

# Inflammatory responses in lungs of rats inhaling coalmine dust: enhanced proteolysis of fibronectin by bronchoalveolar leukocytes

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**ABSTRACT** Chronic exposure to coalmine dust is associated with the accumulation of inflammatory leukocytes in the bronchoalveolar region of the lung and, in the long term, with fibrosis and emphysema of the lung parenchyma. Degradation of connective tissue by inflammatory leukocytes has been implicated in the parenchymal damage that precedes the development of fibrotic or emphysematous lesions in the lung. The ability of inflammatory leukocytes obtained by bronchoalveolar lavage from rats inhaling coalmine dust to degrade fibronectin *in vitro* was assessed. The animals were exposed to an airborne mass concentration of dust similar to the maximum permissible level in United Kingdom collieries. The bronchoalveolar lavage cell population showed changes with duration of dust exposure; there were increases in the total number of leukocytes and in the percentage of polymorphonuclear leukocytes, and the macrophage component of the lavage became increasingly activated, as assessed by the ability of these cells to spread on glass. In addition, degradation of a radiolabelled fibronectin matrix by the coalmine dust exposed bronchoalveolar leukocytes increased with duration of dust exposure. Thus exposure to airborne coalmine dust causes an influx of inflammatory leukocytes to the alveolar region. These cells have enhanced ability to degrade fibronectin and this may be important in subsequent disease development.

Coalworkers' pneumoconiosis is a disease directly attributable to the inhalation of coalmine dust and is characterised by the development in the lungs of fibrotic lesions and emphysema.<sup>1</sup> Patients with coalworkers' pneumoconiosis have increased numbers of inflammatory leukocytes in the bronchoalveolar region<sup>2</sup> and this alveolitis, as in other lung diseases,<sup>3</sup> is likely to be central to the remodelling of lung tissue which leads to the lesions described above. The accumulation of inflammatory leukocytes in the bronchoalveolar region is also one of the earliest manifestations of disease in animals exposed experimentally to coalmine dust,<sup>4</sup> asbestos,<sup>5,6</sup> and silica.<sup>6,7</sup> During inflammation, inflammatory macrophages and neutrophils secrete an array of molecules<sup>8</sup>; included among these are the neutral proteases, active at the normal pH of lung tissue, which are secreted in increased amounts by inflammatory leukocytes<sup>9</sup> and which may damage connective tissue *in vivo*<sup>10</sup> and *in vitro*.<sup>11</sup> Degradation of connective tissue may be

central to the pathogenesis of chronic inflammatory lung disease by enhancing and prolonging the inflammatory and repair responses<sup>12-15</sup> at sites of inflammation in the lung parenchyma. This type of connective tissue damage may occur where neutral protease activity is sufficient to overload the antiprotease screen.<sup>16</sup> This is strongly suggested by the presence of active neutral protease in bronchoalveolar lavage of patients with chronic inflammatory lung disease,<sup>17,18</sup> including coalworkers' pneumoconiosis<sup>19</sup> and, experimentally, in the bronchoalveolar lavage of animals exposed to silica,<sup>20,21</sup> a powerful inflammation generating agent.

In the present study we have investigated the development of pulmonary inflammation in rats exposed to airborne coalmine dust for up to 52 days; rats exposed for 32 days and then allowed to breathe room air for a further 64 days were also studied. Animals were exposed to 10 mg/m<sup>3</sup> airborne coalmine dust; this is of the same order of magnitude as the maximum permissible level in British collieries which is 7 mg/m<sup>3</sup>, measured in the return roadway and about four to six times the level of dust to which miners might

expect to be exposed on a daily basis for their working lives. The leukocyte population of the bronchoalveolar space was obtained by bronchoalveolar lavage and the following parameters assessed: total number, differential count, the ability of the population to degrade fibronectin *in vitro* and the ability of the macrophages to spread on glass as a measure of macrophage activation. Comparisons were made with control rats maintained in room air throughout.

## Materials and methods

### REAGENTS

Alpha-1-protease inhibitor ( $\alpha$ -1-PI) from human plasma, alpha-2-macroglobulin ( $\alpha$ -2M) from human plasma, bovine serum albumin (BSA), human plasma fibronectin, phorbol myristate acetate (PMA), and zymosan were obtained from Sigma Chemical Co Ltd, Poole, Dorset. Ham's F10 medium, fetal calf serum (FCS), and Dulbecco's phosphate buffered saline (PBS) were purchased from Gibco BRL, Paisley, Renfrewshire.  $^{125}$ Iodine ( $^{125}$ I) was obtained from Amersham International, Aylesbury, Buckinghamshire. Microtitre plates were purchased from Sterilin UK Limited, Feltham, Middlesex, and Nembutal from Ceva Limited, Watford. Sodium EDTA was obtained from BDH Limited, Glasgow.

### COALMINE DUST

Dust was collected from the air of a British colliery using dry fabric filters. The dust was irradiated to kill microbial spores (100 KRad) before being generated as a cloud using a Timbrell dust generator. The dust was then passed through a cyclone to produce a respirable fraction that was dispersed as a cloud in a 1 m<sup>3</sup> exposure chamber<sup>22</sup> at an airborne mass concentration of 10 mg/m<sup>3</sup>. Airborne samples collected on to filters showed that the mean target concentration of 10 mg/m<sup>3</sup> was achieved throughout the exposure. The mineralogical composition of the dust, as assessed by x ray diffraction and infra red analysis, was total ash 53.2, quartz 6.7, kaolin 18.1, and mica 0. The dust was low rank with a mass median aerodynamic diameter of 2-3  $\mu$ m, measured by an eight stage cascade impactor.

### INHALATION EXPOSURE OF RATS

Male, syngeneic, PVG rats were obtained from the laboratory animal breeding unit, Institute of Occupational Medicine. Rats were placed in the inhalation chamber and exposed to dust for seven hours a day, five days a week. On days 8, 16, or 32, groups of four rats were removed from the chamber, killed, and the bronchoalveolar leukocyte population obtained by lavage. At each time point, two control rats of identical age, maintained in room air, were also lavaged. One group of four rats was removed from the

chamber on day 32 and maintained in room air for a further 64 days before being lavaged; these will be referred to as "recovery" rats.

### BRONCHOALVEOLAR LAVAGE

Animals were killed by overdose with intraperitoneal pentobarbitone sodium (Nembutal); the thoracic cavity was dissected open to expose the lungs and the trachea cannulated with a blunt 16 g needle. The lungs were then resected and lavaged with four sequential washes of 8 ml saline at 37°C, with gentle massage of the lungs on the second wash to increase the yield of cells; the four washes were pooled and placed immediately on ice.

### CELL COUNTS

The pooled bronchoalveolar lavage fluid was centrifuged and the cell pellet resuspended in phosphate buffered saline. The total cell number was measured using a Neubauer chamber and differential cell counts were performed using May Grunwald Giemsa stained cytocentrifuge preparations.

### SPREADING ASSAY

Cells were resuspended to a concentration of  $1 \times 10^6$ /ml in Ham's F10 medium containing 10% heat inactivated fetal calf serum; 100  $\mu$ l aliquots were placed on 6  $\times$  22 mm glass coverslips and incubated at 37°C for one hour. The coverslip preparations were then washed, fixed, and stained with May Grunwald Giemsa. The maximum diameter of 200 macrophages (any adherent neutrophils were excluded from sizing) was measured using a graphic instruments image analyser, interfaced to an optical microscope.<sup>23</sup>

### NEUTRAL PROTEASE ASSAY

Neutral protease activity was assessed as previously described by measuring the degradation of radioiodine labelled fibronectin adhered as a solid phase matrix<sup>20</sup>; intact cells were cultured on the matrix for four hours at 37°C; solubilised<sup>125</sup>I correlated with increased enzyme concentration. Cells from animals exposed for eight days to coalmine dust were similarly tested for neutral protease activity in the presence of the protease inhibitors,  $\alpha$ -1-PI at concentrations of 0.01, 0.1, and 1.0 mg/ml or  $\alpha$ -2M at concentrations of 0.005, 0.05, or 0.5 mg/ml and also in the presence of fetal calf serum (1%, 5%, or 10%) or ethylene diamine tetra-acetic acid (EDTA) (0.01, 0.1, and 1.0 mg/ml). Triggering experiments with eight day dust elicited cells were carried out in the presence of PMA (0.1, 1.0, or 10  $\mu$ g/ml) or zymosan (1, 10, or 100  $\mu$ g/ml).

### STATISTICAL ANALYSIS

Results were analysed by paired comparisons using Student's *t* test.

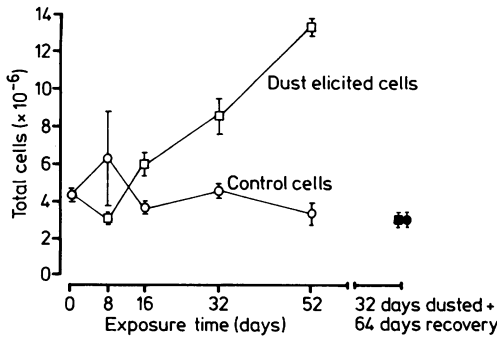


Fig 1 Total leukocytes in bronchoalveolar lavage fluid with duration of exposure to dust. Dust elicited leukocytes were significantly more active than controls after 52 days exposure to dust ( $p < 0.001$ ). Results are mean  $\pm$  SE of four dust exposed or two control rats at each time point.

**Results**

**TOTAL CELLS IN BRONCHOALVEOLAR LAVAGE**

The total number of cells retrieved by bronchoalveolar lavage increased with duration of exposure to dust (fig 1); after 52 days of exposure to dust the total cell number in bronchoalveolar lavage ( $13.2 \pm 0.9$ ) (mean  $\pm$  SE;  $\times 10^6$ ) was significantly greater than bronchoalveolar lavage cells from control animals ( $4.3 \pm 0.8$ ) ( $p < 0.001$ ). Animals exposed to coalmine dust for 32 days followed by recovery in room air for a further 64 days had fewer cells in the bronchoalveolar lavage ( $3.2 \pm 0.2$ ) than animals killed immediately after 32 days exposure to dust ( $8.4 \pm 1.9$ ) ( $p < 0.05$ ), but the difference was not statistically significant.

**PERCENTAGE OF NEUTROPHILS IN BRONCHOALVEOLAR LAVAGE**

No neutrophils were observed in bronchoalveolar lavage of control animals but were present after eight and 16 days of exposure to dust (2.8 and 3.5%

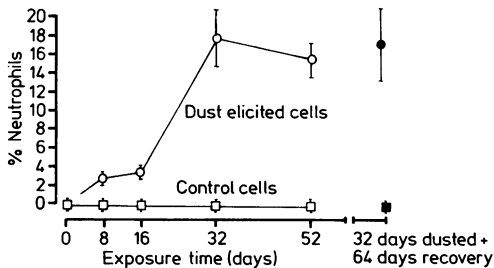


Fig 2 Percentage of neutrophils in bronchoalveolar lavage with duration of exposure to dust. Results are mean  $\pm$  SE of four dust exposed or two control rats at each time point.

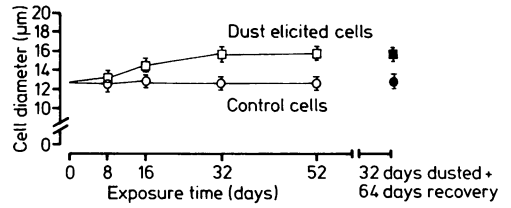


Fig 3 Macrophage spreading with duration of exposure to dust. Spreading was significantly greater than controls at all but eight day time point ( $p < 0.001$ ). Results are expressed as cell diameter in  $\mu$ m and are mean  $\pm$  SE of 200 macrophages counted for each of four dust exposed or two control rats at each time point.

respectively, fig 2). The percentage of neutrophils was greater at the later time points; 32 days: 18%, 52 days: 15.8%. The percentage of neutrophils remained raised (17.8%) in recovery rats exposed to dust for 32 days and maintained in room air for a further 64 days.

**MACROPHAGE ACTIVATION**

The diameter of alveolar macrophages obtained from coalmine dust exposed animals allowed to spread on glass for one hour increased in a time dependent manner from  $13.1 \pm 0.7 \mu$ m on day 8 to  $15.6 \pm 0.2 \mu$ m on day 52 (fig 3). Spreading was significantly greater than the controls ( $12.7 \pm 0.2 \mu$ m) at all but the eight day time point ( $p < 0.001$ ) and remained raised when the 32 day dust exposed animals were allowed to recover for 64 days in room air ( $15.4 \pm 0.4 \mu$ m).

**NEUTRAL PROTEASE ACTIVITY**

The neutral protease activity of coalmine dust elicited leukocytes was significantly greater than that of control cells at all time points ( $p < 0.001$ ) and remained raised on recovery (fig 4). By comparison, there was little increase in the percentage of neutro-

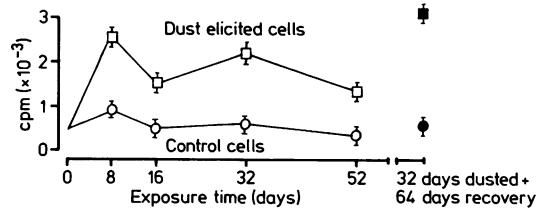


Fig 4 Neutral protease activity of bronchoalveolar leukocytes with duration of exposure to dust. Neutral protease activity of dust elicited cells was significantly greater than the controls at all time points ( $p < 0.001$ ). Results are expressed as counts per minute (cpm) degraded substrate/  $1 \times 10^5$  cells and are mean  $\pm$  SE of three wells for each of four dust exposed or two control rats at each time point.

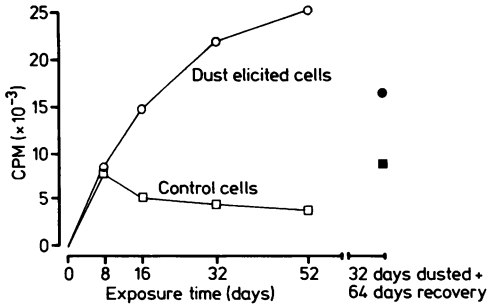


Fig 5 Neutral protease activity as a function of total number of leukocytes in bronchoalveolar lavage. Total neutral protease activity pertaining to whole population of leukocytes from dust exposed animals increased with duration of exposure to dust and was significantly greater than controls at all time points ( $p < 0.001$ ). Results are expressed as mean neutral protease activity, multiplied by total cell number at each time point. Closed symbols represent recovery animals.

phils in the lavage population until day 32. We therefore assumed that both macrophages and neutrophils were contributing to the neutral protease burden and accordingly calculated neutral protease activity as a function of the total number of cells present in lavage (fig 5). The putative total neutral protease activity of the whole population of bronchoalveolar lavage cells increased with duration of exposure to dust and was sixfold greater than that of control cells at day 52.

The neutral protease activity of BAL cells from eight day coalmine dust exposed animals (fig 6) was

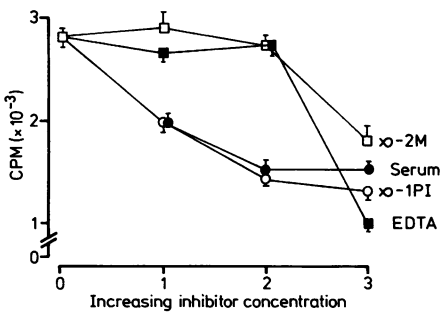


Fig 6 Inhibition of neutral protease activity of eight day dust elicited leukocytes by  $\alpha 1$ -PI and EDTA at 0.01, 0.1 and 1 mg/ml,  $\alpha 2$ -M at 0.005, 0.05 and 0.5 mg/ml, serum at 1%, 5%, and 10%. Results are expressed as counts per minute (cpm) of degraded substrate/ $1 \times 10^5$  cells and are mean  $\pm$  SE of three wells for each inhibitor concentration.  $\alpha 1$ -PI and serum significant reduction at all inhibitor concentrations,  $p < 0.001$ ;  $\alpha 2$ -M-significant reduction at 0.5 mg/ml,  $p < 0.005$ ; EDTA significant reduction at 1.0 mg/ml,  $p < 0.005$ .

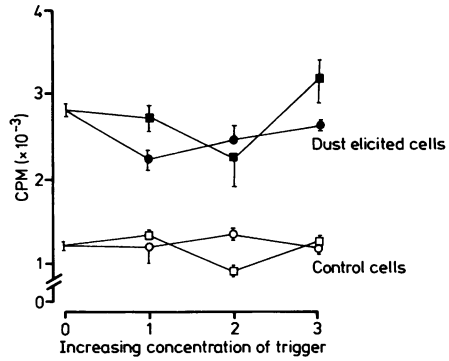


Fig 7 Triggering of neutral protease activity of eight day dust elicited leukocytes and controls. Zymosan at 1, 10, and 100  $\mu$ g/ml, phorbol myristate acetate at 0.1, 1.0, and 10  $\mu$ g/ml. There was no significant enhancement of neutral protease activity by either trigger.

inhibitable in a dose dependent manner by  $\alpha 1$ -PI (significantly reduced at 0.01, 0.1, and 1.0 mg/ml  $p < 0.005$ ),  $\alpha 2$ -M (significant at 0.5 mg/ml,  $p < 0.005$ ), serum (significant at 1%, 5%, and 10%,  $p < 0.005$ ) and by EDTA (significant at 1.0 mg/ml  $p < 0.001$ ). There was no increase in the neutral protease activity of control or bronchoalveolar leukocytes from eight day dust exposed animals on triggering in vitro with PMA or zymosan (fig 7).

## Discussion

The aim of this study was to evaluate the pulmonary inflammatory response to inhaled coalmine dust with particular regard to the proteolysis of fibronectin by inflammatory leukocytes from the bronchoalveolar space. We have shown that chronic inhalation exposure to low levels of coalmine dust leads to the gradual accumulation of inflammatory cells—neutrophils and activated macrophages—in the alveolar region. These results are contrary to those of Bingham *et al*, who reported no increase in the total number of cells in the bronchoalveolar lavage of rats exposed by inhalation to similar levels of coalmine dust.<sup>24</sup> In that study, however, the dusts were obtained by crushing seam coal. This procedure produces a dust that may differ fundamentally from that found in the air of coalmines since it (a) is not produced by coal cutting equipment but is ground and (b) does not include material from the roof and floor that is cut concomitantly with the coal seam. The latter factor may be particularly important, since this is likely to contribute substantially to the non-coal minerals including quartz, which are considered to be important factors in determining the toxicity of any coalmine dust sample.<sup>25,26</sup> In addition, the samples used by

Bingham *et al* were particularly fine dusts compared with the dust used in the present study and would be less likely to deposit in the lower airways.<sup>24</sup> Studies from this laboratory have shown that the inflammatory response to the inhalation of coalmine dust is dose dependent<sup>27</sup> and the finding that alveolar inflammation did result in the study by Bingham *et al* when the dusts were administered by intratracheal instillation,<sup>26</sup> gives added weight to the suggestion that the lack of response to inhaled dust was due to insufficient dust depositing in the alveolar region.

The increased number of leukocytes in the bronchoalveolar lavage of coalmine dust exposed rats in the present study is consistent with the increases observed in lavage of patients with the toxic dust related lung diseases coalworkers' pneumoconiosis, silicosis, and asbestosis.<sup>2,28</sup> Similar increases in total leukocyte numbers in bronchoalveolar lavage fluid have also been shown in experimental studies with silica, where exposure was by intratracheal instillation, in rabbits,<sup>29</sup> rats,<sup>20,30</sup> mice,<sup>31</sup> and guinea pigs,<sup>32</sup> or by inhalation in rats.<sup>6,7</sup>

Inflammatory leukocytes elicited in rat lungs<sup>20</sup> and in the mouse peritoneal cavity<sup>33</sup> have increased proteolytic activity in vitro and bronchoalveolar lavage of cigarette smokers<sup>34</sup> and patients with coalworkers pneumoconiosis<sup>35</sup> yields cells with enhanced secretion of elastase-like activity. In the present study bronchoalveolar lavage leukocytes from animals exposed by inhalation to coalmine dust had greater proteolytic activity than control cells; we confirmed that the measured substrate degradation was due to proteolysis, as reported previously,<sup>20</sup> by inhibition with serum,  $\alpha$ 1-PI, EDTA, and  $\alpha$ 2-M. This study has focused on proteases as these are considered to be key inflammatory cell products in causing injury and remodelling of the lung, both in emphysematous<sup>16</sup> and fibrotic<sup>17,18</sup> lung disease.

The proteolysis assay used in this study has shown that coalmine dust elicited bronchoalveolar leukocytes can damage at least one component of the extracellular matrix, fibronectin; previous studies from this laboratory have shown that inflammatory leukocytes can also degrade other connective tissue components such as collagen and laminin.<sup>11</sup>

We have thus shown that proteolytic activity in coalmine dust inflamed lung can be increased in two ways: (1) there are increased numbers of inflammatory leukocytes in the alveolar region and (2) these cells are up to fivefold more active in degrading fibronectin than the resident alveolar macrophages. The connective tissue damage associated with such increased proteolytic activity may be central to the pathogenesis of the disease process by enhancing inflammation<sup>15,36</sup> and by altering cell to cell and cell to matrix interactions which are important in maintaining normal tissue architecture.<sup>13,14</sup>

The finding that the inflammatory response was sustained during 64 days of recovery after 32 days of exposure to airborne dust may have relevance to the progression of progressive massive fibrosis seen in some coalmine workers even when removed from further occupational exposure to dust<sup>1</sup> and lends support to one report that pulmonary inflammation persists in retired coalminers as measured by clearance of Tc99m DTPA.<sup>37</sup> The persistence of an alveolitis, with neutrophils and activated macrophages, long after cessation of exposure to the coalmine dust is strong circumstantial evidence for the importance of leukocytes in the development of the lesions of coalworkers' pneumoconiosis; such a role for leukocytes in fibrosis due to dusts and other aetiological agents has frequently been suggested.<sup>8,38,39</sup>

Unstimulated control rat macrophages had low levels of fibronectin degrading activity compared with the dust exposed cells. It has been reported previously that triggering with PMA over a similar period to that used in the present study caused enhanced levels of neutral protease release.<sup>40</sup> Phagocytosis of latex beads<sup>41</sup> and colchicine treatment<sup>42</sup> were also shown to enhance neutral protease secretion by mouse peritoneal macrophages, although this was measured over several days. We assessed the roles of phagocytosis and leukocyte activation in inducing enhanced proteolysis of fibronectin by measuring the proteolytic activity of eight day dust elicited cells (97% macrophages) in the presence of a particulate (zymosan) or non-particulate (PMA) trigger. The results were contradictory to those reported previously,<sup>39-41</sup> with no evidence of stimulation; this may be due to differences in the cell types, duration of the experiment, or the proteolysis assays used. In the present study the failure of either trigger to stimulate increased proteolytic activity by the control leukocytes in vitro may reflect a requirement for activation in vivo, by the inflammatory milieu, to prime the leukocytes for increased proteolytic activity on contact with triggers in vitro. Since the proteolytic activity of the coalmine dust elicited leukocytes remained constant throughout the exposure period, it appears that they were maximally triggered in vivo after eight days of exposure to dust and therefore triggering in vitro would not enhance this response.

In conclusion, this study has shown that exposure to 10 mg/m<sup>3</sup> airborne concentration of respirable coalmine dust collected from a British colliery caused inflammation in the lungs of rats. The inflammation was characterised by the presence of an increasing proportion of neutrophils in the bronchoalveolar lavage and the presence of large numbers of activated alveolar macrophages. Proteolysis of fibronectin by the inflammatory leukocytes was increased compared with control cell proteolysis throughout the dusting period and persisted for 64 days of recovery during

which time the rats breathed room air. Alveolitis, as defined by the presence of neutrophils and activated macrophages in bronchoalveolar lavage and an increased ability to degrade fibronectin, also persisted in the recovery animals, although the total number of leukocytes in the bronchoalveolar region was no greater than in control animals. In a previous study in this laboratory rats were exposed to coalmine dust for one year followed by a four month recovery period.<sup>43</sup> In that study both the total number of leukocytes and the percentage of polymorphonuclear leukocytes in the lavage cell population remained raised. The continued presence of inflammatory leukocytes with enhanced ability to degrade extracellular matrix components such as fibronectin could be one important factor in long term pathological change associated with exposure to coalmine dust.

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## Correspondence and editorials

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The journal now also publishes editorials which are normally specially commissioned. The Editor welcomes suggestions regarding suitable topics; those wishing to submit an editorial, however, should do so only after discussion with the Editor.