Gamma-Delta T Cells in BAL Fluid of Chronic Lower Respiratory Tract Infection*

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Gamma-delta (γ/δ) T cells are thought to represent the first line of defense against various pathogenic microorganisms. The aim of the present study was to investigate whether γ/δ T cells were increased in BAL fluid (BALF) of patients with diffuse panbronchiolitis (DPB), a model of chronic lower respiratory tract infection. The study population consisted of four groups, including patients with DPB, sarcoidosis, idiopathic pulmonary fibrosis, and normal subjects. Two-color direct immunofluorescence and flow cytometry were used for analysis of peripheral blood or BALF from these patients. The percentage of peripheral blood or BALF γ/δ T cells relative to the total number of lymphocytes was similar in the four groups. Although the absolute number of γ/δ T cells in BALF was significantly higher in DPB patients compared with the other three groups, the total lymphocyte number in BALF in DPB patients was increased and the number of BALF γ/δ T cells correlated with the total lymphocyte number in BALF. Furthermore, the percentage and number of BALF γ/δ T cells were not related to a certain group of pathogenic organisms or the number of colony-forming units. Our results suggest that γ/δ T cells are unlikely to play a part in chronic lower respiratory tract infection. (CHEST 1997; 111:1697-1701)

Key words: bacterial infection; BALF; diffuse panbronchiolitis; γ/δ T cells

Abbreviations: γ/δ = gamma-delta; BALF = BAL fluid; DPB = diffuse panbronchiolitis; FITC = fluorescein isothiocyanate; IPF = idiopathic pulmonary fibrosis; PBS = phosphate-buffered saline solution; PE = phycoerythin; TCR = T-cell receptor

Most T cells recognize an antigen through the T-cell antigen receptor α/β complex on the T-cell surface. A small group of T cells, bearing an antigen receptor composed of γ and δ subunits, have also been identified. The γ/δ T cells are thought to represent the first line of defense against intracellular microorganisms, including Mycobacterium tuberculosis, M leprae, and Listeria monocytogenes. In addition, a possible role for γ/δ T cells in host defense against other nonintracellular pathogens or viruses has also been reported. Although changes in γ/δ T cell expression have been described in several infectious diseases, the role of these cells in chronic infection of the human respiratory tract has not yet been reported (to our knowledge).

Diffuse panbronchiolitis (DPB) is a chronic infection of the lower respiratory tract common among the Japanese people, with persistent bacterial infection associated with neutrophil retention in the airway. Haemophilus influenzae and Streptococcus pneumoniae are usually isolated from the sputum during the early stages of the disease, but this may change to Pseudomonas aeruginosa as the disease progresses. Histopathologically, the disease is characterized by thickening of the walls of the respiratory bronchioles and infiltration of lymphocytes, plasma cells, and histiocytes. In addition, bronchus-associated lymphoid tissue hyperplasia is observed more often in DPB than in other respiratory diseases. These findings suggest that lymphocytes form an important cellular component of bronchial inflammation in DPB. Therefore, we considered DPB as a model for investigating the significance of γ/δ T cells in chronic infections affecting the respiratory tract.

The present study was undertaken to determine the distribution of γ/δ T cells in peripheral blood and BALF fluid (BALF) of a group of patients with DPB together with normal healthy subjects as a control group, applying the method of immunophenotyping by flow cytometry. To compare the findings in DPB...
with other chronic lung diseases, we also selected patients with sarcoidosis and idiopathic pulmonary fibrosis (IPF).

**Materials and Methods**

**Study Population**

The study population consisted of four groups, including 18 patients with DPB (eight women and 10 men; mean age, 45±16 years; two smokers), 32 patients with sarcoidosis (21 women and 11 men; mean age, 46±16 years; eight smokers and three ex-smokers), 20 patients with IPF (three women and 17 men; mean age, 61±12 years; 11 smokers and five ex-smokers), and 10 nonsmoking healthy volunteers (two women and nine men; mean age, 23±4 years). All patients were not treated with antibiotics, including macrolide antibiotics, or corticosteroids at the time of the investigation. BALF analysis was performed in all patients and healthy volunteers.

Sputum culture was performed in all DPB patients at least on three occasions close to the day of BAL examination, in order to detect, if any, the organism(s) causing airway infection. All DPB patients satisfied the diagnostic criteria for DPB published by the Japanese Ministry of Health and Welfare. The criteria included the following: (1) symptoms—chronic cough, sputum, and dyspnea on exertion; (2) physical signs—mainly coarse crackles, sometimes wheezes, rhonchi, or squawking; (3) chest radiograph/CT—diffusely disseminated fine nodular shadows; (4) respiratory function and blood gas—FEV1 <70%, PaO2 <80 mm Hg; (5) serology—cold hemagglutination >64×; and (6) complication or history—sinusitis. The diagnosis of sarcoidosis and IPF was based on clinical presentation and results of transbronchial lung biopsy or open lung biopsy. All patients with DPB had suffered from cough and sputum for >1 year. There was no history of occupational or domestic exposure to organic or inorganic dusts and no evidence of collagen vascular disease in all four groups.

**BAL and Cell Preparation**

After obtaining an informed consent, each subject was administered atropine (0.5 mg) IM, followed by local anesthesia of the airway with 2% lidocaine. Airway examination was performed using a flexible fiberoptic bronchoscope (BF-P20 type; Olympus Corp; Tokyo). The bronchoscope was wedged into one of the subsegmental bronchi of the right middle lobe and 150 mL of sterile 0.9% NaCl solution at 37°C was infused in three 50-mL aliquots, and gently aspirated immediately after each infusion.

The recovered BALF was pooled, passed through a double layer of gauze to remove gross mucus, then centrifuged. The aliquot was then diluted to a concentration of 1×10⁵ cells per milliliter, and 0.2 mL cell suspension was spun down onto a glass slide at 1,100 rpm for 2 min using a cytocentrifuge (Cytospin 2; Shandon Instruments; Sewickley, Pa). The slides were then dried and stained using the May-Giemsa method. More than 200 cells were identified using a photomicroscope. The remaining BALF cells were resuspended in RPMI-1640 (GIBCO/BRL; Life Technologies Inc; Gaithersburg, Md) with 10% heat-inactivated fetal bovine serum (GIBCO/BRL), and incubated in plastic dishes for 60 min at 37°C in 5% CO₂-humidified atmosphere to remove adherent macrophages that could interfere with accurate cell analysis. More than 90% of nonadherent cells collected for flow cytometric analysis were viable by the trypan blue exclusion test.

**Monoclonal Antibodies**

The following conjugated monoclonal antibodies were used to detect T cells and T-cell subpopulations: fluorescein isothiocyanate (FITC)-conjugated anti-γ/δ T-cell antigen receptor (TCR-γ/δ-1), CD8 (Leu-2a), phycoerythrin (PE)-conjugated anti-CD3 (Leu-4), CD4 (Leu-3a), and CD8 (Leu-2a) antibodies. (They were purchased from Becton Dickinson; Mountain View, Calif.) Mouse IgG1 and IgG2a conjugated with FITC or PE were purchased (from Coulter Immunology; Hialeah, Fla) and used to determine the borderline between stained and unstained cells in the flow cytometric analysis.

**Two-Color Direct Immunofluorescence Staining and Flow Cytometric Analysis**

A total of 100 μL whole blood collected by venipuncture and mixed with ethylenediamine tetraacetic acid was placed into a 12×15-mm polystyrene tube (Falcon Plastics; Oxnard, Calif), and 5 μL of each monoclonal antibody was added. The tubes were incubated for 15 min at room temperature in darkness, and 2 mL of 1X lysing solution (FACS; Becton Dickinson) was added. Cells were mixed vigorously and incubated for 10 min at room temperature, and then washed once in cold phosphate-buffered saline solution (PBS) containing 0.1% sodium azide. The cells were finally resuspended in cold PBS containing 0.5% paraformaldehyde.

BALF cells were adjusted to 1×10⁶ cells per milliliter. A total of 5 µL of each monoclonal antibody was placed into a polystyrene tube, and 100 µL of the cell suspension was added. In the next step, the cells were incubated for 30 min on ice in darkness, washed once in cold PBS containing 0.1% sodium azide, and then resuspended in cold PBS containing 0.5% paraformaldehyde. Fixed cells were kept in the dark at 4°C until analysis.

Stained cells were analyzed on a flow cytometer (FACScan; FACS Division; Becton Dickinson). A computer system (Consort 30; Becton Dickinson) was used for data acquisition and analysis. List mode data for 10,000 to 20,000 events were stored. A cell gate containing lymphocytes was established on the basis of forward and side light scatter. To determine the borderline between stained and unstained cells, cells were also stained with mouse IgG1-conjugated FITC or PE. Percentages were calculated on the basis of the number of lymphocytes found in each quadrant.

**Statistical Analysis**

Data were expressed as mean ± SD. Differences between groups were compared using the two-tailed unpaired Student's t test and Mann-Whitney U test. For multiple group comparisons, one-way analysis of variance and Fisher's PLSD test was employed. A probability <5% was considered statistically significant.

**Results**

**Characteristics of BALF Cells**

Table 1 shows the mean values of different BALF cells for different patient groups and normal subjects. The total number of cells and the percentage of neutrophils were significantly higher in DPB patients, while the proportion of alveolar macrophages was significantly lower in the same patients compared with the other three groups. The percentage
Thus, cells (Table 1), and control with patients was BALF per milliliter) though BALF 4.25±3.53%; IPF, 4.92±3.65%; 3.95±4.61%; IPF, 4.34±5.03%; sarcoidosis, 4.83±3.21%; IPF, 3.95±4.61%; control, 6.41±4.08%). However, the absolute number of γδ T cells in BALF of patients with DPB (6.99±6.62×10³ cells per milliliter) was significantly higher than in patients with sarcoidosis (3.04±3.55×10³ cells per milliliter), IPF (1.72±1.50×10³ cells per milliliter), and control subjects (0.88±0.71×10³ cells per milliliter), the total number of lymphocytes in BALF was much higher in DPB than in healthy subjects (Table 1), and the number of BALF γδ T cells in DPB patients correlated significantly with the total lymphocyte number in BALF (Fig 2). Thus, the increased number of BALF γδ T cells in DPB was not thought to be a selective expansion, but was rather dependent on the increased number of total BALF lymphocytes. Furthermore, as shown in Table 2, there was no correlation between the type and number of colonies of pathogenic microorganisms isolated on sputum culture and the number of BALF γδ T cells in DPB patients.

**Distribution of γδ T Cells in Peripheral Blood and BALF**

Two-color direct immunofluorescence was performed with anti-TCR-γδ-1 (which recognizes all γδ T lymphocytes) and anti-CD3 (pan T-cell) monoclonal antibodies. In peripheral blood, the proportion of CD3+ γδ TCR+ cells (γδ T cells) relative to the total number of peripheral blood lymphocytes was not different among all groups (DPB, 4.34±5.03%; sarcoidosis, 4.83±3.21%; IPF, 3.95±4.61%; control, 6.41±4.05%). In BALF, as shown in Figure 1, the percentage of γδ T cells was also similar in the four groups (DPB, 4.92±3.65%; sarcoidosis, 4.19±3.78%; IPF, 4.25±3.53%; control, 5.59±2.38%). Although the absolute number of γδ T cells in BALF of patients with DPB (6.99±6.62×10³ cells per milliliter) was significantly higher than in patients with sarcoidosis (3.04±3.55×10³ cells per milliliter), IPF (1.72±1.50×10³ cells per milliliter), and control subjects (0.88±0.71×10³ cells per milliliter), the total number of lymphocytes in BALF was much higher in DPB than in healthy subjects (Table 1), and the number of BALF γδ T cells in DPB patients correlated significantly with the total lymphocyte number in BALF (Fig 2).

**Table 1—Mean ± SD BAL Fluid Cellularity and T-Cell Subsets: Comparison Among Patients With DPB, Sarcoidosis, IPF, and Healthy Volunteers**

<table>
<thead>
<tr>
<th></th>
<th>DPB (n=18)</th>
<th>Sarcoidosis (n=32)</th>
<th>IPF (n=20)</th>
<th>Healthy Volunteers (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells, ×10³/mL</td>
<td>11.5±9.8*4</td>
<td>3.4±2.0</td>
<td>4.0±3.3</td>
<td>1.2±0.5</td>
</tr>
<tr>
<td>Macrophages, %</td>
<td>21.4±12.8*4</td>
<td>65.3±20.1</td>
<td>62.5±25.5</td>
<td>84.6±8.5</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>15.1±9.4*</td>
<td>32±18.7*</td>
<td>18.5±19.7</td>
<td>13.7±7.7</td>
</tr>
<tr>
<td>(No., ×10³/mL)</td>
<td>(1.26±0.77)*</td>
<td>(1.17±1.2)*</td>
<td>(0.61±0.77)</td>
<td>(0.18±0.14)</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>63.2±18.7*4</td>
<td>2.3±2.9</td>
<td>13.9±21.1</td>
<td>0.60±0.70</td>
</tr>
<tr>
<td>CD3, %</td>
<td>88.3±5.1*</td>
<td>87.0±9.6</td>
<td>85.2±9.5</td>
<td>81.2±6.8</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>0.58±0.73</td>
<td>3.26±3.58</td>
<td>0.95±0.83</td>
<td>1.03±0.60</td>
</tr>
</tbody>
</table>

*p<0.01 vs healthy volunteers.

^p<0.01 vs IPF.

^p<0.01 vs sarcoidosis.

^p<0.05 vs healthy volunteers.

**Discussion**

A few studies have investigated the role of γδ T cells against extracellular pathogens such as those usually isolated from the sputum of DPB patients. Abo et al reported that when human blood mononuclear cells were incubated with heat-killed bacteria, a significant proportion of γδ T cells was induced by *S pyogenes, Escherichia coli, Staphylo-
in the peritoneal cavity after intraperitoneal inoculation of *E coli* in mice, and suggested that γδ T cells may be stimulated by the self heat shock protein in the damaged tissues and may play a part in the resolution of the inflammatory process. We anticipate from these reports the possible proliferation of γδ T cells at the site of infection in DBP patients and that these cells play a defensive function against certain extracellular pathogens. The present study, however, demonstrated that the proportion of γδ T cells in BALF did not increase in DBP patients, a model for chronic lower respiratory tract infection, compared with patients with sarcoidosis, IPF, or control subjects. This result was contrary to our expectation, based on the above-mentioned previous studies showing a possible role for γδ T cells in host defense against nonintracellular pathogens. It is possible that our failure to detect an increase in peripheral blood and BALF γδ T cells of patients with DBP is dependent on timing. Earlier studies have shown that γδ T cells contribute to the host defense at the early stage of bacterial infection. All of our patients with DBP had been symptomatic for >1 year, and determination of the exact time of onset is virtually impossible in this chronic disease.

Recently, a number of experimental findings have suggested that γδ T cells appear to function as a first line of defense against intracellular pathogens. Several clinical studies have also examined the function of these cells. For example, Ito et al. have demonstrated increased numbers of γδ T cells in patients with pulmonary tuberculosis. However, Balbi et al. reported that only some patients with tuberculosis have increased proportion of peripheral blood γδ T cells, and their number was not increased in pleural effusion. A different finding was reported by Tazi et al. who demonstrated that neither the proportion and absolute number of circulating γδ T cells nor their numbers in granulomas were increased in patients with tuberculosis. This finding was also confirmed recently by Wilsher et al. who reported that γδ T cells were not increased in the peripheral blood of patients with cavitary pulmonary tuberculosis.

Considered together, it is clear from these results that the exact role of γδ T cells in infections with both extracellular or intracellular pathogens is not yet clear. It is possible that the discrepancy in the literature may be due to differences in certain immunologic factors, eg, differences in human and mouse immunologic function and antigen expression, or kinetic factors.

Our results demonstrated that circulatory and BALF γδ T cells were within the normal range in patients with sarcoidosis. These results are different from those reported by Tamura et al. and Raulf et
al. Based on their findings, these investigators hypothesized that γ/δ T cells respond to diseasespecific antigens and that they play a specific role in granuloma formation in sarcoidosis, analogous to that in other granulomatous diseases, including tuberculosis, leprosy, and leishmaniasis. However, in support of our findings, several recent studies have demonstrated that γ/δ T cells were not increased in peripheral blood, BALF, and granulomatous lesions of sarcoidosis patients. However, our results in IPF patients demonstrated that γ/δ T cells were not increased in peripheral blood and BALF, in agreement with earlier published reports. Considered together, our data suggest that γ/δ T cells may not play an important role in the chronic inflammatory lung diseases that are not caused by pathogenic agents such as sarcoidosis and IPF.

In conclusion, our data demonstrated that γ/δ T cells did not increase in peripheral blood and in BALF of patients with DPB regardless of continuous bacterial stimulation. These data suggest γ/δ T cells are unlikely to play a part in chronic lower respiratory tract infection. However, since we focused in this study only on DPB patients as a model of chronic respiratory infection, we believe it is premature to draw firm conclusions on the role of γ/δ T cells. The exact role of γ/δ T cells in other bacterial infections remains to be identified.

REFERENCES