Association of Activated Cytolytic Lung Lymphocytes with Response to Prednisone Therapy in Patients with Idiopathic Pulmonary Fibrosis*

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Previous studies have suggested that immunologic mechanisms may contribute to pathogeneic reactions in certain interstitial lung diseases. Cytolytic lymphocytes are major effector cells of the immune response that have not been extensively studied in these disorders. To investigate the role of activated cytolytic lymphocytes in IPF, we studied B-cell and monocyte/macrophage-depleted lymphocyte preparations isolated from BAL fluid and peripheral blood of patients with this disease (n = 10) and used a lectin-dependent cytotoxicity assay to detect activated cytolytic lymphocytes. In longitudinal studies, those patients who had cytolytic activity of BAL fluid lymphocytes (range, 8 to 35 percent, n = 4) showed significant improvement in pulmonary function (mean increase in diffusing capacity, 30 ± 2 percent) in association with decreased BAL fluid cytolytic lymphocyte activity after prednisone treatment. In contrast, patients who initially lacked cytolytic activity in BAL fluid (n = 6) did not improve with prednisone. Activated cytolytic lymphocytes were not observed in the BAL fluid of healthy subjects. These investigations suggest a causal relationship between activated cytolytic lymphocytes in the lung and disease activity in IPF and that assays of activated cytolytic lymphocytes are helpful in identifying patients who will improve with immunosuppressive therapy.

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IPF = idiopathic pulmonary fibrosis; BAL = bronchoalveolar lavage; NK = natural killer cells; CDCMC = concanavalin A-dependent cell-mediated cytotoxicity; UIP = usual interstitial pneumonia; DIF = desquamative interstitial pneumonia; IAK = interleukin-2-activated killer cells; IL-2 = interleukin-2

Prevalent studies have suggested that immune mechanisms may participate in the pathogenesis of certain interstitial lung diseases (eg, IPF, sarcoidosis, and hypersensitivity pneumonitis). While these diseases may have different initiating events and intermediate phases, they share a common final pathway leading to fibrosis and pulmonary insufficiency. IPF is characterized by accumulation of inflammatory cells in the lung, destruction of normal alveoli, and fibrosis. In contrast to sarcoidosis and hypersensitivity pneumonitis, IPF has not been thought to involve a local cell-mediated response. For example, sarcoidosis and hypersensitivity pneumonitis patients are known to have T-cell alveolitis with an altered distribution of lymphocyte subsets but in IPF, lymphocyte subset ratios have been reported to be normal by Hunninghake and Crystal and to be decreased by Izumi et al.

Caution must be exercised in interpreting lymphocyte phenotyping data to characterize the functional properties of local immunocompetent cells, since both CD4+ and CD8+ T-lymphocytes can have diverse activities. An alternative approach to studying the immunologic components of these interstitial lung diseases therefore has been to examine the functional activity of lymphocytes in BAL fluid. Increased levels of proliferative activity, lymphokine secretion, and NK activity have been observed from BAL fluid lymphocytes of patients with sarcoidosis. In contrast, activated lymphocytes have not been detected in the BAL fluid of IPF patients.

In this study, we investigated the cytolytic activity of lymphocytes from BAL fluid and from peripheral blood of patients with IPF. To measure the level of cytolytic lymphocyte activity, we used a standard lectin-dependent cell-mediated cytotoxicity assay. This assay permits the detection of specific cytolytic cells and interleukin-2 (IL-2)-activated killer cells (IAK), but it does not detect NK cells. The lectin-dependent assay was employed because it is unknown which antigen(s) may be involved in the pathogenesis of IPF. The data obtained in this report demonstrated a subgroup of IPF patients with elevated lectin-dependent killing in BAL fluid. Longitudinal studies of these patients demonstrated an association between improved pulmonary function and decreased lectin-dependent cytolytic activity following prednisone therapy.

Materials and Methods

Patients

Ten consecutive patients with a diagnosis of IPF were recruited...
from Montefiore-North Central Bronx Hospital Center on initial presentation to enter this study. All subjects gave informed consent for the procedures, which were approved by the Institutional Review Board. The diagnosis of IPF was established by a combination of medical history, physical examination, laboratory tests, chest roentgenograms, pulmonary function tests (PFTs), arterial blood gas analyses, and the results of lung biopsies according to previously described criteria.\textsuperscript{14,15} Histologic confirmation of the diagnosis was obtained by transbronchial lung biopsy in four patients and by open lung biopsy in six patients. All patients underwent BAL as described below at the time of bronchoscopy. None of the patients were current smokers at the time of presentation, and seven of ten were lifelong nonsmokers. After diagnosis, all patients were treated with prednisone, 1 mg/kg/day, until their pulmonary function stabilized for one month (average treatment time 2±0.5 months). At this time patients who agreed (eight of ten) underwent a second BAL procedure. At each bronchoscopy 30 ml of heparinized peripheral blood was obtained to determine peripheral blood lymphocyte activity for comparison with BAL lymphocytes.

Eight normal subjects underwent bronchoscopy with BAL and donated 30 ml of peripheral blood for comparative purposes with the study subjects. These normal subjects gave informed consent, and were all nonsmokers who had normal results on PFTs, chest roentgenograms, and were PPD-negative.

**Clinical Laboratory Tests**

**Pulmonary Function Tests:** Full lung volumes, single-breath diffusing capacity for carbon monoxide, and spirometry before and after bronchodilator treatment were performed (Collins DS Plus Pulmonary Testing System). All patients underwent the above studies prior to the initial bronchoscopy. Serial PFT studies were performed two weeks after beginning therapy and monthly thereafter. \(^{22}\) Gallium scintiscans were also obtained on all ten IPF patients.

**Bronchoscopy and Lymphocyte Isolation:** Following topical lidocaine anesthesia, an Olympus BF-10 bronchoscope was passed into the airways transanally and, after inspection of the airways, the bronchoscope was wedged into a segmental orifice of an affected lobe (as assessed by chest roentgenogram). BAL was performed by infusing approximately 150 ml of sterile 0.9 percent NaCl solution in 20- to 30-ml aliquots, with gentle aspiration until a 100-ml fluid return was achieved. At initial bronchoscopy, transbronchial biopsies were obtained from segments different from those used for BAL using standard techniques.

The BAL fluid was centrifuged at 275×g for 10 min and the cell pellet was washed three times with Hank's balanced salt solution without CA +2 and Mg +2 (HBSS, Gibco) and resuspended in 2 to 5 ml of RPMI-1640 medium supplemented with 15 percent fetal cell serum, 5×10\(^{-5}\) M 2-mercaptoethanol, 100 IU penicillin, and 100 \(\mu\)g/ml streptomycin (Gibco). Total and differential cell counts were determined by using a hemocytometer after addition of 40 \(\mu\)l of 0.1 percent crystal violet (in 0.1 M citric acid) to 40 \(\mu\)l of the cell suspension.\textsuperscript{10-12} Viable cell counts were determined by eosin Y dye exclusion. Isolation of blood mononuclear leukocytes on Ficoll-Hypaque cushions were performed.\textsuperscript{11-13} Further purification of BAL and peripheral blood lymphocytes was performed by using nylon fiber columns by the method of Greaves and Brown\textsuperscript{14} to yield a monocyte/macrophage- and B-cell-depleted, nonadherent lymphocyte preparation.\textsuperscript{11-13} Briefly, using aseptic technique, prewashed and presoaked, scrubbed nylon fiber, 3-denier, type 200 (Fenwal Laboratories), was loaded into disposable 10- or 20-ml syringes under pyrogen-free water. The nylon column was then washed with 4 vol of Hank's balanced salt solution, followed by 2 vol of RPMI-complete. BAL or peripheral blood mononuclear cells were placed in the column at concentrations of 1×10\(^6\) cells/ml and then incubated at 37°C in 5 percent CO\(_2\) and 95 percent air. The cells were collected by washing the column with 40 to 50 ml of prewarmed RPMI-complete. These nylon column nonadherent cells were concentrated into a volume of 2 to 5 ml, and total and differential cell counts were determined as described above. Lymphocyte recovery from the column is usually greater than 85 percent. These nonadherent cells contained <1 percent monocytes/macrophages.

**Concanavalin A-Dependent Cell-mediated Cytotoxicity Assay:** The CDCMC assay was used to measure activated lymphocyte killing, as published elsewhere.\textsuperscript{16-18} In brief, the murine mastocytoma cell line P815 (1×10\(^6\) cells/ml) was suspended in 0.3 ml of medium; 0.3 ml of Tris phosphate buffer, pH 7.4; and 100 to 200 \(\mu\)Ci of sodium \(^{51}\)Cr chromate (Amersham Corp). The P815 target cells were then incubated for 30 min at 37°C, washed three times and resuspended in medium at 1.25×10\(^5\) viable cells/ml. Con A, 80 \(\mu\)g/ml, was added to the target cells to give a 2× concentration relative to the final concentration in the assay.\textsuperscript{16-18} One hundred microliters of \(^{51}\)Cr-labeled P815 cells was then dispensed into triplicate wells of a 96 well V-bottom microtiter plate (Nunc). One hundred microliters of viable lymphocytes was added to each well of P815 cells and effector-to-target cells ratio were varied by twofold dilutions from 100:1 to 10:1, with the number of target cells kept constant. The effector cell concentration was 1.25×10\(^5\) cells/ml. After 3 h at 37°C, 100 \(\mu\)l of supernatant from triplicate samples was harvested and the \(^{51}\)Cr radioactivity determined in a gamma spectrophotometer (model 1185, Searle Analytic, Inc). The percent cytotoxicity was calculated by the formula: \([\text{cpm experimental} - \text{cpm spontaneous}] / [\text{cpm maximum} - \text{cpm spontaneous}]\)×100. Spontaneous release was 10 percent of the maximum release. The cytotoxicity data obtained at the above-
indicated effector-to-target cell ratios was a linear ratio, and therefore, we report data only at 100:1. The SD of the mean cpm of experimental maximum and spontaneous groups was usually 10 percent and always <15 percent. The lowest level of activity considered to be detectable is 4 percent.

RESULTS

Clinical Studies

Ten patients with IPF were studied; nine had a pathologic diagnosis of usual interstitial pneumonia (UIP) and one had a diagnosis of desquamative interstitial pneumonia (DIP). Chest roentgenograms showed bilateral diffuse interstitial infiltrates for all patients except the patient with DIP (who had a normal roentgenogram). Gallium scans were variably positive for UIP patients, but negative in the patient with DIP. Pulmonary function tests were consistent with moderate to severe restrictive lung disease (Fig 1, pretreatment pulmonary functions represented as closed symbols).

Initial Cell Counts

The median total and median differential initial cell counts of IPF patients and of normal subjects are shown in Table 1. These data demonstrate increased total cell numbers and percentages of polymorphonuclear leukocytes in BAL fluid of patients with IPF. Some patients with IPF also had increased percentages of lymphocytes. The above findings are consistent with previous reports of IPF alveolitis.17-30

Initial Immunologic Studies

The results of initial CDCMC assays on lymphocytes obtained from BAL fluid of IPF patients are shown in Figure 2 (closed symbols, panel A). CDCMC was detected in the BAL fluid of four of ten patients with IPF. In contrast, cytolytic activity was not detected in BAL fluid of normal subjects (n = 8; CDCMC of BAL fluid = 0). Lectin-dependent cytotoxicity was observed in the peripheral blood of four patients with IPF (Fig 2, panel B, closed symbols); only two of whom had cytolytic activity in BAL fluid (Fig 2). Normal subjects had no detectable lectin-dependent cytolytic activity in peripheral blood (n = 8; CDCMC activity of peripheral blood = 0).

Longitudinal Studies of Pulmonary Function and Cytolytic Lymphocyte Activity

Serial PFTs were performed on all ten IPF patients. Diffusing capacity for carbon monoxide (Dsb) and VC were measured before and during prednisone treatment (Fig 1). Four of the ten IPF patients had improved pulmonary function following therapy (Fig 1). Of importance, these latter patients were the subjects who had initial CDCMC activity in BAL fluid (Fig 2). The mean ± SEM increase in diffusing capacity was 30 ± 2 percent in these latter patients. The improvement in PFTs was associated with improved dyspnea and exercise tolerance (ascertained by clinical history). In contrast, patients without initial CDCMC in BAL fluid (n = 6) did not have a significant increase in diffusing capacity (Fig 1 and 2). The patients with increased pulmonary function also had decreased or absent CDCMC in subsequent analysis of BAL fluid following treatment (Fig 2, open symbols). Patients without CDCMC in initial studies of BAL fluid continued to lack cytolytic activity after prednisone treatment (Fig 2, open symbols). Patients with activity in

Table 1 — Total and Differential Cell Counts in BAL Fluid*  

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Total Cells/ml BAL x 10&lt;sup&gt;-5&lt;/sup&gt;</th>
<th>Differential Cell Counts in BAL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>M</td>
</tr>
<tr>
<td>Idiopathic</td>
<td>10</td>
<td>1.1</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>(0.2-9.0)</td>
<td>(14-98)</td>
</tr>
<tr>
<td>Normal</td>
<td>8</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>(0.4-0.8)</td>
<td>(78-82)</td>
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</tbody>
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*Cells were harvested from BAL fluid and processed as described. M = macrophages, L = lymphocytes, P = polymorphonuclear leukocytes. The median (and range) are reported.

![Figure 2](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21602/ on 06/25/2017)
peripheral blood only did not exhibit improvement in pulmonary function with therapy (Fig 2 and 3). Total and differential cell count data from serial BAL fluid studies are shown in Figure 3. None of these measurements was consistently associated with improved pulmonary function or cytolytic lymphocyte activity.

**DISCUSSION**

Numerous studies of cell-mediated immune phenomena in interstitial lung diseases have been reported. The role of activated cytolytic lymphocytes has not been previously investigated, despite the importance of these as major effector components of the immune response. Ours is the first report, to our knowledge, demonstrating the presence of activated cytolytic cells in the BAL fluid of some patients with IPF. Longitudinal studies indicated that this subgroup of IPF patients responded to prednisone therapy with improved pulmonary function, and, significantly, clinical improvement was associated with decreased lectin-dependent cytolytic lymphocyte activity in BAL fluid. Patients with cytolytic activity in peripheral blood but not in BAL fluid and patients without cytolytic activity from either source did not respond to prednisone therapy. Patients with IPF who had lectin-dependent activity in BAL fluid may constitute a subgroup who have more active inflammation or are in an earlier stage of disease than IPF patients without initial cytolytic activity.

We were able to study only a small number of IPF patients and therefore cannot make a reliable estimate of the prevalence of activated cytolytic lymphocytes in IPF patients. However, the striking and consistent correlation of positive CDCMC activity with steroid responsiveness, even in this small series, strongly suggests that the assay will be useful in assessing prognosis.

Clinical parameters including BAL fluid cell contents and Ga scans were not consistently associated with improvement in pulmonary function in our group of IPF patients. Other investigators have reported that increased percentages of lymphocytes in BAL fluid correlated with subsequent clinical improvement. However, data from longitudinal studies of BAL cell content have not shown consistent correlations between these findings and the patients' clinical course. In our study the initial percentage of lymphocytes did not consistently predict corticosteroid responsiveness. Two patients with increased percentages of lymphocytes had no response to treatment while the patient with the lowest percentage of lymphocytes had the most dramatic response to treatment.

Keough et al also reported that the percentage of neutrophils in BAL fluid decreased when IPF patients responded to corticosteroids. Neither Turner-Warwick and Haslam nor our study confirmed this finding.

The lectin-dependent cytotoxicity assay detects specifically sensitized T-lymphocytes and IL-2 activated killer cell activity (IAK), but not NK cell activity. The finding of activated cytolytic lymphocytes in the lungs of some patients with IPF suggests that a cell-mediated immune response may occur in situ in this disorder. It is possible that cytolytic lymphocytes might be present in the lung of most patients with disease early in the course of the disorder. However, as patients usually present with advanced disease and pulmonary dysfunction, it is currently impossible to test this hypothesis. The data in this report does not permit assignment of the cytolytic activity to either specifically sensitized cytolytic T-cells or IAK cells. Though it is not possible to perform antigen-specific assays on these patients, future studies utilizing IAK-specific target cells will
be useful in further defining the role of functional subsets of cytolytic lymphocytes in this disorder.

This report is the first, to our knowledge, to present data demonstrating a cell-mediated immune response in the lungs of patients with IPF. Significant improvement in pulmonary function was observed in patients with lectin-dependent cytotoxicity in BAL fluid. Assays of activated cytolytic lymphocytes may, therefore, be useful in identifying IPF patients who have improved prognosis on immunosuppressive therapy.

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