Mesothelial Cell Proliferation and Biopersistence of Wollastonite and Crocidolite Asbestos Fibers

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The mesothelial lining is a target for the fibrotic and carcinogenic effects of mineral fibers. Fiber geometry, dimensions, chemical composition, surface reactivity, and biopersistence at the target tissue have been proposed to contribute to these toxic endpoints. We established a dose–response relationship between the number of fibers delivered to the parietal peritoneal lining, inflammation, and mesothelial cell proliferation induced by intraperitoneal injection of crocidolite asbestos fibers in mice. Persistence of these inflammatory and proliferative responses depended on persistence of fibers at the target tissue. Intrapleural injection of wollastonite fibers induced an early inflammatory and proliferative response that subsided after 21 days. Approximately 50% of wollastonite fibers were recovered by bleaching after 21 days and only 2% were recovered after 6 months. In contrast, the number of fibers recovered from tissue digests had not declined 6 months after injection of crocidolite asbestos. These results support the hypothesis that biopersistent fibers cause persistent inflammation and chronic mesothelial cell proliferation.

Human exposure to asbestos fibers causes a range of pathologic reactions of the pleura: benign asbestos pleural effusion, diffuse visceral pleural fibrosis, fibrotic plaques of the parietal pleura, and diffuse malignant mesothelioma (reviewed by Chuang and Green, 1995). The pathogenesis of these lesions is unknown. Pleural effusion and fibrosis of the visceral pleura may be nonspecific responses to active inflammation in the adjacent pulmonary parenchyma because these pathologic reactions also accompany other acute and chronic inflammatory reactions in the lungs (Adamson et al., 1994). Fibrotic scarring of the parietal pleura and dome of the diaphragm are more specific reactions associated with exposure to asbestos fibers; calcified fibrotic pleural plaques as visualized by chest X-ray or computerized axial tomography are considered as markers of prior asbestos exposure (Churg and Green, 1995). Diffuse malignant mesothelioma has been reported to arise in the parietal pleura adjacent to areas with abundant lymphatic drainage. These areas have been described recently as 'black spots' because they preferentially accumulate anthracotic pigment (Boutin et al., 1966).

Direct intrapleural or intraperitoneal injection of natural or man-made fibers has been used to reproduce these fibrotic and malignant reactions in rodents. The pioneering studies of Pott and Stanton and their colleagues suggested that fiber dimensions are important determinants of fiber carcinogenicity (Stanton et al., 1981; Pott et al., 1987). Additional parameters relevant to the biologic activity of fibers are chemical composition, surface reactivity, and biopersistence in the lungs or pleura (reviewed in McClellan et al., 1992; and Warheit et al., 1995). Although numerous studies have evaluated biopersistence of various natural and man-made fibers in the lungs of rodents after intratracheal instillation (Muhle et al., 1994) or inhalation (Hesterberg et al., 1996), few studies have evaluated biopersistence in the pleural or peritoneal lining. A recent investigation after short-term inhalation of refractory ceramic fibers by rats confirms that fibers are rapidly translocated to the pleural space (Gelzleichter et al., 1996a). Previous investigations using intrabronchial instillation of amosite asbestos fibers in dogs provide evidence for migration of fibers into lymphatics (Oberdörster et al., 1988). One marker of fiber migration to the pleural space is induction of an inflammatory response. Intrabronchial or intratracheal instillation of amosite (Oberdörster et al., 1983) or crocidolite asbestos (Li et al., 1994) in rats caused an early acute inflammatory response, followed by a delayed influx of macrophages as well as eosinophils (Gelzleichter et al., 1996b). These investigators developed a novel technique using agarose gel casts to quantitate pleural fiber burden (Bermudez, 1994). Significant numbers of fibers were recovered in the pleural cast, although most were thin and short, <5 μm in length. This recent finding raises questions about the potential pathogenicity of short, thin fibers (Goodglick and Kane, 1990). Other investigators report no apparent translocation of fibers to the visceral pleura after intratracheal instillation (Adamson et al., 1994) or inhalation (Quinlan et al., 1995).
of crocidolite or chrysotile asbestos fibers, although inflammation in the adjacent lung tissue was induced. However, in these studies, fiber localization was assessed by light microscopy and no tissue digests were performed.

A potential biomarker of response to asbestos fibers is proliferation of mesothelial cells. This response has been observed morphologically after intraperitoneal injection of asbestos fibers in rats (Friedmann et al., 1990), by $[^3]$H-thymidine incorporation after intraperitoneal injection of crocidolite asbestos in mice (Moalli et al., 1987), and by BrdU incorporation after intrapleural injection of man-made fibers in rats and hamsters (Rutten et al., 1994). The mechanism responsible for stimulation of acute mesothelial cell proliferation induced by direct injection of fibers into the pleural or peritoneal space is unknown. Proliferation could be a compensatory response to cell injury or apoptosis, a nonspecific reaction to inflammation in the pleural or peritoneal space, or a direct mitogenic effect of fibers. No studies have investigated the relationship between fiber dosimetry, inflammation, and mesothelial cell proliferation.

Pleural fiber dosimetry in humans has been determined using tissue samples obtained by fiberoptic thoracoscopy or at autopsy. Direct measurement of fibers obtained by tissue digestion of samples of the visceral or parietal pleura obtained at autopsy of asbestos workers has shown fibers at these sites in some cases, but not in others (Sebastien et al., 1980; Dodson et al., 1990). Predominantly short chrysotile fibers were found in some cases, although long amphibole fibers were recovered in lung tissue digests from the same worker (Sebastien et al., 1980). A limitation of these studies is the sampling technique: random biopsies of the parietal pleura were used. Boutin et al. (1996) described focal areas of high concentrations of fibers and other particulates near lymphatic vessels in the intercostal spaces of the parietal pleura. In asbestos-exposed workers, $4.1 \pm 1.9 \times 10^6$ fibers/g of dry tissue were recovered from these locations, compared to $0.5 \pm 0.2$ fibers/g of random biopsies of normal pleural tissue. Amphiboles were recovered more frequently than chrysotile fibers in these workers; 22.5% of the fibers were longer than 5 \( \mu \)m (Boutin et al., 1996). Using fiberoptic thoracoscopy, these investigators detected early stages of malignant mesothelioma arising near these areas of lymphatic drainage on the parietal pleural lining (Boutin and Rey, 1993). Examination of these areas by scanning electron microscopy revealed lymphatic channels surrounded by activated macrophages with ruffled surface membranes (Boutin et al., 1996).

We have developed a murine model to study the acute and chronic effects of crocidolite asbestos fibers after direct intraperitoneal injection in mice (Kane and Macdonald, 1993). In this murine model, long asbestos fibers are trapped at sites of lymphatic drainage on the inferior surface of the diaphragm. The initial inflammatory response to fibers trapped at this site is focal accumulation of activated macrophages. This inflammatory response is remarkably similar to the reaction visualized by Boutin et al. (1996) in the human parietal pleura. We have shown that this initial inflammatory reaction is accompanied by a peak of mesothelial cell proliferation 3 days after intraperitoneal injection of 200 \( \mu \)g of crocidolite asbestos fibers. These initial reactions are sensitive to fiber size: neither short crocidolite asbestos fibers nor particulate stimuli such as silica or titanium dioxide particles elicited inflammation or mesothelial cell proliferation (Branchaud et al., 1993; Moalli et al., 1987). It is hypothesized that persistence of long crocidolite asbestos fibers at these sites would lead to persistent inflammation and mesothelial cell proliferation. This hypothesis was tested using a known carcinogenic fiber, crocidolite asbestos, with prolonged persistence in the lungs of humans and experimental animals. In contrast, wollastonite is a naturally occurring calcium metasilicate that has a half-life of only 21 days in the lungs of rats following inhalation (Warheit et al., 1994). Crocidolite asbestos fibers induce malignant mesotheliomas after direct intraperitoneal injection (Pott et al., 1987) or inhalation in rats (McConnell et al., 1994). However, wollastonite did not induce mesotheliomas after intraperitoneal injection of up to 100 mg (Pott et al., 1987) or inhalation by rats (McConnell et al., 1991). The dose–response and temporal relationships between inflammation and mesothelial cell proliferation induced by crocidolite and wollastonite fibers were evaluated in a subchronic assay.

**MATERIALS AND METHODS**

**Animals.** Male C57BI/6 mice, 2 months old (Charles River Laboratories, North Wilmington, MA) were injected intraperitoneally with crocidolite asbestos fibers, wollastonite, or 1 ml of phosphate-buffered saline (PBS). The mice were housed in filter-top plastic cages in a barrier facility maintained at constant temperature and humidity and a 12-hr light–dark cycle. The mice were handled according to the guidelines described in the NIH Guide for the Care and Use of Laboratory Animals as approved by the Brown University Animal Care and Use Committee.

**Fiber samples.** Crocidolite asbestos fibers were obtained from stocks originally prepared and characterized by the Union Internationale Contre le Cancer (Timbrell, 1971/1972) and purchased from Duke Scientific Corporation (Palo Alto, CA). Crocidolite is a member of the amphibole asbestos family: ($Na_2Fe_3^+(Fe^{2+})_3Si_8O_{22}(OH)_2$). NYAD 1250 wollastonite (Lot 11-28-94), a fine particle grade of calcium metasilicate (CaSiO$_2$), was a gift from NYCO (Willsboro, NY). Stock suspensions of crocidolite were prepared and characterized as described previously (Moalli et al., 1987; Goodglick and Kane, 1990). This stock of crocidolite asbestos fibers induced malignant mesotheliomas after weekly intraperitoneal injections in 40–50% of mice (Kane and Macdonald, 1993). The size distribution of NYAD 1250 was described previously (Bellmann and Muhle, 1994).

**Peritoneal lavage.** At the times indicated in the text, mice were terminated with an overdose of ether and the peritoneum was lavaged with a total of 15 ml of PBS as described previously (Macdonald and Kane, 1986). The lavage fluid was centrifuged at 200 g for 5 min at 4°C, resuspended in Dulbecco-
co's modified Eagle's medium, high-glucose formula supplemented with 20 mM L-glutamine, 10 mM sodium pyruvate, 5000 units of penicillin, 5000 mg of streptomycin, and 10% heat-inactivated fetal calf serum (Life Technologies, Inc., Grand Island, NY). The total cell number was counted in a hemacytometer. Cells were plated overnight at 1 x 10^6/ml in 24 multiwell plates containing glass coverslips 1.2 cm in diameter. The coverslips were fixed, stained with May–Grunwald–Giemsa solution, and examined by light microscopy as described previously (Branchaud et al., 1993).

**Fixation and dissection of tissues.** Whole mounts of the diaphragm including the falciform ligament, omentum, and intestinal mesenteries were dissected at 20–70X using a dissecting stereomicroscope as described in Moalli et al. (1987). One hour prior to termination, mice were injected intraperitoneally with 5-bromodeoxyctydine (BrdC; 1 mg/ml, Sigma Chemical Co., St. Louis, MO); BrdC is more soluble than 5-bromodeoxyuridine (BrdU) so the labeling indices are more reproducible (Nüsse et al., 1985). Whole mounts of the diaphragm were fixed overnight in Omni-fix II (An-Con Genetics, Inc., Melville, NY) and then processed for immunohistochemistry. Cross-sections were prepared from the whole mounts, including the falciform ligament, the omentum, and the intestinal mesenteries; embedded in paraffin; and processed for light microscopy as described previously (Moalli et al., 1987). Serial sections were stained with a polyclonal antibody (1:600 dilution) directed against a broad spectrum of cytokeratins (A575; Dako Corp., Carpinteria, CA) that reacts with mature mesothelial cells to confirm the mesothelial origin of surface proliferating cells (Boilen et al., 1986).

**FIG. 1.** BrdU immunohistochemistry on whole mounts of the diaphragm. (A) Three days after ip injection of 200 μg of crocidolite asbestos. (B) 21 days after ip injection of 200 μg of crocidolite asbestos. (C) Three days after ip injection of 2 mg of wollastonite. (D) 21 days after ip injection of 2 mg of wollastonite. ×200.
FIG. 2. Time course of mesothelial cell proliferation after ip injection of crocidolite asbestos (A) or wollastonite (B).

Immunohistochemistry. Incorporation of BrdU by proliferating mesothelial cells on the inferior surface of the diaphragm was detected by immunohistochemistry using fixed whole mounts. The diaphragms were quenched in H2O2-methanol, rinsed, and denatured in 0.75 N HCl for 1 hr at room temperature. The diaphragms were neutralized in 0.1 M borate buffer, pH 8.5, and then incubated with a rat monoclonal anti-BrdU antibody (Accurate Chemical & Scientific Corp., Westbury, NY) at 1:40 dilution in PBS containing 0.1% BSA for 1 hr at room temperature. A secondary biotinylated anti-rat antibody (Organon Teknika-Cappel, Malvern, PA) at 1:250 dilution was applied for 30 min followed by ABC reagent (Vector Laboratories, Inc., Burlingame, CA) and development of a brown precipitate using diaminobenzidine. Twenty areas were selected at the musculotendinous junction and over the muscular region of the diaphragm and counted at 20x magnification using an eyepiece grid. The mean number of proliferating cells that incorporated BrdU was calculated per square millimeter of surface area.

Fiber digests. Samples of the central tendinous region of the diaphragm, the musculotendinous junction, and the falciform ligament were dissected, measured, and digested using bleach (5.25% sodium hypochlorite) for 48 hr at room temperature. This procedure has been verified using crocidolite asbestos and wollastonite fibers (Warheit et al., 1994). All solutions used in the digestion procedure were prefiltered. Recovery of fibers by this digestion technique was determined as follows. A series of increasingly dilute solutions of native fibers of known gravimetric quantities was distributed on nucleopore filters using vacuum filtration. One-half of each filter was prepared for scanning electron microscopy (SEM) and the number of fibers/mm² was counted as described below. A corresponding 1-mm² area was cut from the remaining half of the filter, dissolved in chloroform, and subjected to the digestion and filtration procedure. Recovery ranged from 78% for the most dilute concentration of fibers (5000/mm²) to 89% for the highest concentration of fibers tested (25,000/mm²). No fibers were recovered following digestion of tissues from mice injected with PBS. Fibers were collected on 25-mm nucleopore polycarbonate filters (pore size 0.4 μm), dehydrated, gold coated, and mounted on an aluminum planchet. Filters were examined using an Hitachi 2700 analytical scanning electron microscope equipped with a Link EXL-FQ1 X-ray detector. The WHO/EURO (1985) counting guidelines were followed with these modifications. All fibers and particulates were counted at 4000X. The number of fields counted covered at least 0.18 mm² of the filter surface. Counting stopped when a total of 1000 fibers and nonfibrous particles were reached or when 1 mm² of the filter surface had been examined. Representative photomicrographs of original samples and fiber digests were taken. The chemical composition of all fiber samples before injection and after recovery from tissue digests was verified by energy dispersive X-ray analysis (Macdonald and Kane, 1986).

Statistical analysis. The mean values ± SEM were calculated for three to five mice from each treatment group. Statistical significance of differences between control and treated groups was determined by analysis of variance (ANOVA) using Fisher's PLSD test. A p value < 0.05 was considered as statistically significant.

RESULTS

Mesothelial Cell Proliferation

The time course of mesothelial cell proliferation produced by intraperitoneal injection of crocidolite asbestos fibers or

TABLE 1
Dose Response of Fiber Deposition and Mesothelial Cell Proliferation 3 Days after Intraperitoneal Injection of Crocidolite Asbestos or Wollastonite

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>No. fibers x 10³/mm²</th>
<th>No. labeled cells/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0</td>
<td>21 ± 3.1</td>
</tr>
<tr>
<td>Asbestos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 μg</td>
<td>4.04 ± 2.0</td>
<td>689 ± 243</td>
</tr>
<tr>
<td>200 μg</td>
<td>26.20 ± 4.2</td>
<td>1231 ± 155</td>
</tr>
<tr>
<td>Wollastonite</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 μg</td>
<td>4.25 ± 1.6</td>
<td>98 ± 4.0</td>
</tr>
<tr>
<td>2 mg</td>
<td>19.50 ± 6.1</td>
<td>668 ± 82.8</td>
</tr>
</tbody>
</table>

FIG. 3. Total cell recovery from peritoneal lavage after ip injection of crocidolite asbestos (A) or wollastonite (B).

FIG. 4. Differential cell count recovered from peritoneal lavage after ip injection of 200 μg crocidolite asbestos (A) or 10 mg wollastonite (B). Hatched bars, macrophages; solid bars, neutrophils; open bars, lymphocytes.
FIBER PERSISTENCE AND MESOTHELIAL CELL PROLIFERATION

FIG. 5. May Grünwald–Giemsa stain of cells recovered from peritoneal lavage 3 days after ip injection of saline (A), 200 μg crocidolite asbestos (B), or 2 mg wollastonite (C). \( \times 400 \).

Wollastonite was assessed by immunohistochemistry using whole mounts of the diaphragm to detect incorporation of BrdU into nuclei of proliferating mesothelial cells (Fig. 1). Three days after injection of 200 μg of crocidolite asbestos (Fig. 1A) or 2 mg of wollastonite (Fig. 1C), there was diffuse incorporation of BrdU over the entire inferior surface of the diaphragm. After 21 days, focal areas of mesothelial cell proliferation persisted around clusters of crocidolite asbestos (Fig. 1B). These fiber clusters were located at sites of lymphatic drainage from the peritoneum: the falciform ligament and the lacunar regions of the diaphragm as described previously (Moalli et al., 1987). In contrast, 21 days after injection of 2 mg of wollastonite, scattered areas of mesothelial cell proliferation were observed, mostly at the musculotendinous junction (Fig. 1D). The number of labeled nuclei per square millimeter of diaphragm declined to background levels 56 days after injection of wollastonite fibers. Focal areas of mesothelial cell proliferation persisted for 6 months after injection of 200 μg of crocidolite asbestos: the labeling index in these areas was 2–6% compared to a background level of 0.03–0.05% in saline-injected controls. Incorporation of BrdU by surface proliferating mesothelial cells was confirmed by histologic examination of paraffin-embedded cross-sections of the diaphragm, followed by cytokeratin
The initial proliferative response to intraperitoneal injection of crocidolite asbestos or wollastonite fibers was dose dependent (Fig. 2). After 3 days, mesothelial cell proliferation increased after injection of 20 or 200 μg of crocidolite asbestos; no further increase was noted after injection of 1 mg. Mesothelial cell proliferation was also increased after injection of 200 μg or 2 mg of wollastonite, although even at these higher doses, wollastonite fibers were less potent in inducing mesothelial cell proliferation than crocidolite asbestos fibers after 3 days. At the highest dose of wollastonite fibers tested (10 mg), mesothelial cell proliferation was elevated after 21 days and then declined to background levels after 56 days. There was a dose–response relationship between the number of fibers deposited on the inferior surface of the diaphragm and stimulation of mesothelial cell proliferation 3 days after intraperitoneal injection of crocidolite asbestos or wollastonite (Table 1).

**Inflammatory Responses to Crocidolite Asbestos or Wollastonite Fibers**

Cellular analysis of peritoneal lavage fluid indicated a transient inflammatory response to intraperitoneal injection of crocidolite asbestos or wollastonite fibers. The initial inflammatory response to crocidolite asbestos was dose dependent over the range of 20 μg–1 mg after 3 days (Fig. 3A). As reported previously (Branchaud et al., 1993), this inflammatory reaction declined after 21 days. However, after 56 days, a statistically significant increase in the number of cells recovered by peritoneal lavage was found at all doses of crocidolite asbestos fibers. The magnitude of the initial inflammatory response to wollastonite was dose dependent at 200 μg or 2 mg (Fig. 3B). Similar to crocidolite asbestos, the inflammatory response to wollastonite subsided after 21 days. At later time points, inflammation persisted only after injection of 10 mg of wollastonite.

The initial inflammatory response to 200 μg of crocidolite asbestos or 10 mg of wollastonite was characterized by activated macrophages. A low percentage of neutrophils was recovered 3 and 56 days after injection of crocidolite asbestos at 200 μg (Fig. 4A). After injection of 10 mg of wollastonite, activated macrophages were recovered after 3 or 21 days. At later time points, a statistically significant increase in lymphocytes was found (Fig. 4B). The morphology of inflammatory cells recovered by peritoneal lavage 3 days after injection of saline, crocidolite asbestos, or wollastonite is compared in Fig. 5. Peritoneal lavage cells from saline-
injected mice plated on glass coverslips overnight were smaller and round in comparison with the large, activated macrophages and multinucleated giant cells obtained after injection of 200 μg of crocidolite asbestos. After injection of wollastonite, the peritoneal macrophages were smaller and less adherent in comparison to those obtained after injection of crocidolite asbestos. The morphology and histochemical characteristics of activated macrophages obtained 3 days after intraperitoneal injection of crocidolite asbestos were described previously (Branchaud et al., 1993).

**Histopathology**

Long fibers of crocidolite asbestos or wollastonite are trapped at lymphatic stomata on the inferior surface of the diaphragm. The inflammatory responses to these fibers trapped on the parietal peritoneal lining reflects the distribution of inflammatory cells recovered by peritoneal lavage. Cross-sections of the diaphragm at sites of fiber deposition were compared after intraperitoneal injection of 200 μg of crocidolite asbestos fibers or 2 mg of wollastonite (Fig. 6).
These doses were selected because they produced a similar magnitude of inflammation as reflected by the total cell number recovered by peritoneal lavage. The initial histopathologic reaction to crocidolite asbestos fibers trapped on the inferior surface of the diaphragm is accumulation of macrophages and formation of multinucleated giant cells around long fibers or clusters of short fibers (Fig. 6A). A few lymphocytes, plasma cells, and neutrophils are intermingled within this lesion. A similar, but less intense, inflammatory response was observed at sites of wollastonite deposition on the inferior surface of the diaphragm (Fig. 6B).

Macrophages and multinucleated giant cells persisted at sites of asbestos fiber deposition on the inferior surface of the diaphragm. After 56 days, these lesions became progressively more fibrotic as described previously (Kane and Macdonald, 1993). In contrast, the inflammatory response to intraperitoneal injection of 200 μg or 2 mg of wollastonite resolved completely after 21 days. At the highest dose of wollastonite (10 mg), focal accumulations of mature lymphocytes were observed on the inferior surface of the diaphragm after 21 days (Fig. 6C). Serial sections of the diaphragm confirmed that these focal lesions completely resolved after 6 months.

The inferior surface of the diaphragm is a major site of lymphatic clearance of any particulates or fibers introduced into the peritoneal cavity. At high doses, particulates and fibers are also trapped at milky spots (Kampmeier's foci) at the mesenteric surface. Particles or fibers less than 10 μm in diameter are successfully cleared by the lymphatics (Moalli et al., 1987) and accumulate in lymph nodes within the omentum and mesenteries (Branchaud et al., 1993). The mineral samples used in this study are a mixture of particulates, short fibers, and long fibers. Short asbestos fibers are cleared to mesenteric lymph nodes, while long fibers accumulate in milky spots on the surface of the mesenteries. The inflammatory response to asbestos fibers at these sites is accumulation of activated macrophages and multinucleated giant cells which become progressively fibrotic (Fig. 7A). Short fragments of wollastonite also accumulate in milky spots initially; only a few macrophages and multinucleated giant cells are found 3 days after injection of 200 μg or 2 mg of wollastonite (Fig. 7B). At the highest dose of wollastonite (10 mg), large aggregates of fibers were found loosely adherent to the mesenteric surface; these are readily apparent by dark field illumination (Fig. 7C). Under bright field illumination, few inflammatory cells are found in these large aggregates of wollastonite (Fig. 7D). After 6 months, serial sections of the mesenteries showed no residual evidence of wollastonite aggregates or macrophages, while lymphoid hyperplasia at milky spots persisted.

**Fiber Biopersistence**

Sections of the diaphragm and falciform ligament were dissected, digested in bleach, and analyzed for fiber content at various times after intraperitoneal injection of 200 μg of crocidolite asbestos fibers or 2 mg of wollastonite (Fig. 8).

![FIG. 8. Biopersistence of crocidolite asbestos (closed circles) or wollastonite fibers (open circles) on the diaphragm following ip injection.](image)

After 3 days, approximately 20–25 × 10³ fibers/mm² were deposited on the diaphragm. After 21 days, the number of crocidolite asbestos fibers recovered by bleach digestion increased and then leveled off after 56 days. In contrast, the number of wollastonite fibers recovered by bleach digestion decreased by 50% after 21 days. After 6 months, only 2% of wollastonite fibers deposited after 3 days was recovered. No changes in surface morphology or fiber shape were seen in crocidolite asbestos fibers recovered from the diaphragm at any time point (Fig. 9A). After only 3 days, however, surface etching of wollastonite fibers was visible (Figs. 9B and 9C).

**DISCUSSION**

These results support the hypothesis that persistence of crocidolite asbestos fibers at sites of lymphatic drainage in the peritoneum leads to chronic inflammation and persistent mesothelial cell proliferation. The chronic inflammatory response to crocidolite is characterized by accumulation of activated macrophages and multinucleated giant cells at sites of fiber deposition. Focal areas of persistent mesothelial cell proliferation are localized to sites of asbestos fiber clusters. Although intraperitoneal injection of wollastonite fibers also produced an early, but less intense, inflammatory response, this resolved as the fibers were cleared from or dissolved at their initial sites of deposition around lymphatic stomata and at milky spots in the mesenteries. The initial proliferative response to both crocidolite asbestos fibers and wollastonite depended on the dose of fibers delivered to the diaphragm 3 days after intraperitoneal injection. In contrast to crocidolite asbestos fibers, wollastonite was cleared from the diaphragm after 21 days and mesothelial cell proliferation returned to control levels.
This is the first study that correlates fiber persistence and inflammation with mesothelial cell proliferation in the peritoneal lining. Although the kinetics for clearance of wollastonite from the peritoneum are slower than reported from the lungs after inhalation in rats (Warheit et al., 1994), these fibers are cleared almost completely from the inferior surface of the diaphragm after 6 months. Similar to the results of intratracheal instillation (Muhle et al., 1994) or inhalation studies (Hesterberg et al., 1996), crocidolite asbestos fibers are not modified or cleared from sites of deposition in the peritoneal lining. Transient inflammatory reactions were also reported in the lungs of rats during a short-term inhalation bioassay of wollastonite, while the inflammatory and proliferative responses to crocidolite asbestos persisted in the lungs of rats following inhalation (Warheit et al., 1994; Bérubé et al., 1996).

In addition to biopersistence, chemical composition and surface reactivity of asbestos fibers also contribute to the chronic inflammatory, fibrotic, and proliferative reactions observed in the lungs and mesothelial lining. Reactive oxygen metabolites such as hydroxyl radicals generated by the iron-catalyzed Fenton reaction are hypothesized to mediate the inflammatory and carcinogenic effects of asbestos fibers (Moyer et al., 1994). Amphiboles such as crocidolite asbestos have a high iron content that is bioavailable (Fubini, 1993).

Modification of the shape of wollastonite fibers was noted in the short-term inhalation bioassay described above (Warheit et al., 1994). Surface etching of wollastonite fibers was
also observed following intraperitoneal injection. This surface etching may leave wollastonite fibers more vulnerable to transverse splitting. Although macrophages initially accumulated at sites of crocidolite asbestos and wollastonite fiber deposition in the peritoneal lining, it is unknown whether surface etching, breaking, and dissolution of wollastonite occurred in the peritoneal lining fluid or within macrophage phagolysosomes.

A major caveat in intraperitoneal injection studies is selection of doses that produce a biologic response but do not cause a bolus effect. This was demonstrated by Collier et al. (1994) by injecting a range of doses of glass fibers into the peritoneal cavity of rats. In their study, doses greater than 1.5 mg cause formation of fiber clumps or nodules. This effect did not occur after injection of up to 10 mg of crocidolite asbestos fibers in mice; however, large fiber clumps were produced by injection of 10 mg of wollastonite. In the study reported by Collier et al. (1994), the glass fibers were 2 μm in diameter; this may have contributed to their tendency to form aggregates at lower doses than we observed for crocidolite asbestos or wollastonite in mice. The inflammatory response to the highest dose of wollastonite associated with a bolus effect in mice was different from the response to lower doses. Large aggregates of wollastonite fibers were loosely attached to the mesenteries or floating free in the peritoneal cavity. In contrast to fibers trapped at lymphatic stomata or in milky spots, very few macrophages accumulated on or within these large aggregates. In the Collier et al. study (1994) using glass fibers, these aggregates were the site of a granulomatous inflammatory response. Despite aggregation into clumps, even the high dose of wollastonite fibers injected in mice eventually dissolved completely with no evidence of a granulomatous reaction or fibrosis seen on serial sections of the diaphragm or mesenteries 6 months after the initial injection.

Although most of the wollastonite fibers disappeared from initial sites of deposition, a focal chronic inflammatory response remained 3 weeks after injection of 10 mg on the inferior surface of the diaphragm and 6 months after injection within the mesenteries. This focal chronic inflammatory response to wollastonite was qualitatively different from the initial response to these fibers characterized by accumulation of macrophages. The initial macrophage response resolved quite rapidly and was replaced by focal clusters of mature lymphocytes on the inferior surface of the diaphragm and lymphoid hyperplasia within the mesenteries. No wollastonite fibers were identified in these lesions using darkfield illumination. This lymphocytic hyperplasia may be a response to soluble components released from dissolving wollastonite fibers.

This subchronic assay of biopersistence, inflammation, and mesothelial cell proliferation may provide information about the comparative biologic activities of man-made fibers in the mesothelial lining. Although the mechanism responsible for biopersistence and clearance of fibers from the mesothelial lining may be different from the lungs, recent rodent inhalation studies suggest that man-made fibers are translocated to the pleura following inhalation (Gelzleichter et al., 1996a). There is recent documentation that asbestos fibers also accumulate in the parietal pleura of humans. In this murine model, approximately $1.2 \times 10^7$ crocidolite asbestos fibers per gram of wet tissue were deposited on the diaphragm and induced persistent inflammatory and proliferative responses. This is comparable to approximately $4.1 \times 10^6$ fibers per gram of dry tissue recovered from “black spots” on the parietal pleura of asbestos-exposed workers (Boutin et al., 1996). Additional information is required to assess the biopersistence and biologic activity of fibers in the mesothelial lining. If precautions are taken to avoid a bolus phenomenon, a subchronic intraperitoneal injection assay can rank the biologic effects of man-made fibers relative to those produced by wollastonite, a noncarcinogenic fiber, and crocidolite asbestos, a biopersistent, carcinogenic fiber.

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