Cytotoxicity of Long and Short Crocidolite Asbestos Fibers in Vitro and in Vivo

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ABSTRACT

Fiber length and diameter are important factors in the pathogenicity of asbestos. We examined the relative toxicity of long and short crocidolite asbestos fibers in vitro and in vivo. Both long and short crocidolite asbestos fibers were toxic to elicited macrophages in vitro. Similar to native crocidolite asbestos, long and short fibers stimulated the release of reactive oxygen metabolites from elicited macrophages in vitro. We evaluated whether in vitro cytotoxicity was dependent on the production of reactive oxygen metabolites. In the presence of the reactive oxygen metabolite scavenging enzymes, superoxide dismutase or catalase, the toxicity of long and short crocidolite fibers to macrophages was prevented. Furthermore, macrophages were not killed when either long or short fibers were soaked in the iron chelator, deferoxamine. Native, long, and short crocidolite fibers also caused depolarization of the mitochondrial membrane potential prior to cell death. In vivo, a single i.p. injection of long crocidolite fibers stimulates an intense inflammatory reaction, release of reactive oxygen metabolites near sites of fiber deposition, and cell death. In contrast, these events were minimal after a single injection of short fibers due to the removal of fibers from the peritoneal cavity. After five daily injections of short fibers, however, fibers were present on the surface of the mesothelium and provoked an inflammatory response.

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INTRODUCTION

Exposure to asbestos can potentially cause serious health effects including fibrotic scarring of the lungs (asbestosis) and cancer arising in the lung or mesothelium. The term asbestos refers collectively to naturally occurring hydrated silicate mineral crystals with fibrous morphology. There are two types of asbestos, serpentine and amphiboles. Chrysotile is the only known serpentine, while crocidolite, amosite, anthophyllite, tremolite, and actinolite comprise the family of amphiboles. All types of asbestos consist of fibers of variable lengths and diameters. The length of fibers, for example, can range from approximately 0.1 μm to greater than 200 μm. Fiber dimension is considered a critical factor in the pathogenesis of asbestos-related diseases. Long fibers which are retained in the lungs are associated with chronic lesions in humans and in animal models. In animal models, long fibers have been found to cause a more pronounced inflammatory and fibrotic response than shorter fibers (1–6). Moreover, Stanton et al. observed that any fiber that is ≥8 μm long and ≤0.25 μm in diameter was carcinogenic when implanted within the pleural cavity (7–9).

Shorter fibers may not be harmless, however. Stanton et al. reported that smaller fibers were not necessarily innocuous in their model system, but rather induced tumors much less readily than long, thin fibers. Nevertheless, these observations, as well as others (reviewed in Refs. 1 and 10) suggest that long asbestos fibers are more biologically active and more damaging than shorter fibers. The question arises: are smaller fibers innate less harmful or are smaller fibers less injurious in vivo because they are cleared or sequestered more efficiently than larger fibers from sites of potential damage?

Attempts have been made to correlate in vitro cellular alterations due to asbestos with the in vivo effects of asbestos. These studies present contradictory data on whether long asbestos fibers are more, less, or equally as injurious as short fibers to a number of different cell types in vitro. Various studies have examined the effect of different fiber sizes on macrophages, a cell type which has been implicated in both the initial and more chronic response to asbestos in vivo (10–13). Kaw et al. observed that longer chrysotile, crocidolite, and amosite fibers were slightly more toxic to resident peritoneal macrophages in the presence of serum than were an equal mass of corresponding short fibers (14). Another study reported a slight increase in the toxicity of long asbestos fibers (including amosite and chrysotile) to P388D1 macrophage-like cells (15). Bey and Harington, however, detected no difference in the toxicity of long and short chrysotile or amosite fibers to resident peritoneal macrophages in the presence of serum (16), and Yeager et al. observed that RG 144, a naturally occurring short chrysotile fiber, was more toxic to human alveolar macrophages than UICC chrysotile A (17). The effect of long and short asbestos fibers has also been tested on other cell types. For example, longer fibers (including crocidolite, amosite, and chrysotile) were more effective at inhibiting proliferation and plating efficiency of V79-4 and A549 cells (15, 18, 19) than shorter fibers and longer chrysotile fibers (≥10 μm) were more cytotoxic to tracheal epithelial cells than shorter chrysotile fibers (<2 μm) (20, 21). Both short and long chrysotile fibers, however, were equally efficient at inducing squamous metaplasia in tracheal organ cultures (22). Finally, Hesterberg and Barrett found that milled chrysotile was less effective than native chrysotile in inhibiting the proliferation and morphological transformation of Syrian hamster embryo cells (23).

In this paper we examine the relative cytotoxicity of long and short crocidolite asbestos fibers in vitro and in vivo. We have previously shown that an early event which occurs upon entry of native or long crocidolite asbestos fibers into the peritoneal cavity is an influx of inflammatory macrophages to the sites of fiber deposition and death to mesothelial cells (24) and macrophages. Shorter fibers were cleared from the peritoneal cavity and therefore produced only a limited reaction (24). We hypothesize that the interaction of macrophages with asbestos fibers is important in the early cellular responses to asbestos fibers. Therefore, we first examined the effect of native, long,
and short crocidolite asbestos fibers on macrophages in vitro. Previous results have shown that in vitro, native crocidolite asbestos kills macrophages by an oxidant-dependent mechanism (25, 26). Toxicity was dependent on iron present on the surface of crocidolite fibers. We report here that both short and long crocidolite fibers are acutely toxic to elicited macrophages in vitro. Prior to cell death, we observed that long, short, and native crocidolite asbestos fibers depolarize the mitochondrial membrane. We further evaluated whether cell death due to both long and short crocidolite fibers was dependent on the production of reactive oxygen metabolites and on the presence of iron. In addition, we also addressed the question of whether short crocidolite fibers would be toxic in vivo if they were not effectively cleared from the peritoneal cavity.

MATERIALS AND METHODS

Asbestos Fiber Preparations. Native crocidolite asbestos (Duke Scientific, Palo Alto, CA) was prepared and characterized according to UICC specifications. Native (“mixed”) crocidolite was separated into equations as estimated as follows: the mean length and diameter of each fiber reported (24, 25). Immediately prior to use, fiber suspensions were warmed to 37°C and sonicated for 10 min in a sonicating water bath.

of mixed crocidolite (200 µg/ml and 1 mg/ml), short crocidolite (120 µg/ml and 1 mg/ml) was prepared in PBS (pH 7.4) (Gibco Laboratories, Grand Island, NY) and stored at 4°C as previously reported (24, 25). Immediately prior to use, fiber suspensions were warmed to 37°C and sonicated for 10 min in a sonicating water bath.

The mean surface area of mixed, long, and short crocidolite fibers was estimated as follows: the mean length and diameter of each fiber population was calculated by using the values in Tables 2 and 3, respectively. An estimate of surface area was derived by comparing fibers to rectangles. The surface area of a rectangle is given by the equation

\[ S = 4BL + 2B^2, \]

where \( L \) is the length of the fiber (from Table 2) and \( B \) is the length of the base (i.e., diameters from Table 3). The approximate mean surface areas of mixed, long, and short crocidolite fibers would be 3.56, 7.73, and 8.48 \( \mu m^2/\mu g \), respectively. These values are not meant to reflect the absolute surface area of each population of fibers, but rather should be regarded as rough approximations which can be used for the comparison of mixed, long, and short crocidolite fiber preparations. The estimate of mixed crocidolite surface area, however, if converted to units of \( m^2/g \) (10.4 \( m^2/g \)), is similar to the approximate surface area values obtained by nitrogen absorption used by Rendali (8.1 or 8.7 \( m^2/g \)) (27) and Timbrell (8.3 ± 0.5 \( m^2/g \)) (28).

Cell Culture. Thioglycolate-elicited mouse peritoneal macrophages were obtained as described (25). Cells were plated either onto 12-mm glass coverslips (2.5–5 x 10^6 cells/12-mm coverslip) (Rochester Scientific Co., Rochester, NY) in 100-mm Falcon tissue culture dishes (Becton Dickinson and Co., Lincoln Park, NJ) or into 6-well (35-mm diameter, 9.62 cm²) (Costar, Cambridge, MA) multiwells at a density of 1 x 10^6 cells/well (25).

In Vitro Toxicity Experiments. Toxicity experiments were conducted as published (25). Elicited macrophages plated on 12-mm glass coverslips were placed into 16-mm diameter (2.01 cm²), 24-well plastic multiwells (Costar) and exposed to various doses of short or long crocidolite for up to 22 h. Viability was determined by using FDA (Sigma Chemical Co., St. Louis, MO) (25). SOD and catalase (Sigma) were prepared as reported (25); final concentrations are given in the table legends. SOD was inactivated by using the procedure of Hodgson and Fridovich (29). Deferoxamine (a gift from Ciba Pharmaceutical Co., Summit, NJ) was prepared as described (25). Long crocidolite (120 µg) was presoaked in 2 or 5 mM deferoxamine for 15 min, then rinsed free of excess deferoxamine as previously reported (25), or alternately, fibers were presoaked for a longer time period as described for short fibers below. For long fibers, both procedures (the one above and the one below) gave comparable results. Short crocidolite (30 µg) was presoaked in 5 mM deferoxamine dissolved in DMEM (high glucose formula) (Gibco Laboratories) at room temperature in a sterile polystyrene tube. The fiber suspension was well dispersed for the first 3 h, then the fibers were allowed to settle to the bottom of the tube for approximately 3 h more. After this time, the samples were centrifuged for 30 min at 800 x g, approximately 80% of the supernatant was carefully removed so as not to disturb the fiber pellet, an equal volume of fresh DMEM was added, and the sample was left undisturbed for about 10 h at 4°C. Approximately 60% of the supernatant was again carefully removed and an equal volume of DMEM was added, the sample was centrifuged for 15 min at 800 x g, and 80% of the supernatant was carefully removed and replaced with an equal volume of DMEM. Samples were then added to the cells. The rationale for using the procedure outlined above was to allow short fibers to settle completely. If the washing procedure published previously (25) was used, substantial numbers of short fibers were lost. Finally, this procedure of rinsing away excess deferoxamine leaves at most only 43.7 Mm² of deferoxamine.

H₂O₂ Production. Elicited macrophages (1 x 10⁶ cells/35-mm well) were exposed to various doses of short or long crocidolite for up to 6 h. Phorbol-12-myristate-13-acetate (0.5 µg/ml final concentration) (Sigma) was used as a positive control. H₂O₂ was measured by the horseradish peroxidase-dependent, H₂O₂-mediated oxidation of phenol red as previously described (25, 30). No H₂O₂ was detected when cells were incubated with long or short crocidolite or PMA plus catalase (0.5 µg/ml) (Sigma). In some experiments in which H₂O₂ production was measured (e.g., Fig. 4), the doses of crocidolite used were higher than doses used in the viability or mitochondrial membrane potential assays. This higher dose reflects the higher density of cells which were used as well as the larger surface area of the plastic culture well. In all experiments described in this paper, however, the final number of fibers added per cell or per unit of surface area was kept relatively constant.

Mitochondrial Membrane Potential. The fluorescent probe, rhodamine 123 (Eastman Kodak Co., Rochester, NY), was used to detect changes in mitochondrial membrane potential (31, 32). A concentrated stock of rhodamine 123 (1 mg/ml) dissolved in dimethyl sulfoxide (Sigma) was stored at 4°C in the dark. Elicited macrophages (2.5–5 x 10⁶ cells/12-mm coverslip) were placed in 16-mm diameter plastic multiwells (Costar) and rinsed 3 times with DMEM. Macrophages were pretreated for 10 min with 1 µM rhodamine 123 prior to addition of 50 µg of mixed crocidolite, 30 µg of short crocidolite, or 120 µg of long crocidolite. Cells were incubated with rhodamine 123 with or without crocidolite fibers for various times in the dark at 37°C, in an atmosphere of 5% CO₂-95% air, in DMEM (total volume of 1.0 ml). The dose of rhodamine 123 that we used was not toxic to macrophages nor did the indicator enhance the toxicity of crocidolite fibers over a 1- to 8-h time course.² At the completion of each incubation, coverslips were gently detached from fresh DMEM and were then inverted onto a No. 1 thickness microscope coverglass (Fisher finest premium cover glass, 60 x 24 mm). Care was taken not to expose cells to light for more than five min. Cells were viewed with a Zeiss Axioplan photomicroscope (×400) under epifluorescence illumination with a fluorescein filter set. Macrophages with intact mitochondrial potential had discrete, punctate, or filamentous rhodamine 123 staining. Macrophages with depolarized mitochondria were defined as those cells which contained no discrete rhodamine 123 staining but rather displayed diffuse, cytoplasmic fluorescence (32). Results are expressed as the percentage of cells with discrete rhodamine 123 staining.

In Vivo Reduction of Nitroblue Tetrazolium. Male C57BL/6 mice (Charles River Laboratories, North Wilmington, MA) were given injections i.p. of 1.0 ml of mixed crocidolite (200 µg/ml), long crocidolite

² L. A. Goodpichk and A. B. Kane, unpublished results.
RESULTS

Preparation and Characterization of Long and Short Crocidolite Fibers

Native UICC crocidolite asbestos was used as the starting material for separating long and short fibers. As previously reported (24), these two populations of fibers were obtained through repeated centrifugation. Three populations of fibers were used in our investigation; these populations will be referred to as mixed (native), long, and short crocidolite. The number of fibers per mg for each fiber sample was determined by transmission electron microscopy and is shown in Table 1. The distribution of lengths and diameters of mixed, long, and short crocidolite was also determined by transmission electron microscopy (Tables 2 and 3) (24). Only 1.1% of short crocidolite fibers have lengths >5.0 μm while 8.8% of mixed crocidolite fibers and over 27% of long crocidolite fibers have lengths >5.0 μm.

In Vitro Cytotoxicity of Long and Short Crocidolite Fibers

The cytotoxicity of long and short crocidolite preparations was tested by using thioglycolate-elicited mouse peritoneal macrophages (2.5−5 × 10^6 cells/12-mm coverslip). Both short and long crocidolite were toxic to macrophages in a dose-dependent fashion after a 7-h exposure (Fig. 1). Viability was determined using FDA. At doses below 100 μg, long crocidolite was notably less toxic than short crocidolite. A possible explanation for this phenomenon was that for a given weight, long crocidolite...
CYTOTOXICITY OF LONG AND SHORT ASBESTOS FIBERS

A

Fig. 1. Dose response of short and long crocidolite asbestos toxicity to elicited macrophages. Thioglycolate-elicited peritoneal macrophages were exposed to various doses of short (•) or long (A) crocidolite asbestos fibers for 7 h. Viability was determined by using FDA. Points, mean from triplicate cultures; bars, SD. Untreated control cells were 100 ± 3.2% viable. A, loss of viability due to long or short crocidolite plotted versus µg quantity of crocidolite; B, loss of viability due to long or short crocidolite plotted versus the number of crocidolite fibers (×10⁷) in 5, 10, 25, 50, 100, and 150 µg of long or short crocidolite.

B

Fig. 2. Time course of short and long crocidolite asbestos toxicity to elicited macrophages. Thioglycolate-elicited peritoneal macrophages were exposed to 30 µg of short crocidolite fibers (•) or 120 µg of long crocidolite fibers (A) for the times shown. These doses of long and short crocidolite asbestos contain equal numbers of fibers. Viability was determined by using FDA. Points, mean from triplicate cultures; bars, SD. Untreated cells remained 100 ± 3.3% viable.

contained fewer fibers than a comparable weight of short crocidolite (Table 1). We determined that 30 µg of short crocidolite and 120 µg of long crocidolite contain the same number of fibers as 50 µg of mixed crocidolite. A dose of 50 µg of mixed crocidolite is consistently toxic to 40–60% of elicited macrophages after a 6- to 8-h incubation (25). When macrophages were exposed to 30 µg of short crocidolite for 7 h, 44.7% of the macrophages remained viable, while with 120 µg of long crocidolite, only 8.0% were alive. This result is further demonstrated if the data in Fig. 1A are graphed on the basis of fiber number (Fig. 1B). Based on equal fiber numbers, long crocidolite is apparently more toxic than short crocidolite (Fig. 1B). As shown in Fig. 2, both 30 µg of short crocidolite and 120 µg of long crocidolite produced a time-dependent decrease in macrophage viability; long crocidolite, however, caused a more rapid decline in the percentage of viable cells.

A technical problem encountered in all experiments was the greater time required for short fibers compared to long fibers to settle onto macrophages. We could not centrifuge fibers onto cells since the high speed required to sediment short fibers in itself injures the macrophages. It was, therefore, unclear whether the lag time in short crocidolite toxicity (Fig. 2) represented an innate property of short fibers or rather was merely a result of the delay in settling time. It is notable, however, that after a 24-h incubation, both long fibers (120 µg) and short fibers (30 µg) were equally cytotoxic; 7.0 ± 0.2% of the cells were viable with short fibers and 6.4 ± 1.5% of the macrophages survived with long fibers.

A third way to analyze the data presented in Fig. 1A is on the basis of surface area. The mean surface areas of mixed, long, and short crocidolite were estimated as described in "Materials and Methods" and were determined to be 3.56, 7.73, and 0.84 µm²/fiber, respectively. By our approximation, a given number of long crocidolite fibers would have about 9 times the surface area as an equal number of short crocidolite fibers. Therefore, if macrophages were exposed to doses of long and short crocidolite with equal surface areas, short crocidolite would be at least as toxic as long crocidolite.

Toxicity of Long and Short Crocidolite Fibers Depends on Release of Reactive Oxygen Metabolites

Native, mixed crocidolite is cytotoxic to macrophages by a mechanism dependent on reactive oxygen metabolites (25, 26).
Evidence suggests that iron present in crocidolite may catalyze the formation of the toxic free radical, ‘OH (34). It is not obvious whether short fibers, which are completely phagocytized, and long fibers, which usually are incompletely phagocytized, are necessarily toxic to macrophages by a similar oxidant-dependent mechanism. We examined the mechanism of long and short crocidolite-induced cytotoxicity by determining whether both fiber preparations would induce the release of H$_2$O$_2$ from macrophages and whether SOD, catalase, and deferoxamine would prevent cell death.

Long and Short Crocidolite Fibers Stimulate Release of H$_2$O$_2$ by Macrophages. We first examined whether short and long crocidolite samples stimulated the release of H$_2$O$_2$ from macrophages in vitro. Incubation of macrophages (10$^6$ cells/35-mm well) with long or short crocidolite resulted in release of H$_2$O$_2$ (Figs. 3 and 4). There was an increase in the release of H$_2$O$_2$ when macrophages were incubated with up to 500 µg of long or short crocidolite (Fig. 3). No H$_2$O$_2$ was detected in the presence of catalase. PMA, a potent stimulant of the respiratory burst system, was used as a positive control. Macrophages incubated with 0.5 µg/ml PMA for 2 and 4 h, released 29.4 ± 1.6 and 76.7 ± 4.7 nmol H$_2$O$_2$/10$^6$ cells, respectively. There was an increase in detectable H$_2$O$_2$ over time when macrophages were exposed to equal numbers of long (720 µg) or short (180 µg) crocidolite (Fig. 4). H$_2$O$_2$ release was maximal by 4 h. At all time points, a greater concentration of H$_2$O$_2$ was detected when macrophages were exposed to short crocidolite as opposed to long crocidolite. It is unlikely that the difference in H$_2$O$_2$ release by long and short crocidolite-stimulated cells was due to increased cell death due to long fibers. Under the conditions of this assay, there was no significant difference in the percentage of nonviable cells due to long or short crocidolite at each time point (data not shown).

Toxic Effect of Long and Short Crocidolite Fibers Can Be Decreased with Deferoxamine. Iron can catalyze the formation of the highly unstable hydroxyl radical (‘OH) via a metal-catalyzed Haber-Weiss reaction (36). This reaction uses O$_2$ and H$_2$O$_2$ as substrates. Iron is an integral component of the crystalline structure of crocidolite asbestos (1). In a cell-free system, crocidolite can catalyze the formation of ‘OH from H$_2$O$_2$ (34). The Fe(III) chelator, deferoxamine, prevents this reaction. Moreover, deferoxamine also prevents the toxicity of mixed crocidolite to elicited macrophages (25). We therefore tested whether deferoxamine would prevent the toxicity of short and long crocidolite. Long crocidolite (120 µg) was soaked in 2 or 5 mM deferoxamine and short crocidolite (30 µg) was soaked...
caused a significant decrease in the number of macrophages with discrete inflammatory responses as determined by leakage of albumin into the peritoneal cavity (24). We tested whether short crocidolite fibers would be acutely cytotoxic in vivo if they were not cleared from the surface of the diaphragm. To accomplish this, we gave injections to mice i.p. of 120 µg of short crocidolite once a day for 5 days. This dose of short fibers contains the same number of particles as 200 µg of mixed crocidolite. Similarly, other mice received 5 injections of an equal number of long crocidolite (480 µg), of the nontoxic particle TiO2 (160 µg) (24), or 1.0 ml of PBS as described in “Materials and Methods.” Three days after the last injection of long or short crocidolite, there was an intense inflammatory response as determined by leakage of albumin into the peritoneal cavity (Table 6). Inflammatory cells could also easily be observed on the entire surface of the diaphragm (×100–400) from mice given injections of long or short crocidolite. No inflammatory response was detected after 5 injections of TiO2 or PBS (Table 6); however, TiO2 particles could be observed within lymphatic channels (lacunae) beneath the mesothelial lining of the diaphragm. Long crocidolite fibers

were scattered over the surface of the diaphragm. After 5 injections of long crocidolite, fibrotic adhesions had begun to form between the liver and the diaphragm. Following 5 injections of short crocidolite, accumulations of fibers were also observed on the surface of the diaphragm; the majority of visible fiber clusters were located at the musculotendinous junction. In contrast to this, 1 injection of the cumulative amount of short crocidolite (600 µg) was cleared more rapidly than daily injections. One injection of 600 µg of short crocidolite resulted in fewer fiber clusters on the surface of the diaphragm and a reduced inflammatory response (Table 6). In agreement with previous results (24), 1 injection of 120 µg of short crocidolite produced only a small cluster of fibers on the diaphragm and a weak inflammatory response (Table 6). Single injections of 480 µg or 2.4 mg of long crocidolite produced an intense inflammatory response as determined by leakage of albumin into the peritoneal cavity (Table 6).

Cell injury was assessed after 3 days by trypan blue staining on the surface of the diaphragm. Trypan blue staining was observed at all doses of long and mixed crocidolite (Fig. 6), had no trypan blue staining on the surface of the diaphragm (Table 6). Finally, the presence of inflammatory cells was not sufficient in itself to cause cell death. A single injection of thiglycolate caused an inflammatory response but no trypan blue staining on the surface of the diaphragm (Table 6).

In Situ Reduction of Nitroblue Tetrazolium. NBT was used to detect the production of reactive oxygen metabolites in situ. When NBT is reduced by reactive oxygen metabolites, primarily O₂⁻, a blue precipitate, formazan, is produced (33). Three days after an injection of 200 µg of mixed crocidolite, 480 µg or 2.4 mg of long crocidolite, or 5 daily injections of 480 µg of short crocidolite as described in “Materials and Methods.” Diaphragms were dissected 3 days after the last injection and whole mounts were viewed under a Zeiss Axioplan photomicroscope (×200). Arrows, examples of cells which did not exclude trypan blue dye.

Fig. 6. Light micrograph of trypan blue staining on the surface of the diaphragm removed from mice which had been given injections i.p. (A) once of 480 µg of long crocidolite or (B) 5 times of 120 µg of short crocidolite as described in “Materials and Methods.” Diaphragms were dissected 3 days after the last injection and whole mounts were viewed under a Zeiss Axioplan photomicroscope (×200). Arrows, examples of cells which did not exclude trypan blue dye.

Table 6 In vivo inflammatory response, NBT reduction, and cellular injury due to long and short crocidolite asbestos fibers

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total µg of albumin</th>
<th>NBT reduction</th>
<th>Trypan blue staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no injection)</td>
<td>396</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PBS (1 ml)</td>
<td>588</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thioglycolate (1.5 ml)</td>
<td>1938</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TiO₂ (160 µg)</td>
<td>567</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TiO₂ (800 µg)</td>
<td>586</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Long crocidolite (480 µg)</td>
<td>2255</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Long crocidolite (2400 µg)</td>
<td>2620</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Short crocidolite (120 µg)</td>
<td>1625</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Short crocidolite (600 µg)</td>
<td>1925</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Multiple injections</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PBS (1 ml/day)</td>
<td>768</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TiO₂ (160 µg/day)</td>
<td>904</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Long crocidolite (480 µg/day)</td>
<td>2407</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Short crocidolite (120 µg/day)</td>
<td>2657</td>
<td>+++</td>
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crocidolite, mice were injected i.p. with 0.3 mg/ml NBT and sacrificed 15 min later as described in “Materials and Methods.” With all doses of mixed or long crocidolite, blue formazan precipitate was observed at sites of crocidolite deposition of the surface of the diaphragm (Fig. 7). There was no formazan at sites devoid of fibers. This localized staining was inhibited by SOD, demonstrating that endogenous cellular reductases were not responsible for the formazan formation. In addition, no formazan was observed on the diaphragms of untreated mice or mice given injections of PBS (Table 6). Likewise, the non-degradable, noninflammatory particle TiO₂ induced no formazan staining (Table 6).

The presence of both crocidolite fibers and inflammatory cells was required for NBT reduction. In regions of the diaphragm where inflammatory cells were present but no fibers were observed, there was no formazan staining. In addition, an i.p. injection of thioglycolate, a soluble inflammatory agent which, like crocidolite, elicits macrophages into the peritoneal cavity after 3 days, produced no formazan deposits on the surface of the diaphragm (Table 6).

We further examined whether an injection of short crocidolite would stimulate the release of reactive oxygen metabolites. A single injection of 120 µg of short crocidolite produced little formazan staining on the surface of the diaphragm (Table 6). In some of these animals, we observed some formazan when 1–2 small clusters of fibers were present at the musculotendinous junction. In these situations, NBT reduction was always associated with cells which contained fibers. A similar situation was seen when mice received a single injection of 600 µg of short crocidolite, although under these conditions, we consistently observed small clusters of fibers at the musculotendinous junction. When mice were given daily injections of 120 µg of short crocidolite for 5 days, we detected abundant formazan staining on the surface of the diaphragm (Fig. 7; Table 6). The majority of NBT reduction occurred in areas where fiber clusters were present; formazan staining appeared to be within or in the near vicinity of cells which had engulfed numerous short fibers. Occasionally, cells which did not contain engulfed fibers displayed formazan staining. We suspect that these cells contain a few short fibers which cannot be detected by light microscopy. An injection of SOD prevented NBT reduction on the surface of the diaphragm in response to short crocidolite.

**DISCUSSION**

Fiber dimension is an important parameter in determining the chronic reaction to asbestos. Longer fibers induce a more vigorous acute and chronic inflammatory response than shorter fibers. Longer fibers are also more fibrogenic and carcinogenic than shorter fibers. The exact basis for these size-dependent differences is unclear. Are short fibers intrinsically more inert than long fibers or are shorter fibers cleared more efficiently from the lungs and serosal cavities? One gauge of biological activity is fiber cytotoxicity. Crocidolite asbestos is toxic to macrophages both *in vitro* and *in vivo*. Macrophages are an initial cell to interact with asbestos following inhalation into the lungs (12, 13) or entrance into the peritoneal or pleural cavities (24). The prevailing opinion is that longer asbestos fibers *in vitro* are more cytotoxic to macrophages (and other cell types) than shorter fibers (1, 10).

In this report we presented evidence that both long and short crocidolite fibers are toxic to thioglycolate-elicited mouse peritoneal macrophages *in vitro*. On the basis of equal weight, short crocidolite was more toxic to macrophages than long crocidolite (Fig. 1A). A given weight of short crocidolite, however, contains more fibers than a comparable weight of long crocidolite. To normalize the ratio of fibers per cell, long and short crocidolite were also added to macrophages at equal fiber numbers. Under these conditions, long crocidolite was more cytotoxic than short crocidolite (Figs. 1B and 2). After 24 h, however, long and short crocidolite (at equal fiber numbers) were equally lethal to macrophages. Finally, based on equal surface areas, short crocidolite appeared to be equally, if not more toxic than long crocidolite. We suspect that surface area is an important parameter in determining fiber cytotoxicity. Crocidolite toxicity depends on fiber-bound iron (25) and the amount of exposed reactive iron on the surface of crocidolite fibers is proportional to surface area.

**Previous Investigations of Long and Short Fiber Cytotoxicity.** The difference between our *in vitro* results and those reported
previously by other groups reflects differences in experimental conditions. There are several points to consider when trying to compare various studies. These include the following: (a) Fiber dose: some studies examine toxicity on the basis of either equal fiber weight (15, 17) and/or equal fiber surface area (14). Limited dose-response studies were performed in certain investigations (14, 16, 17). In this paper, we demonstrate that cytotoxicity can differ depending on whether macrophages are exposed to an equal mass, equal number, or equal surface area of crocidolite fibers. (b) Length of incubation: the length of time that cells are exposed to asbestos varies in different investigations. Times can range from a 1-h incubation with mineral fibers (17), to a 48- (15, 16) or 72-h exposure (14, 16). In some studies serum is present (14, 16, 17); serum can delay asbestos cytotoxicity (1). (c) Type of asbestos: the relative cytotoxicity of amphibole versus serpentine asbestos is a debated topic. The cytotoxicity of long and short amphiboles may or may not be the same as the relative toxicity of long and short serpentine asbestos. (d) The source of short fibers: in some reports, preparations of short asbestos fibers were produced by milling longer fibers (15; reviewed in Ref. 1). Milling asbestos, however, alters fiber surface properties (10). (e) Macrophage population: different populations of macrophages used in various models include human alveolar macrophages (17), resident mouse (14) and Syrian hamster peritoneal macrophages (16, 17), and P388D1 macrophage-like tumor cells (15). We chose to use elicited (i.e., nonspecifically activated) macrophages since we have observed that activated macrophages are present in early and chronic lesions caused by crocidolite asbestos in vivo.6 (f) Assay of viability: assays which have been used to determine viability include membrane permeability (i.e., trypan blue exclusion, erythrosin B staining, FDA and release of lactate dehydrogenase) (14, 15, 17), uptake of tritiated amino acids (14), and inhibition of growth (reviewed in Ref. 1) in the presence of macrophage growth factors (16). These assays may not necessarily correlate with one another (39). Furthermore, some assays, such as growth inhibition and lack of 3H-amino acid uptake, do not necessarily measure loss of viability. (g) Cell culture conditions: there are variations from study to study on medium used and other cell culture conditions.

Proposed Mechanism of Long and Short Crocidolite Cytotoxicity in Vitro. Native (mixed) crocidolite fibers induce oxidant-dependent toxicity to macrophages (25). We hypothesize that iron present on the surface of crocidolite fibers catalyzes the formation of 1O2. We observed that short and long crocidolite also killed macrophages via an oxidant-dependent mechanism. Short and long crocidolite were found to stimulate the release of H2O2 from macrophages (Figs. 3 and 4). Catalase, SOD, and deferoxamine decreased the toxicity due to both crocidolite samples (Tables 4 and 5).

Disruption of mitochondrial function is postulated to play a role in oxidant-induced cell death (reviewed in Ref. 37). One indicator of mitochondrial function is membrane potential. We therefore examined whether macrophages exposed to crocidolite asbestos would lose mitochondrial membrane potential. We observed that incubation of macrophages with mixed crocidolite disrupted the mitochondrial membrane potential as determined by the fluorescent probe rhodamine 123 (Fig. 5). Depolarization of the mitochondria preceded cell death due to crocidolite. Catalase prevented both mitochondrial membrane depolarization (not shown) and cell death. Similar to mixed crocidolite, both long and short crocidolite preparations induced a time-dependent decrease in the number of macrophages with discrete rhodamine 123 staining (Fig. 5). It remains unclear whether loss of mitochondrial membrane potential is causally related to crocidolite-induced cell death. While mitochondrial depolarization and release of mitochondrial calcium due to agents such as the proton ionophore carbonyl cyanide p-trifluoromethoxyphenyldrazone, are not directly lethal to macrophages over 7 h (40), mitochondrial dysfunction coupled with other oxidant-induced injury may cause irreversible injury. We are currently investigating other potential sites of crocidolite-induced oxidant injury as well as the mechanism by which crocidolite produces loss of mitochondrial membrane potential.

In Vivo Cell Injury Due to Long and Short Crocidolite Fibers. We further examined in vivo injury caused by long, short, and mixed crocidolite fiber preparations. Two early events associated with the injection of mixed or long crocidolite asbestos into the peritoneal cavity of mice are an inflammatory response, characterized by an influx of macrophages, and death of mesothelial cells (24). Injection of short crocidolite into the peritoneal cavity causes only a slight inflammatory reaction and minimal cell injury on the surface of the diaphragm compared to long or mixed crocidolite (24). The majority of short fibers are cleared from the surface of the diaphragm (24). In this paper, we tested whether short crocidolite fibers would be cytotoxic in vivo if they were not cleared from the peritoneal cavity. To accomplish this, we gave injections to mice once each day for 5 consecutive days of 120 µg of short crocidolite. Our rationale was that multiple injections of short crocidolite would saturate the lymphatic drainage system of the peritoneal cavity. We speculate that short fibers were not efficiently cleared from the peritoneal cavity under these conditions due to the large number of fibers injected and a continual recruitment of inflammatory cells; a large accumulation of inflammatory cells on the surface of the diaphragm may occlude lymphatic openings. In support of this latter point, a single injection of 600 µg of short crocidolite did not produce as potent an inflammatory response as a single injection of 600 µg of short crocidolite (Table 6). We postulate that after a single injection, the majority of short fibers are cleared prior to the onset of an inflammatory response. Interestingly, neither single nor multiple injections of TiO2 particles induce an inflammatory response. TiO2 particles have diameters <5 µm and are readily cleared by lymphatic channels (24). Since 98.9% of short fibers also have lengths ≤5 µm (Table 2), it would seem that fiber length alone cannot account for the intensity of the inflammatory response and for the reduced clearance of short fibers after multiple injections. We hypothesize that the chemical composition of short crocidolite fibers plays an important role in eliciting an inflammatory response, reducing fiber clearance, and causing cell death. Additionally, fiber diameter may contribute to the biological effects.

Multiple injections of short crocidolite produced cell injury on the surface of the diaphragm as assessed by cellular trypan blue uptake. Trypan blue staining in vivo has previously been shown to correlate with cell injury as determined by scanning electron microscopy (24). One interpretation of these results is that uncleared short crocidolite fibers deposit on the surface of the diaphragm and are cytotoxic. The presence of inflammatory cells alone cannot account for this cell injury since an injection of thioglycolate caused no trypan blue staining (Table 6; Ref. 24). Furthermore, it could be argued that in our short crocidolite preparation, 1.1% of the fibers have lengths greater than 5 µm and that these longer fibers may be responsible for the effects observed in vivo. This seems unlikely, however, since 5

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injections of short fibers produced more injury and more NBT reduction than a single injection of the same dose of short fibers. At sites on the diaphragm where we observed cell injury due to crocidolite, we also observed the production of reactive oxygen metabolites using NBT. NBT apparently detects $O_2^-$ or some reaction product of $O_2^-$ since the injection of SOD prevented NBT reduction. Formazan was produced on the surface of the diaphragm after injection of mixed or long crocidolite, and after multiple injections of short crocidolite (Table 6; Fig. 7). It is unclear what cell type is producing these reactive oxygen metabolites. The most likely, though not only, candidate is the macrophage. Three days after an i.p. injection of crocidolite, the predominant inflammatory cell is the macrophage (24). In vitro, activated macrophages have been shown to have a potent respiratory burst mechanism which is triggered to release $O_2^-$ during phagocytosis or after stimulation by soluble agents such as phorbol esters or endotoxin (36, 41). Arachidonic acid metabolism can also produce reactive oxygen metabolites (41). In addition, we (Fig. 3 and 4) (25) and others (42–45) have demonstrated that macrophages produce reactive oxygen metabolites during phagocytosis of asbestos fibers in vitro. Nevertheless, other types of inflammatory cells, such as neutrophils, are capable of producing oxygen metabolites (36). It remains to be determined whether mesothelial cells can also release reactive oxygen metabolites in response to asbestos. At the present time we can only speculate that the release of reactive oxygen metabolites might contribute to cell injury in vivo; we are currently addressing this question in our model system. Results from other types of model systems have suggested that reactive oxygen metabolites are responsible for cell injury in vivo (46–48).

Conclusions and Further Questions. In conclusion, both short and long crocidolite are toxic to macrophages in vitro. Similar to mixed crocidolite, long and short crocidolite fibers are lethal to macrophages via an oxidant-dependent mechanism; iron present in crocidolite is also required for toxicity. Prior to cell death, mixed, long, and short crocidolite disrupt the mitochondrial membrane potential. It remains to be determined whether this early sign of cell injury is causally related to cell death. Furthermore, the mechanism by which reactive oxygen metabolites are toxic to macrophages needs to be elucidated. Finally, an interesting question to resolve will be whether reactive oxygen metabolites released from macrophages can damage mesothelial cells. In our in vivo model of occupational exposure to crocidolite asbestos, small quantities of short crocidolite fibers are rapidly removed from the peritoneal cavity and therefore appear relatively inert. If the clearance mechanisms are overburdened, however, short fibers become trapped on the surface of the peritoneal lining and are cytotoxic. Longer fibers which cannot easily pass through stomata are not efficiently removed from the peritoneal cavity and are also cytotoxic. Our data do not necessarily conflict with other in vivo studies which report that long fibers are more pathogenic than shorter fibers (reviewed in Refs. 1 and 10). Fiber size is important in determining fiber deposition, translocation, and clearance in both the lung (49) and the peritoneal cavity (7–9, 24). Fiber size is also proportional to surface area and thus is relevant to fiber surface chemistry. The potential importance of fiber surface chemistry is becoming more apparent. In cell-free systems, crocidolite, chrysotile, amosite, and glass fibers, have all been shown to catalyze the formation of hydroxyl radicals from $H_2O_2$ (34, 50, 51).

Within 3 days after an i.p. injection of mixed or long crocidolite asbestos fibers, mesothelial cells start to regenerate and replace cells which have been killed (24). This proliferative response is maximal after 7 days; 14–21 days after a single injection, the mesothelial lining has been restored (24). We hypothesize that repeated episodes of mesothelial cell death followed by regeneration is important for subsequent chronic complications. In other model systems, cell proliferation has also been implicated as a necessary step in the early stages of carcinogenesis (52, 53). Mesothelial regeneration occurs in a "prooxidant" environment as documented by our in situ NBT assay. Directly and/or indirectly, reactive oxygen metabolites can cause mutations, DNA strand breaks, chromosomal breaks, other nuclear alterations, and epigenetic changes (41, 54). Fibers may also cause cellular alterations by directly interacting with mesothelial cells (55). Furthermore, inflammatory macrophages and other cells in asbestos-induced lesions, can potentially secrete a variety of factors which regulate cell growth and migration, as well as the deposition and turnover of extracellular matrix components (56–60). One or more of these events may contribute to the induction of malignant mesotheliomas by asbestos fibers.

Finally, are short fibers a health hazard to humans? The data presented in this paper cannot directly address this issue; we can only speculate that with prolonged or extensive occupational exposure to short asbestos fibers, saturation of clearance mechanisms could potentially lead to the accumulation of fibers in the lungs or serosal cavities. Recently, we have observed that weekly i.p. injections of 120 µg of short crocidolite produces tumors in 71.4% of mice after 50–60 weeks. It remains to be elucidated whether unclered short fibers are cytotoxic, fibrogenic, or carcinogenic in humans.

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Cytotoxicity of Long and Short Crocidolite Asbestos Fibers in Vitro and in Vivo

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