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Activated alveolar macrophages in subclinical pulmonary inflammation in collagen vascular diseases

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ABSTRACT A study was initiated to determine whether alveolar macrophages from patients with collagen vascular diseases but free of pulmonary symptoms were spontaneously activated and whether they released various mediators related to the pathogenesis of pulmonary fibrosis. Alveolar macrophages obtained by bronchoalveolar lavage from 32 patients with proved collagen vascular disease but no evidence of lung disease were compared with those from 10 patients with collagen vascular disease with interstitial lung disease (CVD-ILD) and from 10 healthy controls. The total number of alveolar macrophages did not differ between patients with collagen vascular disease and controls but were substantially increased in the CVD-ILD group. Alveolar macrophages from 31 of the 32 patients with collagen vascular disease and from all 10 in the CVD-ILD group had at least one criterion of activation. Neutrophil chemotactic activity was detected in supernatants from alveolar macrophage culture in 23 of the 32 patients with collagen vascular disease and from nine of the 10 in the CVD-ILD group; fibronectin secretion by alveolar macrophages was increased in 12 of the 32 patients with collagen vascular disease and in nine of the 10 in the CVD-ILD group. Furthermore, alveolar macrophages from 20 of the 32 patients with collagen vascular disease and four of the 10 CVD-ILD patients spontaneously released increased amounts of superoxide anion. Thus alveolar macrophages were spontaneously activated in a high proportion of patients with collagen vascular disease.

Collagen vascular diseases are frequently associated with interstitial pulmonary fibrosis.[1] Since interstitial fibrosis is generally an irreversible process, an understanding of the mechanisms leading to fibrosis is necessary for the development of treatment strategies to prevent irreversible alveolar damage. Current concepts of the pathogenesis of pulmonary fibrosis emphasise the role of alveolitis. The accumulation of inflammatory cells in the alveolus is believed to mediate in injury and the process of fibrosis.

Bronchoalveolar lavage allows a simple determination of effector cell populations and their state of activation in the alveolitis. The alveolitis associated with interstitial pulmonary fibrosis associated with collagen vascular disease is characterised by the presence of both neutrophils and alveolar macrophages.[2] There is evidence that alveolar macrophages are activated and spontaneously secrete mediators concerned in the pathogenesis of pulmonary fibrosis, and that in addition they direct the activity of various other cells that play a part.[3–5] Recent reports have shown that an inflammatory alveolitis may be present in symptomless patients with collagen vascular disease.[6–11] We recently showed that alveolitis was present in a high proportion of patients with collagen vascular disease who had no clinical or radiological evidence of pulmonary disease.[9] The distribution of immune and inflammatory cells in the lower respiratory tract of these patients was similar to that reported in patients with interstitial pulmonary fibrosis associated with collagen vascular disease.[10 12–14] In addition, the presence of neutrophils in the lavage fluid from untreated patients was associated with a progressive deterioration of their pulmonary function.[9] Together

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these data are consistent with the hypothesis that alveolitis precedes changes in lung connective tissue,[2 11-15] and that early detection of alveolitis might predict which patients are at risk of developing clinically apparent disease.

A study was initiated to determine whether alveolar macrophages from patients with collagen vascular disease who had no overt pulmonary disease spontaneously released products relevant to the pathogenesis of pulmonary fibrosis. Comparisons were made with cells from patients with collagen vascular disease and associated interstitial lung disease (CVD-ILD) and from healthy controls.

Methods

SUBJECTS

We studied 32 patients with proved collagen vascular disease. Thirty were female and all were non-smokers with ages ranging from 21 to 65 (mean 36·7) years. The diagnosis of collagen vascular disease was made on the basis of well established criteria for primary Sjögren's syndrome (n = 9), progressive systemic sclerosis (n = 12), rheumatoid arthritis (n = 5), dermatopolymyositis (n = 3), and mixed connective tissue disease (n = 3).[16-20] None had symptoms or signs suggesting that the collagen vascular disease had affected the respiratory tract, and all had normal chest radiographs as judged by two radiologists who were unaware of the clinical data. None had a history of occupational or drug exposure known to be associated with interstitial lung disease. A history of pulmonary disease and clinical or chest radiograph abnormalities were exclusion criteria. The results of pulmonary function measurements (mean (SEM)%) were: total lung capacity (TLC) 96 (4·6) predicted, forced vital capacity (FVC) 97 (4·2) predicted, forced expiratory volume in one second (FEV_1), 105 (4·3) predicted, and diffusing capacity for carbon monoxide (transfer factor, TLCO) 109 (6·1).

The control groups consisted of 10 healthy non-smokers and 10 non-smoking patients with CVD-ILD (8 progressive systemic fibrosis, 1 rheumatoid arthritis, 1 dermatopolymyositis). The interstitial lung disorder was defined by clinical, physiological, radiological and histological criteria.[1 21]

BRONCHOALVEOLAR LAVAGE

Bronchoalveolar lavage was performed, after premedication with atropine and under local anaesthesia with lignocaine, with a wedged fibreoptic bronchoscope (Olympus model BF-B3, Olympus Corporation of America, New York). A total of 250 ml of sterile normal saline was instilled in five 50 ml aliquots with immediate gentle vacuum aspiration after each aliquot.[20] Total and differential cell counts were determined and the viability of the alveolar macrophages was assessed by trypan blue exclusion. Bronchoalveolar lavage fluid was filtered through several layers of sterile surgical gauze and centrifuged at 400 *g* for 10 minutes at 4°C. After three washings the pellet was resuspended at a cell concentration of $1·5 \times 10^6$/ml in Hanks' balanced salt solution (HBSS). Informed consent was obtained from all subjects.

ALVEOLAR MACROPHAGE ISOLATION

Two millilitre volumes of the cell suspension were cultured at 37°C in humidified air (5% carbon dioxide) in 35 mm diameter plastic culture dishes. After two hours' incubation non-adherent cells were removed. According to differential cell counts of adherent cells stained by Wright's technique there were at least 95% alveolar macrophages and no basophils or mast cells.[22] Viability at the end of the incubation period was over 95%. Serum free RPMI-1640 (3 ml) was then added to each culture and incubation was continued for three hours in the same conditions as before.

Supernatants from cultures were collected and filtered (0·2 μmol/l filter Gelman Sciences Inc, Ann Arbor, Michigan) and stored at −30°C until they were assayed. All supernatants were assayed within two weeks of being harvested.

NEUTROPHIL CHEMOTACTIC ACTIVITY ASSAY

Neutrophil chemotactic activity derived from alveolar macrophages was measured by counting the number of neutrophils that passed through a 3 μm micropore filter (Nucleopore Corporation, Pleasanton, California) from the upper component of a 48 well microchemotaxis chamber (Neuro Probe, Cabin John, Maryland) in response to the macrophage supernatant in the lower chamber.[23] To distinguish chemokinesis from chemotaxis supernatants on both sides of the filter membrane were studied. There was no difference from the negative controls (buffer), which indicated a chemotactic process in the experimental wells. The number of neutrophils that had migrated through the filter was determined microscopically under oil immersion. Four fields were read in each well and each supernatant was studied in quadruplicate. The results were expressed as the difference between the mean number of cells per field in the experimental well and the mean number of cells per field in the control well (migration toward medium). As a positive control, migration towards FMLP 0·1 μmol/l (Peninsula Laboratories Inc, San Carlos, California) was determined.

FIBRONECTIN ASSAY

Fibronectin was determined by radioimmunoassay.[24] The results were expressed as ng fibronectin/10^6 cells per hour.

SUPEROXIDE ANION ASSAY

Spontaneous generation of superoxide anion was assayed by a lucigenin dependent chemiluminescence method adapted from Williams and Cole,[25 26] in which lucigenin (*bis*-*N*-methylacridinium nitrate; Sigma Chemical Co, St Louis, Missouri) served as a chemilumigenic probe. Lucigenin 10^{4}M was dissolved in HBSS buffered with 18 mmol/l HEPES (*N*-2-hydroxyethyl-piperazine-*N*-2-ethane sulfonic acid). Alveolar cells were centrifuged at 800 *g* (10 min, 4°C) and resuspended in HBSS-HEPES to a concentration of 1×10^{6} viable alveolar macrophages/ml. The suspension was kept on ice in a siliconised glass container until use. Chemiluminescence was measured at 37°C using a Luminometer (Lumac System AG, Basel). A 500 μl aliquot of the alveolar macrophage suspension was added to each phial; each phial contained a 900 μl aliquot of the lucigenin solution and a 50 μl aliquot of a 3% gelatin solution. A 100 μl aliquot from a 120 μg/ml superoxide dismutase solution was added to one phial. The total volume in each phial was brought to 1·650 ml by adding the appropriate amount of HBSS-HEPES. All the phials were incubated in parallel. Intensity of luminescence was integrated for 60 seconds after a 12 minute incubation (37°C). The chemiluminescence response were determined by subtracting background chemiluminescence values from the mean recorded chemiluminescence. The results are expressed in relative luminescence units (RLU)/$0{\cdot}5 \times 10^{6}$ viable alveolar macrophages.

STATISTICAL ANALYSIS

Most of the data were non-parametric and comparisons were made by means of the Mann-Whitney U test.

Results

NUMBER AND TYPE OF BRONCHOALVEOLAR CELLS

The volume of bronchoalveolar lavage fluid recovered was not significantly different between patients and controls ($p > 0{\cdot}04$). The CVD-ILD group had significantly greater cell counts than patients with collagen vascular disease alone (collagen vascular disease group) or controls (table 1). The percentage of alveolar macrophages was significantly less in the CVD and in CVD-ILD groups than in controls, though absolute numbers of alveolar macrophages did not differ between CVD and control groups (table 1). An increased total count and proportion of lymphocytes and neutrophils were found in the collagen vascular disease and CVD-ILD groups. This was largely because 12 of the collagen vascular disease patients and four of the CVD-ILD group had increased proportions of lymphocytes (> 18%)—ranging from 18% to 59% of the recovered cells. The proportion of neutrophils in the lavage fluid was increased (> 4%) in 14 of the collagen vascular disease and in seven of the CVD-ILD group. Overall, bronchoalveolar lavage fluid studies in collagen vascular diseases showed that 13 of the collagen vascular

Bronchoalveolar lavage cells from patients with collagen vascular disease without pulmonary disease (CVD), from patients with collagen vascular diseases and interstitial lung disorders (CVD-ILD), and from controls

	n	*Total cell count* (10^{4}/ml)	*Alveolar macrophages* (%)	*Alveolar macrophages* (10^{4}/ml)	*Lymphocytes* (%)	*Lymphocytes* (10^{4}/ml)	*Neutrophils* (%)	*Neutrophils* (10^{4}/ml)	*Eosinophils* (%)	*Eosinophils* (10^{4}/ml)
CVD	32	14·5 (5·4–48)	78·3* (38–97)	10·8 (4·8–38)	17·6* (2–59)	2·87* (0·16–17)	3·56* (0–12)	0·64* (0–4·2)	0·4 (0–5)	0·08 (0–0·76)
Primary Sjögren syndrome	9	13·7 (5·5–35)	68·8 (38–88)	8·3 (4·8–13·9)	27·2 (4–59)	4·7 (0·2–17·5)	3·55 (0–12)	0·64 (0–4·2)	0·33 (0–1)	0·04 (0–0·16)
Progressive systemic sclerosis	12	14·2 (5·4–34·2)	82·7 (68–96)	11·7 (4·8–31·1)	12·8 (2–25)	1·8 (0·22–4·8)	3·9 (0–9)	0·55 (0–2·4)	0·33 (0–2)	0·1 (0–0·76)
Rheumatoid arthritis	5	13·7 (7·3–20)	78·4 (64–97)	10·4 (5·1–14)	18·4 (2–31)	2·75 (0·16–6·2)	3·2 (1–5)	0·47 (0·08–1)	0 (0)	0 (0)
Dermato-polymyositis	3	22·3 (6–48)	88 (80–93)	18·6 (5·5–38·4)	9 (4–14)	2·6 (0·52–6·7)	2·7 (0–5)	0·9 (0–2·4)	0·33 (0–1)	0·16 (0–0·48)
Mixed connective tissue disease	3	15·6 (15–17)	73·3 (68–79)	11·5 (10·2–12·4)	20 (14–24)	3·1 (2·1–3·7)	5 (3–7)	0·78 (0·4–1·05)	1·6 (0–5)	0·25 (0–0·75)
CVD-ILD	10	34·6* (7·3–80)	76·6* (41–94)	26·7* (6·6–44)	13·7* (4–52)	3·6* (0·9–15)	7·2* (0–18)	3·16* (0–0·13)	2·5* (0–7)	1·05* (0–4·2)
Controls	10	9·86 (2·9–21)	91·2 (87–98)	9·1 (2·8–18)	7·7 (2–14)	0·83 (0·2–2·5)	0·7 (0–2)	0·05 (0–0·14)	0·4 (0–1)	0·03 (0–0·1)

Values expressed as mean (range).
*Difference from control is statistically significant ($p < 0{\cdot}05$).

disease group had normal bronchoalveolar lavage fluid, five had pure lymphocyte alveolitis, and 14 had a neutrophil alveolitis (with associated alveolar lymphocytosis in seven).

ALVEOLAR MACROPHAGE FUNCTION

Spontaneous release of neutrophil chemotactic activity by alveolar macrophages was detected in 23 of the 32 patients in the CVD group (fig 1). All but one of the patients with increased proportions of neutrophils had alveolar macrophages that spontaneously released neutrophil chemotactic activity. The release of neutrophil chemotactic activity also occurred in 10 patients without neutrophil alveolitis. There was no significant relationship between the percentage or total number of neutrophils in bronchoalveolar lavage fluid and the generation of neutrophil chemotactic activity by alveolar macrophages. Of the CVD-ILD patients, nine had increased neutrophil chemotactic activity in their supernatants.

Alveolar macrophages from 12 patients in the CVD group released substantial amounts of fibronectin (fig 2), compared with nine of 10 CVD-ILD patients. None of the healthy controls had evidence of fibronectin release. Fibronectin was spontaneously released by alveolar macrophages irrespective of the nature of the alveolitis—that is, of whether they had a neutrophil, lymphocyte, or normal cell pattern in their broncho-

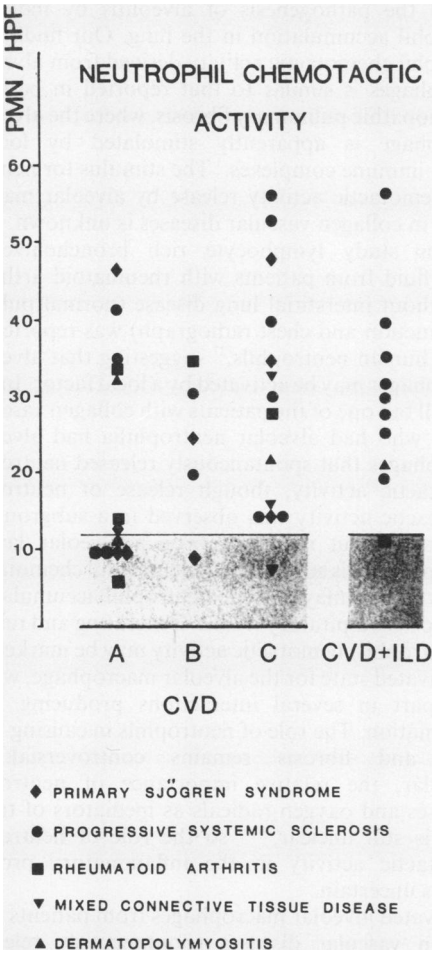

Fig 1 *Spontaneous neutrophil chemotactic activity (NCA) generation by alveolar macrophages from patients with collagen vascular disease (CVD) but without lung disease: A—denotes patients with normal bronchoalveolar lavage differential; B—patients with alveolar lymphocytosis (> 2 × 10^4 lymphocytes/ml); C—patients with alveolar neutrophilia (> 0·1 × 10^4 neutrophils/ml) with or without increased percentage of lymphocytes, and from patients with collagen vascular disease and associated interstitial lung disease (CVD + ILD). The hatched area indicates normal values obtained from healthy non-smokers.*

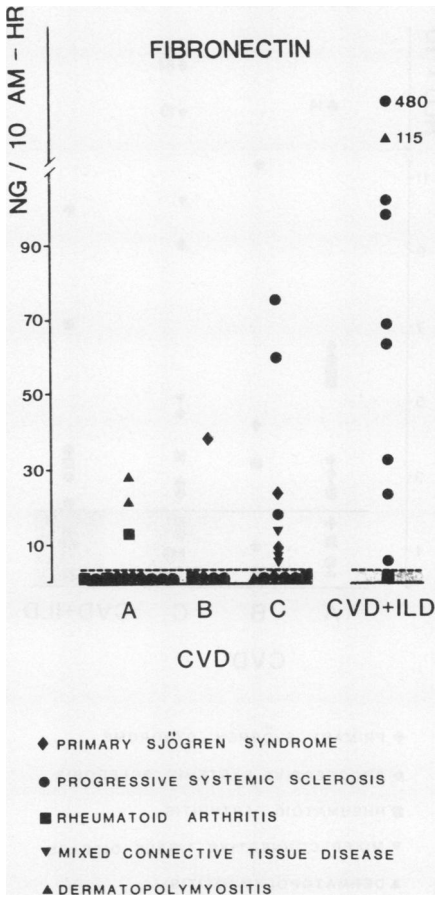

Fig 2 *Spontaneous fibronectin secretion of alveolar macrophages from patients with collagen vascular disease (CVD) but without lung disease. Key as in figure 1.*

alveolar lavage fluid. Alveolar macrophages from 10 of 12 patients in the CVD group that spontaneously released fibronectin also released neutrophil chemotactic activity.

Superoxide anion was spontaneously released by alveolar macrophages from 21 patients in the CVD group (fig 3) and by six in the CVD-ILD group. The pattern of alveolar inflammation did not affect the release of superoxide by alveolar macrophages.

There was no correlation between the level of the three alveolar macrophage activation markers and the total number of alveolar macrophages.

Discussion

Our data clearly show that alveolar macrophages from patients with collagen vascular disease but no lung disease are spontaneously activated and release various inflammatory mediators. The pattern of alveolar inflammation, in terms of the type of cells recovered from the lower respiratory tract and their state of activation, did not differ among patients with collagen vascular diseases between those who had overt lung manifestations and those who did not.

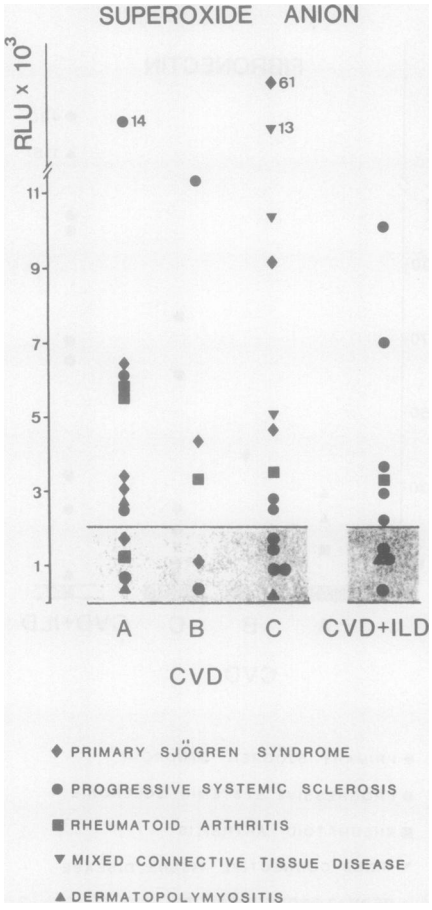

Fig 3 *Spontaneous superoxide anion release by alveolar macrophages from patients with collagen vascular disease (CVD) but without lung disease. Key as in figure 1.*

In this study three effector functions of alveolar macrophages were evaluated. The release of neutrophil chemotactic activity is thought to play a crucial part in the pathogenesis of alveolitis by inducing neutrophil accumulation in the lung. Our finding of neutrophil chemotactic activity derived from alveolar macrophages is similar to that reported in patients with idiopathic pulmonary fibrosis, where the alveolar macrophage is apparently stimulated by locally formed immune complexes.[3] The stimulus for neutrophil chemotactic activity release by alveolar macrophages in collagen vascular diseases is unknown. In a previous study lymphocyte rich bronchoalveolar lavage fluid from patients with rheumatoid arthritis but without interstitial lung disease (normal pulmonary function and chest radiograph) was reported to attract human neutrophils,[27] suggesting that alveolar macrophages may be activated by a local factor. In our study all but one of the patients with collagen vascular disease who had alveolar neutrophilia had alveolar macrophages that spontaneously released neutrophil chemotactic activity, though release of neutrophil chemotactic activity was observed in a subgroup of patients without increased bronchoalveolar lavage neutrophils. This suggests that neutrophil chemotactic activity release may precede neutrophil accumulation of the lower respiratory tract.[11] Generating and releasing neutrophil chemotactic activity may be markers of the activated state for the alveolar macrophage, which takes part in several interactions producing lung inflammation. The role of neutrophils in causing lung injury and fibrosis remains controversial; in particular, the relative importance of neutrophil proteases and oxygen radicals as mediators of tissue injury is still unclear,[28–30] so the role of neutrophil chemotactic activity in the inflammatory process remains uncertain.

Activated alveolar macrophages from patients with collagen vascular diseases spontaneously released increased amounts of fibronectin, which may have a role in the interaction between macrophages and fibroblasts, since macrophages appear to have the capacity both to stimulate and to suppress fibroblasts.[31 32] Fibronectin, a large glycoprotein, is known to mediate cell matrix interaction through various functions, including its chemotactic properties and as a competence factor for fibroblast growth. Increased production of fibronectin by alveolar macrophages is a common feature of several interstitial lung diseases,

implying that increased production of this macromolecule may be a general response of the alveolar macrophage to various stimuli.[4] In our study all but one patient with collagen vascular disease associated with interstitial lung disease showed increased spontaneous release of fibronectin. In contrast, only a subgroup of those with collagen vascular disease alone had alveolar macrophages that spontaneously released increased amounts of fibronectin. The fibronectin produced by the human alveolar macrophage can act as a chemoattractant for lung fibroblasts and might contribute to the development of fibrosis. Since these patients had normal chest radiographs and normal pulmonary function, close follow up is required to determine whether they are at risk of developing symptomatic interstitial lung disease in the future.

Spontaneous superoxide release by alveolar macrophages as assessed by chemiluminescence was noted in a high proportion of patients with collagen vascular diseases. This may reflect increased metabolic activity of the phagocytes in collagen vascular diseases. There is evidence suggesting that increased superoxide generation reflects active pulmonary inflammation.[33-36] The mechanisms responsible for alveolar macrophage activation in the lower respiratory tract of patients with collagen vascular diseases is unknown. Possibly they are activated primarily as a consequence of an immune process or as a non-specific inflammatory response at a distance from an inflammatory site.[37 38]

We have shown that subclinical alveolar inflammation, comprising activated alveolar macrophages and lymphocytes or neutrophils (or both), is present in a high proportion of patients with collagen vascular diseases. The spontaneous generation of superoxide anion and of neutrophil chemotactic activity may reflect the activated state of alveolar macrophages, whereas the spontaneous release of fibronectin may be important in the pathogenesis of interstitial lung disease. Our results suggest that fibronectin release increases but superoxide anion generation decreases as interstitial lung disease develops in patients with collagen vascular disease. The presence of asymptomatic alveolitis in collagen vascular diseases does not imply that these patients will develop overt pulmonary fibrosis in the future. Since interstitial lung disease is frequently associated with collagen vascular diseases, this group presents a unique opportunity to characterise changes in the alveoli that may precede the permanent damage to lung parenchyma. A detailed follow up of such patients is required to determine the relationship of our findings to the development of interstitial lung disease.

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