-1 promoter (19).

In summary, we have demonstrated that both in vitro and in vivo asbestos exposure upregulated the attachment of RPLs to RPMCs and that VCAM-1 appeared to be important for the adhesion process. Because neutrophils and macrophages constitute a potent source of toxic reactive oxygen and nitrogen species, the formation of which is increased after asbestos exposure (24, 29, 37), it is conceivable that increased pleural leukocyte adhesiveness may play a role in initiating and/or potentiating asbestos-induced injury to the pleural mesothelium.

This study was supported by National Heart, Lung, and Blood Institute Grant HL-54196.

Address for reprint requests and other correspondence: E. Kagan, Dept. of Pathology, Uniformed Services Univ. of the Health Sciences, F. Edward Hébert School of Medicine, 4301 Jones Bridge Rd., Bethesda, MD 20814-4799 (E-mail: ekagan@usuhs.mil).

Received 7 January 1999; accepted in final form 29 March 1999.

REFERENCES


23. Liberek, T., N. Topley, W. Luttmann, and J. D. Williams. Adherence of neutrophils to peritoneal mesothelial cells:


