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Reactive Oxygen Metabolite Production Induced by Asbestos and Glass Fibers: Effect of Fiber Milling

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Abstract: Particle stimulated chemiluminescence (CL) production by human polymorphonuclear leucocytes (PMN) has been utilized to evaluate the pathogenicity of mineral and glass fibers with the understanding that reactive oxygen metabolites (ROM) production as measured by CL is etiopathogenically related to fiber toxicity. In the present study to investigate the specific pathogenic role of fiber number and dimensions, CL production from PMN exposed to anthophyllite asbestos mineral and glass fiber samples milled for different time periods was measured. Almost all the fibrous particles in the glass fiber sample were destroyed after milling for 30 minutes. With anthophyllite, the total number of fibrous particles remained almost constant for up to 240 minutes of milling, although the size of fibrous particles was reduced. CL produced by the same mass of glass fiber was elevated after milling for 15 minutes, but then declined when the milling time was further increased. Similarly, with anthophyllite, the production of CL was elevated at the first period of milling for 30 minutes, but then declined at the longer milling times. The level of CL produced was not correlated to the total number of fibrous particles, for both the glass fiber and the anthophyllite samples. Likewise for the glass fiber and anthophyllite samples, no specific range of fiber dimension was correlated to the peak hight CL production. These findings indicate that neither the total number, nor the specific range of fiber dimension solely determines CL production. As a consequence, it may be concluded that other physiochemical factors, such as the surface reactive characteristics of milled fibers, may be more closely related to CL production by PMN.

Key words: Chemiluminescence, Free radicals, Polymorphonuclear leucocytes, Glass fiber, Asbestos, Anthophyllite, Milling, Fiber size

Introduction

Stanton *et al.* recognized that various durable mineral fibers with length larger than 8 μm and with width smaller than 1.5 μm induced pleural sarcoma similar to human mesothelioma in rats when fiber samples were administered intrapleurally[1]. They proposed that the carcinogenicity of mineral fibers was dependent on fiber dimension and durability rather than on physicochemical properties. Inhalation studies have also supported the hypothesis[2, 3].

The relationship between fiber dimensions and tissue reaction against fibrous materials has been explored with a variety of *in vitro* methods, including cell viability[4], superoxide production[5, 6], chromosomal aberration[7], and TNF release[8, 9]. The production of reactive oxygen metabolites such as superoxide anion by phagocytes can be detected as luminol dependent chemiluminescence (CL)[10–12]. CL is not only a marker of cell viability[5, 6], but an indicator of tissue reactions against mineral fibers[11, 13] related to inflammation and carcinogenesis[14].

We have examined the stimulatory effect of mineral anthophyllite asbestos and glass fibrous particles of differing

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Table 1. Number and size distribution of milled samples with various milling time duration

	Glass fiber				Anthophyllite			
Duration of milling (minutes)	0	15	30	60	0	30	60	240
Number $\times 10^{-6}$/mg	540	590	80	19	2400	1800	2400	2600
Length (μm)								
25 percentile	1.2	1.1	1.1	–	0.77	0.84	0.63	0.32
50 percentile	1.8	1.9	1.6	–	1.6	1.5	1.4	0.64
75 percentile	3.5	3.5	2.7	–	3.3	2.6	2.6	0.96
Width (μm)								
25 percentile	0.17	0.15	0.19	–	0.12	0.12	0.11	0.07
50 percentile	0.41	0.22	0.32	–	0.17	0.16	0.15	0.13
75 percentile	0.61	0.37	0.53	–	0.29	0.20	0.19	0.18
Related figure for size*	1 a	1 b	1 c	1 d	2 a	2 b	2 c	2 d
Related figure for the CL**	3 a	3 b	3 c	3 d	4 a	4 b	4 c	4 d

*Distribution of width (μm) and length (μm) of each sample is represented in Figs. 1 and 2. **Each alphabet corresponds to the curve depicted in Figs. 3 and 4, which represents time course of CL production induced by the sample.

dimensions upon CL production of human polymophonuclear cells (PMN). Samples containing fibrous particles of various dimensions were prepared by ball mill using different milling times. The total number and distribution in dimensions of fibrous particles were estimated using transmission electron microscopy analysis. The specific purpose of the present study is to clarify whether the total number of fibrous particles or fibrous particles of specific dimensions, are closely correlated to the CL production of PMN. If CL production of PMN is not adequately explained by either the total number or size distribution of fibrous particles, it suggests that CL production of PMN depends upon alternative factors such as the surface reactive properties of mineral fibers.

Materials and Methods

Preparation and evaluation of milled fiber samples

One gram of JM100 microglassfiber (Johns Manville) was milled in a ball mill (Pascall) with 70 medium balls and 50 small balls at 65 rpm for 0, 15, 30, 60 minutes. One gram of UICC standard anthophyllite asbestos sample was milled for 0, 30, 60, 240 minutes. The time of milling was selected after several trials in order to obtain suitable samples with appropriate wide ranges of fiber dimensions. The number and dimensions of fibrous particles and their distribution in relation to the time of milling were obtained by direct measurements of transmission electron microphotographs. Particles of length greater than three times their width were defined as fibrous particles and counted. One ml of 50 μg /ml anthophyllete sample suspension in water was collected and dried on a 227 mm^2 area of a Millipore® filter. The filter was spattered with carbon and removed with acetone, and samples were observed on a mesh with magnification of $\times$ 3000. Photographs taken were further enlarged 2.36 times. Glass fiber samples were similarly treated.

PMN chemiluminescence measurements

PMN was obtained from heparinized blood of an author (T.I.) by sedimentation and hemolysis and preincubated with luminol in Krebs-Ringer-Hepes solution at 37°C for 5 minutes[11]. Fiber sample suspension in Krebs-Ringer-Hepes buffer was added and chemiluminescence was recorded in Biolumat LB 9505 luminometer (Berthold) at 37°C. The final observation mixture contained 1.0×10^6 /ml PMN, 100 μM luminol, and 400 μg/ml fiber sample in Krebs-Ringer-Hepes buffer solution (pH 7.35)[11]. Sequential time measurements of CL production from simultaneous observations of the milled and unmilled samples run in parallel were represented in one trace figure. Each set of fibers experiments were performed in triplicate, which confirmed that the order of the peak heights in one figure did not vary among replicate experiments. Using the PMN samples obtained from conveniently recruited four persons, consistency of the results over different donors was examined. Typical results of the replicate experiments are shown to illustrate the time course of CL production.

Results

The total numbers of fibrous particles in the untreated and milled samples are shown in Table 1. The total number of fibrous particles in the glass fiber samples did not change

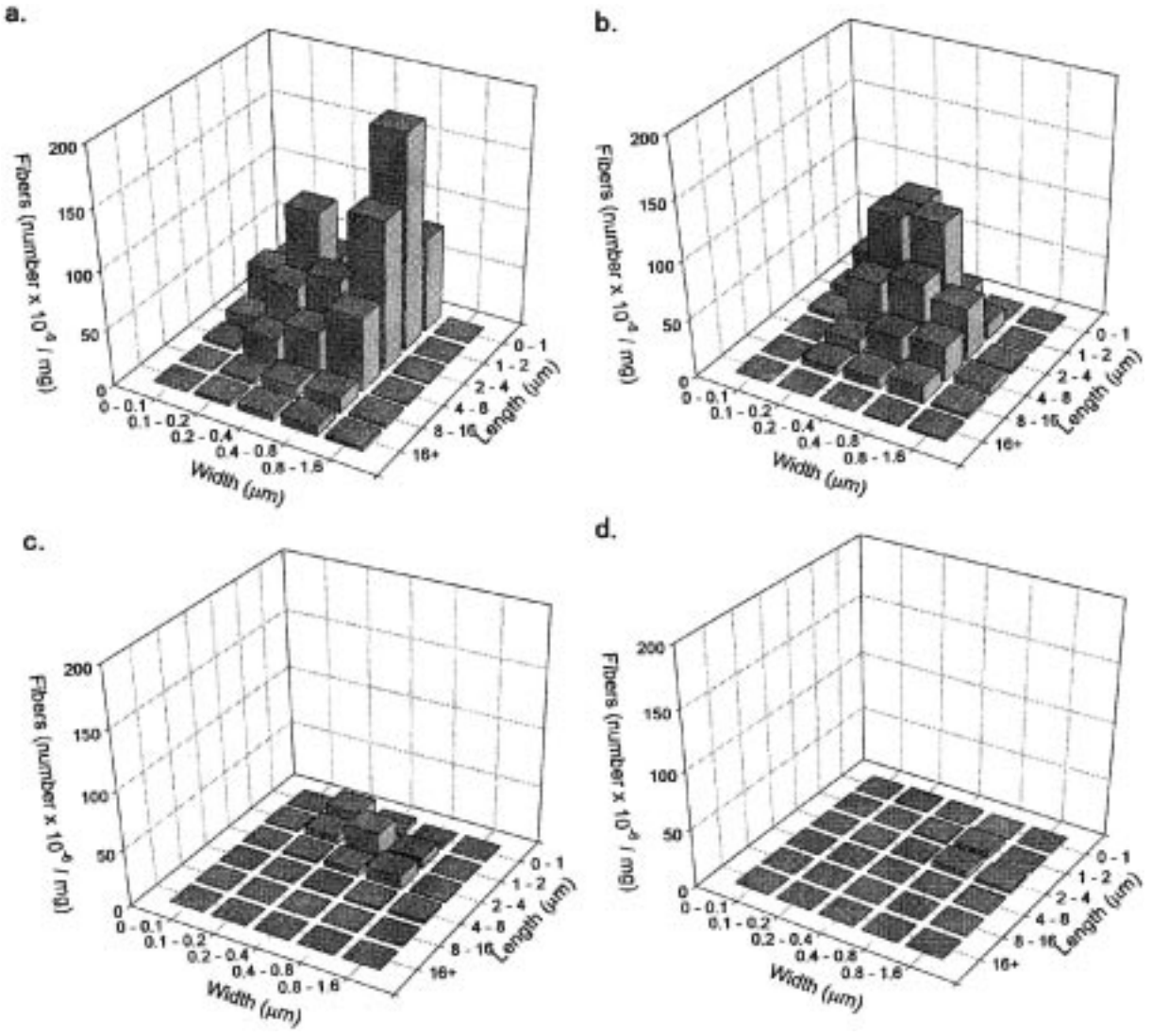

Fig. 1. Changes in the size distribution of glass fiber samples with duration of the milling.
Aliquot of ball-milled glass fiber samples was photographed using transmission electron microscope. The length and width of each fibrous particle was determined. Histogram of particulate number with certain length (0<length≤1 μm, 1 μm<length≤2 μm, 2 μm<length≤4 μm, 4 μm<length≤8 μm, 8 μm<length≤16 μm, or, 16 μm<length) and width (0<width≤0.1 μm, 0.1 μm<width≤0.2 μm, 0.2 μm<width≤0.4 μm, 0.4 μm<width≤0.8 μm, or, 0.8 μm<width≤0.16 μm) contained in 1 mg of sample is shown. Times of milling were: (a), 0 minutes; (b), 15 minutes; (c), 30 minutes; (d), 60 minutes.

much after 15 minutes, but decreased following longer milling times. The total number of fibrous particles decreased to 4% of the unmilled sample after milling for 60 minutes. In the case of the anthophyllite sample, the total number of fibrous particles remained stable up to the end of four hours' milling.

Changes in dimensions of fibrous particles in the untreated and milled samples are shown in Table 1. The numbers of the longer glass fibers diminished and the width of glass fibers decreased. With anthophyllite samples, length and width decreased continuously with milling time to the final lowest level attained after 240 minutes of milling. Changes in particle size distribution profiles of glass fiber and anthophyllite samples in relation to the time of the milling are presented in Figs. 1 and 2. The original glass fiber sample contained large numbers of fibrous particles with widths larger than 0.4 μm and smaller than 0.8 μm. After 15 minutes of milling, this particular fraction disappeared, and fiber numbers increased among the fractions with widths larger than 0.1 μm and smaller than 0.4 μm, and lengths smaller than 2 μm. After 30 minutes of milling only a few fibrous glass particles remained. Fibrous particles of anthophyllite

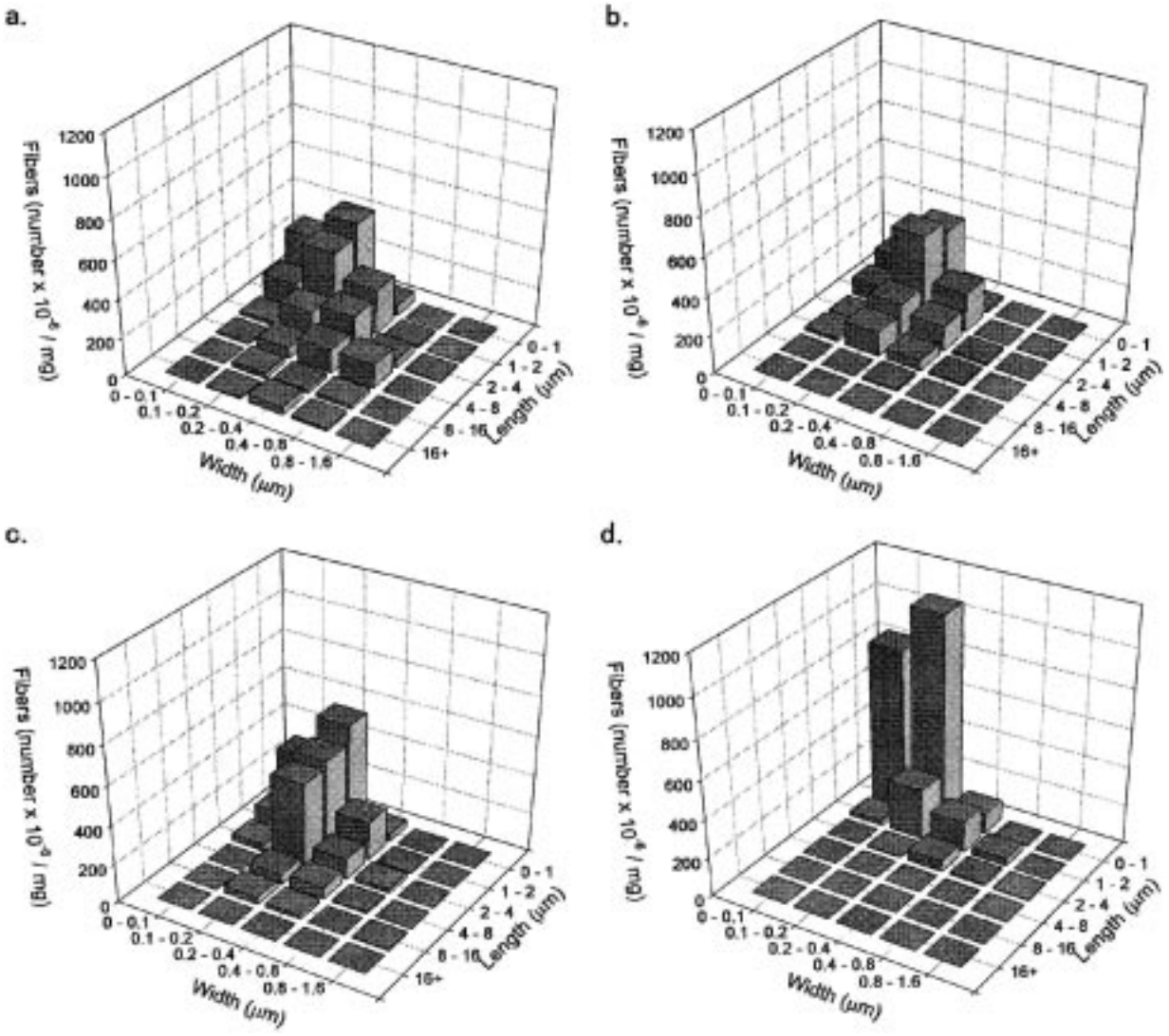

Fig. 2. Changes in the size distribution of anthophyllite samples with duration of the milling.
Aliquot of ball-milled anthophyllite samples was photographed using transmission electron microscope. The length and width of each fibrous particle was determined. Histogram of particulate number with certain length (0<length≤1 μm, 1 μm<length≤2 μm, 2 μm<length≤4 μm, 4 μm<length≤8 μm, 8 μm<length≤16 μm, or, 16 μm<length) and width (0<width≤0.1 μm, 0.1 μm<width≤0.2 μm, 0.2 μm<width≤0.4 μm, 0.4 μm<width≤0.8 μm, or, 0.8 μm<width≤0.16 μm) contained in 1 mg of sample is shown. Times of milling were: (a), 0 minutes; (b), 30 minutes; (c), 60 minutes; (d), 240 minutes.

decreased mainly in length during the milling period of 30 to 60 minutes. Anthophyllite sample milled for 240 minutes contained mainly fibrous particles with width smaller than 0.2 μm and length smaller than 1 μm.

Aliquots of unmilled or milled fibrous samples at the same final mass concentration of 0.4 mg/ml were added to the PMN in all the experiments. Production of CL increased rapidly after the addition of glass fiber samples, producing a peak at about 5 minutes (Fig. 3) and remained high for over 25 minutes. The peak height of CL produced by the glass fiber sample milled for 15 minutes was the highest and the peak height for 60 minutes' milling was the smallest, while the peak height produced for 30 minutes milling was a similar level as that of the unmilled sample. These observations were conserved among PMN samples from further recruited 4 subjects. The maximal peak height of CL produced by the anthophyllite samples was significantly larger than that produced by the glass fiber samples. In contrast, the CL production peaked more rapidly at about 1 minute and exhibited a more pronounced biphasic response, but had almost completely ceased after 20 minutes from the addition of the anthophyllite sample (Fig. 4). The level

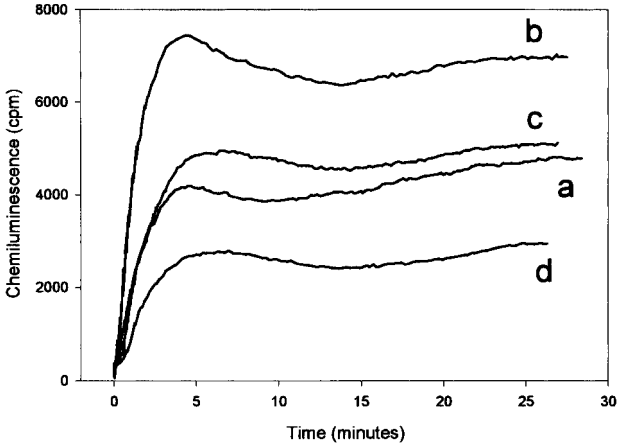

Fig. 3. CL produced by milled glass fiber samples.
Luminol-dependent CL of 1.0×10^6 human PMN stimulated with glass fiber samples was recorded with Biolumat LB 9505 luminometer. Milling time of each sample was: (a), 0 minutes; (b), 15 minutes; (c), 30 minutes; (d), 60 minutes.

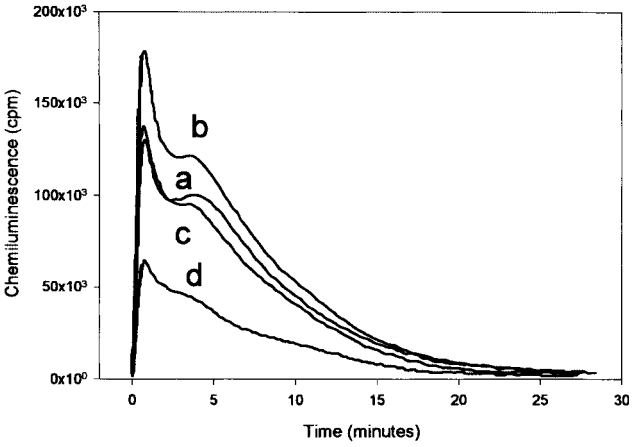

Fig. 4. CL produced by milled anthophyllite samples.
Luminol-dependent CL of 1.0×10^6 human PMN stimulated with anthophyllite samples was recorded with Biolumat LB 9505 luminometer. Milling time of each sample was: (a), 0 minutes; (b), 30 minutes; (c), 60 minutes; (d), 240 minutes.

of CL production by the anthophyllite sample milled for 30 minutes was larger than that by the untreated sample. This change was consistently observed with all PMN samples prepared from the four recruited subjects. However after 30 minutes of milling, the level of CL production decreased in relation to the duration of milling. All the time courses of CL production by anthophyllite milled for various times were broadly similar.

Discussion

In both samples of glass fiber and anthophyllite, the level of CL produced increased with the shorter time of milling, and decreased with the longer time of milling.

Starting from the same material, the particulate samples we compared differed only in the time of ball milling for preparation, and they were found to contain a different size distribution of fibrous particles. If physicochemical changes had occurred on the fiber surface, they may also be expected to be dependent on the time of milling. Some studies have used purified samples prepared by repeated centrifugation[5] or by dielectrophoresis[15] to compare the effect of fibers differing only in their length. However, these fibers were prepared by milling before the purification that the surface physicochemical properties might have been changed as well. Therefore, it cannot be ruled out that the physicochemical properties have some effect.

CL production would be expected to be proportional to the total number of fibrous particles if PMN interact with each the individual fiber. Coffin *et al.*[16] showed, in comparison between amosite and ferroactinolite toxicity, that hemolysis and viability data agreed with in vivo tumorigenesis data best when these in vitro data were expressed as total fiber number. The present study indicates that the number of glass fiber particles decreased markedly after milling for 30 minutes, while the level of CL produced by the sample was at the same level as that by unmilled sample. However for the anthophyllite, the number of fibrous particles remained at the same level in the milled as the unmilled sample, while the level of CL decreased. These results indicate that the CL production is apparently not exclusively determined by the total number of fibrous particles.

Another possibility, by which the CL production is determined, may be by a specific size range of fibrous particles. Blake *et al.*[15] reported that among five fiber length categories, a fraction with a mean length of 17 μm was the most active in injuring cells. We have previously reported that number of fibers contained in glass fiber thin fractions corresponded well with the CL production[17]. In this study, we employed the same glass fiber samples and independently prepared anthophyllite samples, and obtained length and width distribution of each samples, resulting in the different interpretation described below. With our glass fiber samples, the level of CL produced by the sample milled for 60 minutes remained almost 60% of the level of CL produced by untreated sample, and the sample milled for 30 minutes was similar to that of the unmilled sample. These results indicate

that CL is being produced by non-fibrous glass particles. In the sample milled for 15 minutes, the number of fibrous particles with widths between 0.1 μm and 0.4 μm and lengths smaller than 2 μm increased, although this increase in number did not seem to be sufficient to explain the increase in the amount of CL produced by the sample milled for 15 minutes.

With the anthophyllite milled samples, the number of fibrous particles greater in length and width decreased, and the number of smaller size fibrous particles increased, which indicated that anthophyllite samples were split both lengthwise and widthwise by the milling. Short and thin anthophyllite fibers contained in the sample milled for 240 minutes were considered to have weak potency to produce CL, because the sample produced only a low level of CL but still contained a large number of fibrous particles. Changes in the levels of CL by samples milled for up to 60 minutes were not explicable by the size distribution of length and width. Peak height of CL production by the sample milled for 30 minutes was larger than that by unmilled sample. Peak height of CL production by the sample milled for 60 minutes was smaller than that by the sample milled for 30 minutes. This rank was consistently observed throughout the experiments using PMN from different subjects. However, we cannot indicate specific responsible range of particles among the Figs. 2a, 2b, and 2c.

As was shown for both amorphous glass fiber and crystalline anthophyllite, short time milling produce the largest level of CL, and samples milled for a longer time lost their CL stimulatory ability as the milling time increased. The milling process inevitably produces ablation and friction that may result in modification to the fiber surface physiochemical characteristics. With amosite asbestos originally prepared by milling, it is reported that the oxidative state and site occupancy of Fe is different between long and short fibers[18]. Sakabe *et al.*[19] has reported that the toxicity of quartz particles to peritoneal mononuclear cells decreased when the particle sample was milled, and that the toxicity reappeared by acid or alkaline leaching of milled samples. They concluded that the decrease in the toxicity was related to associated changes of the particle surface as was revealed by x-ray diffraction analysis, electron microanalysis, and infrared adsorption spectra. Using the standard UICC chrysotile B sample, Koshi *et al.* reported that cytotoxicity and hemolysis potential was reduced following milling and heating of the sample[20], and furthermore, heat treatment of chrysotile B sample was found to induce changes in the surface crystal structure[21]. In future studies on the effect of particle size, surface characters of fiber samples would need to be controlled for.

In summary, the findings of the present study indicate that change in the level of CL production by milled fiber samples was determined neither by the total number of fibers, nor by the size distribution of fibrous particles. This result suggests that changes in the level of CL production may therefore be potentially determined by non-dimensional fiber-related factors such as the surface characteristics of the milled particles. Consequently, subsequent investigations using particle surface-sensitive analytical techniques e.g. X-ray diffraction, would be a requisite to elucidate with greater precision and specificity the PMN CL particulate stimulatory activity.

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