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Effect of Particle Size of Intratracheally Instilled Crystalline Silica on Pulmonary Inflammation

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Abstract: Effect of Particle Size of Intratracheally Instilled Crystalline Silica on Pulmonary Inflammation: Takayoshi KAJIWARA, *et al*. Department of Environmental Health Engineering, University of Occupational and Environmental Health— Crystalline silica, known as a causal substance of silicosis, has been carefully evaluated for its carcinogenicity and fibrogenicity. In this study, we instilled crystalline silica of two different size ($S_{1.8}$:1.80 μm (S.D. 2.0), $S_{0.7}$:0.74 μm (S.D. 1.5)) into the trachea of rats to evaluate the size effects of the particles on pulmonary inflammation. $S_{1.8}$ and $S_{0.7}$ samples were administered to rats by a single intratracheal instillation (2 mg/ 0.4 ml saline). At three days, 1 wk and 1, 3 and 6 months after the instillation, the blood, bronchoalveolar lavage fluid (BALF), and pulmonary tissues were analyzed. Six images per HE-stained section were digitally captured and examined by the point counting method (PCM). Polymorphonuclear leukocyte (PMN)-in-blood specimens and cytospin specimens from BALF were stained immunohistochemically with BrdU. At six months after the instillation, the effects on inflammatory cells in the pulmonary tissues and BALF tended to be more marked in the rats instilled with $S_{1.8}$ than those instilled with $S_{0.7}$. Particularly, clear differences were observed in the number of inflammatory cells in BALF. Even if the particles are of the same chemical composition, the results suggest that, their biological effects vary depending on their particle size. Therefore, when such particles are used in workplaces, strict control systems should be established according to the risks present by different sizes of particles.

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Key words: Crystalline silica, Instillation, Particle size, Rat, Inflammation

Particle size (surface area, length, etc.) is an important factor in the evaluation of the biological effects of respirable and insoluble particulate matter (PM)[1, 2]. It is said particles less than 10 μm of aerodynamic diameter, reaching deeply into the pulmonary alveoli, may be critical in the development of pneumoconiosis. It has also been suggested that the smaller the particle size the greater the inhalation toxicity to the lung[3, 4]. It has been reported that, regulations for airborne dust, should cover finer particles from PM_{10} down to $PM_{2.5}$[5]. Epidemiological studies have shown that an increase in the concentration of airborne dust (PM_{10} or $PM_{2.5}$) in the atmosphere leads to an increase of pulmonary and cardiovascular disorders[3, 6–8]. In addition, developments in nanotechnology and nanomaterials are rapidly proceeding ahead of a clear understanding of their potential health effects and environmental impacts. Therefore, in the workplace, assessment of the environment should include respirable particles.

Crystalline silica, known as a causal substance of silicosis, has been carefully evaluated for its carcinogenicity and fibrogenicity. It is known that the initial response of the lung to silica involves an inflammatory process with polymorphonuclear leukocyte (PMN) recruitment to the alveoli[9]. Our previous report[10] showed that crystalline silica instillation to the rabbit lung induces immature PMN from the bone marrow, like other inflammatory sources such as cigarette smoking[11], ambient particulate matter[12], or pneumococcal pneumonia[13]. Our hypothesis was that these immature PMN could play a role as an

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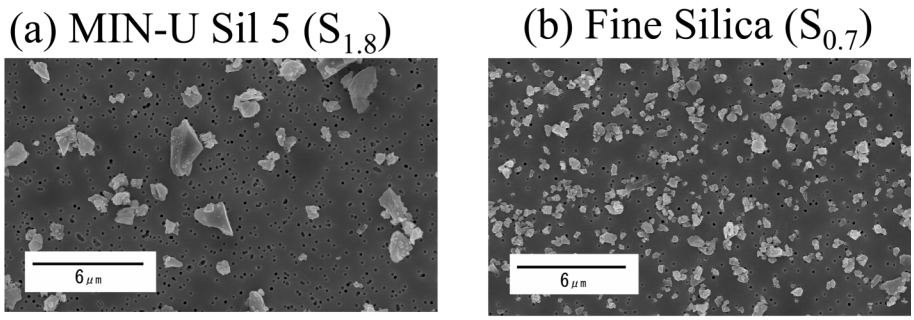

Fig. 1. Scanning electron micrograph: (a) MIN-U Sil 5, (b) fine silica obtained by liquid centrifugation of original MIN-U Sil 5.

indicator of the inflammatory impact of silica exposure. In this study, we instilled crystalline silica of two different size (micron-size vs. submicron-size) into the trachea of rats to evaluate the size effects of the particles. Evaluation was based on the kinetics of immature neutrophils that were newly produced from the bone marrow.

Material and Methods

Materials (Fig. 1)

We used commercial crystalline silica (MIN-U-Sil-5: U.S. Silica Co., Berkeley Springs, WV) and fine particles separated from the MIN-U-Sil-5 by liquid phase centrifugation. To prepare fine silica, MIN-U-Sil-5 was suspended in distilled water. After centrifuging the suspension (500 G: 10 min), the supernatant was filtered through a cellulose filter (pore size, 0.1 μm) and air dried at 50°C. The particle size distribution was determined by using a particle size analyzer (Microtrac FRA, Model FA500, Nikkiso). The particle size distribution is shown in Fig. 2. The geometric mean diameter was 1.80 μm (S.D.=2.0) and 0.74 μm (S.D.=1.5) for MIN-U Sil 5 ($S_{1.8}$) and fine silica ($S_{0.7}$), respectively.

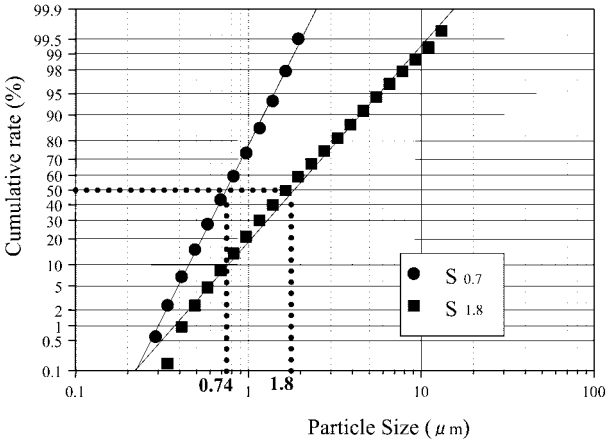

Fig. 2. Size distribution curve of crystalline silica. The particle size distribution was determined by using a grain size analyzer (Microtrac FRA, Model FA500, Nikkiso). The geometric mean diameter was 1.80 μm (S.D.=2.0) and 0.74 μm (S.D.=1.5) for MIN-U Sil 5 ($S_{1.8}$) and fine silica ($S_{0.7}$), respectively.

Experimental animals

Ten-week-old male Wistar rats were fed standard diet, and were maintained at the Animal Research Center of the University of Occupational and Environmental Health, in Japan. All of the experimental procedures were approved by the Experimentation Committee of the University of Occupational and Environmental Health, Kitakyushu, Japan.

Intratracheal instillation

$S_{1.8}$ and $S_{0.7}$ samples were suspended in saline at concentrations of 2 mg per 0.4 ml and administered to 35 rats per group (105 rats in total three groups) by a single intratracheal instillation. Control animals were given only physiological saline. At three days, 1 wk and 1, 3 and 6 months after exposure, 7 rats per group were euthanatized and subjected to autopsy. Blood samples, bronchoalveolar lavage fluid (BALF) and pulmonary tissues were collected from each rat.

Blood and bronchoalveolar lavage fluid (BALF)

At autopsy, rats were anesthetized with an intraperitoneal injection of ketamine (90 mg/kg) and xyladine (10 mg/kg), and their blood was taken from the heart. The number of cells was counted by an automatic blood cell counter (Celltac MEK 5204 Nihon Koden, Tokyo, JAPAN). Blood smears were stained by Diff-Quik kit (International Reagent Co., Kobe, Japan). By

randomly counting 200 white blood cells (WBC) cells per smear, the number of PMN was calculated.

BALF were recovered by injection of 15 ml of saline into the right lung while clamping the left main bronchus. After centrifugation (1,500 rpm; 10 min), the number of WBC were counted by means of a cell counter. PMNs in the BALF were also counted by preparing cytospin specimens.

Morphological procedures of pulmonary tissue

Extracted lungs were fully inflated and fixed by an intratracheal infusion of 10% buffered formalin at 25 cm water column pressure. After fixation, the left lung was sectioned into 3 blocks and then embedded in paraffin. Sections were stained with hematoxylin-eosin (HE). The procedure for evaluation of inflammatory areas has been described elsewhere[14]. Six images per HE-stained section were digitally captured at random. By placing a 300-point grid over each image on the PC screen, the inflammatory areas were examined by the point counting method (PCM). Excluding airspaces, the percentage of inflammatory areas in the pulmonary parenchyma which was inflamed was calculated. Results were then categorized into four grades according to the percentage of inflammation.

Measurement of PMN cells newly produced from bone marrow

Four days before each autopsy, bromodeoxyuridine (BrdU:SIGMA, 0.1 mg/g) was injected via the caudal vein to label the neutrophils that were newly produced from the bone marrow. In order to separate PMNs for the BrdU staining, the blood was collected in tubes containing acid citrate dextrose (ACD), mixed with its 4% dextran and allowed to stand for 30 min at room temperature. Leukocyte suspension (LRP) separated from the upper layer was laid on 4 ml of lymphocyte separation medium (LSM) (ICN Biomedicals: specific gravity 1.077) and centrifuged at room temperature for 30 min (1,600 rpm). Osmotic Shock was added to the cell sediment of the lowest layer (red blood cell and PMN) of the 4 layers to destroy the red blood cells, and the supernatant was removed by aspiration. The cell suspension was adjusted to 2000 cells/μl by adding PMN buffer (containing 2% FBS), and 75 μl (150,000 cells) was taken for preparation of cytospin specimens and fixed with methanol for 10 min.

Immunological staining

PMN-in-blood specimens and cytospin specimens from BALF were stained with BrdU by the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method. Mayer's hematoxylin was used for counter staining. BrdU-positive PMNs were determined by the method described by Mukae[12] and Ogami[10]. After randomly selecting fields under a light microscope, 200 cells per specimen were examined. Cells with even slightly stained nuclei were judged to be positive.

Statistical significance

Data are expressed as the mean ± standard error (SE). Differences between the three groups were analyzed by analysis of variance (ANOVA). A p value of 0.01 or less, was judged as a significant difference.

Results

Blood

For the total white blood cell counts (Fig. 3A) and the number of PMNs (Fig. 3B) , there were no marked differences among the groups. For the number of PMNs, expressed as the number of BrdU positive PMNs, newly produced from the bone marrow, similarly, there were no remarkable differences among the three groups (Fig. 3C).

Lung tissues

At three days after instillation, obvious inflammatory findings such as the infiltration of inflammatory cells or an increase of neutrophils were not detected in the pulmonary tissues in any group (Fig. 4). However, after 6 months, in the $S_{1.8}$ group, inflammatory changes such as alveolar proteinosis, an increase of foamy alveolar macrophage in the pulmonary alveoli, neutrophilic infiltration, and a thickening of the alveolar wall were marked. In contrast, in the $S_{0.7}$ group, these findings were less evident, but aggregation of inflammatory cells such as granuloma cells was more frequently seen mainly around the small vessels. The inflammation score tended to increase with time (Fig. 5). The increase was particularly evident at and after 1 month following exposure. Assessment of inflammation by the point counting method showed that, although the inflammation score tended to increase particularly at and after 1 month following exposure, there was no significant difference between the $S_{1.8}$ group and the $S_{0.7}$ group.

Bronchoalveolar lavage fluid (BALF)

The numbers of white blood cells in BALF in the exposure groups were significantly increased compared with that in the control group (Fig. 6A). This increasing tendency was more remarkable in the $S_{1.8}$ group than in the $S_{0.7}$ group. The number of PMNs in BALF of each group is shown in Fig. 6B. In the control group, few PMNs were detected during the observation period. In the exposure groups, the number of PMNs tended to increase over time, particularly in the $S_{1.8}$ group when compared with the $S_{0.7}$. These tendencies were similar to those for the white blood cells as shown in Fig. 6A. The number of BrdU-positive PMNs in BALF gradually and significantly increased in the exposure groups when compared with the control, particularly in the $S_{1.8}$ group

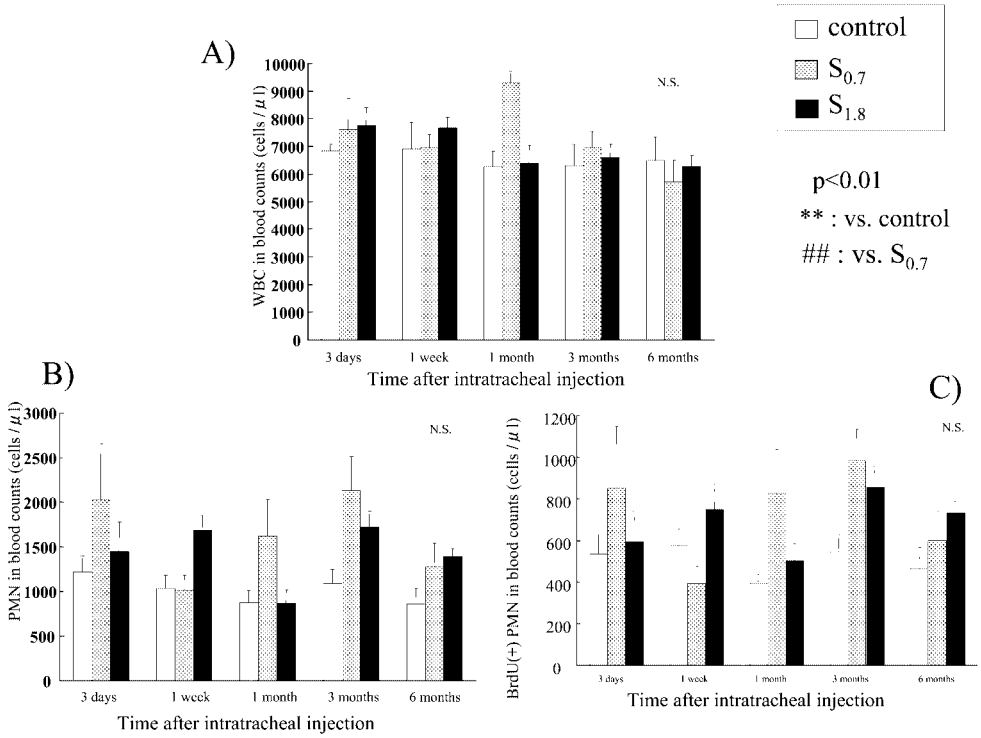

Fig. 3. A) The numbers of WBC in blood after intratracheal instillation. B) The number of PMN in blood after intratracheal instillation. C) The number of BrdU(+) PMN in blood after intratracheal instillation. There was no significant difference among the groups. Each bar shows the average cell counts and standard error of 7 rats, respectively. There was no accidental death or disease of rats during the observation period.

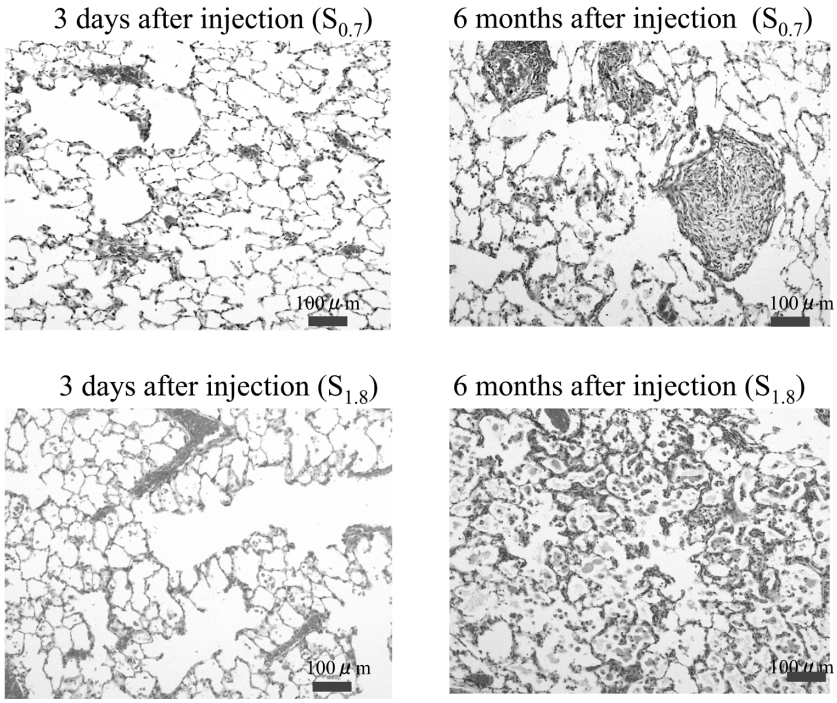

Fig. 4. Lung tissue after intratracheal instillation. Inflammatory changes in the lung were observed in rats at 6 months after instillation in both exposed groups. Three months after instillation, lipoproteinosis, thickening of the alveolar wall, and foamy macrophages were markedly observed.

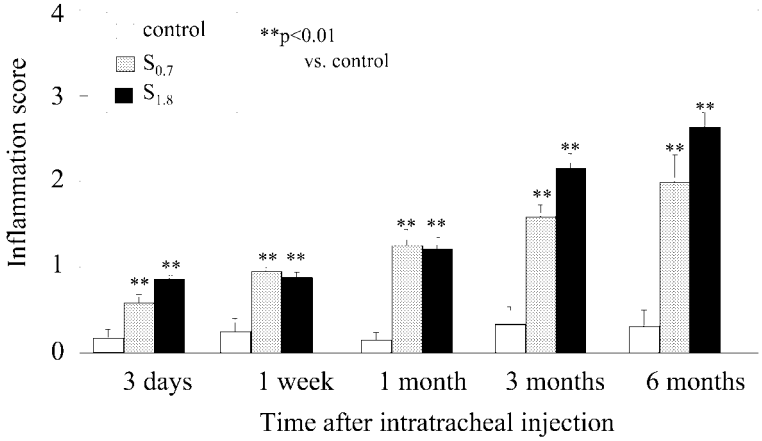

Fig. 5. Inflammation score after intratracheal instillation. Lung pathological changes were confirmed using an inflammation score. There was no significant difference between $S_{1.8}$ and $S_{0.7}$. The inflammation score gradually and significantly increased in both exposed groups. Each bar shows the average inflammation score and standard error of 7 rats, respectively.

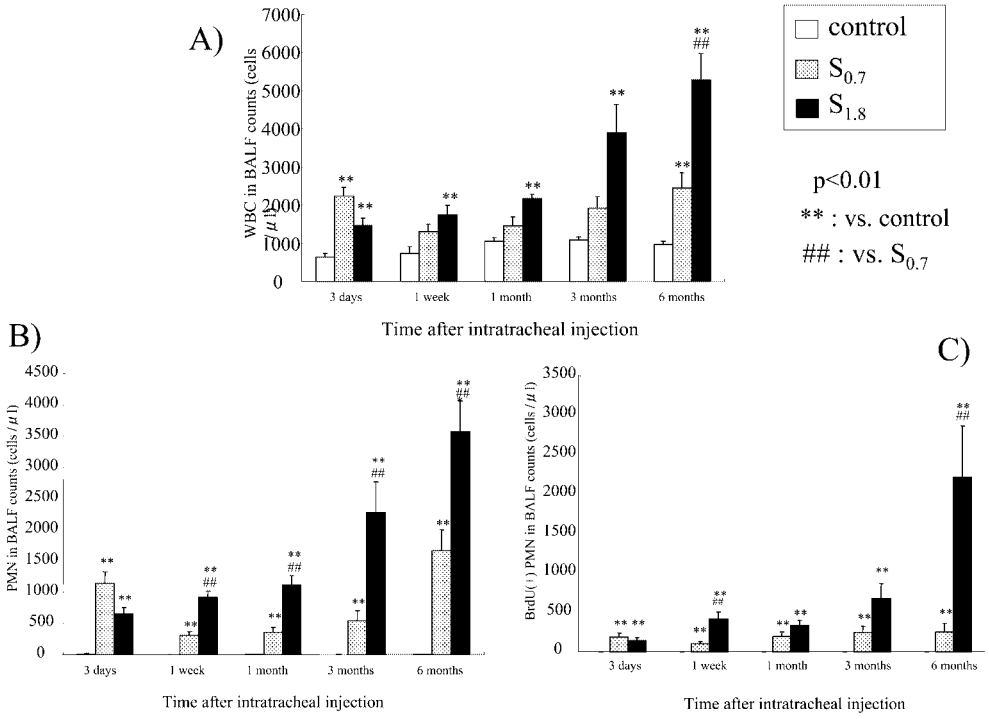

Fig. 6. A) The numbers of WBC in BALF after intratracheal instillation. In the control, WBC counts showed little change. In the exposed groups, the WBC counts of BALF gradually and significantly increased especially in $S_{1.8}$. B) The numbers of PMN in BALF after intratracheal instillation. In the control, few PMN were observed throughout the experiment. In the exposed groups, the PMN counts of BALF gradually and significantly increased especially in $S_{1.8}$. C) The numbers of BrdU(+) PMN in BALF after intratracheal instillation. In the control, few BrdU(+) PMN were observed throughout the experiment. In the exposed groups, the BrdU(+) PMN counts of BALF gradually and significantly increased, especially in $S_{1.8}$. Each bar shows the average cell counts and standard error of 7 rats, respectively.

(Fig. 6C).

Discussion

In our experiments, the difference in the biological effects due to particle size was more evident in white blood cell counts and neutrophils in the bronchoalveolar lavage fluid. In contrast, no differences were noted in the number of inflammatory cells in the blood compared with the control group. In an experiment evaluating the biological effects of crystalline silica by particle size, Wiessner *et al.*[15] demonstrated that particles with a diameter of more than 1 μm severely affected the lungs of mice, which is consistent with our results.

There were no differences between the exposure groups and the control group in the change in the number of inflammatory cells in the blood with time. These results suggest that the inflammatory alteration in the lung induced by instillation of crystalline silica is only a local response and not so extensive as to affect the number of circulating blood cells.

In the BALF findings, the increase in the number of inflammatory cells was more obvious in the $S_{1.8}$ group than in the $S_{0.7}$ group (Fig. 6A and 6B). Microscopic findings (Fig. 4) and inflammation score also indicated that the inflammation of lung tissue (Fig. 5) tended to be slightly greater in the $S_{1.8}$ group than in the $S_{0.7}$ group. That is, for crystalline silica, it was demonstrated that larger particles induced more extensive inflammatory changes to the lung morphologically. On the other hand, it was a characteristic of the $S_{0.7}$ group, that inflammatory areas were detected mainly in the perivascular area. The inflammatory areas were probably caused by the retention of fine particles in the perivascular interstitial tissues or lymph nodes in relation to the clearance of particles (Fig. 4). Moreover, the increase of PMN in BALF of the $S_{0.7}$ group was maintained at 6 months after instillation. Therefore, it seemed that the inflammation due to the fine fraction progressed with time.

A finding from a cohort of 2,670 men employed in nine North American sand-producing plants with pure quartz exposure and followed from 1994 showed that there was a strong indication of excess mortality from chronic non-malignant renal disease[16]. Thus, it is suggested that silica affects not only the lung but other organs. Although the kinetics of the inhaled particle are not well understood, it is thought that the difference in the translatability to the other organs depends on the size of the particle. Further evaluation is required to determine whether inflammation of the lung is extended to other systemic organs.

In this study, we evaluated the difference in the biological effects with respect to silica particle size by comparing the number of PMN that were newly produced from the bone marrow. Concerning the role of PMN newly produced by the bone marrow in the inflammatory areas, it was reported that they contribute to the progress of the morbid condition in the inflammatory response of the lung caused by acute respiratory distress syndrome (ARDS) etc.[13, 17–19]. In our previous study using rabbits, we confirmed immature PMNs were produced by the bone marrow after an intratracheal instillation of crystalline silica[10].

Although we aimed to evaluate the contribution of neutrophils to the extent of the inflammation in the present study, no remarkable differences in the number of BrdU-positive PMNs in the blood attributable to crystalline silica were noted between the exposure groups and the control group (Fig. 3C). In BALF, the number of BrdU-positive PMNs was significantly higher in the exposure groups compared to the control. Furthermore, the increase of BrdU-positive PMNs with time was evident in the $S_{1.8}$ group. However, this increase was not marked in the $S_{0.7}$ group during the observation period (Fig. 6C). The increase of these newly produced young PMNs (BrdU-positive PMNs) by the bone marrow in the pulmonary alveoli suggests that the inflammation observed in the lung of the $S_{1.8}$ group is a diffuse alteration which advances with time and that transfer of inflammatory cells from interstitial tissues to alveolar cavities accelerates. On the other hand, in the $S_{0.7}$ group, young PMNs were not increased in BALF as the inflammation was localized in the perivascular areas. These findings suggest that the inflammation site is related to the clearance of the particles and probably reflects the difference in types of inflammatory response to different particle sizes.

Though not investigated in the present study, it has been proposed that various cytokines (TNF-α, IL-8, IL-6, IL-1, etc.) increase after intratracheal exposure to crystalline silica, and the release of these cytokines triggers inflammatory responses[20–22]. We therefore suggest that the difference in types of inflammatory response to different particle sizes is attributable to the qualitative or quantitative difference in cytokine release. Technological progress has enabled us to produce 'nano-sized' industrial materials, however, we still do not have much knowledge about the inhalation of the nano-sized particles in actual workplaces or their biological influence. The possibility of exposure to such ultrafine particles may increase in the workplace in the near future. Therefore, it is necessary to promote the risk assessment of fine and ultrafine particles from various viewpoints. Future studies are needed to evaluate the differences in the amount of cytokine release associated with different particle sizes.

Conclusion

Crystalline silica of different particle sizes was administered by intratracheal instillation into the lungs of rats, and its biological effects were evaluated based on the number of inflammatory cells in the blood and in

BALF at various time points after administration. At six months after instillation, the effects on inflammatory cells in the rats tended to be more marked in the group exposed to silica of 1.8 μm diameter $S_{1.8}$ than silica of 0.7 μm diameter. Especially, clear differences were observed in the number of inflammatory cells in BALF. Even if the particles have the same chemical composition, the results suggest that their biological effects will vary depending on their particle size. Therefore, when such the particles are used in the workplace, strict control systems should be established according to risks present by different particle sizes.

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