Density of phenotypic markers on BAL T-lymphocytes in hypersensitivity pneumonitis, pulmonary sarcoidosis and bronchiolitis obliterans with organizing pneumonia


ABSTRACT: In vitro studies have shown that the density of surface antigens reflects the degree of activation of T-cells. We therefore studied the density of surface antigens on T-cells from bronchoalveolar lavage fluid (BALF) and blood in patients with sarcoidosis, hypersensitivity pneumonitis (HP) and bronchiolitis obliterans with organizing pneumonia (BOOP). BALF cells were stained with anti-CD3, anti-CD4, anti-CD8 and anti-human leucocyte antigen-DR (HLA-DR) monoclonal antibodies, and were analysed by cytofluorometry. The density was evaluated by measuring the Mean Channel fluorescent intensity of the stained cells.

The results demonstrated a significant increase in the CD3 density in patients with hypersensitivity pneumonitis (108±20.2 MC), compared with those with pulmonary sarcoidosis (51±12.6 MC), BOOP (74.5±9.3) and healthy controls (57±1.5 MC). Similar results were obtained for the CD4 and CD8 density in patients with HP. Although the number of HLA-DR positive cells was increased, the density was lower in patients with sarcoidosis (75±15.6) and hypersensitivity pneumonitis (74±14.8 MC) than in healthy controls (72±15 MC). Comparable changes were not observed in peripheral blood.

These results suggest that T-cell activation in hypersensitivity pneumonitis may be associated with an increase in the CD3, CD4, and CD8 density on BALF T-cells.

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A prominent accumulation and recruitment from the blood of activated T-cells has been found in the lung of patients with certain interstitial lung diseases, most notably hypersensitivity pneumonitis (HP) [1].

A profound proliferation of antigen specific T-cells, at least in part, causes this accumulation within the lungs [2]. According to the now recognized general mechanisms of the immunological processing of antigens, the suspected antigen, after being processed to peptides, is presented by monocytes (mo)/macrophages (MA), binds to the antigenic receptor on T-cells, and induces an interaction of the receptor with the CD3 antigen complex, leading to an effective signal transduction via the membrane into the cytoplasm [3].

By using an anti-CD3 monoclonal antibody (MoAb), which mimics the antigenic stimulus, CD3 expression on activated T-cells during the activation process in vitro, was investigated [4-6]. Results showed a down modulation of CD3 on activated T-cells in healthy individuals, due to a decrease in density. Therefore, not only different phenotypic markers on T-cells, but also the degree of activation of these cells should be evaluated, in order to elucidate pathogenetic processes in patients with interstitial lung diseases.

In some interstitial lung diseases, a large increase in CD3 positive T-cells in the lungs is a common finding. Furthermore, a differential increase in T-cell subsets has been found, with a profound increase in CD4+ T-cells in patients with sarcoidosis [7], and CD8+ T-cells in patients with HP [8, 9] and bronchiolitis obliterans with organizing pneumonia (BOOP) [10-12]. Not only different surface phenotypes of T-cells, but also the degree of activation of the cells should be evaluated, in order to elucidate pathogenetic processes. Therefore, as potential indicators of activation, we have examined the CD3, CD4, CD8 and human leucocyte antigen-DR (HLA-DR) density on T-cells, which were freshly prepared from bronchoalveolar lavage fluid (BALF) samples from patients with interstitial lung diseases and healthy individuals.

Patients and material

Study population

Twelve patients with HP (nine with summer-type HP, one with bird fanciers' HP, and two with HP due to
air-conditioning), 26 with pulmonary sarcoidosis, and 13 with BOOP were selected for our study. Diagnosis was made by using transbronchial lung biopsy in addition to clinical findings. BOOP cases were diagnosed by open lung biopsy and were not treated with corticosteroids. In all cases of summer-type HP, precipitating antibodies to *Trichosporon cutaneum* were detected in sera, a restrictive pulmonary function pattern was present, and ground glass opacity without signs of fibrosis was found on chest radiographs. In sarcoidosis, only active cases were included in the study. Active cases were defined as those who had abnormalities on chest radiographs persistent for more than 5 yrs, elevated levels of serum angiotensin converting enzyme (ACE), and increase in BALF lymphocytes percentages. Only four cases showed impaired lung function. No corticosteroids were administered. The investigated population is detailed in table 1.

The lavage study of human subjects received approval from the Ethics Committee of Chest Disease Research Institute, Kyoto University.

**Methods**

**Preparation of BALF and blood cells**

Lavage was performed by a routine method as described previously [13]. Briefly, 300 ml of warmed (37°C) physiological saline was instilled into the lungs via a bronchofiberscope (Olympus P20: 5 mm in diameter), which was wedged into the right middle lobe. The recovered lavage fluid was filtered through gauze, washed twice, and centrifuged for 10 min at 4°C. The total cell number was determined, and cells were resuspended in Eagle’s minimum essential medium (MEM) (Nissui, Tokyo).

Blood mononuclear cells were prepared from peripheral blood by using Ficoll Hypaque centrifugation.

**Cytoflowmetry**

BALF and blood mononuclear cell staining procedures (2x10^6 ml^-1) were carried out in Eagle’s MEM supplemented with 10% foetal calf serum (FCS), using saturating concentrations of antibodies. All incubation were performed on ice. Cells were incubated with tenfold diluted OKT3,4,8, and OKIa1 MoAbs (Ortho Diagnostic, Raritan, NJ, USA) for 30 min.

Cells were washed twice to remove excessive antibodies, and in the next step were stained with 80 times diluted fluorescein isothiocyanate (FITC) conjugated anti-mouse immunoglobulin G (IgG) (Tago, Burlingame, CA, USA) for 30 min at 4°C. After washing the cells, the percentage of positive cells compared with controls omitting the primary monoclonal antibody, and the intensity of fluorescence emission for the lymphocyte population (as an indicator of antigenic density) were measured by using an Orthospectrum type III cytoflowmeter. The intensity of fluorescence emission was expressed as the Mean Channel intensity of FITC (MC).

**Statistics**

All results were expressed as mean±sd. The significance of differences between the study groups was evaluated by using the one-way analysis of variance. To assess the difference between BALF and blood data within the same study group, the paired t-test was used, and a p value <0.05 was considered significant [8].

**Results**

**Basic BALF findings**

Basic BALF cell findings are summarized in table 2. Compared to healthy nonsmokers, the total cell recovery and the lymphocyte % were significantly increased in patients with sarcoidosis, HP, and BOOP.

**Table 1.** Population investigated in this study

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
<th>Significant differences between groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sarcoidosis</strong></td>
<td>26</td>
<td>12</td>
<td>13</td>
<td>40</td>
<td>AC (p&lt;0.05)</td>
</tr>
<tr>
<td><strong>HP</strong></td>
<td>37±11</td>
<td>45±12</td>
<td>58±9</td>
<td>42±11</td>
<td></td>
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<tr>
<td><strong>BOOP</strong></td>
<td>25/1</td>
<td>11/1</td>
<td>12/1</td>
<td>40/0</td>
<td></td>
</tr>
<tr>
<td><strong>Healthy</strong></td>
<td>31.6±9.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td><strong>Chest radiograph</strong></td>
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<td>14</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>II</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td></td>
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</tr>
</tbody>
</table>

Data for age and SACE are presented as mean±sd. HP: hypersensitivity pneumonitis; BOOP: bronchiolitis obliterans with organizing pneumonia; NS: nonsmokers; S: smokers; SACE: serum angiotensin converting enzyme; ND: not determined.
Table 2. — Basic BALF findings

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
<th>Significant differences between groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovered cells</td>
<td>1.50±0.90</td>
<td>10.92±17.80</td>
<td>3.22±3.65</td>
<td>0.68±0.36</td>
<td>BD, CD (p&lt;0.001)</td>
</tr>
<tr>
<td>x10⁶/ml¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AB (p&lt;0.05)</td>
</tr>
<tr>
<td>Macrophage %</td>
<td>61.7±20.5*</td>
<td>26.1±21.9</td>
<td>57.6±26.1</td>
<td>90.7±10.6</td>
<td>AD, BD, CD, AB (p&lt;0.001)</td>
</tr>
<tr>
<td>Lymphocyte %</td>
<td>37.8±20.5</td>
<td>65.8±19.4</td>
<td>35.0±23.3</td>
<td>8.6±9.8</td>
<td>BC (p&lt;0.01)</td>
</tr>
<tr>
<td>Neutrophil %</td>
<td>0.32±0.83</td>
<td>6.45±7.17</td>
<td>4.37±5.64</td>
<td>0.45±1.5</td>
<td>AD, BC, CD, AB (p&lt;0.001)</td>
</tr>
<tr>
<td>Eosinophil %</td>
<td>0.12±0.27</td>
<td>0.79±1.09</td>
<td>3.39±3.36</td>
<td>0.24±0.59</td>
<td>BC, AC, CD (p&lt;0.05)</td>
</tr>
</tbody>
</table>

*: data are given as the percentage of total cells. For abbreviations see legend to table 1.

Table 3. — Percentage of lymphocyte phenotypes in BALF and blood

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
<th>Significant differences between groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BALF cells</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3 %</td>
<td>87.5±5.9</td>
<td>88.7±11.0</td>
<td>80.4±14.7</td>
<td>70.9±15.9</td>
<td>AD (p&lt;0.01)</td>
</tr>
<tr>
<td>CD4 %</td>
<td>67.6±15.9</td>
<td>30.4±22.1</td>
<td>27.3±16.4</td>
<td>48.1±13.9</td>
<td>AD, AB, AC (p&lt;0.001)</td>
</tr>
<tr>
<td>CD8 %</td>
<td>16.2±11.3</td>
<td>55.7±17.0</td>
<td>46.4±23.5</td>
<td>22.1±6.6</td>
<td>AB, BD, CD (p&lt;0.001)</td>
</tr>
<tr>
<td>HLA-DR%</td>
<td>36.4±10.9</td>
<td>41.2±14.9</td>
<td>40.5±14.8</td>
<td>24.6±11.5</td>
<td>AD, BD, CD (p&lt;0.01)</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>6.87±5.10</td>
<td>0.59±0.51</td>
<td>1.30±1.90</td>
<td>2.73±1.59</td>
<td>AD, BD, CD, AB, AC (p&lt;0.001)</td>
</tr>
<tr>
<td><strong>Blood cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3 %</td>
<td>59.6±9.6</td>
<td>66.6±7.3</td>
<td>57.9±15.2</td>
<td>55.9±8.0</td>
<td>AD (p&lt;0.01)</td>
</tr>
<tr>
<td>CD4 %</td>
<td>35.1±6.9</td>
<td>43.1±5.7</td>
<td>40.2±14.5</td>
<td>40.8±8.5</td>
<td>AD, AB, AC (p&lt;0.001)</td>
</tr>
<tr>
<td>CD8 %</td>
<td>23.5±6.6</td>
<td>23.4±6.6</td>
<td>18.9±7.1</td>
<td>26.1±7.3</td>
<td>AB, BD, CD (p&lt;0.001)</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>24.5±6.6</td>
<td>19.6±4.2</td>
<td>18.2±6.2</td>
<td>19.7±5.3</td>
<td>AD, BD, CD, AB, AC (p&lt;0.001)</td>
</tr>
</tbody>
</table>

Data are given as the percentage of total lymphocytes. BALF: bronchoalveolar lavage fluid; HLA-DR: human leucocyte antigen-DR.

Percentage of lymphocyte phenotypes in BALF and blood

The percentages of lymphocytes expressing the studied phenotypic markers are shown in table 3. The percentages of CD3 positive T-cells were always higher in BALF than in blood.

A significant increase in BALF CD3+ T-cells was found in sarcoidosis and HP, compared with healthy nonsmokers (p<0.01). BALF CD4+ T-cells were increased in sarcoidosis, whereas BALF CD8+ T-cells were increased in HP and BOOP, leading to corresponding changes in the CD4/CD8 ratio. HLA-DR+ lymphocytes tended to be increased in BALF, when compared with blood lymphocytes. A significant increase in BALF HLA-DR+ lymphocytes was found in HP, compared with healthy controls (p<0.05). Since B-cells in BALF are usually below 5% of all lymphocytes, the measured proportion of HLA-DR+ lymphocytes usually reflects T-cells.

CD3 density on BALF and blood T-cells

The CD3 density on BALF and blood T-cells was examined by using a cytoflowmeter, and results are shown in figure 1. The CD3 density on blood T-cells was always higher than that on BALF T-cells, despite the fact...
that the percentages of T-cells expressing CD3 were always higher in BALF. When the CD3 density on BALF T-cells was compared between the study groups, HP showed the highest MC. An increase in MC was also found in patients with BOOP, when compared with healthy controls, but it was significantly less than in patients with HP. No increase was found in patients with sarcoidosis. In blood, an increase in MC was found in HP, when compared with sarcoidosis or BOOP.

**CD4 density on BALF and blood T-cells**

The CD4 density on BALF and blood T-cells is shown in figure 2. The CD4 density on BALF T-cells in patients with HP was significantly higher than that in healthy controls, sarcoidosis and BOOP. The density on BALF T-cells in patients with sarcoidosis was decreased, when compared with healthy controls. A difference in the density between BALF and blood T-cells was detected in patients with sarcoidosis, BOOP and healthy controls, but no difference was found in patients with HP.

**CD8 density on BALF and blood T-cells**

The CD8 density on BALF and blood T-cells showed a wider range of MC, when compared to the MC of the other antigens (fig. 3). The MC of CD8 on BALF T-cells in HP was significantly increased compared to healthy controls. In patients with HP, the MC was higher on BALF than on blood T-cells and showed an increase when compared to that in patients with sarcoidosis and BOOP.

**HLA-DR density on BALF and blood lymphocytes**

The MC of HLA-DR on BALF lymphocytes was lower than that on blood lymphocytes, as was the case with CD3 (fig. 4). A significant decrease in HLA-DR on BALF lymphocytes, when compared to healthy controls, was shown in patients with sarcoidosis and HP.
Discussion

It has been reported that a significant accumulation of T-cells in the lungs is a characteristic of patients with interstitial lung diseases, especially in HP and sarcoidosis [1, 7, 10]. In a previous study, we found an increase in the number of BALF lymphocytes in patients with BOOP [11, 12]. Furthermore, the subsets of BALF T-cells increased differently, in both quality and quantity, in all studied diseases. This suggests that different pathogenetic processes of T-cell activation and accumulation may occur in patients with interstitial lung diseases. In addition, a functional heterogeneity of T-cell subsets is considered to be present [9].

There are two possible mechanisms by which the number of lung T-cells increases in patients with interstitial lung diseases. Firstly, various chemotactic factors, which attract lymphocytes produced in the lungs, play a role in the preferential recruitment of T-cells from blood to the lungs [2, 14]. Secondly, a proliferation of T-cells, induced by an interaction with accessory cells, including lung macrophages, may occur in the lungs.

In this study, we obtained fresh cell populations from the lung by lavage, and measured the CD3 density both on lavage and blood T-cells by cytofluorometry. A significant increase in the CD3 density was found on BALF T-cells in patients with HP, compared to that in patients with sarcoidosis and BOOP. There was no difference between sarcoidosis and healthy non-current smokers. Blood T-cells showed less marked differences in the CD3 density between the study groups.

These results suggest that the micromilieu in the lungs may determine the CD3 density on T-cells in patients with HP. The CD3 density of recruited T-cells may be influenced by various locally-produced mediators, which either down-regulate or enhance the CD3 density. It is not yet known, however, which cytokine or biologically active material critically determines the CD3 density on T-cells.

Another possibility is that a preferential recruitment of CD3+ T-cells with increased CD3 density may occur in the lung of patients with HP. No reports have so far evaluated whether such a selection of T-cell subsets, which show an increased CD3 density, occurs in vivo. There is no knowledge available on possible chemoattractants produced in the lungs, which may recruit special T-cell subsets with increased CD3 density into the lung of patients with interstitial lung diseases.

In vitro studies, on the other hand, have revealed that a decrease in the CD3 density (down-modulation) occurs when T-cells are stimulated by some mitogens or antigens [4, 5]. One study demonstrated that the phosphorylation of CD3 (gamma chain) mediates a down-regulation of the T-cell receptor (TCR)/CD3 complex. But how this down-regulation is co-ordinated with the antigen specific T-cell activation remains to be resolved. Furthermore, platelet-activating factor (PAF), for instance, is an important mediator in cell-cell interactions, and may be involved in down-regulating CD2 and CD3 on peripheral human T-cells, in time- and dose-dependent manners [15]. Reports, which describe the functional modulation of T-cells by anti-CD3 MoAb, show contradictory results; in some, the anti-CD3 MoAb is mitogenic [16], but in others inhibitory [17] to T-cells.

As another index of T-cell activation, the HLA-DR expression was measured in this study. The findings of increased percentages of T-cells expressing HLA-DR in patients with HP or sarcoidosis were consistent with previous reports [8, 9, 18]. In marked contrast, however, the density of HLA-DR expression on lavage T-cells was found to be reduced in patients with HP or sarcoidosis in this study. This may indicate, that in disease with a high number and proportion of activated HLA-DR+ T-cells accumulating in the lungs, the intensity of HLA-DR antigen expression by each individual HLA-DR+ T-lymphocyte is reduced, for reasons which are still unknown.

Another important result of our study was that the CD3 density was higher in all groups in blood than in lavage, although the percentages of CD3 positive cells in blood were lower. This suggests that there might be a preferential recruitment of CD3+ T-cells to the lungs, but that, even in normal individuals, CD3 density is down-regulated because of local factors. This finding may also indicate that BALF T-cells are activated even in healthy individuals, compared to blood T-cells, when seen in the context of the knowledge that the CD3 density is closely related to an activated state of T-cells in vitro [4–6]. In particular, when activated by antigen, the CD3 density decreases. The CD3 subunit, together with the antigen, tends to internalize within the cell membrane. In our study, the significant difference in the CD3 density on BALF T-cells between HP and sarcoidosis patients may reflect a difference in antigenic stimulation: a temporal stimulation in HP, and a persistent one in sarcoidosis.

The densities of CD4 and CD8 on BALF T-cells were increased in parallel with the CD3 density on BALF T-cells in patients with HP. This was not found in blood, either in patients with sarcoidosis or BOOP. CD8 positive T-cells are considered to play a cytotoxic or suppressive role in patients with HP [8, 9]. CD4 positive T-cells usually enhance immune responses in the lungs as effector T-cells (helper or inducer). Even in patients with HP, CD4 positive T-cells may be important in local immune responses. A comprehensive explanation of the prominent accumulation of CD8 positive T-cells in the lungs of HP has yet to be found. From the observation of a high percentage of lung lymphocytes bearing the very late activation antigen-1 (VLA-1) in HP, one report proposed that CD8 positive T-cells represent a long-term activated and homing population in the lung of patients with HP, possibly as the result of a continuous stimulation [19].

In summary, the present study shows that the process of T-cell activation in HP is associated with an increase in the density of CD3, CD4, and CD8 antigen expression on BALF T-cells. Since similar changes were not observed in sarcoidosis and BOOP, the pathogenetic mechanisms involved in T-cell activation may be different in these diseases.
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References