Silica-induced chemokine expression in alveolar type II cells is mediated by TNF-\(\alpha\)

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Barrett, Edward G., Carl Johnston, Günter Oberdörster, and Jacob N. Finkelstein. Silica-induced chemokine expression in alveolar type II cells is mediated by TNF-\(\alpha\). Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L1110–L1119, 1998.—Recent evidence has suggested that epithelial cells may contribute to the inflammatory response in the lung after exposure to crystalline silica through the production of and response to specific growth factors, chemokines, and cytokines. However, the exact cellular and molecular responses of epithelial cells to silica exposure remains unclear. Using a murine alveolar type II cell line (murine lung epithelial (MLE)-15 cell line), we measured the early changes in various cytokine and chemokine mRNA species after exposure of the cells to 4–35 \(\mu\)g/cm\(^2\) of silica (cristobalite), interferon (IFN)-\(\gamma\), tumor necrosis factor (TNF)-\(\alpha\), and lipopolysaccharide (LPS) alone or in combination. Total mRNA was isolated and assayed with an RNase protection assay after 6 and 24 h of exposure. Cristobalite exposure alone led to an increase in monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-2, and regulated on activation normal T cells expressed and secreted (RANTES) mRNAs. Treatment with IFN-\(\gamma\) alone increased MCP-1 mRNA levels. Treatment with TNF-\(\alpha\) or LPS alone led to an increase in MCP-1 and MIP-2 mRNA. The combination of cristobalite plus TNF-\(\alpha\) led to an additive increase in MCP-1 and MIP-2, whereas cristobalite plus IFN-\(\gamma\) or LPS had a synergistic effect. We also found with a TNF-\(\alpha\)-neutralizing antibody that TNF-\(\alpha\) plays a major role in mediating the type II cell chemokine response to cristobalite exposure. The results indicate that the cristobalite-induced chemokine response in the lung epithelium is mediated in part by TNF-\(\alpha\) and can be enhanced by macrophage- and lymphocyte-derived inflammatory mediators in an additive and synergistic fashion.

Airway epithelium; interferon-\(\gamma\); lipopolysaccharide; monocytic chemotactic protein-1; macrophage inflammatory protein-2; regulated on activation normal T cells expressed and secreted; tumor necrosis factor-\(\alpha\).

INHALATION OF CRYSTALLINE SILICA can lead to the development of pulmonary inflammation and fibrosis (16). Studies (15, 16, 22) examining the early inflammatory response in the lung after silica exposure have typically focused on the contribution of lymphocytes, macrophages, neutrophils, and their products. However, an additional or alternate means by which silica may initiate an inflammatory response is through direct interaction with the pulmonary epithelium. Type II cells are uniquely situated at the interface between the alveolar air space of the lung and the capillary circulation, allowing them to respond to airborne stimuli and interact with various cell types such as endothelial and mesenchymal cells, alveolar macrophages, and other inflammatory cells (26). Type II cells are known to play an important role in lung injury through the synthesis and secretion of pulmonary surfactant and by acting as the stem cell for the replacement of damaged type I epithelial cells (26). Recently, using an established cell line and primary rat type II cells in an in vitro silica exposure model, Driscoll and co-workers (13) observed that type II cells respond directly to silica (\(\alpha\)-quartz) by increasing expression of the chemokine mRNA species macrophage inflammatory protein (MIP)-2 and cytokine-induced neutrophil chemoattractant (CINC). More importantly, the same study also showed the localization of MIP-2 mRNA expression in type II cells after in vivo silica exposure. Thus it is likely that type II cells play a key role in inflammatory cell recruitment through the release of specific chemokines; however, the exact molecular and cellular events leading to the type II cell response remain unclear.

The release of soluble inflammatory mediators such as tumor necrosis factor (TNF)-\(\alpha\) and interferon (IFN)-\(\gamma\) from silica-activated alveolar macrophages and lymphocytes will likely influence the nature of the type II cell chemokine response to silica in vivo. Increased expression of chemokines in the lung has been linked to the release of the cytokines TNF-\(\alpha\) and interleukin (IL)-1 from macrophages, which act in an autocrine and/or paracrine fashion to stimulate the release of chemokines from cells including macrophages, epithelial cells, and fibroblasts (12, 13, 23, 30, 31). Exposure of the lung to silica also leads to an influx of T lymphocytes, which are a source of IFN-\(\gamma\) production (21, 33). A study (24) that used cocultured primary rat type II cells and alveolar macrophages observed that cell-cell interactions modified the direct type II cell-particle response to coal dust as measured by the synthesis and assembly of extracellular matrix proteins. Various stimuli are known to cause type II cells to produce inflammatory mediators such as IFN, MIP-2, CINC, IL-6, IL-8, and monocytic chemotactic protein (MCP)-1 (8, 13, 12, 17, 23, 30, 31). Thus we hypothesize that the in vivo response of type II cells to silica exposure will depend on direct particle-cell interactions and on paracrine stimulation by other silica-activated cells such as macrophages and lymphocytes. In the context of the studies described here, direct particle-cell interactions are defined as any particle effect, such as formation of oxidants or binding to the cell, that involves only one cell type (i.e., type II cell).
In the present study, we utilized an in vitro model to examine the mechanisms through which crystalline exposure in the lung increases the expression of specific cytokines and chemokines in type II cells. We conducted studies examining both the direct particle-type II cell interactions and the effects of crystalline-induced macrophage- and lymphocyte-derived inflammatory mediators on type II cell cytokine and chemokine mRNA expression. We also examined whether these mediators or other inflammatory stimuli such as LPS had a synergistic or additive effect on epithelial chemokine expression when combined with silica exposure. In addition, using an anti-TNF-α antibody, we investigated the role of TNF-α in particle-induced alterations in type II cell chemokine expression. Our results support the hypothesis that silica-induced alterations in chemokine expression in type II cells are mediated by particle-cell interactions and paracrine stimulation by other silica-activated cells involving, at least in part, the production of TNF-α.

**MATERIALS AND METHODS**

**Particles and reagents.** Cristobalite, a form of crystalline silica (a gift from Dr. D. Hemenway, University of Vermont, Burlington), was size selected with a cascade cyclone sampler (series 280 Cyclade, Sierra Instruments, Carmel Valley, CA) with an ~1.2-µm particle diameter cutoff. The average particle diameter was measured with a scanning electron microscope equipped with an imaging software package where 50 particles were measured and the average was calculated. Mean particle size was 0.64 ± 0.05 µm, and particle size range was 0.08–1.5 µm. Silica was baked at 180°C for 16 h to inactivate any possible contaminating endotoxin. Silica particle suspensions were sonicated for 30 s before addition to cells.

**Cell culture.** The murine lung epithelial (MLE)-15 cell line was immortalized from lung tumors of transgenic mice containing the Simian virus 40 large T antigen under the control of the simian virus 40 large T antigen promoter and contains the Simian virus 40 large T antigen under the control of the simian virus 40 large T antigen promoter and retains a single copy of the murine leukemia virus genome. The MLE-15 cell line maintains many of the morphological characteristics and gene expression patterns consistent with those seen in nontransformed bronchial and alveolar type II epithelial cells. More specifically, the MLE-15 cell line maintains a typical polygonal epithelial cell morphology and retains multilamellar inclusion bodies (19, 36). The MLE-15 cell line is used in studies of type II cell biology and is an excellent model system for studying pulmonary diseases such as idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease (COPD). The MLE-15 cell line is maintained in Dulbecco's modified Eagle's medium-Ham's F-12 medium (DMEM-F12) containing 5% fetal bovine serum and 10 µg/ml of gentamicin. For stimulation experiments, cells were plated (2 × 10⁶ cells) in 60-mm cell culture dishes (DMEM-F12-1% FBS) coated with type I collagen and allowed to grow for 48 h (~5 × 10⁶ cells). After the growth period, cells were washed with Hank's balanced salt solution (without calcium chloride, magnesium chloride, magnesium sulfate, and phenol red) and the medium was replaced with fresh DMEM-F12-0% FBS. Silica and/or the agent of interest was added immediately after medium replacement.

Prepare of a silica-macrophage-conditioned medium was carried out with the use of the mouse monocyte-macrophage cell line RAW 264.7 (RAW; American Type Culture Collection). RAW cells were maintained in DMEM-F12 medium with 10% FBS and 10 µg/ml of gentamicin. For stimulation experiments, cells were plated (2 × 10⁶ cells) in 60-mm cell culture dishes (DMEM-F12-5% FBS) and allowed to grow for 48 h (~5 × 10⁶ cells). Silica was added at 35 µg/cm² for 6 h. After the exposure period, the medium was removed and centrifuged at 350 g for 10 min to remove any particles or cells. Conditioned medium was then added to MLE-15 cells for 3 h. For TNF-α antibody experiments, the antibody was added to the RAW cells 1 h before the end of the silica exposure period.

**Cytotoxicity analysis.** Cell death was measured by evaluating lactate dehydrogenase (LDH) activity in the culture medium. Cells were cultured for either 6 or 24 h with varying doses of silica, and then the cells were assayed for total protein with the microbichoninic acid assay (Pierce, Rockford, IL). Total RNA was isolated from MLE-15 cells with TRIzol Reagent (GIBCO BRL, Grand Island, NY). RNA protection assay. Total RNA was isolated from MLE-15 cells with TRIzol Reagent (GIBCO BRL, Grand Island, NY). RNase protection analysis was carried out with the use of a previously described protocol (18). Briefly, unlabeled sense RNA encoding for eotaxin, IL-6, IFN-γ, IFN-β, interferon-inducible protein (IP)-10, lymphotoxin (LTN), lymphotoxin (LT)-β, MIP-1α, MIP-1β, MIP-2, MCP-1, regulated on activation normal T cells expressed and secreted (RANTES), T cell activated (TCA)-3, TNF-α, TNF-β, mouse ribosomal protein L32 (L32), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased in kit form (RiboQuant Kit, Pharmingen, San Diego, CA). For synthesis of radiolabeled antisense RNA, the final reaction mixture (10 µl) contained 70 µCi of [α-32P]UTP (3,000 Ci/mmol; NEN, Cambridge, MA); 10 µmol of UTP; 500 µmol each of GTP, ATP, and CTP; 10 µmol of dithiothreitol, 1 x transcription buffer; 12 U of Rnasin; 8 U of T7 polymerase (all from Promega, Madison, WI); and an equimolar pool of linearized template (500 ng total). After 1 h at 37°C, the mixture was treated with DNase buffer (90 µl) and RNase-free DNase (2 U; Promega) for 30 min at 37°C, and the probes were purified by extractions with phenol-chloroform and chloroform and precipitation as above, dissolved in 5 µl of gel loading buffer, and electrophoresed on 5% acrylamide-8 M urea sequencing gels. Dried gels were placed on XAR film (Kodak, Rochester, NY) with intensifying screens and were developed at −80°C for 18 h. For certain data displays, dried gels were developed on phosphorimager screens, and gel band intensity was analyzed with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).
Statistics. Results from LDH and phosphorimager analyses are reported as means ± SE. Statistical comparisons were made with a one-way ANOVA with Tukey-Kramer multiple comparisons test, with significance defined as $P < 0.05$.

**RESULTS**

Cristobalite-induced alveolar epithelial cell injury. Cristobalite caused a dose- and time-dependent increase in LDH release from MLE-15 cells (Fig. 1). Exposure of MLE-15 cells to 18 and 35 µg/cm² of cristobalite for 6 h resulted in small but significant LDH releases. These LDH responses were 13 and 29%, respectively, of the total LDH release as determined by cell lysis of MLE-15 cells with sonication. LDH release from the MLE-15 cells was significantly increased for the 9 (12% total LDH), 18 (30% total LDH), and 35 µg/cm² (52% total LDH) particle concentrations after exposure to cristobalite for 24 h.

Effect of cristobalite exposure on epithelial cell chemokine expression. To investigate whether chemokine gene expression in murine alveolar type II epithelial cells could be induced by direct interactions with cristobalite particles, we performed in vitro exposure studies with MLE-15 cells. mRNA for the chemokines eotaxin, IP-10, LTN, MCP-1, MIP-1α, MIP-1β, MIP-2, and TCA-3 was not detected in untreated cultures of MLE-15 cells (Fig. 2). When MLE-15 cells were exposed to 18 or 35 µg/cm² of cristobalite for 6 h, MCP-1 and...
MIP-2 mRNA levels increased significantly. However, after 24 h of cristobalite exposure, both MCP-1 and MIP-2 mRNA levels were reduced in the 18 µg/cm² exposure group and absent in the 35 µg/cm² exposure group. Time-course experiments that used the 18 µg/cm² dose showed even more dramatically the rise and fall of MCP-1 and MIP-2 mRNA expression (Fig. 2). Unlike MCP-1 and MIP-2, RANTES mRNA expression did not rise until after 9 h of cristobalite exposure and continued to increase after 24 h of exposure (Fig. 2). mRNA for the chemokines eotaxin, IP-10, LTN, MIP-1α, MIP-1β, and TCA-3 was not observed at any time point or dose tested. Transcription of the GAPDH and L32 genes were constitutive and were not altered by cristobalite exposure.

Effects of alveolar macrophage- and lymphocyte-derived cytokines on chemokine expression in epithelial cells. The release of alveolar macrophage- and lymphocyte-derived inflammatory mediators such as TNF-α and IFN-γ will likely influence the type II cell response to silica in vivo. To investigate the influence of these mediators on type II cell expression of chemokines, we exposed MLE-15 cells to recombinant murine IFN-γ and TNF-α either alone or in combination with cristobalite. Treatment with TNF-α alone led to a dose-dependent increase in MCP-1 and MIP-2 mRNAs (Fig. 3). The combination of TNF-α with increasing concentrations of cristobalite led to a dose-dependent increase in both MCP-1 and MIP-2 mRNAs (Fig. 3). Unlike the cristobalite-only exposures in which particle doses >18 µg/cm² were required to initiate a chemokine response, the combination of cristobalite and TNF-α led to MCP-1 and MIP-2 mRNA responses at a particle dose of 9 µg/cm². This response at a lower particle concentration did not, however, correlate with an increase in cellular cytotoxicity because LDH levels remained the same after the combined treatment compared with cristobalite treatment alone (data not shown). Quantitative measurements of changes in MCP-2 and MIP-1 mRNA levels are shown in Fig. 4, A and B, respectively. For all treatment plus cristobalite (9 µg/cm²) groups, statistical comparisons were made with the treatment control group (i.e., TNF-α, IFN-γ, LPS) because cristobalite (9 µg/cm²) treatment alone does not induce chemokine expression. Unlike the seemingly synergistic MCP-2 mRNA response seen with the combination of 9 µg/cm² of cristobalite and TNF-α, the combination of 18 µg/cm² of cristobalite and TNF-α was additive compared with the sum of cristobalite (18 µg/cm²) alone and TNF-α alone (Fig. 4A). A similar additive response was seen for MCP-1 mRNA levels after the same combination of 18 µg/cm² of cristobalite and TNF-α (Fig. 4B).

Unlike TNF-α, treatment with IFN-γ alone or in combination with cristobalite only altered MCP-1 mRNA levels (Figs. 3 and 4). Also, both 9 µg/cm² and 18 µg/cm² particle doses in combination with IFN-γ led to a synergistic increase in MCP-1 mRNA levels. As with the TNF-α-cristobalite exposures, the IFN-γ-cristo-

![Fig. 3. Measurement of chemokine mRNA after various treatments (6-h exposure) of Sc, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and lipopolysaccharide (LPS) with RNase protection assay. Unless otherwise indicated, TNF-α = 1 ng/ml, IFN-γ = 100 U/ml, and LPS = 5 µg/ml. Gel is representative of 3 separate experiments. LTN, lymphotactin; IP-10, interferon-inducible protein-10; TCA-3, T cell activated-3.](http://ajplung.physiology.org/)
Baltite exposures also showed no increase in LDH levels compared with cristobalite-treated cells (data not shown). Addition of both IFN-γ and TNF-α to MLE-15 cell cultures led to a synergistic increase in MCP-1, MIP-2, and RANTES (Figs. 3 and 4).

Treatment with the inflammatory agent LPS led to an increase in MIP-2 and MCP-1 mRNA levels in the MLE-15 cells (Figs. 3 and 4). Although it appears in Fig. 3 that TNF-α (1 ng/ml) is the more potent inducer of MCP-1 (vs. LPS), when the treatment groups were normalized to GAPDH levels and quantitated with the phosphorimager, LPS was actually slightly more effective. Treatment with LPS in combination with cristobalite (9 and 18 µg/cm²) led to a synergistic increase in MIP-2 and MCP-1 mRNA levels compared with the sum of LPS alone and cristobalite alone (Fig. 4, A and B, respectively). Interestingly, whereas treatment with LPS in combination with TNF-α led to a synergistic increase in MIP-2 mRNA, the same treatment only led to an additive increase in MCP-1 mRNA levels. Also, similarly to TNF-α-IFN-γ treatment, LPS-IFN-γ treatment led to a synergistic increase in MCP-1, MIP-2, and RANTES mRNA levels (Figs. 3 and 4).

None of the other chemokines (eotaxin, IP-10, LTN, MIP-1α, MIP-1β, or TCA-3) was detected after any treatment combination of TNF-α, IFN-γ, cristobalite, and LPS tested. Transcription of the GAPDH and L32 genes were constitutive and were not altered by TNF-α/IFN-γ/cristobalite/LPS exposure.

To further explore the silica-induced paracrine interactions between epithelial cells and macrophages, we used a macrophage cell line to generate a silica-macrophage-conditioned medium (described in MATERIALS AND METHODS). Treatment of the MLE-15 cells with the conditioned medium led to an increase in MCP-1 and MIP-2 mRNA levels (Fig. 5). Although virtually undetectable here, MCP-1 mRNA levels can be detected more clearly if the RNase protection blots are exposed to autoradiograph film for 2 days (results not shown). The pattern of MCP-1 and MIP-2 expression was similar to that seen after cristobalite or TNF-α treatment of MLE-15 cells. We (unpublished data) and others (7) have shown that the RAW macrophage cells used to generate the silica-macrophage-conditioned medium produce TNF-α protein after exposure to silica.

To examine the role that TNF-α played in regulating MCP-1 and MIP-2 mRNA expression in the MLE-15 cells after treatment with the silica-macrophage-conditioned medium, we added a TNF-α-neutralizing antibody to the medium 1 h before its addition to the MLE-15 cells. The results in Fig. 5 show that treatment with the TNF-α antibody completely inhibited the increase in MCP-1 mRNA and markedly attenuated the increase in MIP-2 mRNA.

Contribution of epithelium-expressed cytokines in regulating cristobalite-induced chemokine expression. Previous studies suggested that type II cells have the ability to produce the inflammatory mediators IFN (18) and IL-6 (8) after various stimuli. To investigate whether the observed silica-induced alterations in chemokine mRNA were the result of autocrine interactions with other inflammatory mediators produced by the MLE-15 cells, we used the RNase protection assay to measure mRNA levels for the following cytokines: IL-6, IFN-γ, IFN-β, LT-β, TNF-α, TNF-β, transforming growth factor (TGF)-β1, and TGF-β2 after various treatment conditions (Fig. 6). Results show that after treatment with cristobalite, LPS, and TNF-α, there was an in-
crease in TNF-α mRNA in the MLE-15 cells (Fig. 6). After correction for gel loading, TGF-β1 was found to be constitutively expressed and was not altered after any treatment (Fig. 7). An increase in IFN-γ and LT-β mRNA could be detected in all treatment groups except the control and IFN-γ groups if the RNase protection blots were exposed to autoradiograph film for 2 days (results not shown). Next, we treated the MLE-15 cells with cristobalite and/or an anti-TNF-α antibody to assess whether TNF-α was involved in regulating the cristobalite-induced alterations in MCP-1 and MIP-2 mRNAs. Antibody treatment completely eliminated the increase in MCP-1 and MIP-2 mRNAs (Fig. 5).

DISCUSSION

The contribution of the type II cell to the pathogenesis of silicosis remains largely unknown. However, increasing evidence implicates the type II cell as a potential mediator of pulmonary recruitment and activation of inflammatory cells through the release of a variety of chemokines (17, 24, 30, 31). Recently, Driscoll and co-workers (13) reported that rat type II cells express mRNA for the chemokines MIP-2 and CINC in response to direct interactions with α-quartz. The same authors also found that pretreatment with an anti-MIP-2 antiserum before intratracheal instillation of α-quartz in rats reduced by 60% the accumulation of neutrophils in bronchoalveolar lavage fluid (BALF) (13). In the present study, we confirm and extend these findings by using an in vitro murine type II cell silica exposure model. We observed an increase in mRNA levels for the chemokines MCP-1, MIP-2, and RANTES in a time- and dose-dependent fashion after cristobalite exposure in MLE-15 cells. These chemokines can be
alveolar macrophages after treatment with LPS, TNF-α. MCP-1 gene expression and secretion by rat whole lung tissue of rats exposed to carbon black (11). Patients with coal worker’s pneumoconiosis (4) and in such as basophils (22), eosinophils (20), and mast cells (22, 27), although they also act on other cell types such as basophils (22), eosinophils (20), and mast cells (2).

Previously, MCP-1 has been detected in BALF, alveolar macrophages, fibroblasts, and type II cells of patients with coal worker’s pneumoconiosis (4) and in whole lung tissue of rats exposed to carbon black (11). Also, MCP-1 gene expression and secretion by rat alveolar macrophages after treatment with LPS, TNF-α, and IL-1β has been reported (5). However, until this study, the silica-induced expression of MCP-1 and RANTES mRNAs in any lung cell type has not been reported. Increases in RANTES gene expression have not been previously associated with particle exposure in the lung. Other inflammatory lung disorders such as respiratory syncytial virus infection have been shown to induce RANTES in infected airway epithelial cells (3). Our results along with those of others provide strong evidence for the participation of type II epithelial cells in the recruitment of monocytes, neutrophils, and T lymphocytes to the lung after silica exposure.

A critical question is whether the observed increases in cytokine and chemokine message are independent of cristobalite-induced cell death. A previous study (13) has shown that a rat epithelial cell line exposed to 11.1 mg/cm² of α-quartz can elevate CINC and MIP-2 mRNA levels without a corresponding increase in LDH levels. Our data suggest that at least at low doses of cristobalite (9 µg/cm²) and in the presence of TNF-α or IFN-γ, a chemokine response can be generated, with no increase in LDH levels. At higher doses of cristobalite (18 and 35 µg/cm²), interpretation of the results in terms of a cell death-mediated component becomes complicated. For example, we might speculate that the decrease in MCP-1 and MIP-2 mRNAs after 24 h is due to the high toxicity seen at this time. However, at the same time MCP-1 and MIP-2 mRNA levels are decreasing, RANTES levels are rising, thus contradicting the hypothesis that increased toxicity is attenuating the chemokine response.

The direct response of type II cells to silica observed in vitro will likely account for only part of the response observed in vivo where paracrine stimulation by other silica-activated cell types is extremely important. In the present study, we examined the effects of the macrophage- and T lymphocyte-derived cytokines TNF-α and IFN-γ on the cristobalite-induced chemokine response in the MLE-15 cells. The observation that TNF-α treatment in the MLE-15 cells led to an increase in MIP-2 and MCP-1 mRNA levels is consistent with previous studies performed in human and rat epithelial type II-like cell lines (13, 30). Previous studies that used the human lung carcinoma A549 (1) and bronchial epithelial BEAS-2B (32) cell lines have also shown an increase in RANTES mRNA and protein after at least 6 h of TNF-α or IFN-γ treatment. The fact that the duration of our TNF-α and IFN-γ exposures was only 6 h likely explains why we did not observe an increase in RANTES mRNA expression. However, using the MLE-15 cells, we did confirm a previous study (32) showing that the combination of TNF-α and IFN-γ displays a marked synergism in inducing RANTES mRNA expression in BEAS-2B cells. We also showed TNF-α-IFN-γ and LPS-IFN-γ synergistically elevated MCP-1 and MIP-2 mRNAs. These results suggest that the type II cell will respond to multiple paracrine interactions with other surrounding cells, such as alveolar macrophages and T lymphocytes, through the release of various chemokines.

When given in combination with cristobalite, TNF-α or IFN-γ lowers the dose at which a particle-induced alteration in chemokine expression is observed in MLE-15 cells. This suggests an in vivo situation where silica-activated alveolar macrophages and T lymphocytes can modulate the sensitivity and pattern of the epithelial chemokine response through the release of TNF-α or IFN-γ. Our silica-macrophage-conditioned medium experiments further this hypothesis by showing the ability of the conditioned medium to stimulate a chemokine response in the MLE-15 cells. In addition, TNF-α appears to be the primary cristobalite-induced macrophage-derived mediator orchestrating this macrophage-induced epithelial chemokine response, as evidenced by our TNF-α antibody experiments in which the chemokine response induced by conditioned medium is markedly attenuated after antibody treatment.
Our observation that bacterial endotoxin in combination with cristobalite can also synergistically increase the MCP-1 and MIP-2 mRNA responses in MLE-15 cells suggests that other inflammatory stimuli can sensititize or “prime” the epithelium, thereby enhancing the particle response. This concept is in agreement with a previous study (9) that found pretreatment of human alveolar macrophages with LPS followed by exposure to silica dust led to an enhanced production of thromboxane A$_2$ and leukotriene B$_4$. These data suggest that the induction of MCP-1 and MIP-2 gene expression in murine type II cells after in vivo exposure to cristobalite will, at least in part, be influenced by interactions with alveolar macrophages and other inflammatory stimuli such as endotoxin.

We have found (unpublished results) that the in vitro activation of MLE-15 chemokine expression could not be achieved with the less inflammatory dust titanium dioxide. This result suggests that the chemokine response observed after cristobalite exposure is not the result of a generic particle-cell interaction. Other investigators have also found that the in vitro activation of epithelial chemokine expression, although not unique to silica, is not the property of all particles. For example, a previous study (13) found that the highly inflammatory dust α-quartz and crocidolite asbestos fibers led to an increase in the epithelial expression of CINC and MIP-2, whereas the less inflammatory dust titanium dioxide or man-made vitreous fibers (MMVF)-10 did not increase chemokine expression. The same study speculated that the differential effect of α-quartz, crocidolite, titanium dioxide, and MMVF-10 glass fibers on CINC and MIP-2 expression may be explained by the fact that both α-quartz and crocidolite can give rise to reactive oxygen species.

The precise mechanism by which direct interactions between cristobalite particles and lung epithelial cells lead to increased MCP-1 and MIP-2 gene expression remains uncertain. Silica can give rise to reactive oxygen species as a result of its surface chemistry and/or by stimulating the cellular generation of oxidants (6). In this respect, reactive oxygen species have been shown to act as second messengers, stimulating the translocation of the stress-responsive transcription factor nuclear factor (NF)-κB (29). The promoter regions for MCP-1 and MIP-2 contain the binding element for NF-κB (34, 35). A recent study (14) demonstrated an increase in nuclear translocation of NF-κB in rat lung epithelial cells exposed to α-quartz in vitro as well as the ability of antioxidants to attenuate the α-quartz-induced NF-κB translocation and MIP-2 gene expression in a rat lung epithelial cell line. Cristobalite-induced MCP-1 gene activation may also involve activator protein (AP)-1 binding inasmuch as previous studies (25, 27, 37) that used endothelial cells have shown both AP-1 and NF-κB binding is required for maximal gene induction after IL-1 or H$_2$O$_2$ exposure. Our study examined whether direct cristobalite-induced alterations in MCP-1 and MIP-2 mRNAs were the result of autocrine interactions with other inflammatory mediators produced by the MLE-15 cells. The results of our study suggest that cristobalite-induced activation of epithelial TNF-α plays a critical role in the activation of the MCP-1 and MIP-2 genes. Driscoll and co-workers (12) have shown that passive immunization of mice against TNF-α markedly attenuates the increases in lung MIP-2 mRNA seen in response to α-quartz. The question of whether cristobalite-induced activation of TNF-α or the subsequent TNF-α-induced activation of the MCP-1 and MIP-2 genes is the result of oxidant stress was not examined in the present study. However, evidence exists showing antioxidants inhibit TNF-α-mediated stimulation of IL-8, MCP-1, and collagenase expression in cultured human synovial cells (28).

Our results suggest that there are multiple mechanisms involved in cristobalite-induced expression of the chemokines MCP-1, MIP-2, and RANTES. For example, one potential mechanism is that chemokine expression is mediated by the cristobalite-induced expression of TNF-α. This mechanism allows us to explain our conflicting results showing a synergistic effect of TNF-α plus cristobalite treatment on MCP-1 and MIP-2 mRNA levels at a 9 µg/cm$^2$ particle dose and an additive effect at an 18 µg/cm$^2$ particle dose. We believe the observed synergism after the 9 µg/cm$^2$ particle dose is in fact an additive effect. The combination of cristobalite-induced TNF-α and recombinant TNF-α increases the level of TNF above a threshold level, subsequently leading to the increased expression of MCP-1 and MIP-2 mRNAs. This hypothesis is further supported by our observations showing that the chemokine response is eliminated after addition of a TNF-α antibody. In contrast, our results showing that IFN-γ in combination with cristobalite leads to a synergistic increase in MCP-1 mRNA but has no effect on cristobalite-induced MIP-2 mRNA levels suggest that an alternate non-TNF-α-mediated pathway is stimulated. The argument could be made, however, that the observed synergy between IFN-γ and cristobalite is just the sum of the effects mediated by IFN-γ enhancing the previously described TNF-α-mediated particle-activated pathway plus the effects of IFN-γ treatment alone. However, if this argument were true, we should have also seen an effect of IFN-γ treatment on cristobalite-induced MIP-2 mRNA levels. Thus, in the presence of IFN-γ, cristobalite appears to be able to stimulate an alternate non-TNF-α-mediated pathway, leading to the expression of MCP-1.

In summary, the present study demonstrates that the early inflammatory response in the lung after exposure to cristobalite, a form of crystalline silica, is a dynamic process involving multiple cell types. The type II cell plays a crucial role in this inflammatory response by interacting directly with cristobalite particles or by interacting with macrophage- and lymphocyte-derived mediators. In both cases, the interactions lead to the generation of the chemokines MCP-1, MIP-2, and RANTES from the type II cell. Our in vitro results also suggest that cristobalite-induced TNF-α from either the alveolar macrophage or the type II cell is a key mediator of the type II cell chemokine response.
REFERENCES


