

## IN VITRO INJURY TO ELEMENTS OF THE ALVEOLAR SEPTUM CAUSED BY LEUKOCYTES FROM THE BRONCHOALVEOLAR REGION OF RATS EXPOSED TO SILICA

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### INTRODUCTION

Quartz exposure is associated with lung fibrosis (silicosis) and Type II alveolar epithelial cell hyperplasia is also commonly present.<sup>1</sup> Bronchoalveolar lavage studies using rats in our own laboratories,<sup>2</sup> and in humans,<sup>3</sup> have demonstrated that there is leukocyte recruitment into the lungs following inhalation exposure to silica. Studies on other fibrotic lung diseases have stressed the importance of the leukocytes of the alveolitis in the progression of disease via release of important mediators.<sup>4</sup> We have therefore set out to examine the ability of bronchoalveolar leukocytes from rats exposed to silica by a single intratracheal instillation, to cause injury to the extracellular matrix and cellular elements of the alveolar septum *in vitro*. Leukocytes from rats exposed to two other inflammogenic particulates—a heat killed bacterial preparation and a yeast cell wall preparation (zymosan)—were similarly assessed, for comparison with quartz.

### MATERIALS AND METHODS

#### Animal Model of Silicosis

Syngeneic PVG rats, SPF bred, were exposed by intratracheal instillation to 1 mg of DQ<sub>12</sub> standard quartz. As controls, the heat killed bacterial preparation *Corynebacterium parvum* was also injected as was the yeast cell wall preparation zymosan; both of these particulates are known to cause inflammation. Bronchoalveolar leukocytes were obtained by lavage as described in detail elsewhere<sup>5</sup> at various time points after injection. In this model quartz exposure causes fibrosis, Type II epithelial cell hyperplasia and alveolar lipoproteinosis beyond 1 month exposure which are evident in histological sections of exposed lung.

#### Assay of Leukocyte-Mediated Type II Alveolar Epithelial Cell Injury

This assay is described in detail elsewhere<sup>6</sup> and involves labelling of Type II alveolar cell line (A549) with <sup>51</sup>Cr. Bronchoalveolar leukocytes are then added to the labelled cells in microtitre wells and co-cultured for 4 hours; the ability of the leukocytes to cause lysis or detachment of the epithelial cells is assessed.

#### Assay of Leukocyte-Mediated Proteolysis of Fibronectin

Leukocyte-mediated proteolysis of fibronectin was assessed using a solid phase assay of <sup>125</sup>I-labelled fibronectin in microtitre plate wells. This assay has been described in detail elsewhere<sup>7</sup> and measures protease-mediated injury. The leukocyte-mediated proteolytic activity shown here against fibronectin is also active against <sup>125</sup>I-labelled collagen and laminin. Leukocytes are cultured on the solid phase of <sup>125</sup>I-labelled fibronectin and allowed to degrade the matrix for 4 hours; products of proteolysis of fibronectin are measured as free counts in the supernatant.

#### Leukocyte Separation

Whole inflammatory bronchoalveolar leukocyte populations from quartz-exposed rats were separated by centrifugation through Septra-Cell medium into macrophage and neutrophil-enriched fractions.

#### Statistical Analyses

Results were analysed by analysis of variance and differences in treatments compared for significance using a 't' test.

### RESULTS

#### Inflammation Caused by a Single Injection of Silica, *C. parvum* or zymosan

Figures 1 and 2 show the total number of bronchoalveolar leukocytes and percentage neutrophils lavaged from rats injected intratracheally with quartz, *C. parvum* or zymosan. All three particles caused initial burst of inflammation characterized by recruitment of large numbers of leukocytes containing high proportions of neutrophils. In the case of *C. parvum* and zymosan this initial alveolitis was followed by a return to the normal situation where no neutrophils were present although the numbers of macrophages remained raised indicating a mild macrophage alveolitis. In the case of quartz, however, an intense macrophage/neutrophil alveolitis persisted until at least one month. Previous studies have shown that this alveolitis persists for up to three months.<sup>8</sup>

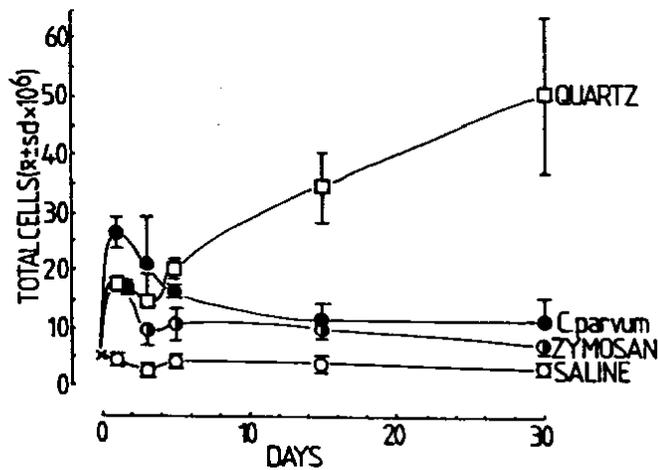


Figure 1. Total leukocytes in bronchoalveolar lavage up to 30 days after instillation of saline, quartz, *C. parvum* or zymosan into the lungs of rats. Data is mean  $\pm$  standard deviation from 3 rats. Significant ( $P < 0.01-0.001$ ) increases with all particulates compared to saline.

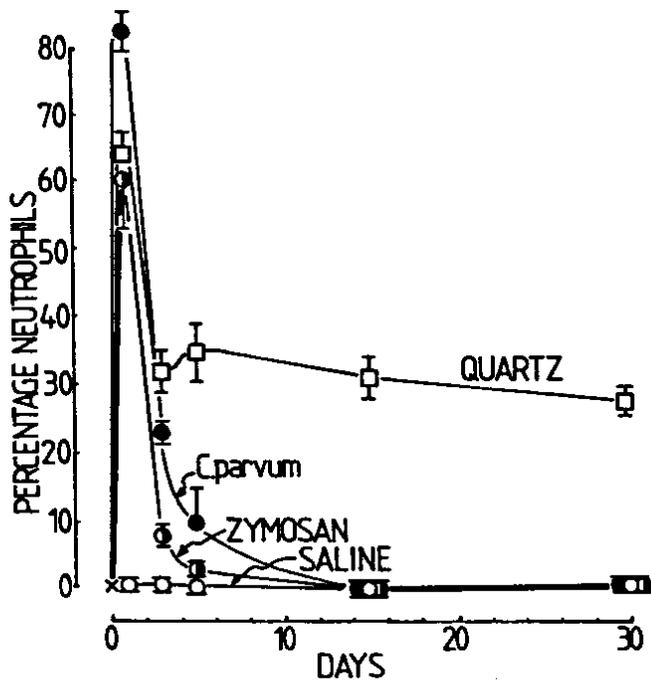


Figure 2. Percentage neutrophils in bronchoalveolar lavage up to 30 days after instillation of saline, quartz, *C. parvum* or zymosan. Data derived as in legend to Figure 1. Significant ( $P < 0.01-0.001$ ) increases in percentage neutrophils, compared to saline, for quartz at all time points and for *C. parvum* and zymosan at 1, 3 and 5 days.

**Activity of Bronchoalveolar Leukocytes in Breaking Down Fibronectin**

As shown in Figure 3 the bronchoalveolar leukocytes obtained from the lungs following injection of different parti-

cles showed varying abilities to break down fibronectin. During the acute inflammatory phase the leukocytes from lung exposed to all three particulates were capable of breaking down fibronectin. However, only quartz was capable of eliciting a sustained high level of proteolysis, in keeping with the persistence of the inflammation in quartz-exposed lung. It was notable that the ability to break down fibronectin correlated strongly with the presence of neutrophils.

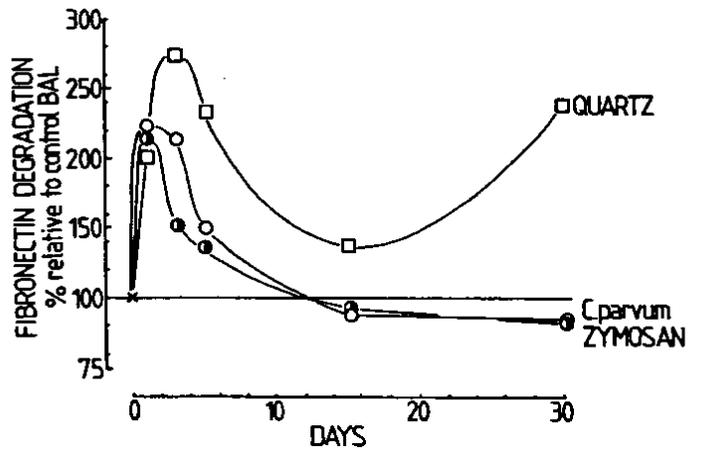


Figure 3. Proteolytic activity against fibronectin shown by bronchoalveolar leukocytes from rats injected with the indicated particulates. Data expressed as a percentage of the activity shown by control bronchoalveolar leukocytes.

**Injury to Alveolar Epithelial Cells Caused by Bronchoalveolar Leukocytes**

Bronchoalveolar leukocyte populations elicited with quartz or *C. parvum* were tested for their ability to cause injury to cells of an alveolar epithelial cell line *in vitro*. Both *C. parvum*-elicited bronchoalveolar lavage cells obtained after one day (70-90% PMN) and 5-day quartz leukocytes (50% macrophages/50% neutrophils) were capable of causing the target cells to detach from the sub-stratum (Figure 4). There was no lytic injury to the target cells and the detachment injury could be completely inhibited by protease inhibitors such as alpha 1-protease inhibitor.<sup>6</sup>

We have also examined the ability of leukocytes from the lungs of rats chronically inhaling coalmine dust to mediate injury. This showed that rats exposed, by inhalation, for 48 days to coalmine dust collected from the air of a British colliery<sup>5</sup> also caused epithelial injury and degradation of fibronectin (Figure 5).

**Cellular Origin of Epithelial Cell Detaching Injury in Quartz-Elicited Bronchoalveolar Leukocyte Populations**

As shown above, high proportions of neutrophils seem to accompany fibronectin-degrading and epithelial-injuring activity in the inflammatory leukocyte populations which we have examined. To determine whether the macrophages could also be producing proteolytic activity against fibro-

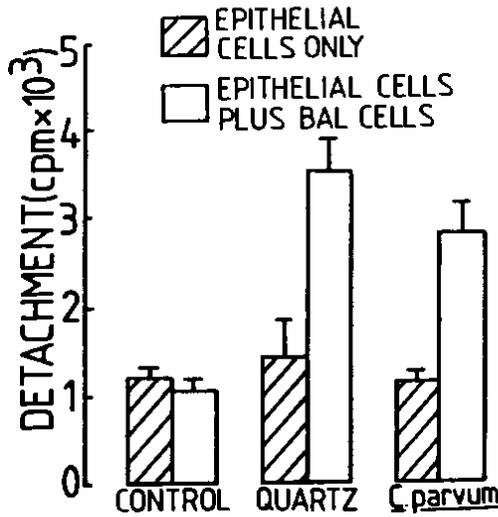


Figure 4. Detachment injury caused to alveolar epithelial cells *in vitro* by co-culture with control, quartz or *C. parvum* elicited bronchoalveolar leukocytes. All data given as mean + SEM of triplicate cells in 3 separate experiments. Significantly increased detachment caused by quartz and *C. parvum* treatment ( $p < 0.001$ ).

very high levels of epithelial injury being caused by the neutrophil-enriched fraction. However, despite the macrophage-enriched fraction containing only 5% PMNs, this population caused 5-fold more detachment injury than control alveolar macrophages.

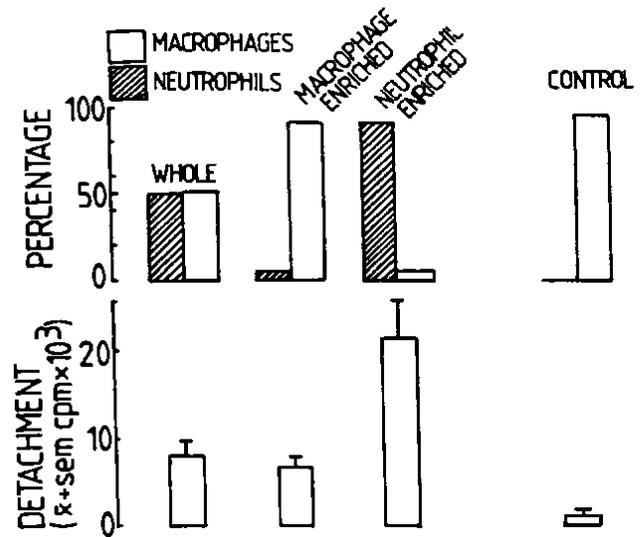


Figure 6. Cellular composition (upper panel) of, and detachment injury (lower panel) caused by, whole quartz-elicited bronchoalveolar leukocytes and both macrophage-enriched and neutrophil-enriched fractions obtained from it. Proportions of neutrophils and macrophages shown as mean percentage. Detachment injury shown as mean + SEM of cpm in detached cells.

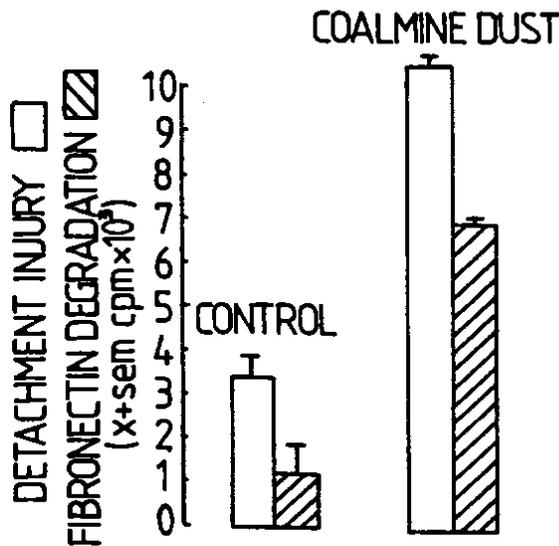


Figure 5. Detachment injury and fibronectin degradation caused by control bronchoalveolar leukocytes and bronchoalveolar leukocytes from rats inhaling coalmine dust for 45 days. Significant increases ( $P < 0.001$ ) in both parameters with coalmine dust exposed bronchoalveolar leukocytes compared to controls.

nectin, and causing detachment injury, the 5 day quartz bronchoalveolar leukocytes were separated into macrophage-enriched and neutrophil-enriched populations. These were then tested for their ability to cause epithelial cell detachment injury. Figure 6 demonstrates that separation of the mixed population into the enriched populations resulted in

### DISCUSSION

This study has shown that a single injection of silica into the rat lung causes a long-term alveolitis. The alveolitis is characterized by a 3–12 fold increase in bronchoalveolar leukocytes comprising 30–40% neutrophils. Intratracheal instillation of a heat-killed bacterial preparation (*C. parvum*) or yeast cell walls (zymosan) also caused large scale burst of inflammation immediately following injection but these resolved quickly, returning to near normal levels by 15 days. Thus the initial severity of the alveolitis is not the main factor determining the persistence of silicotic inflammation in the intratracheal model.

The exact events which engender persistent inflammation with silica are speculative but cytotoxicity of quartz towards alveolar macrophages might be central. The consequence of silica-induced alveolitis is likely to be fibrosis since the ability of inflammatory leukocytes to mediate further damage and pathological change in the lung is well established for a range of aetiologic agents.<sup>4</sup> In an attempt to understand which leukocyte-derived injurious factors might be important in the development of quartz-related pathology we examined the ability of the leukocytes from quartz-exposed lung to break down fibronectin. During the acute inflammation engendered

by *C. parvum* and zymosan there were high levels of proteolytic activity present; the levels of protease however returned to normal within 15 days. An examination of the proteolytic activity of quartz-elicited leukocytes showed that this proteolytic activity, capable of breaking down fibronectin and other connective tissue elements<sup>7</sup> and so generating chemotaxin<sup>10</sup> and causing epithelial injury and basement membrane damage,<sup>6</sup> was 4, present persistently, and in increased quantities, for up to 1 month following quartz instillation; previous studies suggest that this inflammation and hence the increased protease burden persist for up to 3 months and possibly longer. The total proteolytic burden of the lung is not reflected adequately as the increase, on a per cell basis, in dust-elicited bronchoalveolar leukocytes since the total number of leukocytes is also increased. If the increase in cell numbers is taken into consideration (a 16-fold increase on day 30) this produces a greater than 30-fold increase in the total protease burden of the lung following silica exposure for 30 days. Although the present study has utilized intratracheal instillation we have found that inhalation exposure to a pneumoconiotic dust (coalmine dust containing quartz) also caused an alveolitis producing greatly enhanced lung burdens of fibronectin-degrading activity.<sup>11</sup>

The ability of inflammatory bronchoalveolar lavage leukocytes to injure epithelial cells correlates with proteolytic activity<sup>6</sup> and so we examined this aspect of injury production by quartz bronchoalveolar leukocytes. The quartz bronchoalveolar leukocytes caused detachment injury which appeared to be mediated by both macrophages and neutrophils as shown by separation studies where the different leukocyte types were obtained in enriched form. It is therefore possible to conclude that the bronchoalveolar macrophages from quartz-exposed lung are activated with regard to proteolytic activity. The time course studies with *C. parvum* revealed a modest macrophage alveolitis present beyond 15 days but this population was not activated with regard to protease production. The fact that the cell numbers were increased compared to controls argues for the fact that this did indeed represent an inflammatory population albeit one which was not characterized by increases in neutrophils. It is possible therefore that only inflammatory macrophages from mixed populations, where neutrophils are also present, show increased proteolytic activity. A likely explanation for this is that the alveolar macrophages from such populations have internalized neutrophil elastase as has been previously reported.<sup>12</sup> It was notable that the neutrophil-enriched fraction had twice the proportion of neutrophils found in the whole population but produced a 3-fold increase in detaching activity. This suggests that either the separation procedure caused activation of neutrophils or that macrophages suppressed neutrophil proteolytic activity in the mixed population.

This study has shown that a single deposition of 1 mg of quartz in the rat lung causes a prolonged and intense alveolitis characterized by increased proteolytic activity of bronchoalveolar leukocytes, capable of causing injury to the epithelial and matrix elements of the alveolar septum. The results strongly suggest that leukocytes from rats exposed by inhalation to pneumoconiosis-producing dust also have these properties and that both macrophages and neutrophils

express this injurious proteolytic activity, although in the case of macrophages this may be due to sequestered neutrophil elastase.

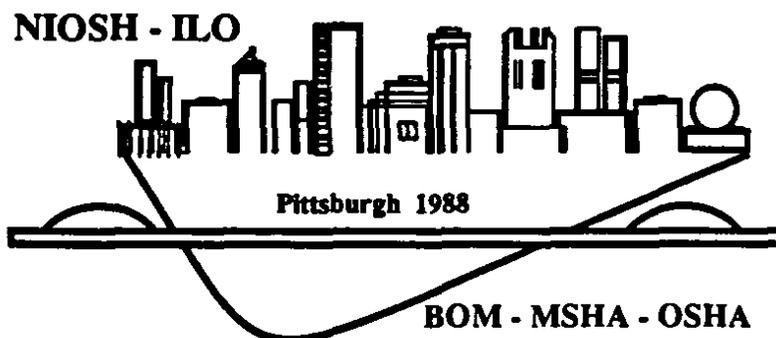
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