THE ROLE OF FIBER LENGTH IN CROCIDOLITE ASBESTOS TOXICITY *IN VITRO* AND *IN VIVO*

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INTRODUCTION

Inhalation of asbestos fibers during occupational exposures is associated with the development of pleural plaques and effusions, diffuse interstitial fibrosis, and an increased incidence of cancer including bronchogenic carcinoma and malignant mesothelioma. The geometry and dimensions of asbestos fibers are important factors in the pathogenesis of these diseases. Short fibers or spherical mineral particles which reach the alveoli are rapidly cleared from the lungs. Long, straight fibers characteristic of amphibole asbestos are translocated to the pleural and peritoneal linings. In contrast, curly serpentine fibers are trapped in the upper respiratory tract or at tracheobronchial bifurcations. Serpentine fibers also fragment and are gradually cleared, while unmodified amphiboles persist in the lungs.[1,2]

These differences in deposition, translocation, and clearance may account for the different pathogenicity of short and long asbestos fibers after inhalation. Alternatively, long fibers may have intrinsically different effects on potential target cells in the lung than short fibers. Support for this alternate mechanism is based on numerous *in vitro* models of asbestos toxicity and transformation. In many of these *in vitro* models, long fibers are more biologically active than short fibers or spherical particles as monitored by acute cell lysis, disruption of the cytoskeleton, inhibition of cell proliferation, stimulation of various enzyme activities, and transformation *in vitro*.[3-9]

We have re-examined the importance of fiber length using two models of crocidolite asbestos toxicity *in vitro* and *in vivo*. Acute toxicity of long or short fibers was demonstrated in primary cultures of thioglycollate-elicited mouse peritoneal macrophages *in vitro*. In contrast, after direct intraperitoneal injection of crocidolite asbestos fibers, long fibers were more toxic than short fibers. This differential toxicity *in vivo* is due to more effective lymphatic clearance of short fibers from the peritoneal lining. However, if lymphatic clearance was prevented, short fibers were toxic, as well as carcinogenic, in this *in vivo* model.

MATERIALS AND METHODS

Preparation and Characterization of Asbestos Fibers

Crocidolite asbestos which was prepared and characterized according to the Union Internationale Contre Le Cancer (UICC) was used to prepare samples enriched in short and long fibers as described previously.[10]

In Vitro Toxicity Protocol and Assay for H_2O_2 Release

Thioglycollate-elicited mouse peritoneal macrophages were plated onto 12-mm glass coverslips ($2.5–5 \times 10^4$ cells per coverslip) and exposed to various doses of short or long crocidolite fibers for up to 24 hours. Viability was determined using fluorescein diacetate (FDA). Superoxide dismutase (SOD) and catalase were prepared as reported; final concentrations are given in the table legends. Deferoxamine-coated fibers were prepared as described.[11] For assay of H_2O_2 release, elicited macrophages (10^6 cells per 35-mm well) were exposed to various doses of short or long crocidolite fibers for up to 6 hours. H_2O_2-mediated oxidation of phenol red was assayed as previously described.[11]

In Vivo Injury

Male C57B1/6 mice were injected intraperitoneally with mixed crocidolite (200 μg), short crocidolite (120 μg), or phosphate buffered saline (PBS) using the procedure of Moalli et al.[10] These doses of short and mixed crocidolite contained an equal number of fibers. Mice were sacrificed after 3 days. Peritoneal lavage fluid was used to measure lactate dehydrogenase (LDH) activity. The diaphragm was dissected and stained with trypan blue as described previously.[10] In some experiments, mice were injected intraperitoneally with agarose blue A spherical beads. Two days later, 120 μg of short crocidolite fibers were injected intraperitoneally. Mice were sacrificed 3 days later.

NBT Reduction *In Vivo*

Mice were injected with 1.5 ml thioglycollate (4% w/v), 200 μg of titanium dioxide, 200 μg of crocidolite asbestos alone, or 200 μg of crocidolite asbestos which had been presoaked in 10 mM deferoxamine and then rinsed as described above. Mice were sacrificed 3 days later following a 15 minute exposure to 0.3 mg/ml nitroblue tetrazolium (NBT). Fixed diaphragms were dissected and viewed under a dissecting stereomicroscope (2–80 x). Reduced NBT formed a blue precipitate (formazan).

Induction of Mesotheliomas

Male C57B1/6 mice were injected intraperitoneally with crocidolite asbestos. Two series of experiments were conducted: in Series A, 20 mice were injected weekly with either 200 μg/ml of long crocidolite or 200 μg/ml of mixed crocidolite fibers (40 mice total). In Series B, 15 mice were injected weekly with either 120 μg/ml of short crocidolite

or 480 μg/ml of long crocidolite fibers (30 mice total). The doses of long and short crocidolite used in Series B contained the same number of fibers as 200 μg/ml of mixed crocidolite. At the intervals indicated, complete autopsies were performed on all mice.

RESULTS

Preparation and Characterization of Long and Short Fibers

Native UICC crocidolite asbestos was used for separation of long fibers and short fibers by repeated centrifugations. The number of fibers per mg of each fiber sample was determined by transmission electron microscopy and is shown in Table I. Greater than 60% of the long fiber preparation is longer than 2.0 μm, while 90% of the short fiber preparation is shorter than 2.0 μm. The doses used in these *in vitro* and *in vivo* experiments were adjusted to contain an equal number of native, long, or short fibers. The doses listed in Table I correspond to 2.8–5.6 × 10^9 fibers/10^6 cells in the *in vitro* experiments and approximately 5.7 × 10^8 fibers/10^6 resident peritoneal macrophages in the *in vivo* experiments. For the *in vitro* experiments, the dose was kept constant with respect to cell number and surface area of the culture dish. The LD_{50} corresponds to approximately 25 μg of native crocidolite asbestos fibers/cm; 2 complete time and dose response curves were published previously.[11] In the *in vivo* experiments to produce mesotheliomas, approximately 40 weekly injections of 200 μg of native crocidolite asbestos fibers were used. The total dose delivered to each mouse over this time period is approximately 2 × 10^{10} fibers. This dose is comparable to human occupational exposures to asbestos fibers estimated at 10^{10}—10^{11} fibers during a lifetime.[12] In contrast, previously reported models to induce mesotheliomas in rodents use a single dose of fibers ranging from 10–25 mg injected intrapleurally or intraperitoneally.[1,13-17]

In Vitro Toxicity of Long and Short Fibers

Long or short fiber preparations of crocidolite asbestos caused a dose-dependent decrease in viability of thioglycollate-elicited mouse peritoneal macrophages. As shown in Table II, equal numbers of long and short fibers killed 20–30% of the cells after six hours. After 24 hours, only 7.0 ± 0.2% of the cells exposed to short fibers were viable, while 6.4 ± 1.5% of the cells exposed to long fibers remained viable. At earlier time points, there is a lag in short fiber toxicity most likely due to the delayed time in settling onto the cultured cells.

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

Previous studies have shown that acute asbestos toxicity in a variety of *in vitro* models is mediated by reactive oxygen metabolites.[11,18,19] While phagocytosis of any particle triggers the release of superoxide anion and H_2O_2, the toxicity of asbestos fibers is postulated to depend on the iron-catalyzed generation of the hydroxyl radical. We tested whether a similar oxidant-dependent mechanism is responsible for acute toxicity of long and short crocidolite asbestos fibers in our *in vitro* model system.

We tested whether long and short fiber preparations stimulated the release of H_2O_2 from elicited mouse peritoneal macrophages in vitro. As shown in Table II, equal numbers of long and short fibers produced similar release of H_2O_2 after six hours of exposure. Both long and short fiber preparations also stimulated the production of superoxide anion as shown by the reduction of NBT.

Acute toxicity of native crocidolite asbestos fibers to macrophages is prevented by exogenous superoxide dismutase and catalase which detoxify superoxide anion and H_2O_2, respectively, or by coating fibers with deferoxamine

Table I

Characteristics of Crocidolite Asbestos Fiber Preparations

Sample	# of Fibers per mg x 10^9	% of Fibers ≥ 2.0 μm long	*In Vitro* Dose (μg)	*In Vivo* Dose (μg)
Mixed (native) fibers	2.93	23.6	50	200
Long fibers	1.22	60.3	120	480
Short fibers	4.64	9.4	30	120

Short and long asbestos fibers were separated from native crocidolite asbestos by differential centrifugation as described in Materials and Methods.

Table II

Viability and Release of H_2O_2 by Elicited Mouse Peritoneal Macrophages Exposed to Short or Long Crocidolite Asbestos Fibers for Six Hours *In Vitro*

Sample	Viability	nmoles $H_2O_2/10^6$ cells
Control (untreated)	100 ± 3.2[a]	0
Long fibers	19.0 ± 7.5	25.7 ± 9.8
Short fibers	31.3 ± 5.8	23.6 ± 4.8

[a] Mean ± SD.

Thioglycollate-elicited mouse peritoneal macrophages (2.5-5.0 x 10^4 cells per 12-mm glass coverslip) were exposed to equal numbers of short (30 µg) or long (120 µg) crocidolite asbestos fibers. After six hours, viability was determined by the ability to hydrolyze and retain fluorescein diacetate as described in Materials and Methods. For determination of H_2O_2 release; 10^6 cells per 35-mm multiwell were exposed to equal numbers of short (180 µg) or long (720 µg) crocidolite asbestos fibers. After six hours, release of H_2O_2 was measured as described in Materials and Methods. No H_2O_2 was detected when 0.5 mg/ml catalase was included in the reaction mixture.

which prevents the iron-catalyzed formation of the hydroxyl radical.[11] As shown in Table III, exogenous superoxide dismutase or catalase decreased the toxicity of long or short fibers. Deferoxamine-coated long or short fibers were also less toxic in this *in vitro* model.

In Vivo Toxicity of Crocidolite Asbestos Fibers

We have previously characterized the acute mesothelial reactions to a single intraperitoneal injection of 200 µg of crocidolite asbestos fibers in mice. The morphological reactions to asbestos fibers were studied by scanning electron microscopy of the diaphragm. Between 1-3 days after injection of asbestos fibers, mesothelial cells become swollen, develop blebs, and detach from the surface of the diaphragm.[10] Macrophages phagocytizing asbestos fibers also show morphologic evidence of injury. Three days after injection of native crocidolite asbestos fibers, there was increased trypan blue staining of the diaphragm and increased LDH activity recovered in the peritoneal lavage fluid (Table IV). A single intraperitoneal injection of PBS, thioglycollate broth, or titanium dioxide or silica particles did not injure the peritoneal lining.

Reactive Oxygen Metabolites are Released from Macrophages Exposed to Asbestos Fibers *In Vivo*

A single injection of crocidolite asbestos fibers has been shown to stimulate an inflammatory response characterized by accumulation of macrophages on the mesothelial surface.[10] Nitroblue tetrazolium (NBT) was used to detect production of reactive oxygen metabolites *in situ*. Three days after injection of native crocidolite asbestos fibers, mice were injected intraperitoneally with NBT and sacrificed 15 minutes later. Blue formazan precipitates were found at sites of asbestos fiber deposition on the surface of the diaphragm. This localized staining was completely inhibited by SOD. A nondegradable particle, titanium dioxide, or a soluble inflammatory agent, thioglycollate broth, did not cause reduction of NBT at the mesothelial lining. Peritoneal macrophages collected by lavage three days after a single injection of 200 µg of native crocidolite asbestos fibers showed spontaneous release of H_2O_2 when assayed *in vitro* (39.2 ± 2.8 nmoles $H_2O_2/10^6$ cells/hour). Neither macrophages collected three days after injection of PBS or thioglycollate broth nor resident peritoneal macrophages released any detectable H_2O_2 when assayed *in vitro*.

Exogenous Scavenging Enzymes or Deferoxamine Reduces Crocidolite-Induced Injury *In Vivo*

We next investigated whether the enzymes SOD and catalase decreased crocidolite-induced injury *in vivo*. SOD and catalase conjugated to polyethylene glycol (PEG) were used to prolong their stability *in vivo*. Both PEG-catalase and PEG-SOD significantly decreased the number of trypan blue-positive cells on diaphragms exposed to crocidolite (Table IV). Treatment of crocidolite-injected mice with PEG-SOD or PEG-catalase also reduced LDH activity compared to crocidolite alone (Table IV). It is unlikely that the protection

afforded by PEG-SOD and PEG-catalase was due to the nonspecific adsorption of proteins onto crocidolite fibers. Inactivated PEG-catalase did not prevent crocidolite-induced injury and PEG-conjugated bovine serum albumin did not protect cells from crocidolite-induced damage.

We tested whether the iron chelator, deferoxamine, could decrease crocidolite-induced injury *in vivo*. Mice were injected intraperitoneally with deferoxamine-coated crocidolite and then sacrificed after 3 days. Deferoxamine significantly decreased the number of trypan blue stained cells on the diaphragm compared to crocidolite alone (Table IV) and produced a dose-dependent decrease in recovered LDH activity.

In Vivo Toxicity of Long and Short Fibers

We next compared the acute toxicity of short, long, and mixed crocidolite *in vivo*. In contrast to mixed or long crocidolite fibers, a single intraperitoneal injection of 200 μg of short crocidolite fibers results in only a mild inflammatory response and little cellular injury. Longer fibers, on the other hand, are trapped on the surface of the diaphragm at the lymphatic stomata. We tested whether short crocidolite would be acutely cytotoxic *in vivo* if fibers were not cleared from the peritoneal cavity. To obstruct stomata, mice were injected intraperitoneally with Amicon agarose blue A beads (50–150 μm in diameter) and 2 days later injected with 120 μg of short crocidolite fibers. Recovered LDH activity was the same when mice were injected with short crocidolite fibers plus agarose beads or when mice were injected with mixed crocidolite fibers (Table V). Agarose beads alone did not increase recovered LDH activity. Similarly, trypan blue staining on the surface of the diaphragm was similar after injection of mixed crocidolite alone or short crocidolite fibers plus agarose beads.

Carcinogenicity of Long and Short Fibers

Crocidolite asbestos fibers are not only toxic to mesothelial cells *in vitro* and *in vivo*, but are also carcinogenic. On the basis of our previous observation that short fibers can injure the mesothelial lining if lymphatic clearance is obstructed, we tested whether repeated exposures to short fibers would obstruct lymphatic clearance and produce mesotheliomas. Mice were injected weekly with equal numbers of native, long, or short crocidolite asbestos fiber

Table III

Protection Against Toxicity Caused by Long or Short Crocidolite Asbestos Fibers by Superoxide Dismutase or Catalase

Sample	Viability	
Control (untreated)	100 ± 9.8	[a]
Long fibers	3.3 ± 0.5	
Long fibers + catalase	61.6 ± 14.2	[b]
Long fibers + SOD	74.2 ± 3.5	[c]
Short fibers	38.8 ± 8.1	
Short fibers + catalase	97.6 ± 9.1	[d]
Short fibers + SOD	94.2 ± 4.9	[b]

[a] Mean ± SD.
[b] P< 0.01 as compared to long or short fibers alone.
[c] P< 0.001 as compared to long fibers alone.
[d] P<0.002 as compared to short fibers alone.

Thioglycollate-elicited mouse peritoneal macrophages were exposed to equal numbers of long (120 μg) or short (30 μg) crocidolite asbestos fibers for six hours. Where indicated, superoxide dismutase (SOD; 420 μg/ml) or catalase (50 μg/ml) was added. Viability was determined as described in Materials and Methods. Cultures treated with SOD or catalase alone showed no loss of viability.

Table IV

Trypan Blue Staining and LDH Activity in Peritoneal Lavage Fluid Three Days after Intraperitoneal Injection of Native Crocidolite Asbestos Fibers Alone or with Exogenous Scavengers

Treatment	Number of Trypan Blue Stained cells per 0.5 cm^2	LDH activity (Units/ml)
Control (PBS)	73 ± 24 [a]	44.0 ± 13.0 [a]
Crocidolite asbestos	798 ± 105 [b]	154.0 ± 13.0 [b]
Crocidolite + PEG-SOD	247 ± 49 [c]	90.3 ± 7.5 [c]
Crocidolite + PEG-catalase	349 ± 46 [d]	66.7 ± 6.5 [c]
Crocidolite + deferoxamine	381 ± 14 [d]	83.0 ± 6.0 [c]

[a] Mean ± SEM of triplicate mice.
[b] $P < 0.001$ compared to control (PBS).
[c] $P < 0.002$ compared to crocidolite alone.
[d] $P < 0.02$ compared to crocidolite alone.

Mice were injected with 1.0 ml of PBS or 200 µg of native crocidolite asbestos fibers. Where indicated, mice also received an injection of 500 units of PEG-SOD or daily injections of 500 units of PEG-catalase as described in Materials and Methods. Finally, mice were injected with 200 µg of crocidolite asbestos fibers which had been presoaked in 10 mM deferoxamine, then rinsed as described in Materials and Methods. Peritoneal lavage fluid was collected three days later and assayed for lactate dehydrogenase (LDH) activity. The dissected diaphragms were stained with trypan blue and counted as described in Materials and Methods. Three mice were used in each treatment group. Injection of PEG-SOD or PEG-catalase did not significantly increase the extent of trypan blue staining as compared to controls.

preparations. After 22–60 weekly injections, animals were sacrificed as they developed ascites or evidence of intestinal obstruction. After injection of native crocidolite asbestos fibers, 37.5% of the mice developed mesotheliomas. In comparison, 23.5% of mice injected with long fibers and 50.0% of mice injected with short fibers had mesotheliomas.

DISCUSSION

In this report, we present evidence that both long and short crocidolite asbestos fibers are toxic to thioglycollate-elicited mouse peritoneal macrophages *in vitro*. Both fiber preparations stimulated release of H_2O_2 from these cells. As shown in previous investigations, reactive oxygen metabolites mediate acute asbestos toxicity.[11,18,19] Long and short fiber preparations of crocidolite asbestos also killed macrophages via a similar, oxidant-dependent mechanism. As with native crocidolite asbestos fibers, catalase, superoxide dismutase, or deferoxamine decreased the toxicity of long or short fibers.

Numerous *in vitro* studies have reported that long asbestos fibers are more biologically active than short asbestos fibers.[3-9] The differences between these previously published studies and the data reported here reflect different experimental protocols and different definitions of acute toxicity. In these experiments, we exposed primary cultures of elicited mouse peritoneal macrophages to equal numbers of fibers in the absence of serum. Toxicity, as defined by hydrolysis and retention of fluorscein diacetate, was monitored up to 24 hours. Similar results were obtained using erythrosin B staining or trypan blue uptake. Under these conditions, similar to peritoneal macrophages *in vivo*, these cells do not proliferate. Finally, we obtained our short fiber preparation by centrifugation, not by milling which alters the surface properties of asbestos fibers.[20]

Table V

In Vivo Injury Caused by Intraperitoneal Injection of Mixed or Short Crocidolite Asbestos Fibers Alone or with Argarose Beads

Treatment	Extent of Trypan Blue Staining	LDH Activity (units/ml)
Control (PBS)	−	44.0 ± 13.0 [a]
Control (beads)	−	59.0 ± 4.9
Short fibers	+	93.5 ± 18.8 [b]
Short fibers + beads	+++	168.0 ± 30.0 [c]
Mixed fibers	+++	164.0 ± 32.0 [c]

[a] Mean ± SEM of triplicate mice.
[b] P< 0.02 compared to control (PBS).
[c] P< 0.05 compared to short fibers alone.

Mice were injected intraperitoneally with 1.0 ml of PBS, 120 µg of short crocidolite asbestos fibers, or 200 µg of mixed (native) crocidolite asbestos fibers. Where indicated, two days before injection of short crocidolite asbestos fibers, mice were injected intraperitoneally with agarose beads as described in Materials and Methods. After 3 days, the mice were sacrificed. Peritoneal lavage fluid was collected and assayed for LDH activity and the diaphragms stained with trypan blue as described in Materials and Methods.

Fiber dimensions are also an important factor in the chronic reactions to asbestos fibers. In animal models, long fibers are more inflammatory and fibrogenic than short fibers.[1] More effective clearance of short fibers from the lungs may be responsible for these different reactions to long and short fibers. However, even with direct implantation of fibers into the pleural or peritoneal cavity, long fibers induce mesotheliomas more effectively than short fibers or spherical mineral particles.[13,14] In previous studies, we confirmed that direct intraperitoneal injection of long fibers produced more mesothelial cell injury *in vivo* than injection of short fibers or spherical mineral particles. Long fibers are not as readily cleared through lymphatic stomata at the peritoneal lining, while short fibers and spherical mineral particles accumulate in regional lymph nodes.[10] In this report, we present evidence that short fibers are also cytotoxic *in vivo* and carcinogenic if lymphatic clearance is obstructed or saturated. It is not known whether occupational exposure to massive doses of short rated. It is not known whether occupational exposure to massive doses of short fibers or other particulates may also saturate pulmonary lymphatic clearance mechanisms and increase the risk of developing mesotheliomas.

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This research was supported by research grants ES 03721 and ES 03189 from the National Institutes of Health

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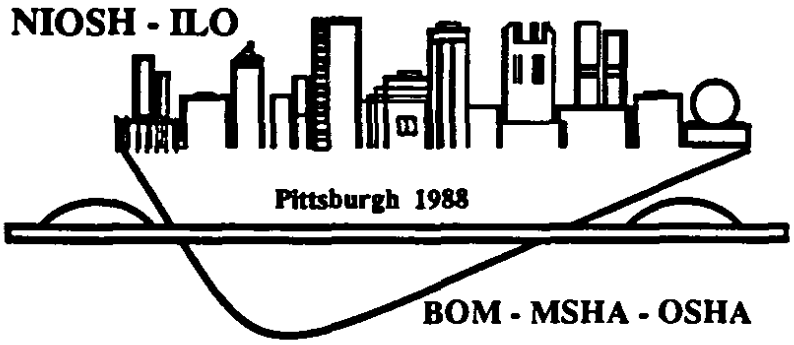

Pittsburgh, Pennsylvania, USA—August 23–26, 1988
Pittsburgh, Pennsylvanie, Etats-Unis—23–26 aout 1988
Pittsburgh, Pennsylvania EE. UU—23–26 de agosto de 1988

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September 1990

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DHHS (NIOSH) Publication No. 90-108 Part I