Collagenase and fibronectin in bronchoalveolar lavage fluid in patients with sarcoidosis

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ABSTRACT Bronchoalveolar lavage fluid from 43 patients with biopsy proved sarcoidosis and 10 control subjects were assayed for fibronectin and collagenase activity. Fibronectin was significantly increased in the group with sarcoidosis and was found to be positively correlated with angiotensin converting enzyme activity, protein concentration, percentage of T cells and helper:suppressor ratios in the lavage fluid. Increased fibronectin in the bronchoalveolar lavage fluid was not related to functional or radiographic indices of interstitial disease and did not identify patients subsequently requiring treatment. Latent collagenase was present in bronchoalveolar lavage fluid from 16 patients with sarcoidosis but not in any control sample. There was no association between the collagenase activity and the cell profiles of the lavage fluid. Yet carbon monoxide transfer factor was decreased in patients with bronchoalveolar lavage fluid collagenase. Ten of 16 patients with bronchoalveolar lavage fluid collagenase had radiographic class III or IV disease and a disease duration of more than two years. On follow up 62% of patients with bronchoalveolar lavage fluid collagenase required subsequent treatment, compared with only 23% of patients without collagenase. These results indicate an association between bronchoalveolar lavage fluid collagenase and progressive, prolonged disease in sarcoidosis, whereas increased bronchoalveolar lavage fluid fibronectin is associated with indices of disease activity.

Pulmonary sarcoidosis is often a self limiting disease, with spontaneous resolution occurring in about 80% of patients. In the remaining 20% parenchymal injury and fibrosis is frequently observed. This progression to fibrosis, with loss of lung function, represents the most serious manifestation of the disease. Current concepts of the pathogenesis of pulmonary fibrosis suggest that two events are required for the development of fibrosis—(a) a derangement of the normal alveolar architecture and (b) an increase in the number and level of activation of lung fibroblasts.1 Studies of patients with idiopathic pulmonary fibrosis suggest that considerable damage to alveolar structure is due to the release of hydrolytic enzymes, most notably collagenase, by neutrophils, which are present in the lungs of these patients.2 Alveolar macrophages from patients with idiopathic pulmonary fibrosis produce increased quantities of fibronectin and growth factors that stimulate the proliferation and activation of fibroblasts.34 Alveolar macrophages from patients with sarcoidosis also release these factors, suggesting that the mechanism of recruitment and activation of fibroblasts is similar in the two diseases.34 The events that cause alveolar derangement in sarcoidosis are unclear. There is conflicting evidence on whether collagenase is present in lavage fluid from patients with sarcoidosis.35 Some workers suggest that alveolar damage is caused by the influx of inflammatory cells and granuloma formation,7 whereas others suggest that neutrophils may have an important role in the fibrotic process in sarcoidosis.89 The present study set out to assess the levels of two putative fibrogenic markers—fibronectin and collagenase—in the lavage fluids from a group of patients with sarcoidosis and to compare these levels with physiological, radiological, immunological, and biochemical indices of disease severity or activity.

Methods

STUDY POPULATION

Forty three patients with sarcoidosis (32 of them male), mean age 35-3 (range 20–68) years, underwent
bronchoalveolar lavage. The mean duration of disease from diagnosis was 2-9 (SD 4.4, range 0-20) years. Diagnosis was confirmed by biopsy of various tissues (37 lymph node, four lung, and two Kveim biopsies) performed at the time of diagnosis. Thirteen patients had had corticosteroid treatment previously but had received no treatment for more than six months at the time of lavage. Eleven were current smokers. Four healthy volunteers and six hospitalised patients with lung disease were included in the study as a control group. Pulmonary function tests, performed before lavage, indicated normal lung function in control subjects. All subjects gave their informed consent for lavage.

**BRONCHOALVEOLAR LAVAGE**

Before bronchoscopy patients were given intramuscular atropine (0.6 mg) and pethidine (50 mg) and the upper respiratory tract was anaesthetised with lignocaine spray and lignocaine 2% solution. A fibreoptic bronchoscope (Olympus Model B3R) was securely wedged in a subsegmental bronchus in the right middle lobe, 180 ml of sterile 0-9% saline at 37°C was infused in three 60 ml aliquots, and gentle suction was applied after each infusion. The volume of the aspirated fluid was recorded and the fluid strained through sterile surgical gauze to remove mucus. The fluid was then centrifuged at 400 g for 15 minutes and the supernatant stored at −20°C for subsequent analysis.

**ANALYSIS OF LAVAGE CELLS**

The cells recovered from the lavage fluid were washed twice in Hanks’ balanced salt solution (HBSS) without Ca ++ or Mg ++ (Gibco Ltd, Scotland), resuspended in HBSS containing 0.5% bovine serum albumin, and counted. The cells were adjusted to a density of 2 × 10⁶ cells/ml and analysed for the presence of cells positive for Leu-1 (pan T-cells), Leu-2a (cytotoxic/suppressor), Leu-3a (helper/inducer), and HLA-Dr (macrophages and B lymphocytes) with the use of murine monoclonal antibodies (Becton-Dickinson, Mechelen, Belgium). Cells were incubated with the respective monoclonal antibody at 4°C for 45 minutes. They were then washed twice in Dulbecco’s phosphate buffered saline (PBS, Oxoid, UK) containing 20 mM sodium azide. FITC conjugated goat anti-mouse IgG (Tago Inc, California) was added and samples were incubated at 4°C for a further 30 minutes. The cells were washed, resuspended in a small amount of 90% glycerol and placed on a microscope slide. Fluorescent labelled cells were then counted on a Leitz Dialux 22AB epifluorescent microscope. At least 200 cells were counted on each slide. Differential counts of lymphocytes, macrophages, and neutrophils were also performed on Wright Giemsa stained cell smears by a second observer. Where a substantial difference (> 10%) was noted between the percentage of T lymphocytes as estimated by monoclonal analysis and the percentage of lymphocytes counted after Wright Giemsa staining, an esterase stained smear was used to distinguish between small macrophages and large lymphocytes.

**ANALYSIS OF LAVAGE FLUID**

Before analysis lavage fluids were centrifuged at 1000 g for 15 minutes; phenylmethylsulphonylfluoride (PMSF, final concentration 0.1 mmol/l) added to prevent protease digestion and concentrated (∗ × 20) by ultrafiltration in CF25 Centriflo membrane cones (Amicon Corporation, Danvers, USA). To minimise protein loss on concentration, the fluids were first concentrated by a factor of 50-100 and the cone membranes washed three times with aliquots of the filtrate. These were then combined to give final × 20 concentrates. Substrate for collagenase assay was prepared by labelling type I acid soluble calf skin collagen (Sigma, Poole, Dorset) with 4C-labelled acetic anhydride (Amersham, Bucks). The assay system was essentially that described by Linblad and Fuller.11 Labelled substrate (100 μg, total cpm 30 000) in 0.05 M tris containing 0.005 M CaCl₂ and 0.2 M NaCl (pH 7.5) was incubated with 100 μl of concentrated lavage fluid at 35°C for 90 minutes. p-Amino-phenylmercuric acetate (APMA) at a final concentration of 1 mmol/l was included to activate latent enzyme.12 The reaction was terminated by the addition of 100 mM EDTA and 100 μg of unlabelled collagen. Incubation was continued for a further 30 minutes to allow complete denaturation of the cleaved fragments.

Four hundred microlitres of dioxane: methanol: water (4:1:5 volume) was then added to precipitate undigested collagen and the samples were centrifuged at 2500 g for 60 minutes. The supernatant, containing digested collagen fragments, was counted on a Beckman Rack Beta scintillation counter (Model 1217-001) after the addition of 10 ml of scintillation fluid (4 g/l PPO in toluene:triton X, 2:1 v/v). Samples were assayed in duplicate and a control, to which EDTA and unlabelled collagen were added before incubation, was included for each sample. The difference in counts between test and control samples was used to calculate units of collagenase activity as micrograms of collagenase degraded per minute per millilitre of lavage fluid. The sensitivity limit of the assay was 0.35 units/ml.

Fibronectin was measured by an enzyme linked immunosorbent assay (ELISA).13 Purified human fibronectin and antibody to human fibronectin was obtained from Boehringer Mannheim GmbH (Mannheim, West Germany). Protein was determined by a modification of the Lowry method.14 Angiotensin converting enzyme (ACE) activity was measured in × 20 concentrates of lavage fluid.15
NORMALISATION OF BRONCHOALVEOLAR LAVAGE FLUID PROTEINS

No satisfactory reference component for normalisation of lavage fluid proteins is currently available. Albumin, a useful reference in normal subjects, is unsatisfactory in disease states where alveolitis or capillary leakage occur. Expression of protein concentrations with respect to volume of fluid recovered also presents problems in patients with sarcoidosis as variation has been noted both between control and sarcoidosis groups and between subgroups of patients divided according to disease activity and severity. To minimise this problem, urea, a small molecular weight compound that diffuses freely across the capillary endothelium, was chosen as a reference component. Urea concentrations in the bronchoalveolar lavage fluid of controls and patients with sarcoidosis were similar (control: 3-1, range 1-4–9-1 µg ml; sarcoid: 3-3, range 0-9–12-5 µg/ml). Within the sarcoidosis group no association was noted between urea concentrations in bronchoalveolar lavage fluid and disease activity or severity. Thus the problems associated with albumin and volume in normalising bronchoalveolar lavage fluid protein concentrations were largely overcome by using bronchoalveolar lavage fluid urea as reference. Problems associated with variation in bronchoalveolar lavage fluid urea owing to the dwell time of infused saline, however, still remain.

PULMONARY FUNCTION TESTS

Measurements of forced vital capacity (FVC), forced expiratory volume in one second (FEV₁) and carbon monoxide single breath diffusion capacity (transfer factor, TLCO) were performed using a vitalograph Model S spirometer and a PK Morgan transfer test Model D. The percent predicted values for FVC, FEV₁ and TLCO were calculated as described by Cotes et al., corrected for use with the Morgan transfer test instrument.

RADIOLOGY

A chest radiograph was available for all patients at the time of lavage. We used the 1980 UC/ILO 12 point scale to grade the profusion of small opacities as the Siltzbach classification did not quantitate the range of infiltrates seen.

STATISTICAL METHODS

The data on lavage fluid are expressed as medians and absolute ranges. For comparison between the control and sarcoidosis groups the Wilcoxon rank sum test was used. Correlation between two measurements within a group was assessed according to Pearson’s test. Yates’s corrected χ² test was used for comparison of proportions. Pulmonary function data are expressed as means with standard deviations in parentheses.

Results

The collagenase assay was shown to measure true mammalian collagenase activity on analysis of the reaction products by SDS polyacrylamide gel electrophoresis (fig 1). The electrophoretic profile of the cleaved collagen was typical of that observed for collagenase digestion of type I collagen. Preliminary experiments indicated that APMA was slightly more effective than trypsin in activating latent collagenase in lavage fluid (APMA activation 1-8 (SD 0-67) units/ml; trypsin activation 1-26 (0-5) units/ml, n = 4), so APMA activation was used throughout the study. Inclusion of PMSF in the lavage samples and APMA in the reaction mixture protected against interference by serine proteases.

BRONCHOALVEOLAR LAVAGE FLUID

The detection limit for the ELISA was 5 ng/ml. Fibronectin was detected in bronchoalveolar lavage fluid.

![Fig 1 SDS-Polyacrylamide gel electrophoresis profiles of (A) native undigested type I collagen; (B) collagen cleavage products after incubation with 100 µl lavage fluid at 35°C for 90 minutes (standard assay conditions); and (C) collagen cleavage products after incubation with 100 µl lavage fluid for 21 hours at 35°C (total digestion conditions).](http://thorax.bmj.com/Thorax: first published as 10.1136/thx.43.5.393 on 1 May 1988. Downloaded from http://thorax.bmj.com/)
Fig 2  (A) Fibronectin concentrations in lavage fluid from control subjects and patients with sarcoidosis. ----- indicates the mean values in control subjects + 2 SD. (B) Collagenase concentrations in lavage fluid from control and sarcoid groups. △ indicates current smoker.

Fig 3 Correlation between lavage fluid concentrations of fibronectin and (A) angiotensin converting enzyme (ACE) ($r = 0.58, p < 0.01$); (B) protein ($r = 0.68, p < 0.001$).

levels and both lavage ACE and protein (figs 3a and 3b) in patients with sarcoidosis but not in the control group. There was no similar association between bronchoalveolar lavage fluid collagenase levels and ACE or protein.

LAVAGE FLUID CELLS
There was no difference between the control and sarcoidosis groups in the total number of cells recovered by lavage, but the percentage of T lymphocytes (Leu-1 positive) was significantly greater...
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Table 1 Cell profiles of bronchoalveolar lavage fluid from control subjects and patients with sarcoidosis (median: range)

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<thead>
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<th>Control (n = 10)</th>
<th>Sarcoidosis (n = 43)</th>
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<tr>
<td>Total cells recovered (× 10⁹)</td>
<td>9.35±4.4–30.3</td>
<td>15.3±1.4–52.4</td>
</tr>
<tr>
<td>% T lymphocytes</td>
<td>7.25±0.4–13.9</td>
<td>22.0±1.0–58.0**</td>
</tr>
<tr>
<td>Helper: suppressor cell ratio</td>
<td>1.30±0.1–3.5</td>
<td>2.4±0.2–11.8*</td>
</tr>
<tr>
<td>% Alveolar macrophages</td>
<td>95.0±89–99.5</td>
<td>74.0±35.0–98.0**</td>
</tr>
<tr>
<td>% Neutrophils</td>
<td>1.0±0.3–2.0</td>
<td>1.6±0.3–22.0</td>
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*p < 0.02; **p < 0.01 Wilcoxon rank sum test.

than in the sarcoidosis group, as was the ratio of Leu-3a positive (helper-inducer) to Leu-2a positive (suppressor-cytotoxic) cells (table 1). Total bronchoalveolar lavage fluid fibronectin levels were positively correlated with both %T cells (r = 0.49, p < 0.01) and helper:suppressor ratios (r = 0.59, p < 0.001), suggesting an association between high fibronectin concentrations and “active” disease. No correlation was observed between fibronectin concentrations and total numbers of cells or macrophage numbers in the bronchoalveolar lavage fluid. The bronchoalveolar lavage fluid cell profiles of patients with lavage collagenase were similar to those of patients without collagenase and no correlation was observed between bronchoalveolar lavage fluid collagenase activity and any cellular measurement, whether expressed in absolute numbers of cells or as a proportion of total cells.

PULMONARY FUNCTION

Mean values of FEV₁ % predicted (90.0 (SD 19.4) and FVC % predicted (91.4 (SD 19.4) were within the normal range. Only eight of the 43 patients had an FVC below 80% predicted. Thirty one patients had normal TLCO values (mean 92.7% (22.2%) predicted) and 12 had values below 80%. Patients with bronchoalveolar lavage fluid collagenase had lower TLCO % predicted levels than those without bronchoalveolar lavage fluid collagenase (table 2). This was not associated with smoking as no relationship between smoking status and TLCO % predicted was observed. There was no association between bronchoalveolar lavage fluid fibronectin levels and pulmonary function, nor did lavage fluid cellular profiles of those with functional impairment differ significantly from those with normal pulmonary function.

RADIOGRAPHIC PROFILES

In the patients, radiographic profusion scores ranged from 1 to 9 on the UC/ILO scale with 66% of patients having a profusion score of 3 or less. There was no association between lavage collagenase or fibronectin and profusion score. There was, however, an association between bronchoalveolar lavage fluid collagenase and advanced disease as assessed by radiographic classification scores (table 3). Ten (63%) of the patients with bronchoalveolar lavage fluid collagenase had class III or class IV disease compared to seven (26%) of those without bronchoalveolar lavage fluid collagenase (p < 0.05); all five patients with radiographic evidence of fibrosis (class IV) were collagenase positive. A similar proportion (62%) of patients with bronchoalveolar lavage fluid collagenase had a disease duration of over two years and eight of the 13 patients who had previously received corticosteroid treatment were collagenase positive. No association between fibronectin levels and radiographic classification or disease duration was found.

FOLLOW UP

Follow up information on functional and clinical progress was available for 35 of the 43 patients (mean 16.9 months, range 6–30 months). Thirteen patients were treated with corticosteroids because of functional impairment, the decision being taken on clinical grounds and without knowledge of bronchoalveolar

Table 2 Pulmonary function of patients with and without lavage fluid collagenase

<table>
<thead>
<tr>
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<th>FEV₁ % pred</th>
<th>FVC % pred</th>
<th>TLCO % pred</th>
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<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>&lt;80%†</td>
<td>Mean (SD)</td>
</tr>
<tr>
<td>Collagenase negative</td>
<td>94.9 (16.3)</td>
<td>5</td>
<td>96.4 (15.6)</td>
</tr>
<tr>
<td>Collagenase positive</td>
<td>83.1 (21.9)</td>
<td>5</td>
<td>83.8 (22.4)</td>
</tr>
</tbody>
</table>

*p < 0.05.
†No of patients with <80% predicted.
FVC—forced vital capacity; TLCO—Carbon monoxide transfer factor.
lavage fluid collagenase or fibronectin levels. Of the 35 patients, only 13 (37%) had elevated bronchoalveolar lavage fluid collagenase levels, yet these patients constituted 62% of the patients who were subsequently treated (table 4). The proportion of patients with raised bronchoalveolar lavage fluid fibronectin concentrations was similar in the treated and untreated patients, as was the proportion of patients with over 30% of T cells and helper:suppressor ratios of more than 4 in their lavage fluid.

Discussion

Rennard et al observed raised fibronectin concentrations in lavage fluid from patients with sarcoidosis and suggested that fibronectin might contribute to the development of fibrosis in sarcoidosis by acting as a chemotactrant for fibroblasts. In the present study bronchoalveolar lavage fluid fibronectin concentrations in patients with sarcoidosis were positively correlated with bronchoalveolar lavage fluid angiotensin converting enzyme concentrations, percentages of T cells, and helper:suppressor ratios, all of which have been shown to be increased in the "active" alveolitis stage of disease. This suggests that high bronchoalveolar lavage fluid fibronectin concentrations are associated with "active" disease and may reflect an additional aspect of the inflammatory response observed in sarcoidosis. The alveolar macrophage would appear to be the most likely source of bronchoalveolar lavage fluid fibronectin. Increased production of fibronectin by alveolar macrophages from patients with sarcoidosis was found by Rennard et al. In this study no correlation was observed between bronchoalveolar lavage fluid fibronectin concentration and numbers of alveolar macrophages. This may, however, be due to the heterogeneous nature of the alveolar macrophage population, which comprises monocytes and macrophages at various stages of maturation and activation. "In vitro" stimulation of normal alveolar macrophages fails to elicit an increase in fibronectin production, suggesting that a specific "in vivo" stimulus or a particular subset of "primed" alveolar macrophages may be responsible for the observed increase in fibronectin. Increased fibronectin concentrations in the bronchoalveolar lavage fluid was not related to functional or radiographic indices of disease severity and did not identify patients subsequently requiring treatment. Thus, despite an association between bronchoalveolar lavage fluid fibronectin and active alveolitis, no evidence of a link between bronchoalveolar lavage fluid fibronectin and subsequent fibrosis was observed.

Latent collagenase activity was detected in lavage fluid from 16 of the 43 patients studied. Gadek et al failed to detect collagenase, either active or latent, in bronchoalveolar lavage fluid from nine patients with sarcoidosis. By contrast, Cordier and coworkers detected collagenolytic activity in three patients with sarcoidosis. The key to these discrepancies may lie in the profiles of the patients. The group studied by Gadek et al had normal lung volumes and diffusing capacities. Pulmonary function data are not given by Cordier et al, but lung biopsy showed evidence of intense fibrosis in two of the three patients. In our study most (56%) patients with bronchoalveolar lavage fluid collagenase had diffusing capacities of less than 80% predicted and five had radiographic evidence of fibrosis. In addition, a high proportion of these patients had been diagnosed over two years previously, half had received previous corticosteroid treatment, and 62% required treatment after lavage. These results suggest an association between bronchoalveolar lavage fluid collagenase activity and prolonged, progressive disease in sarcoidosis.

Collagenase activity has also been detected in bronchoalveolar lavage fluid from patients with idiopathic pulmonary fibrosis and the adult respiratory distress syndrome, diseases in which interstitial fibrosis occurs more rapidly than in sarcoidosis. It is suggested that digestion of interstitial collagen by collagenase in the lower respiratory tract contributes to the derangement of alveolar structures and the disordered pattern of collagen deposition seen in these diseases. Alveolar derangement and collagen accumulation also occurs in some patients with sarcoidosis, though usually over a prolonged period. Our results suggest that the role of collagenase in the development of interstitial fibrosis may be similar in all three diseases.

The source of bronchoalveolar lavage fluid collagenase in patients with sarcoidosis remains unclear. Three cells present in the lower respiratory tract are capable of producing collagenase—the macrophage, the fibroblast, and the neutrophil. In idiopathic pulmonary fibrosis lavage fluid collagenase is associated with an increase in neutrophils. Increased numbers of neutrophils are also observed in the adult respiratory distress syndrome, though Christner et al indicate that production of collagen-
Collagenase and fibronectin in bronchoalveolar lavage fluid in patients with sarcoidosis

Collagenase and fibronectin of proportion disease. Neutrophils disease and by fibroblasts our collagenase tion collagenase in produce tion between neutrophil collagenase 29; activation state. 03' Another subjects. 6 Another investigators to recovered can also after perturbation of secreted by collagenase inhibitor, 29 in lavage fluid collagenase patients with sarcoidosis. The latent form of patients with progressive, prolonged disease had collagenase activity in their lower respiratory tracts, suggesting that collagenase may have a role in the development of fibrosis in sarcoidosis. The cellular source of this collagenase is unclear.

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