Degradation of connective tissue components by lung derived leucocytes in vitro: role of proteases and oxidants

GERALDINE M BROWN, K DONALDSON

From the Institute of Occupational Medicine, Edinburgh

ABSTRACT Inflammatory leucocytes are implicated in connective tissue damage during chronic inflammatory lung disease. In an investigation of the role of leucocytes in connective tissue derangements in the lung, inflammatory leucocytes were generated in rat lungs by intratracheal instillation of inflammatory agents and retrieved by bronchoalveolar lavage. The proteolytic activities of control macrophages and of two inflammatory cell populations were compared; iodinated collagen, laminin, and fibronectin matrices were used. The inflammatory cells caused consistently and substantially more degradation of the matrices than the controls on a per cell basis. The oxidant scavengers superoxide dismutase and catalase did not inhibit matrix degradation, but α_1 protease inhibitor and α_2 macroglobulin were inhibitory. It is concluded that matrix damage in this assay is enhanced by inflammatory cells and is mediated principally by serine protease activity.

Accumulation of inflammatory leucocytes in the alveolar region of the lung is characteristic of disorders leading to fibrosis or destruction of lung parenchyma; inflammatory macrophages and polymorphonuclear leucocytes have been implicated in the pathogenesis of both types of disease.[1] In the adult respiratory distress syndrome large numbers of polymorphonuclear leucocytes (neutrophils) accumulate in the alveoli and are believed to have a major role in the disease process.[2] In interstitial lung fibrosis alveolitis is the initiating event in a process that leads, ultimately, to remodelling of the lung parenchyma.[3] Inflammatory cells are also implicated in the degradation of lung tissue and subsequent loss of alveoli in emphysema.[4]

Polymorphonuclear leucocytes and activated macrophages secrete proteases and reactive oxygen intermediates at sites of inflammation[5][6] and, although beneficial during short term inflammation, the persistence of increased numbers of inflammatory cells in the alveolar region may lead to an excessive burden of these products. Indeed, concentrations of proteases are raised in the bronchoalveolar lavage fluid of patients with adult respiratory distress syndrome,[7] idiopathic pulmonary fibrosis,[8] and sarcoidosis.[9]

Proteolytic enzymes and oxidants can damage connective tissue components in vitro[10][11][12][13] and in vivo.[14][15] Thus structural derangements of the lung parenchyma that occur during chronic inflammatory lung disease may be mediated by inflammatory cell derived proteases and reactive oxygen intermediates.

In the present study we have measured the ability of lung derived macrophages and polymorphonuclear leucocytes to degrade connective tissue components in vitro. We prepared iodinated matrices, using connective tissue components that occur in lung basement membrane and extracellular matrix. Proteolytic activity was assessed by measuring degradation of the matrices by three populations of lung derived leucocytes—control and two populations of inflammatory cells. To assess the role of proteases and oxidants in matrix degradation, we examined the inhibitor profile of matrix destruction caused by the three cell populations, using protease inhibitors and oxidant scavengers.

Methods

REAGENT
Rat tail (type 1) collagen, human plasma fibronectin, α_1 protease inhibitor, α_2 macroglobulin, catalase, superoxide dismutase, and bovine serum albumin were obtained from the Sigma Chemical Company (Poole, Dorset). Mouse laminin, phosphate buffered

saline (PBS) and serumless medium (N and T) were purchased from Gibco BRL (Paisley, Renfrewshire). Ethylene diamine tetra-acetic acid (EDTA) was obtained from BDH, Poole, Dorset, and *Corynebacterium parvum* from Wellcome Reagents Ltd (Hither Green, London). Iodine-125 (^{125}I) was purchased from Amersham International (Aylesbury, Buckinghamshire). The quartz used was the DQ$_{12}$ standard sample.

ANIMALS
Specific pathogen free, syngeneic, female *PVG* rats were obtained from the breeding unit of the Institute of Occupational Medicine, Edinburgh.

CELL PREPARATIONS
Production of inflammatory cells was induced in rat lungs by intratracheal instillation of 1 mg *C parvum* or quartz cells were retrieved by bronchoalveolar lavage of the resected lungs 16 hours after *C parvum* or five days after quartz instillation; control cells were obtained similarly from untreated rats. The resected lungs were cannulated with a blunt 16G needle and lavaged with four sequential 8 ml volumes of saline at 37°C. The lungs were gently massaged during each wash and the recovered lavage fluid (5 ml from the first lavage, 6–7 ml thereafter) was pooled in plastic universal containers and placed immediately on ice. The bronchoalveolar lavage cells were pelleted by centrifugation at 800 g, washed once in 30 ml of ice cold PBS and resuspended in the appropriate medium for the assay. To ensure that non-specific activation did not occur, the cells were kept ice cold throughout the preparation procedure and all manipulations were carried out with plastic pipettes and containers.

SUBSTRATE IODINATION AND MATRIX PREPARATION
Collagen, laminin, and fibronectin were iodinated by the method of McConahey and Dixon,[16] except that L-cysteine was substituted for sodium meta-bisulphite. Unbound iodine was removed by chromatography on a Sephadex G25 column (PD10, Pharmacia, Milton Keynes, Bucks). The labelled proteins showed activities of 5–25 × 10^6 cpm/µg protein. Collagen labelled with iodine-125 (^{125}I) was diluted in 0·1 M acetic acid and 100 µl aliquots (10000 cpm) were placed in microtitre removawells and dried on at 45° C, a temperature at which collagen is denatured to gelatin. Laminin labelled with ^{125}I was diluted in PBS and 10000 cpm aliquots were dried on to removawells as above; ^{125}I fibronectin was also diluted in PBS and 40000 cpm aliquots similarly dried onto removawells.

PROTEOLYSIS ASSAY
All assays of fibronectin proteolysis were carried out in N and T medium containing 2% bovine serum albumin; N and T medium without bovine serum albumin was used in the collagen and laminin degradation experiments. To reduce background counts, the removawells coated with ^{125}I substrate were presoaked for two hours immediately before use with 200 µl of assay medium alone and were then washed once with 300 µl of PBS. In all experiments except the dose-response study of fibronectin proteolysis, 1 × 10^5 cells were added to each removawell in a final volume of 200 µl medium. In all but the dose (6 h) and time response of fibronectin proteolysis, matrix degradation was assessed following four hours' incubation at 37° C. To assess release of ^{125}I labelled degradation products, 150 µl of supernatant medium was harvested from each well and counted by gamma counter.

INHIBITORS
The role of oxidants in matrix degradation was assessed in inhibition studies, the hydrogen peroxide scavenger catalase and superoxide dismutase, a scavenger of superoxide anion, being used. Similarly, the role of proteases was assessed by means of the protease inhibitors α$_1$ protease inhibitor and α$_2$ macroglobulin. Catalase, superoxide dismutase, and α$_1$ protease inhibitor were tested at final concentrations of 0·01, 0·1 and 1·0 mg/ml; α$_2$ macroglobulin was used at 0·005, 0·05, and 0·5 mg/ml.

STATISTICAL ANALYSIS
The effect of inhibitors on fibronectin matrix degradation was analysed by means of the Genstat linear interpolation program.[17] All other results were assessed by analysis of variance; the Minitab statistical package was used. The significance of differences between mean values at specific times and concentrations were tested by Student's *t* test.

Results

BRONCHOALVEOLAR LAVAGE CELL POPULATIONS
Both groups of treated rats showed alveolar inflammation, evidenced by greater numbers of cells in the bronchoalveolar lavage fluid compared with controls and by changes in the proportion of cells present (fig 1). In the control population the average yield of cells per rat was 7·5 (SEM 2·0) × 10^6, of which over 95% were macrophages and the remainder lymphocytes. The population of cells elicited by *C parvum* showed the greatest increase in cell numbers—to 62·6 (22·1) × 10^6, of which over 75% were neutrophils, about 20% macrophages, and about 2% lymphocytes. Cell numbers in the quartz lavage fluid were also greater than in the control lavage fluid—23·78 (7·26) × 10^6, of which
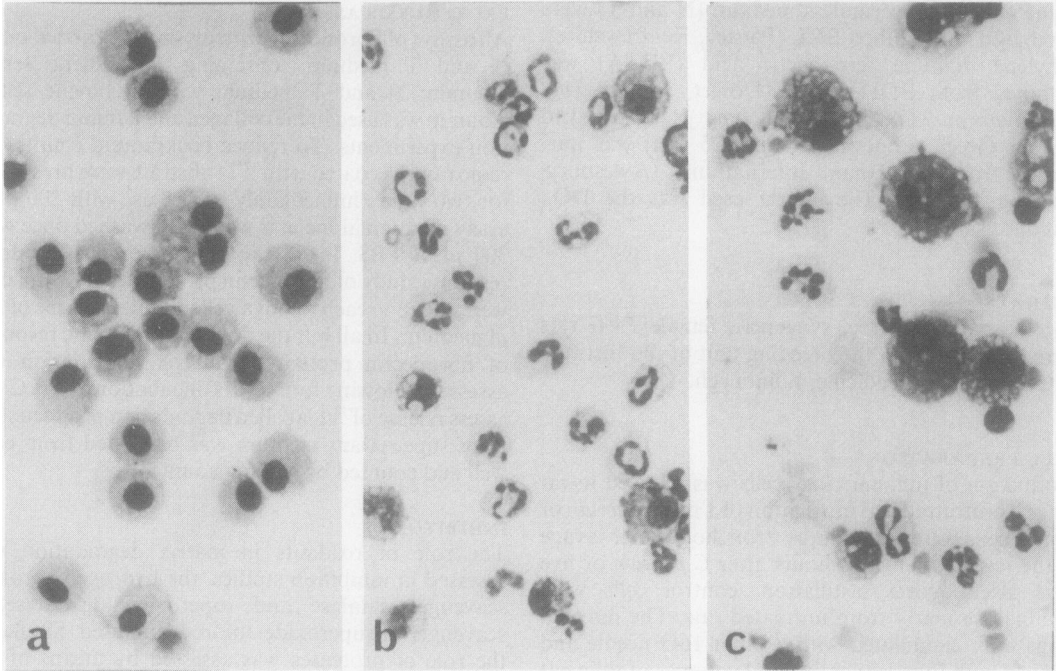
Fig 1   *Bronchoalveolar lavage populations from rat lungs. (a) Control cells (>95% macrophages); (b)*
Corynebacterium parvum elicited cells (>75% leucocytes, ≃ 20% macrophages, ≃ 2% lymphocytes); (c) quartz
elicited cells (≃ 50% macrophage, ≃ 50% polymorphs).

about half were neutrophils and about half macrophages.

VALIDATION OF MATRIX DEGRADATION ASSAY

Proteolysis of fibronectin, denatured collagen, and laminin matrices were assessed in preliminary validation experiments with the enzymes trypsin, elastase, and collagenase. The matrices were equally susceptible

to dose dependent proteolysis by the three enzymes (results not published).

DOSE-RESPONSE STUDY

The proteolytic activity of each lavage population was assessed at four concentrations (0·001, 0·01, 0·1, and 1·0 × 10^5 cells/well) with an ^{125}I fibronectin matrix, and all showed some ability to degrade the matrix (fig 2).
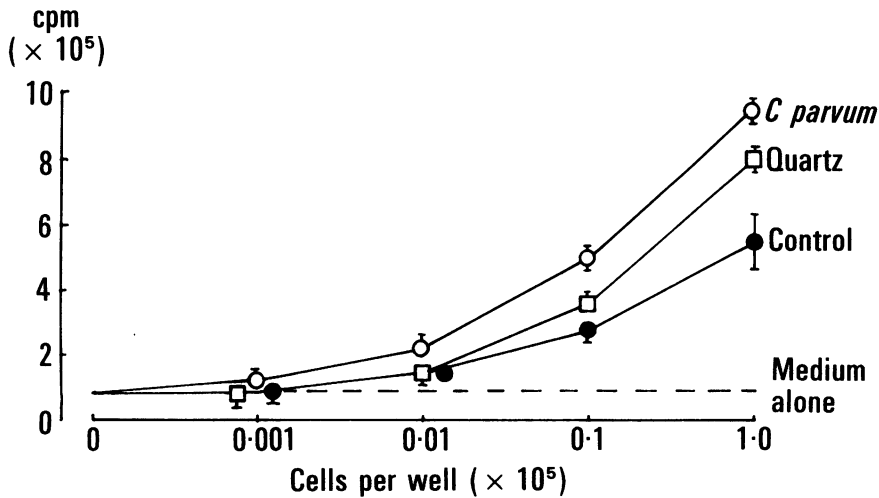

Fig 2   *Dose-response relationship of fibronectin matrix degradation by control cells and cells elicited by Corynebacterium parvum and quartz: counts released into the medium after six hours' incubation. Results are the means and standard errors of three experiments, with triplicate samples in each.*

The degradative capacity of each cell population increased in a dose dependent manner. The release of ^{125}I degradation products by the control and quartz populations was significantly greater than the background level only at concentrations of 0.1 and 1.0×10^5 cells/well (controls $p < 0.0025$ and $p < 0.005$ respectively, quartz $p < 0.005$); but at 1×10^5 cells/well the amount of radioactivity released by the quartz cells was almost double that of the control cells. The cells elicited by *C parvum* were more actively proteolytic than either the control or the quartz elicited cells, releasing significantly more radioactivity than the background level at 0.01×10^5 cells/well ($p < 0.025$). Although the cells elicited by *C parvum* caused greater matrix proteolysis than the quartz population at each cell concentration, the difference between the two populations of inflammatory cells was significant only at a concentration of 0.1×10^5 cells/well ($p < 0.005$).

TIME-RESPONSE STUDY

The three lavage cell populations were tested at a concentration of 1×10^5 cells/well for periods of 2, 4, 6 and 24 hours with an ^{125}I fibronectin matrix. Each cell population produced significantly more release of radioactivity than medium alone at every time point ($p < 0.025-< 0.005$) (fig 3). The proteolytic activity of all three lavage cell populations showed a time dependent increase, which continued up to 24 hours for both inflammatory populations, but plateaued at 6 hours with the controls. Both inflammatory populations were significantly more active than the controls at all time points ($p < 0.01-p < 0.005$) and similarly the cells elicited by *C parvum* were significantly more

active than the quartz elicited cells at each time point ($p < 0.025-< 0.005$).

DENATURED COLLAGEN AND LAMININ

In addition to fibronectin, we tested the proteolytic activity of the lavage cells with two alternative connective tissue components that occur in lung extracellular matrix—laminin and denatured collagen. As with fibronectin degradation, the two inflammatory cell populations caused greater proteolysis of collagen and laminin than control cells, and collagen degradation produced by *C parvum* elicited cells was greater than that produced by quartz elicited cells (fig 4); but there was no difference in laminin degradation between *C parvum* and quartz elicited cells.

ROLE OF PROTEASES AND OXIDANTS

To examine the role of proteases and oxidants in matrix degradation, we studied the effect of protease inhibitors and oxidant scavengers on the proteolysis of a fibronectin matrix produced by control cells and by cells elicited by *C parvum* and quartz. The oxidant scavengers superoxide dismutase and catalase had no effect on the matrix proteolysis caused by any of the lavage cell populations (fig 5). Similarly, the protease inhibitors α_1 protease inhibitor and α_2 macroglobulin had no effect on control cell proteolysis but caused a dose dependent reduction in matrix proteolysis by both inflammatory cell populations (table). α_1 protease inhibitor was equally effective in inhibiting matrix proteolysis by *C parvum* and quartz elicited cells, causing a significant reduction of both cell populations at 0.1 and 1 mg/ml ($p < 0.001$) and at 0.01 mg/ml ($p < 0.01$). α_2 macroglobulin significantly reduced
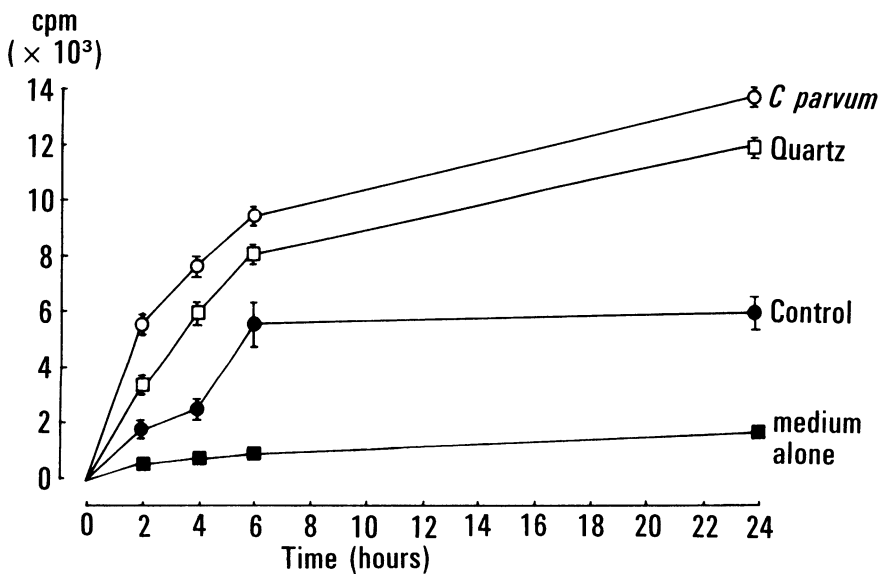
cpm
$(\times 10^3)$

Fig 3 *Time response of fibronectin matrix degradation by control cells and cells elicited by Corynebacterium parvum and quartz: counts released into the medium by 1×10^5 cells per well. Results are the means and standard errors of three experiments, with triplicate samples in each.*
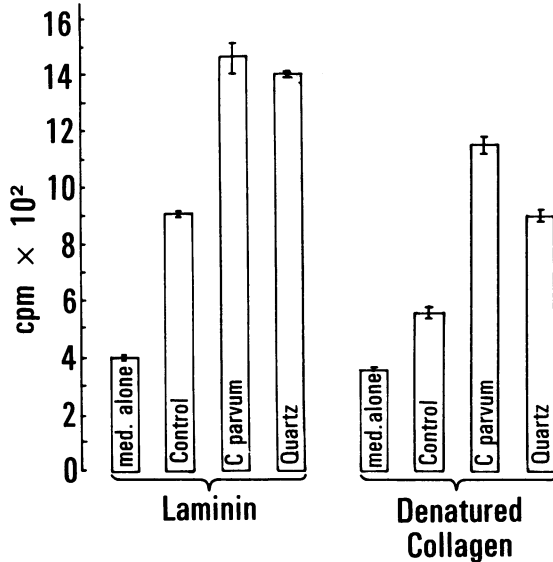
Fig 4 *Degradation of laminin and denatured collagen matrices: counts released into the medium by control cells and cells elicited by Corynebacterium parvum and quartz after four hours' incubation with 1 × 10⁵ cells per well. Results are the means and standard errors of three experiments, with triplicate samples in each.*

fibronectin matrix proteolysis by the quartz elicited cells at 0·05 mg/ml (p < 0·05) and 0·5 mg/ml (p < 0·001) but was less effective in reducing proteolysis produced by *C parvum* elicited cells, achieving a statistically significant effect only at a concentration of 0·5 mg/ml (p < 0·001).

Discussion

The aim of the present study was to assess the role of inflammatory leucocytes in connective tissue damage in the lung and to examine the relative contributions of reactive oxygen intermediates and proteases to this damage.

The connective tissue components collagen, laminin, and fibronectin occur extensively in the lung parenchyma[18] and have diverse properties, relevant to normal lung functioning, which may be altered during chronic lung disease.[19 20] Alteration of the conformation of connective tissue structure leads to the loss of basement membrane[21] and breakdown of cell to cell and cell to matrix interactions,[22-27] which are important in growth control and the maintenance of normal tissue structure. In addition, protease generated connective tissue fragments are chemotactic for leucocytes.[28 29] Thus proteolytic damage to connective tissue may enhance and prolong the inflammatory process and lead ultimately to fibrosis or emphysema.[20]

In this study, we assessed the ability of lung derived inflammatory cells to damage connective tissue components normally present in lung extracellular matrix and basement membrane. All matrices were susceptible to damage by control and inflammatory bronchoalveolar leucocytes, the latter being consistently and substantially more active. Fibronectin degradation by the control cell population plateaued by 6 hours, which may indicate a transient activation of the cells during preparation. On a per cell basis, the quartz population produced twice as much neutral protease as the controls and the *C parvum* population three times more than the controls. The increased
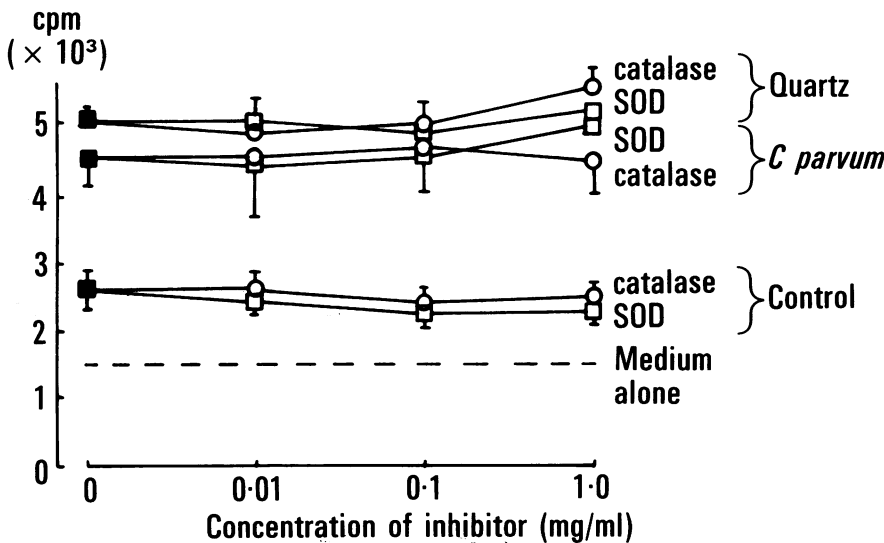

Fig 5 *Dose-response relationship of the effect of the oxidant scavengers superoxide dismutase (SOD) and catalase on degradation of a fibronectin matrix by control cells and cells elicited by Corynebacterium parvum and quartz. Results are the means and standard errors of three experiments, with triplicate samples in each. ■ no inhibitor.*

Degradation of a fibronectin matrix by control bronchoalveolar cells and those elicited by Corynebacterium parvum and quartz in the presence of the protease inhibitors α_1 protease inhibitor and α_2 macroglobulin (results (means (SEM)) derived from triplicate samples in three separate experiments)

Inhibitor	Final conc (μg/ml)	Control cells	C parvum elicited cells	Quartz elicited cells
None		2603 (299)	4587 (456)	5108 (304)
α_1 protease inhibitor	0·01	2380 (251)	2885 (569)**	3963 (374)**
	0·1	2428 (261)	2795 (379)**	3260 (335)***
	1·0	2333 (235)	2097 (230)***	2531 (274)***
α_2 macroglobulin	0·005	2787 (321)	4298 (418)	4648 (335)
	0·05	2456 (281)	4157 (571)	4198 (348)**
	0·5	2286 (288)	3528 (418)***	3534 (275)***

**Significant reduction compared with no inhibitor: p < 0·01.
***Significant reduction compared with no inhibitor: p < 0·001.

protease appeared to be related to an increased proportion of neutrophils in the bronchoalveolar lavage fluid. Recalculation of the data on the basis of the differential cell count suggested that the neutrophils in the two inflammatory populations were secreting similar amounts of neutral protease; this was estimated to be five times more than the amount secreted by the bronchoalveolar macrophages.

To determine the nature of the process of matrix degradation, we tested the effects of inhibitors of protease and of reactive oxygen intermediates in the assay. In vitro studies have suggested that reactive oxygen intermediates may be important in connective tissue damage,[30 31] but in our assay neither catalase nor superoxide dismutase reduced matrix proteolysis, thus indicating that neither hydrogen peroxide nor superoxide anion are involved in damage to the extracellular matrix. These results are in general agreement with the findings of several in vitro studies discounting reactive oxygen intermediates as a source of extracellular matrix degrading activity,[10 32 33] although in one study H_2O_2 was implicated.[32] To confirm that oxidant injury did not contribute to matrix proteolysis in the present study we tested exogenous hydrogen peroxide and superoxide anion and no proteolysis occurred (results not published). These results are in agreement with the findings of a previously published study in this laboratory, which indicated that reactive oxygen intermediates are not a major arbiter of tissue injury in mineral dust inflammation.[33]

To elucidate the mechanisms of proteolysis further we examined the inhibitor profile of the three cell populations, using protease inhibitors. The low level of proteolysis shown by control cells could not be inhibited, possibly owing to the presence of cysteine proteases[34] or to exclusion of soluble inhibitors from sites of close contact between leucocytes and the extracellular matrix.[10 35 36]

The C parvum population (>95% neutrophils) and the quartz population (50% neutrophils, 50% macro-

phages) showed similar inhibition profiles in response to α_2 macroglobulin and to α_1 protease inhibitor. Macrophage neutral proteases have been reported to be largely metalloproteases[37 38] and thus not capable of inhibition by α_1 protease inhibitor.[39] The inhibition profiles of the two inflammatory populations should therefore have been different in terms of their response to α_1 protease inhibitor if the macrophages in the quartz population were secreting metalloproteases. Since α_1 protease inhibitor inhibited the two inflammatory populations to the same extent, we tested inhibition of exogenous serine protease (porcine elastase) and metalloprotease (bacterial collagenase) in the fibronectin assay and confirmed that in our assay system serine proteases but not metalloproteases were inhibited by α_1 protease inhibitor (results not published). These results suggest that the proteolytic activity of the quartz population may be due to a serine protease, or to cysteine proteases as suggested above.

In summary, we have elicited two distinct populations of inflammatory cells in rat lungs, one containing largely neutrophils and the other composed equally of macrophages and neutrophils. The proteolytic activity of the two populations of inflammatory cells in vitro was substantially greater on a per cell basis than that of control bronchoalveolar cells. By carrying out inhibitor studies we have discounted the role of reactive oxygen intermediates in matrix degradation and have indicated that the proteolysis is mediated by serine protease activity.

Our results show that inflammatory leucocytes from the bronchoalveolar region of the lung have substantial connective tissue proteolytic activity; we have also shown that this activity can be inhibited by α_2 macroglobulin and α_1 protease inhibitor. These two inhibitors are found in the alveolar region of the lung, but the presence of active proteases in the bronchoalveolar lavage fluid of patients with chronic lung disease[7-9] suggests that the presence of large numbers of inflammatory cells in the lung parenchyma overloads this antiprotease screen. Protease activity

derived from inflammatory leucocytes may thus be a major arbiter of connective tissue damage during chronic inflammatory lung disease.

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