EXPERIMENTAL STUDIES ON THE EFFECT ON THE IMMUNE SYSTEM OF EXPOSURE TO COALMINE DUST AND QUARTZ.

Y. KUSAKA, M.D. • R. T. Cullen, Ph.D • K. Donaldson, Ph.D

Institute of Occupational Medicine 8
Roxburgh Place, Edinburgh EH8 9SU

INTRODUCTION

Immune effects of mineral dusts may influence the development and progression of pneumoconiosis. In coalworkers' pneumoconiosis, progressive massive fibrosis and Kaplan's Syndrome are said to be related to immunologic abnormalities.[1] In addition, experimental studies have revealed that fibrogenic mineral dust alter immune responses.[2,3]

As part of a programme to examine the effect of silica and coalmine dust on the immune system, two approaches were taken: firstly immunocompetent cells from the rat spleen were exposed to dust *in vitro* and their mitogenic responses were assessed; secondly, dusts were intratracheally administered to rats and the effects of elicited bronchoalveolar leukocytes on splenocyte mitogenesis were studied.

MATERIALS AND METHODS

Animals

Twelve to fifteen-week old, female, SPF-maintained, inbred PVG rats were supplied by the Institute of Occupational Medicine breeding unit.

Dusts

Four kinds of dusts were used in the experiments: two were coalmine dusts collected from the air of British collieries mining 1) anthracite coalmine dust (A) and 2) low rank coalmine dust (L), 3) titanium dioxide (TiO_2; rutile, Tioxide Ltd. Stockton on Tees), a dust of low biological activity, 4) quartz dust (DQ12 standard).

Splenocyte Mitogenesis

Rats were killed by intraperitoneal injection with Nembutal and spleens were aseptically removed and disaggregated with a glass homogenizer. After lysing the erythrocytes, splenocytes were suspended in Hepes-buffered RPMI 1640 medium supplemented with 50mM 2-Mercaptoethanol, 2mM glutamine, 100mg/l kanamycin and 10% fetal calf serum (cRPMI). Finally 2×10^5 splenocytes, in medium were delivered to each well of 96-well microtiter plates.

The splenocytes, with or without dust suspensions, supernatant or bronchoalveolar cells, were cultured in the presence or absence of a suboptimal dose of phytohemagglutinin (PHA, 10 µg/ml) for three days at 37°C in 5% CO_2. The cultures were then pulsed with 0.25uCi tritiated thymidine, incubated overnight, and the uptake of ^3H Thymidine was determined by liquid scintillation counting.

Effect of Dusts on Splenocyte Mitogenesis

The four kinds of dusts were autoclaved and suspended in cRPMI. Each aliquot was added to splenocytes to obtain a final concentration in the well of 10, 50 or 100 µg/ml. They were then co-cultured at 37°C in 5% CO_2 for 24 hours and stimulated with suboptimal PHA for a further three days in culture. A preliminary study showed that 24 hours of co-culture of the splenocytes with dusts led to the optimal response to PHA. The cultures were assessed for mitogenesis as described above.

Effect of Supernatants on Mitogenesis

Splenocytes were adjusted to 1×10^6 cells/ml and aliquots of 5ml were delivered to plastic flasks. The splenocytes were allowed to adhere for six hours (adherence efficiency 27 ± 10%, $\bar{x} \pm$ sd) and non-adherent cells were removed by washing. The adherent splenocytes were cultured with dusts at a final concentration of 100 µg/ml for 24 hours and supernatants were collected which were spun, filtered and frozen until use. The supernatants, at various dilutions were delivered to wells containing 2×10^5 splenocytes and these were cultured and harvested as described above.

Interleukin-1 Activity in Spleen Cell Supernatants

Three-fold dilutions of supernatants from cultures of adherent spleen cells exposed to dust at 100 µg/ml, were incubated with C3H mouse thymocytes at 6×10^5 per well in microtiter plates. PHA was added at a final concentration of 4 µg/ml and the plates cultured for 72 hours. Thymocyte proliferation was determined by the incorporation of tritiated thymidine added during the final 16 hours of culture. Supernatant from unfractionated spleen cells cultured with 10 µg/ml Concanavalin A (Con A) served as a positive control. Con A activity was neutralized with methylmannoside before use in the thymocyte assay.

Effect of Bronchoalveolar Leukocytes from Dust-Exposed Rats on Splenocyte Mitogenesis

Rats were intratracheally instilled with 1mg of the four different kinds of dusts suspended in 0.5ml Phosphate Buffered Saline (PBS). PBS alone was injected into rats as a control. Bronchoalveolar cells (BAC) were obtained by lavage seven days later. BAC were washed with RPMI164O and sus-

pended in cRPMI. BACs from quartz-treated rat were separated into a macrophage and neutrophil-enriched populations by density gradient centrifugation through Sepra-cell medium. Total and differential counting was done on Diff-quick stained cytospin preparations and viability was assessed by trypan blue exclusion. Total or separated BACs were added to splenocytes at final ratios of from 1:4 to 1:128. The cultures were incubated and assessed for mitogenesis as above.

Statistical Analysis

Since variation between experiments was large, the data were expressed as mitogenic indices for each condition: the mitogenic indices were obtained by dividing the suboptimal PHA-driven splenocyte mitogenesis with dust, supernatant or bronchoalveolar leukocytes, by the mitogenesis without these treatments. The differences in mean values of mitogenic indices between treated and untreated were tested by paired t-test. The differences were considered as significant if values were less than 0.05. In the IL-1 assay, the ^3H uptake by the cultures with various supernatants were compared to those with control supernatant (no dust treatment).

RESULTS

Effect of Dusts on Splenocyte Mitogenesis *In Vitro*

Both quartz at 10, 50 or 100 μg/ml and low rank coalmine dust L at 50 and 100 μg/ml significantly enhanced mitogenesis. Quartz especially augmented splenocyte proliferation even without mitogen (data not shown). On the contrary, both TiO_2 and coalmine dust A suppressed splenocyte proliferation in a dose-dependent manner (Figure 1).

Effect of Supernatant from Dust-Exposed Adherent Splenocytes on Mitogenesis

The supernatant, tested at various dilutions did not cause enhancement of mitogenesis and, in fact, supernatant from splenocytes treated with quartz at a high dose were inhibitory to mitogenesis (typical results of 1:16 dilution shown in Figure 2).

Interleukin-1 Activity in Supernatants

Despite the lack of enhancement in the spleen cell mitogenesis assay, the thymocyte assay did show interleukin 1-like activity in the quartz supernatant diluted at 1:7.5 (Figure 3).

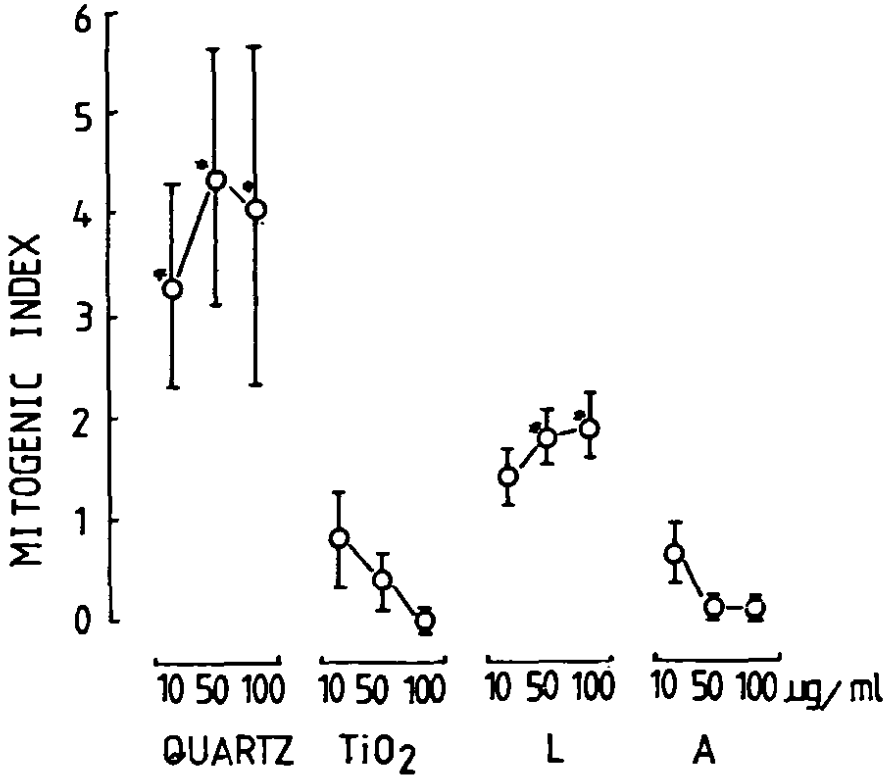

Figure 1. Mitogenic indices (means with standard errors) of splenocytes cultured with four kinds of dusts. Mitogenic index derived as the ratio of mitogenesis with dust:mitogenesis without dust. An asterisk denotes a significant ($p<0.05$) difference from the control.

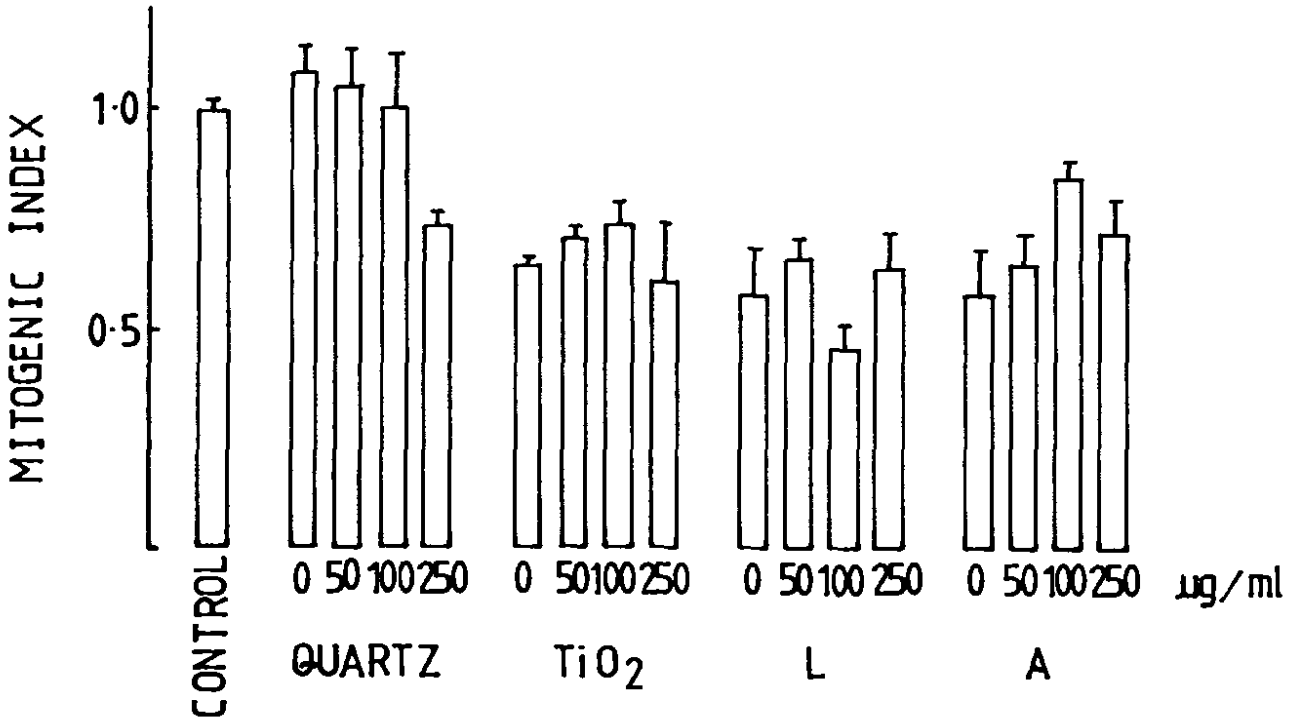

Figure 2. Effects of supernatants from dust-exposed adherent splenocytes on mitogenesis. Data are shown as mitogenic indices (means with standard deviations). Mitogenic index derived as the ratio of mitogenesis with supernatant from dusted or not-dusted adherent splenocytes:mitogenesis without supernatant.

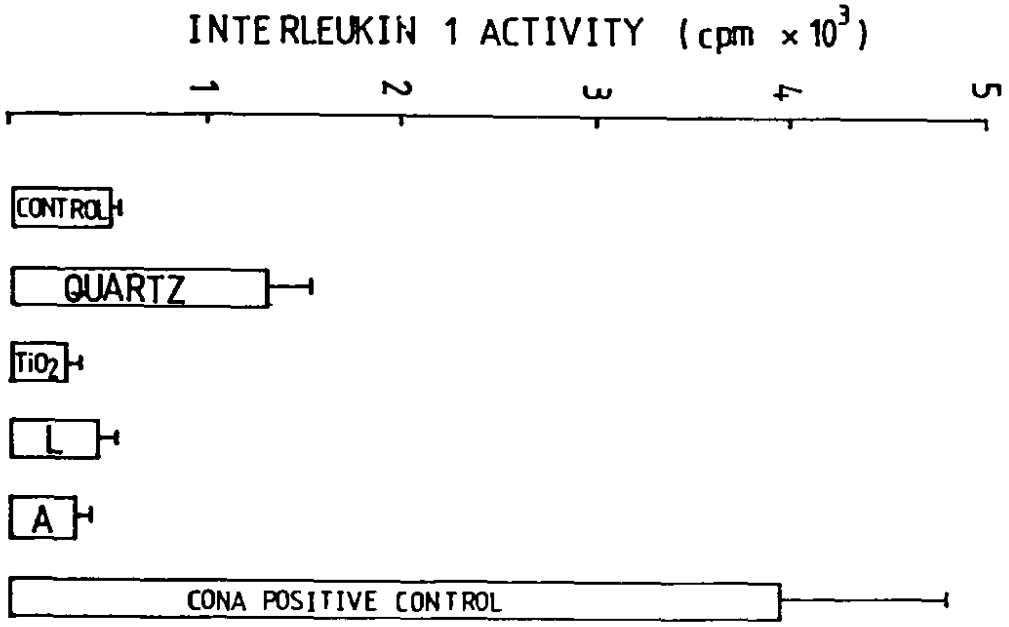

Figure 3. Interleukin 1 activity in supernatants from dust-exposed adherent splenocytes. An asterisk denotes a significant ($p<0.05$) difference from the control supernatant.

Higher concentrations of supernatant were inhibitory and supernatant from spleen adherent cells treated with other dust had no detectable Il-1 activity.

Effect of Bronchoalveolar Leukocytes from Dust-Exposed Lungs on Mitogenesis

Control, PBS-elicited bronchoalveolar leukocytes (total 4.16 $\pm$ 0.33 $\times$ 10^6 ($\bar{x}$ $\pm$ sd) cells per rat, macrophages >98%, viability >95%) showed an inhibitory effect on splenocyte mitogenesis which was effector:indicator cell ratio-dependent. The bronchoalveolar leukocytes from coal dust L (total 3.08 $\pm$ 0.76 $\times$ 10^6 cells per rat, macrophages 96 $\pm$ 2%, neutrophils 3 $\pm$ 2%, viability >91%), coalmine dust (A) (total 2.58 $\pm$ 0.33 $\times$ 10^6 cells per rat, macrophages >99%, viability >92%) or TiO_2 (total 4.61 $\pm$ 0.33 $\times$ 10^6 macrophages >99%, viability >93%) did not affect mitogenesis. Figure 4 shows the results for BAC from coalmine dust A.

The BAC from quartz-treated rats (total 16.83 $\pm$ 4.64 $\times$ 10^6 cells per rat, macrophages 42 $\pm$ 4%, neutrophils 57 $\pm$ 4%) was significantly less inhibitory to splenocyte proliferation, at ratios of 1:64, 1:32 and 1:16, than the control. After the separation into macrophage- and neutrophil-enriched fractions (recovery rate 60 $\pm$ 1%), the macrophage-enriched population (macrophages 89 $\pm$ 5%) also showed less inhibition at ratios of 1:64 and 1:32. In contrast to the inhibitory effect of the total leukocytes or separated macrophages, the neutrophil-enriched population (neutrophils 82 $\pm$ 2%) markedly enhanced mitogenesis compared to control BAC (Figure 5).

DISCUSSION

In our rat model system, we have examined the effects of exposure to mineral dusts on the immune system. The splenic lymphocytes were taken as indicator cells for the direct effect of dust on the immunomodulatory role of leukocytes within the lung.

Both quartz and coalmine dust with a high (>5%) quartz component, enhanced splenocyte mitogenesis *in vitro*. Supernatant from adherent splenocytes, presumed to be mostly macrophages, treated with quartz showed increased IL-1 activity, whilst supernatant from coalmine dust or TiO_2-treated macrophages had no such activity. None of these supernatants caused enhanced mitogenesis. These apparently conflicting findings may be explained as follows. Adherent macrophages secrete, in addition to IL-1, a variety of substances including prostaglandins and hydrogen peroxide, which are inhibitory to lymphocyte proliferation.[4,5] Subsequently the ability of any supernatant to influence mitogenesis is likely to be the product of both the inhibitory and enhancing activity present in it. Evidence that inhibitory factors were present, and could be diluted out was

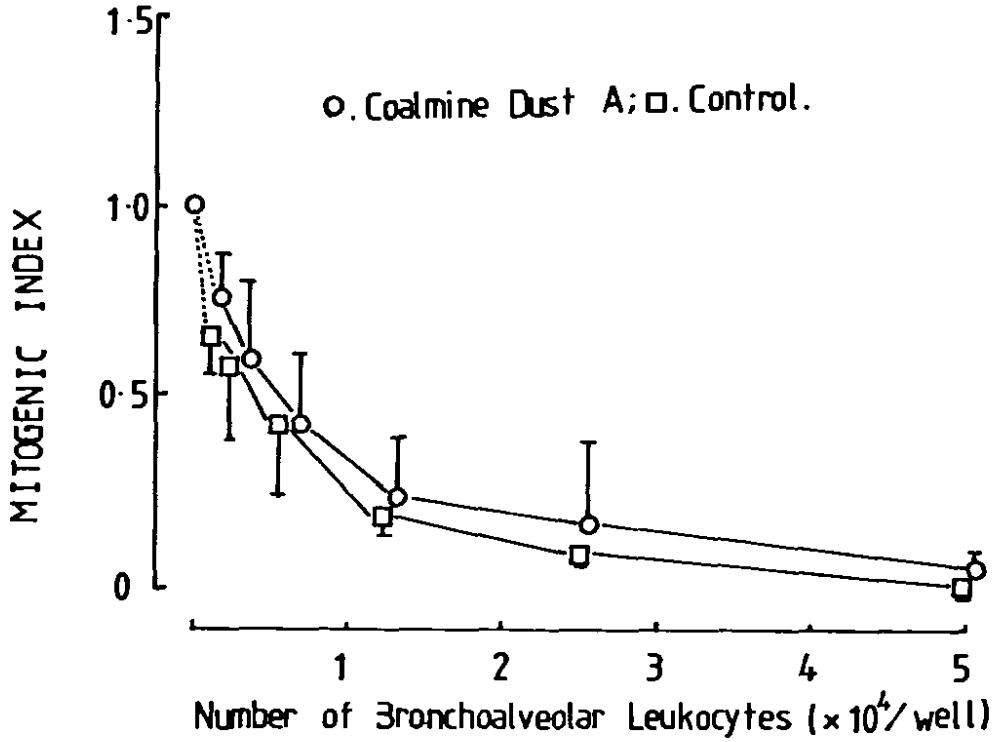

Figure 4. Bronchoalveolar leukocytes from coalmine dust L—instilled rats inhibited splenocyte mitogenesis in a dose-dependent manner. No significant differences from control leukocytes were present. Mitogenic index derived as the ratio of mitogenesis with bronchoalveolar leukocytes:without leukocytes.

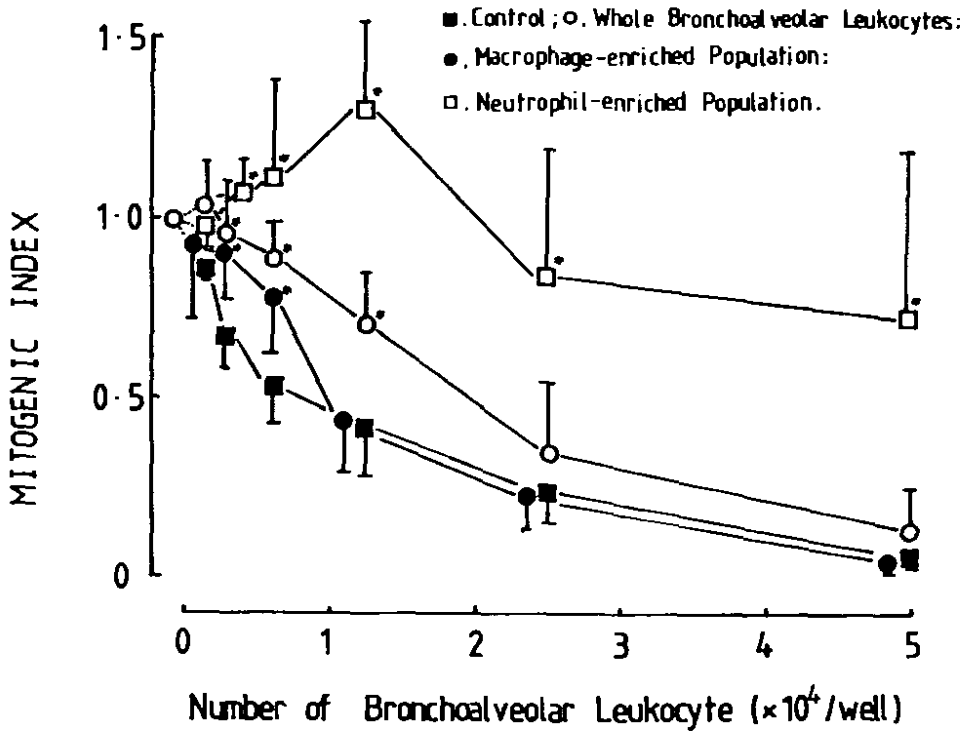

Figure 5. Effects of bronchoalveolar leukocytes from quartz-injected rats on splenocyte mitogenesis. Data are shown as mitogenic indices (means with standard deviations). An asterisk denotes a significant difference ($p<0.05$) from controls treated with PBS.

shown by the fact that IL-1 activity in the supernatant from quartz-exposed splenocytes was expressed only at higher dilutions. Further studies are needed to elucidate the mechanism of enhanced mitogenesis by quartz and low rank coalmine dust *in vitro* including further characterization of the secreted product present in supernatant form dust-treated macrophages.

Alveolar macrophages are situated at the air-tissue interface, strategically located for initial contact with inhaled particulates. They also play a crucial role in pulmonary immune responses. Alveolar macrophages in some species including rats are said to be poor accessory cells for mitogen or antigen-derived lymphocyte proliferation.[6] In our study normal bronchoalveolar macrophages inhibited splenocyte mitogenesis in a dose-dependent manner. TiO_2 and two kinds of coalmine dusts did not affect this down-regulatory function of alveolar macrophages. However the whole BAC and the alveolar macrophage-enriched population from quartz-treated rats inhibited lymphocyte response to a lesser extent than control BAC although this may be due to contaminating neutrophils as described below. Further studies are needed to confirm whether alveolar macrophages elicited by exposure to quartz have altered immunomodulatory properties, as suggested by this study.

The neutrophils separated from quartz BAC strikingly enhanced mitogenesis and this could be mediated through protease[7] or an Il-1 analogue which has been described in secretions from peritoneal neutrophils.[8]

Inhalation exposure to asbestos fiber, another type of fibrogenic dust, causes recruitment of Ia-positive alveolar macrophages and secretion of Il-1 by alveolar macrophages.[9,10] Additionally, alveolar macrohpages from asbestos-exposed rat enhanced T lymphocyte proliferation *in vivo*.[11] *In vitro* fibrogenic dust such as asbestos and silica stimulated alveolar macrophages to secrete IL-1.[12] Inhalation exposure to silica also causes secretion of IL-1 by alveolar macrophages when stimulated with endotoxin.[13] These studies suggest that fibrogenic dusts have immunostimulatory effects on alveolar macrophages and our results partially support these findings. However the complex effect of recruitment of newly exudated, monocyte-derived populations with altered cytokine production and the role of neutrophils, which are also found in dust exposed lung,[14] remain to be resolved.

This study suggests that, in the lungs of individuals inhaling quartz or quartz-containing coalmine dusts, the alveolar macrophages may be affected by phagocytosed dust to release a range of mediators which could modulate lymphocyte responses in the local environment of the lung. Additionally, neutrophils which are recruited into dust-exposed lung could also enhance immune responses leading to localized immunodulation. Any "adjuvant-type" effect on the immune system could contribute directly to heightened responses within the lung both to dust itself and to infectious agents, both of which could contribute to the tissue injury and fibrosis found in pneumoconiosis.

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