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# Production of fibronectin by the human alveolar macrophage: Mechanism for the recruitment of fibroblasts to sites of tissue injury in interstitial lung diseases

(inflammation/fibrosis/chemotaxis)

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ABSTRACT **Because cells of the mononuclear phagocyte system are known to produce fibronectin and because alveolar macrophages are activated in many interstitial lung diseases, the present study was designed to evaluate a role for the alveolar macrophage as a source of the increased levels of fibronectin found in the lower respiratory tract in interstitial lung diseases and to determine if such fibronectin might contribute to the development of the fibrosis found in these disorders by being a chemoattractant for human lung fibroblasts. Production of fibronectin by human alveolar macrophages obtained by bronchoalveolar lavage and maintained in short-term culture in serum-free conditions was demonstrated; *de novo* synthesis was confirmed by the incorporation of [$^{14}$C]proline. This fibronectin had a monomer molecular weight of 220,000 and was antigenically similar to plasma fibronectin. Macrophages from patients with idiopathic pulmonary fibrosis produced fibronectin at a rate 20 times higher than did normal macrophages; macrophages from patients with pulmonary sarcoidosis produced fibronectin at 10 times the normal rate. Macrophages from 6 of 10 patients with various other interstitial disorders produced fibronectin at rates greater than the rate of highest normal control. Human alveolar macrophage fibronectin was chemotactic for human lung fibroblasts, suggesting a functional role for this fibronectin in the derangement of the alveolar structures that is characteristic of these disorders.**

Fibronectin, a large glycoprotein found in plasma and tissues, is thought to mediate cell–matrix interactions (1–4) through its ability to bind to cells (5, 6), collagen (7, 8), and other connective tissue components and to attract cells by virtue of its chemotactic properties (9, 10). Several cell types are known to produce this macromolecule, including cells of the mononuclear phagocyte system (11, 12). Together, these observations lead to the hypothesis that mononuclear phagocytes at sites of tissue injury produce increased amounts of fibronectin which, in turn, recruits fibroblasts to aid in the repair process.

The lung is a common site of tissue injury and is one of the few sites where human tissue mononuclear phagocytes are readily available for study. In addition, recent studies have demonstrated increased levels of fibronectin in the lower respiratory tract of patients with interstitial lung diseases (13). In this context, the present study was designed to answer several questions, including (*i*) do human alveolar macrophages produce fibronectin? (*ii*) do alveolar macrophages from patients with chronic tissue injury produce increased amounts of fibronectin? and (*iii*) is the fibronectin produced by the human alveolar macrophage chemotactic for human lung fibroblasts?

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## MATERIALS AND METHODS

**Study Population.** The study population consisted of 48 patients with interstitial lung disease and 7 normal volunteers. Patients were selected from consecutive patients evaluated for interstitial lung disease at the National Institutes of Health and for whom adequate material was available. All patients and normals were studied under approved protocols at the Clinical Center after informed consent had been obtained. All data are presented as mean ± SEM.

The patients are summarized in Table 1. Idiopathic pulmonary fibrosis was diagnosed (14) in 21 patients. All had the disease in midcourse; five were smokers, and seven were taking prednisone (mean dose, 15.1 ± 1.1 mg daily). Sarcoidosis was diagnosed (15) in 17 patients. Two were smokers; five were taking prednisone (20 ± 10 mg daily). Ten patients were grouped as having "other" chronic interstitial lung disorders including: chronic interstitial lung disease associated with progressive systemic sclerosis (one), chronic interstitial lung disease associated with mixed connective tissue disease (one), lymphocytic infiltrative disorder (one), histiocytosis X (two), asbestosis (one), chronic hypersensitivity pneumonitis (one), and undiagnosed interstitial lung disease (three) (16). One smoked and two were taking prednisone (20 ± 10 mg daily).

Seven normal volunteers (four men and three women; mean age, 23.4 ± 2.5 yr; two smokers, five nonsmokers) had no history or physical findings suggesting lung disease, and all had normal chest roentgenograms and normal results on pulmonary function tests.

**Fibronectin Production by Alveolar Macrophages.** Alveolar macrophages were obtained by using bronchoalveolar lavage as described (17). Cells were separated from lavage fluid by centrifugation, washed four times, and resuspended in RPMI-1640 medium at $10^7$ cells per ml. An aliquot of cells was then subjected to cell differential count by using a Wright–Giemsa stained cytocentrifuge preparation.

To evaluate whether alveolar macrophages were capable of producing fibronectin, the lavage cells (86% macrophages, 8% neutrophils, and 6% lymphocytes) were plated at $2 \times 10^5$ cells per well in 0.2 ml of RPMI-1640 medium without serum in 6-mm-diameter microtiter wells (Falcon) and cultured at 37°C in 95% air/5% $CO_2$; the nonadherent cells were removed by washing with RPMI-1640 after 4 hr in culture. The medium was then replaced and the cultures were incubated for up to 48 hr longer. The supernatant medium was aspirated at various times and

Abbreviation: ELISA, enzyme-linked immunoassay.

Table 1. Patient population: Clinical data

| Diagnosis | *n* | M | F | Age, yr | VC, %* | TLC, %* | DC, %* | $FEV_1/FVC$, % | Resting $Pa_{O_2}$, torr |
|---|---|---|---|---|---|---|---|---|---|
| Idiopathic pulmonary fibrosis | 21 | 13 | 8 | 46.5 ± 2.8 | 59.8 ± 5.3 | 61.1 ± 4.7 | 68.0 ± 6.8 | 79.6 ± 2.1 | 81.6 ± 2.7 |
| Sarcoidosis | 17 | 10 | 7 | 35.4 ± 4.0 | 76.7 ± 5.2 | 76.9 ± 4.3 | 96.5 ± 3.9 | 73.6 ± 4.7 | 94.2 ± 5.2 |
| Other (see text) | 10 | 4 | 6 | — | 72.1 ± 8.2 | 75.3 ± 5.9 | 72.8 ± 10.5 | 76.1 ± 4.2 | 78.4 ± 5.8 |

Data are shown as mean ± SEM. VC, vital capacity; TLC, total lung capacity; DC, diffusing capacity; $FEV_1/FVC$, forced 1-sec expiratory volume/forced vital capacity.
* As percentage of volume predicted.

stored in liquid nitrogen vapor until use. For harvesting larger volumes of supernatant fluid, the cells were plated in serum-free RPMI-1640 at a density of $2 \times 10^6$/35-mm dish and handled in a similar fashion.

Fibronectin was identified and measured in the macrophage supernatants by using an enzyme-linked immunoassay (ELISA) for human fibronectin (18).

Fibronectin production by alveolar macrophages was confirmed by labeling purified cells for 24 hr in culture with [$^{14}$C]proline (5 μCi/ml; 1 Ci = $3.7 \times 10^{10}$ becquerels; Amersham). Supernatants were harvested and the fibronectin was purified either by immunoprecipitation with antifibronectin antibody (19) or by batchwise adsorption to gelatin-Sepharose beads (8) at 37°C for 1 hr. Gel buffer containing 3% 2-mercaptoethanol was added directly to the immunoprecipitates and the pelleted beads to solubilize the bound fibronectin, and sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed (20). After electrophoresis, the gels were dried and autoradiograms were made.

To confirm the *de novo* synthesis of fibronectin by alveolar macrophages, a purified population of alveolar macrophages was obtained as described above and cultured for up to 48 hr in serum-free medium with or without cycloheximide (25 μg/ml, Calbiochem). The supernatant media from these cultures were then evaluated for fibronectin by ELISA.

Further confirmation of macrophage fibronectin production was obtained by evaluating alveolar macrophages by immunofluorescence with antifibronectin antiserum (21).

**Fibronectin Production by Macrophages from Patients with Interstitial Lung Disease.** Fibronectin production by alveolar macrophages from patients with interstitial lung diseases was compared to production by macrophages from normal individuals by plating the cells obtained at bronchoalveolar lavage in microtiter wells as described above. After 24 hr at 37°C, the media were aspirated and fibronectin was measured as described above. All studies were done in quadruplicate.

**Fibronectin Production by Other Immune and Inflammatory Cells.** To determine whether lymphocytes, neutrophils, or eosinophils, variably present in the lavage cell population, could produce fibronectin under the study conditions, fibronectin production by each of these cell types was evaluated directly. Lymphocytes and neutrophils were obtained from the blood of normal volunteers as described (22) and were >95% pure. Eosinophils were obtained from guinea pigs by the methods of Gleich and Loegering (23) and Pincus (24); these cells were >95% pure. Each of these cell populations was cultured in microtiter wells in RPMI-1640 medium without serum under similar conditions to those used for alveolar macrophages. In addition, in parallel studies, the lymphocyte population was stimulated by allogeneic lymphocytes treated with mitomycin C (40 μg/ml for 30 min), and the neutrophils were activated with phorbol myristate acetate at 75 ng/ml. For the lymphocytes and neutrophils, the supernatants were evaluated by ELISA. For the eosinophil supernatants, ELISA also was used except that the antigen used as a standard was guinea pig plasma fibronectin and the antiserum was rabbit anti-guinea pig fibronectin.

**Chemoattractant Activity of Macrophage Fibronectin.** Supernatants from cultured alveolar macrophages were evaluated for chemotactic properties for diploid human lung fibroblasts (HFL-1, American Type Culture Collection CCL 153) by the assay described by Postlethwaite *et al.* (25). To verify that it was macrophage fibronectin that was chemotactic for fibroblasts, the macrophage supernatant was depleted of fibronectin by incubation with gelatin-coupled Sepharose beads followed by centrifugation at 10,000 × *g*. The supernatant fluid was carefully aspirated and saved for the chemotaxis assay. The beads were then washed twice with phosphate-buffered saline, and purified macrophage fibronectin was eluted with 1.0 ml of 6 M urea. The urea extract was dialyzed twice for 2 hr against 1000 vol of RPMI-1640 medium and then immediately assayed for chemotactic activity for fibroblasts. Checkerboard analysis was carried out to determine if the attractive properties of macrophage fibronectin were chemotactic, and not simply chemokinetic (9).

**Statistical Evaluations.** All comparisons between patient groups were made by using the Wilcoxon–Mann–Whitney rank order test (26).

## RESULTS

**Production of Fibronectin by Human Alveolar Macrophages.** With ELISA, fibronectin could easily be detected in the supernatants of cultured alveolar macrophages (Fig. 1). Plasma and fibroblast fibronectin were indistinguishable, in the ELISA, from macrophage-produced fibronectin—i.e., fibronectin produced by alveolar macrophages inhibited the binding of antifibronectin antibody in a manner identical to that of plasma and fibroblast fibronectin. Crude macrophage supernatant did not interfere with the antigen coating the ELISA plate—i.e., the crude supernatants did not degrade or disrupt the antigenic determinants bound to the plastic surface as determined by subsequent binding of antifibronectin antibody. Furthermore, the addition of protease inhibitors to macrophage supernatants did not change the values determined.

Fibronectin accumulation in the medium of the alveolar macrophages was linear for at least 24 hr (Fig. 1). *De novo* synthesis of fibronectin by alveolar macrophages was demonstrated by two methods. (*i*) Addition of [$^{14}$C]proline to the macrophage cultures resulted in the production of a radiolabeled molecule that had the characteristic monomer molecular weight of fibronectin and both precipitated with antifibronectin antibody and bound to gelatin (Fig. 1 *Inset*). (*ii*) Addition of cycloheximide

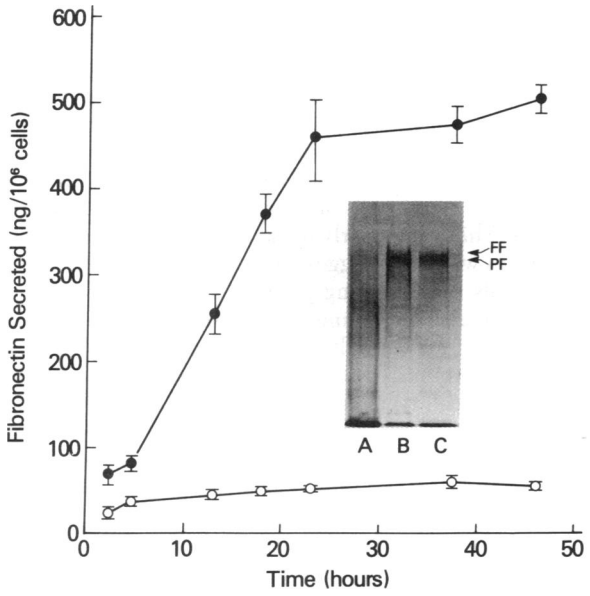


FIG. 1. Production of fibronectin by human alveolar macrophages. Macrophages isolated from lavage fluid of a patient with idiopathic pulmonary fibrosis were cultured in RPMI-1640 medium without serum; the supernatant fluid was collected and assayed for fibronectin. Amount of fibronectin secreted (ng/$10^6$ cell per hr) is plotted against time in culture. ●, Macrophages alone; ○, macrophages cultured in the presence of cycloheximide. (*Inset*) Fibronectin produced by macrophages labeled by incubation with [$^{14}$C]proline for 24 hr. Lanes: A, trichloroacetic acid precipitate of macrophage supernatant included for comparison; B, macrophage supernatant bound to gelatin-Sepharose; C, macrophage supernatant immunoprecipitated with antifibronectin antibodies. Standards: FF, fibroblast fibronectin; PF, plasma fibronectin.

to the culture medium abolished both the accumulation of fibronectin in the medium as measured by ELISA and the incorporation of [$^{14}$C]proline into fibronectin (not shown).

Several lines of evidence strongly suggested that the alveolar macrophage is the only inflammatory and immune effector cell found in the human lower respiratory tract which produces significant quantities of fibronectin.

(*i*) Human lymphocytes produced no detectable fibronectin (<0.3 ng/$10^6$ cells per hr) whether cultured directly or stimulated in mixed lymphocyte culture.

(*ii*) Human neutrophil supernatants appeared to produce small quantities of fibronectin (equivalent to 1.0 ng/$10^6$ cells per hr measured in the ELISA). However, in contrast to the alveolar macrophage fibronectin, the pattern of inhibition of this material in the ELISA was not identical in pattern to the inhibition produced by serum or fibroblast fibronectin, suggesting that, in the case of the neutrophil, the inhibition of the ELISA test was not detecting fibronectin antigenically similar to other fibronectins. Moreover, the inhibition produced in the ELISA by neutrophil supernatants could be blocked by the inclusion of protease inhibitors. Together, these observations suggest that the "apparent" fibronectin produced by neutrophils consisted mostly of proteases that degraded the fibronectin coating the walls of the ELISA plates—i.e., these proteases released fibronectin from the ELISA plate which subsequently prevented binding of the antifibronectin antibody to the surface of the plate. Thus, fibronectin production by these cells probably was much less than 1.0 ng/$10^6$ cells per hr.

(*iii*) Human eosinophils were not available for testing, but guinea pig eosinophils produced no fibronectin (data not shown).

(*iv*) Immunofluorescence studies with antifibronectin antibody of cells recovered by bronchoalveolar lavage showed that alveolar macrophages were the only cells demonstrating intracellular staining (not shown). No cells were stained with antiprocollagen type I antibodies, suggesting a lack of contaminating fibroblasts or epithelial cells (cells known to produce both collagen and fibronectin).

**Production of Fibronectin by Alveolar Macrophages in Patients with Interstitial Lung Disease.** Alveolar macrophages obtained from normal individuals produced fibronectin at a mean (±SEM) rate of 0.49 ± 0.23 ng/$10^6$ cells per hr over a 24-hr period. In contrast, macrophages of patients with idiopathic pulmonary fibrosis produced 10.5 ± 1.7 ng of fibronectin per $10^6$ cells per hr ($P < 0.01$ compared to normal) and those from sarcoidosis patients produced 5.4 ± 2.4 ng/$10^6$ cells per hr ($P < 0.02$ compared to normal). The average value for the fibronectin production by alveolar macrophages from 10 patients with various other types of interstitial lung disease was 3.5 ± 1.3 ng/$10^6$ cells per hr (Fig. 2). Although this did not reach statistical significance for the group as a whole ($P > 0.1$ compared to normal), 6 of 10 patients had macrophage fibronectin production rates that exceeded the highest normal value. The numbers of patients in each diagnostic category, however, were too small to permit further subdivision and separate statistical analysis.

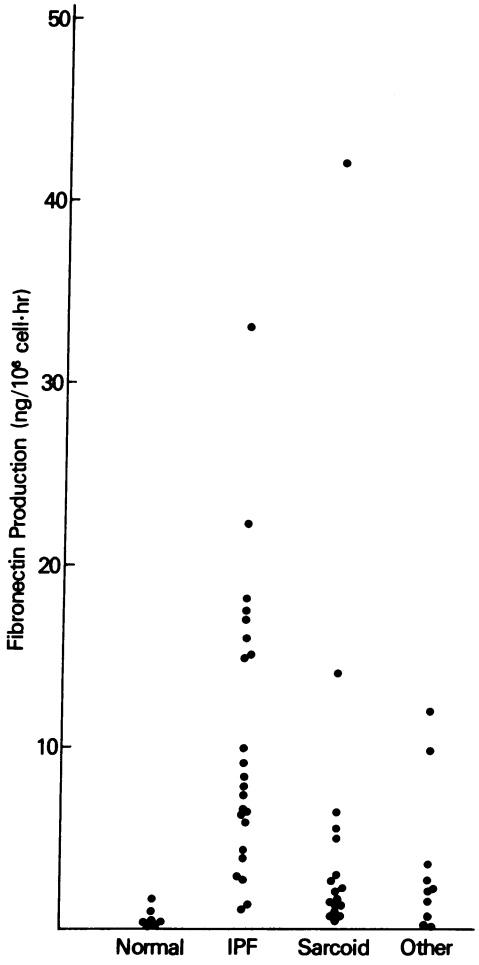


FIG. 2. Production of fibronectin by alveolar cells of normals and patients with interstitial lung disease. Cells were cultured for 24 hr in RPMI-1640 medium without serum at a density of $10^6$ cells per ml. Four groups were evaluated: normals, patients with idiopathic pulmonary fibrosis (IPF), patients with pulmonary sarcoidosis (Sarcoid), and patients with other interstitial lung diseases (Other). All values represent the mean of four determinations.

All groups tested included smokers but smoking did not account for the increased fibronectin production observed in cultures of macrophages from patients with interstitial disease. The mean production rate for smokers with interstitial disease was 7.9 ± 2.3 ng/$10^6$ cells per hr compared to 6.5 ± 1.5 ng/$10^6$ cells per hr for nonsmokers with interstitial disease ($P > 0.2$). Similarly, no effect of medication was observed; patients taking steroids produced fibronectin at a rate of 8.2 ± 1.9 ng/$10^6$ cells per hr compared to 7.0 ± 1.1 ng/$10^6$ cells per hr for patients not taking steroids ($P > 0.2$).

**Capacity of Alveolar Macrophage Fibronectin to Attract Fibroblasts.** Supernatants from human alveolar macrophages were chemotactic for human lung fibroblasts (Fig. 3*A*). This activity was removed from the macrophage supernatants by gelatin affinity adsorption which depletes the supernatants of fibronectin. In addition, the fibronectin purified from the macrophage supernatants was chemotactic for fibroblasts (Fig. 3*B*). Moreover, the chemotactic activity of macrophage supernatants for fibroblasts was present in the void volume of a Sephadex G-25 column and was not dialyzable (data not shown). Thus, this chemotactic factor activity was distinct from the low molecular weight neutrophil chemotactic factor also produced by alveolar macrophages. In addition, checkerboard analysis of macrophage fibronectin revealed that the fibronectin stimulated directed migration of fibroblasts (data not shown). Thus, the macrophage fibronectin fulfilled the criteria for being a chemotactic factor.

## DISCUSSION

Human alveolar macrophages produce fibronectin that is similar in molecular weight and antigenicity to plasma and fibroblast fibronectins. In addition, macrophages from patients with interstitial lung disease produce fibronectin at an increased rate, and this macrophage-produced fibronectin is chemotactic for human lung fibroblasts. These observations suggest that alveolar macrophages may play a role in the reorganization of the alveolar structures in the interstitial lung diseases through the secretion of fibronectin with subsequent recruitment of fibroblasts to areas of local inflammation.

**Alveolar Macrophage Production of Fibronectin.** Several lines of evidence demonstrate that alveolar macrophages are capable of *de novo* synthesis of fibronectin and are not merely releasing fibronectin that originated at a different site. First, when measured by ELISA, macrophages do not produce fibronectin in the presence of cycloheximide, an inhibitor of protein synthesis. Second, macrophages incubated in the presence of [$^{14}$C]proline secrete a labeled macromolecule that has the same molecular weight as fibronectin monomer (220,000) and is antigenically and functionally similar to plasma and fibroblast fibronectins. Third, the nearly linear secretion of fibronectin by macrophages with time suggests that production of fibronectin by these cells is an ongoing process. Furthermore, although other inflammatory and immune effector cells were present in some of the macrophage cultures (lymphocytes in normals; lymphocytes, neutrophils, or eosinophils in patients with interstitial disease), these cell types contribute little, if any, to fibronectin production. In this context, direct evaluation of purified lymphocytes and eosinophils demonstrates that they do not produce fibronectin under the same culture conditions. Neutrophils have been reported to produce fibronectin (27) but, based on their maximal production rate and the maximal proportions of neutrophils present in the cultures (i.e., for patients with idiopathic pulmonary fibrosis the maximum was 25% with a mean of 8.9 ± 1.7%), neutrophils could not account for more than 1% of the fibronectin found in the culture supernatants of cells recovered by lavage.

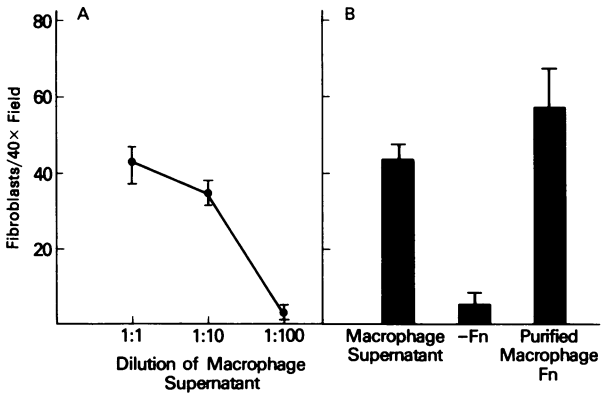


FIG. 3. Chemotactic function of human alveolar macrophage fibronectin for lung fibroblasts. (*A*) Alveolar macrophages were cultured at $10^6$ cells per ml in RPMI-1640 medium without serum and the supernatant fluid was tested for chemotactic activity for fibroblasts. Ordinate shows number of fibroblasts migrating across the chemotaxis membrane in response to macrophage supernatant. (*B*) Fibroblast chemotaxis caused by whole macrophage supernatant, supernatant depleted of fibronectin by affinity adsorption (−Fn), and purified fibronectin from macrophage supernatant that had bound to gelatin-Sepharose.

Thus, alveolar macrophages not only produce fibronectin but also are the major inflammatory and immune effector cell of the lower respiratory tract that produces this macromolecule. In support of this, Villiger *et al.* (28) also demonstrated that normal alveolar macrophages are capable of fibronectin production. That alveolar macrophages should be capable of producing fibronectin is not unexpected because human peripheral blood monocytes (10) and murine peritoneal macrophages (11), also cells of the mononuclear phagocyte series, produce this macromolecule.

Although the macrophage form of fibronectin is antigenically similar to plasma and fibroblast fibronectins and is functionally similar in its ability to bind denatured collagen (i.e., gelatin) it is possible that it will have minor differences compared to these other fibronectins. In this context, it is known that the fibroblast and plasma fibronectins differ in that the fibroblast form has a greater degree of glycosylation than the plasma form (1–4). In addition, although equally active in attaching cells to collagen, the fibroblast fibronectin is more active as a hemagglutinin and in reversing the abnormal morphologic features of transformed cells in culture (29).

**Production of Fibronectin by Alveolar Macrophages in the Interstitial Lung Diseases.** Macrophages from a large proportion of patients with various interstitial lung diseases secrete fibronectin at an increased rate. Furthermore, although in some cases the macrophages conceivably could be releasing fibronectin produced elsewhere, the fibronectin produced by alveolar macrophages most likely represents *de novo* synthesis because cycloheximide inhibited it in all cases studied. The higher levels of macrophage fibronectin production are comparable to about 10% of the production rate by fibroblasts (500–2000 ng/$10^6$ cells per hr) (18, 30). This increased production rate is found for macrophages from patients with idiopathic pulmonary fibrosis, pulmonary sarcoidosis, and, although the numbers of patients available was too small for further subdivision, in 6 of 10 patients with other interstitial lung diseases. Thus, the increased production of fibronectin by the alveolar macrophage does not appear to be specific for one disease process but rather may represent a common property of the alveolar macrophage in the interstitial lung diseases.

The mechanisms that stimulate the alveolar macrophage to produce fibronectin at an increased rate in the interstitial lung disorders are not clear. However, it is known that in idiopathic pulmonary fibrosis the macrophages are activated by locally produced immune complexes (31). In sarcoidosis, the alveolar macrophages are also activated (e.g., they produce lymphocyte activating factor), but the stimulus for this activation is unknown (32). The production of fibronectin by the alveolar macrophage at an increased rate in both disorders implies that increased production of this macromolecule may be a general response of the macrophage to various conditions. Preliminary results show that the production of fibronectin by alveolar macrophages in these disorders, however, may require a specific stimulus because usual immunologic and phagocytic stimuli have failed to increase fibronectin production by alveolar macrophages under our assay conditions.

**Functional Role for Alveolar Macrophage Fibronectin.** The fibronectin produced by the human alveolar macrophage can act as a chemoattractant for lung fibroblasts and thus contribute to the development of fibrosis in the interstitial lung disorders. Because fibroblasts produce collagen, the recruitment of fibroblasts by activated alveolar macrophages could lead to a local accumulation of collagen and hence the development of local fibrosis. In addition, because activated human alveolar macrophages also produce a growth factor that stimulates lung fibroblasts to replicate (33), the consequences of macrophage activation in the interstitial lung diseases would include the recruitment of fibroblasts to the local area, together with a local expansion of their numbers. Furthermore, because fibronectin mediates the adhesion of fibroblasts to the extracellular matrix (1–3), the increased production of this macromolecule in the fibrotic disorders of the lung may play an important role in modulating the topography of the alveolar fibroblasts in these conditions.

Fibronectin has also been shown to be an opsonin (4, 34) and is thought to mediate the phagocytosis and clearance of collagenous connective tissue debris. This is of particular relevance to the interstitial lung disorders in which connective tissue turnover is likely increased (35). It is known, for example, that patients with idiopathic pulmonary fibrosis have active collagenase present in their lower respiratory tract (36). In this setting, the fibronectin produced by the alveolar macrophage may participate in clearing partially degraded connective tissue components within the alveolar structures. Thus, by virtue of its ability to recruit and orient fibroblasts and its role as an opsonin, fibronectin could affect the metabolism of both the cellular and extracellular matrix components of connective tissue. In this context, the increased production of fibronectin in the interstitial lung disorders may be important in the development of the fibrosis found in these diseases.

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