Expression of surface antigens distinguishing “naive” and previously activated lymphocytes in bronchoalveolar lavage fluid

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Abstract

Studies in animals suggest that the initial activation of unprimed (“naive”) T lymphocytes by inhaled antigens may occur outside the lung with later recruitment to the lung. If this is true all lymphocytes present in the lung should show evidence of prior activation. To test this hypothesis for lymphocytes present on the alveolar surface, the expression of surface antigens, which distinguish unprimed from previously activated cells (CD45RA, CD29, Leu-8), were measured on T lymphocytes recovered from blood and bronchoalveolar lavage fluid from normal subjects and patients with sarcoidosis. Few T lymphocytes from the lavage fluid of normal subjects and patients with sarcoidosis expressed the Leu 8+ or CD45RA (bright) phenotype expected for “naive” cells; more cells had the CD29 (dark) phenotype expected for “naive” cells, though five of eight subjects had under 2% of such cells. These findings support the conclusion that the only T lymphocytes present on the surface of the respiratory tract are those recognising antigens that have been previously encountered by the individual. Further studies are required to determine whether “naive” T lymphocytes are present in other lung compartments.

T lymphocytes are prominent in lavage fluid recovered from the lower respiratory tract of normal individuals and are present in the lung in increased numbers in patients with various lung diseases. These cells are thought to have an important role in protecting the normal lung against microorganisms and foreign antigens, in addition to producing lung damage in disorders characterised by uncontrolled immune responses.1–3

Despite their importance, little is known about the factors controlling the recruitment of T lymphocytes to the human lung. Studies in animals suggest that the activation of “naive” lymphocytes induced by inhaled antigens occurs in regional and central lung lymph organs (hilar nodes, spleen) rather than in the lung.4–6

Once activated, the lymphocytes are recruited to the lung, a process amplifed by the presence of an immune-inflammatory reaction within the lung and by the presence of specific antigen.6–15

If this hypothesis is correct, virtually all lymphocytes present in the lung should show evidence of prior activation. Previous studies have shown that relatively few T lymphocytes present on the alveolar surface express antigens associated with recent activation, such as HLA-DR, transferrin receptors, or receptors for interleukin.2,16,17 These activation antigens, however, are only transiently expressed. Thus, although these studies indicate that most T lymphocytes in lavage fluid have not been activated recently, it remains unclear whether the remaining cells are “naive” T lymphocytes or “memory” T lymphocytes, which have previously been activated but have lost their expression of markers of recent activation. In supporting the idea that many T lymphocytes present on the alveolar surface may have been previously activated is the fact that more than 20% of T lymphocytes in lavage fluid from normal subjects express the “late” activation antigen VLA-1.18

Recently several monoclonal antibodies have been described that detect surface antigens on a subpopulation of circulating blood T lymphocytes (Leu-8, CD45RA, CD29).19–24 These antibodies were initially thought to recognise functionally distinct subpopulations of T lymphocytes, but there is now evidence to suggest that the expression of these antigens is modulated by cell activation. Activation of “naive” T lymphocytes reduces expression of CD45RA and increases expression of CD29, and these phenotypic changes appear to be stable.25–28 Similarly, activation of Leu-8 cells reduces the expression of this antigen by most cells.26

To assess whether T lymphocytes on the alveolar surface have the phenotype of “naive” or of previously activated cells, we examined the expression of antigens detected by Leu-8, CD45RA, and CD29 monoclonal antibodies on CD4+ (helper-inducer) and CD8+ (suppressor-cytotoxic) T lymphocytes recovered from the lung by bronchoalveolar lavage from normal subjects and patients with sarcoidosis.

Methods

SUBJECTS

We studied seven non-smoking normal volunteers (two men and five women), mean age 29 (SD 5) years, with no history of past or current lung disease, and seven untreated patients with sarcoidosis (four men and three women), age 33 (7) years; three of the latter were non-smokers and four current smokers.
(10 (10) cigarettes/day). All had compatible clinical and radiological findings at presentation. There was no evidence of mycobacterial or fungal infection and no history of exposure to agents known to cause granulomatous disease. Non-caseating granulomas were found in biopsy material from six patients; the remaining patient presented with hilar adenopathy, erythema nodosum, and polyarthritis, which resolved without treatment. The duration of disease, evaluated as described previously,3 ranged from one week to nine years. Chest radiographic abnormalities at the time of evaluation were as follows: stage 1, four patients; stage 2, two; stage 3, one.

CELL PREPARATION
Bronchoalveolar lavage and the isolation of mononuclear cells from lavage fluid and peripheral blood were performed as described previously. Bronchoalveolar lavage was performed on all patients with sarcoidosis and on four of the normal volunteers. All patients and volunteers undergoing lavage gave informed consent. The total and differential cell counts of cells recovered by lavage from the two groups were similar to those in previous studies. The percentages of T lymphocytes were 54 (SD 10) CD4+ and 42 (12) CD8+ in the control subjects and 70 (23) CD4+ and 25 (24) CD8+ in the patients with sarcoidosis (as determined by indirect immunofluorescence microscopy).

IMMUNOFLUORESCENT STAINING
CD4 (OKT4) and CD8 (OKT8) hybridomas were obtained from the American Type Culture Collection (Rockville, Maryland). Antibodies were purified from ascitic fluid or culture medium by protein-A sepharose chromatography and were biotinylated. Leu-8 (Becton Dickinson, Sunnyvale, California), CD45RA+ (2H4, Coulter Immunologic, Hialeah, Florida), CD29 (4B4, Coulter), and CD14 (My4, Coulter) were obtained from commercial sources in the phycoerythrin conjugated form. The structures recognised by these antibodies are shown in table 1.

Unseparated blood mononuclear cells and lavage cells were prepared for two colour immunofluorescence analysis. Cells were incubated simultaneously with appropriate dilutions of one biotinylated mouse monoclonal antibody (CD4, CD8, or appropriate controls) and one phycoerythrin conjugated mouse monoclonal antibody (Leu-8, CD45RA, CD29, CD14, or appropriate control antibodies), washed, incubated with 5 μg/ml fluorescein isothiocyanate (FITC) conjugated avidin, washed again, fixed in phosphate buffered saline containing 1% formaldehyde, and stored in the dark at 4°C until analysis.

ANALYSIS OF IMMUNOFLUORESCENCE
Immunofluorescence was evaluated with an Epics C cytofluorograph (Coulter) fitted with a 5 W argon laser (Coherent, Palo Alto, California). CD4+ and CD8+ lymphocytes were identified by gating on three parameters: (1) narrow angle light scatter appropriate for lymphocytes; (2) 90° angle light scatter appropriate for lymphocytes; and (3) a positive specific green fluorescence signal (for example, green fluorescence of cells treated with biotinylated CD4 or CD8 monoclonal antibodies greater than that of cells treated with biotinylated isotype matched control antibodies). Less than 1% of blood and lavage cells identified by these criteria were positive for the CD14 surface molecule expressed on monocyte/macrophages (data not shown). The profile of orange fluorescence (resulting from binding with phycoerythrin conjugated monoclonal antibodies Leu-8, CD45RA, CD29, or CD18) of 5000 cells meeting the above three criteria was then evaluated.

Data were analysed by the Easy 88 data management system (Coulter). The percentage of Leu-8 positive cells was calculated by using a subtraction mode program (Immuon, Coulter Electronics). Two of the antibodies used in the study (CD45RA and CD29) distinguish subpopulations of blood T lymphocytes based on the relative intensity of staining with these antibodies as most cells are “positive” with these antibodies, though the expression varies from low to high. Although the distribution is usually bimodal (for example, cells with low and high expression), cells expressing intermediate levels of these surface antigens were also present. To quantify the expression of these surface antigens on lymphocytes, cursors were placed at the channels of the “raw” fluorescence histogram for blood lymphocytes containing the largest number of cells with “low” and “high” expression (see example in fig 1A). This permits the definition of three populations: cells expressing small surface antigen (“dull”), cells expressing intermediate amounts of antigen (“intermediate”), and cells expressing large amounts of antigen (“bright”). The same cursors were then used to separate the “raw” fluorescence histogram of

<table>
<thead>
<tr>
<th>Table 1 Monoclonal antibodies used in the study</th>
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<tbody>
<tr>
<td><strong>Cluster designation</strong></td>
</tr>
<tr>
<td>CD4</td>
</tr>
<tr>
<td>CD8</td>
</tr>
<tr>
<td>CD14</td>
</tr>
<tr>
<td>CD29</td>
</tr>
<tr>
<td>CD45RA</td>
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<tr>
<td>None</td>
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Expression of surface antigens distinguishing "native" and previously activated lymphocytes in bronchoalveolar lavage fluid

lavage fluid lymphocytes stained with the same monoclonal antibody into the three populations of cells (see example in fig 1B).

STATISTICAL ANALYSIS

Results are presented as mean (SD) percentages of the total CD4⁺ or CD8⁺ lymphocytes with a given phenotype. Statistical comparisons were made with the Mann-Whitney rank order test. p < 0.05 was considered significant.

Table 2. Surface phenotypes of blood and lavage fluid T lymphocytes from normal subjects and patients with sarcoidosis (mean (SEM) percentages of CD4⁺ or CD8⁺ lymphocytes)

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Blood</th>
<th>CD4⁺</th>
<th>CD8⁺</th>
<th>Lavage fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-8</td>
<td>CD45RA</td>
<td>CD29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Volunteers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study group</td>
<td>Leu-8 positive</td>
<td>dull</td>
<td>intermediate</td>
<td>bright</td>
</tr>
<tr>
<td>Blood</td>
<td>CD4⁺</td>
<td>82 (5)</td>
<td>25 (9)</td>
<td>57 (4)</td>
</tr>
<tr>
<td></td>
<td>CD8⁺</td>
<td>55 (8)</td>
<td>16 (5)</td>
<td>62 (3)</td>
</tr>
<tr>
<td>Lavage fluid</td>
<td>CD4⁺</td>
<td>1 (1)</td>
<td>74 (27)†</td>
<td>25 (25)</td>
</tr>
<tr>
<td></td>
<td>CD8⁺</td>
<td>1 (1)</td>
<td>42 (21)†</td>
<td>57 (20)</td>
</tr>
<tr>
<td>Sarcoidosis Patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>CD4⁺</td>
<td>73 (20)</td>
<td>39 (23)</td>
<td>49 (16)</td>
</tr>
<tr>
<td></td>
<td>CD8⁺</td>
<td>22 (26)‡</td>
<td>12 (8)</td>
<td>66 (8)</td>
</tr>
<tr>
<td>Lavage fluid</td>
<td>CD4⁺</td>
<td>1 (1)</td>
<td>44 (40)</td>
<td>55 (39)</td>
</tr>
<tr>
<td></td>
<td>CD8⁺</td>
<td>1 (1)</td>
<td>17 (13)</td>
<td>79 (13)</td>
</tr>
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</table>

*p < 0.05 in the comparison of normal subjects and patients with sarcoidosis.
†p < 0.05, ‡p < 0.01 in the comparison of T lymphocytes obtained from blood and bronchoalveolar lavage fluid within the same study group.

The numbers in square brackets indicate numbers of individuals studied.

Results

EXPRESSION OF LEU-8 ON BLOOD AND LAVAGE FLUID T LYMPHOCYTES

Most CD4⁺ blood T lymphocytes from normal subjects and patients with sarcoidosis expressed the Leu-8 surface antigen (normal (mean (SD)) 0.05, 82 (5), n = 7; sarcoid 73 (20), n = 7; p > 0.2). In contrast, few of the CD4⁺ T lymphocytes recovered by lavage from normal subjects or patients with sarcoidosis were Leu-8⁺ (normal 1 (1), n = 4; sarcoid 1 (1), n = 7).

About half of the CD8⁺ blood T lymphocytes from normal subjects were Leu-8⁺ (mean 0.55 (18)) but significantly fewer of those from patients with sarcoidosis (0.05 positive 22 (26); p < 0.05 in the comparison with normal subjects); in four of the seven patients less than 10.0% of CD8⁺ blood T lymphocytes were Leu-8⁺. Very few CD8⁺ lymphocytes were observed among cells recovered by lavage in either group (0.0% in normal subjects 0.4 (0.3) and in patients 0.6 (0.4); p > 0.2).

EXPRESSION OF CD45RA ON BLOOD AND LAVAGE FLUID T LYMPHOCYTES

CD4 cells

The expression of CD45RA on CD4⁺ blood T lymphocytes had a bimodal distribution as described previously, although many cells expressed intermediate levels of this antigen (fig 1A). When CD4⁺ blood T lymphocytes were divided into three populations based on their expression of CD45RA (CD45RA⁺, CD45RA⁺, CD45RA⁺), the proportion of cells in the three populations was similar in the two study groups (table 2).

The distribution of CD45RA on lavage fluid CD4⁺ T lymphocytes showed a unimodal distribution, most cells being CD45RA⁺ or CD45RA⁺ and a broad "tail" of cells expressing greater amounts of the antigen (fig 1B). Very few of the CD4⁺ cells from normal subjects or patients with sarcoidosis were CD45RA⁺ (table 2; % in normal subjects 2 (2), n = 4; sarcoidosis 1 (2), n = 4; p > 0.2).
**Figure 2**  Expression of CD29 on CD4⁺ blood (A) and lavage fluid (B). T lymphocytes: cell populations analysed by fluorescence cytometry (for details see legend to fig 1).

### CD8 cells
The expression of CD45RA on CD8⁺ blood T lymphocytes from normal subjects and patients with sarcoidosis also showed a bimodal distribution; the proportion of cells with a CD45RA<sub>dull</sub>, CD45RA<sub>intermediate</sub> and CD45RA<sub>bright</sub> phenotype was similar in the two groups (table 2). Few CD8⁺ lavage fluid lymphocytes had the CD45RA<sub>bright</sub> phenotype in either normal subjects (blood < 0.001) or patients with sarcoidosis, with no difference between the two groups (p > 0.2).

### EXPRESSION OF CD29 ON BLOOD AND LAVAGE FLUID LYMPHOCYTES

#### CD4 cells
The expression of CD29 on CD4⁺ blood T lymphocytes from normal subjects and patients with sarcoidosis showed a broad bimodal distribution in most individuals, many cells displaying an intermediate phenotype (fig 2A). The proportion of CD29<sup>dull</sup>, CD29<sup>intermediate</sup>, and CD29<sup>bright</sup> cells in the two study groups was similar (table 2, p > 0.2 for each comparison).

Expression of CD29 on CD4⁺ lavage fluid lymphocytes was unimodal, most cells being CD29<sup>bright</sup> (CD4⁺ CD29<sup>dull</sup> lymphocytes: % in normal subjects 8 (8); sarcoidosis 1 (1); p < 0.01 and <0.05 respectively in the comparison of CD29<sup>dull</sup> cells in lavage fluid and blood for the two groups). Although CD4⁺ lavage fluid lymphocytes considered to be CD29<sup>dull</sup> were identified, some individuals had very few such cells (two of four normal subjects and three of four patients with sarcoidosis had less than 2%).

#### CD8 cells
The expression of CD29 on CD8⁺ blood T lymphocytes also had a bimodal distribution. The expression of CD29 on CD8⁺ lavage lymphocytes was unimodal, and generally few cells with the CD8⁺ CD29<sup>dull</sup> phenotype were observed (table 2); half the subjects had less than 2% of such cells.

### INTENSITY OF EXPRESSION OF CD4 AND CD8 ON BLOOD AND LAVAGE FLUID T LYMPHOCYTES
The intensity of expression of CD4 on blood T lymphocytes from normal subjects and patients with sarcoidosis was homogeneous in its distribution. CD4⁺ lavage fluid lymphocytes also stained strongly with the CD4 antibody, but the channel of peak fluorescence was less here than in blood T lymphocytes (normal subjects: channel number for blood 152 (19), lavage fluid 113 (25); sarcoidosis: blood 153 (22), lavage fluid 136 (20)). The intensity of staining with CD8 monochlonal antibody was similar for blood and lavage lymphocytes.

### Discussion
The most striking finding of this study is that most CD4⁺ and CD8⁺ T lymphocytes recovered by bronchoalveolar lavage have the Leu-8⁺, CD45RA<sub>intermediate</sub> CD29<sup>intermediate</sup> phenotype, whereas Leu-8⁺, CD45RA<sub>bright</sub>, CD29<sup>dull</sup> T lymphocytes, present in substantial numbers in the blood, are very scanty in the lung. Recent studies suggest that the expression of these surface antigens changes with differentiation. "Naive" T lymphocytes are thought to express the Leu-8⁺ CD45RA<sub>bright</sub> CD29<sup>dull</sup> phenotype. Activation of such lymphocytes via their T cell receptor is associated with a progressive change in surface phenotype to Leu-8⁺ CD45RA<sub>intermediate</sub> CD29<sup>bright</sup> 25-50 The findings of this study are therefore compatible with the hypothesis that essentially all lymphocytes present on the surface of the lower respiratory tract have been previously activated. If "naive" T lymphocytes were to be activated in situ on the alveolar surface, large numbers of "naive" cells would have to be present to ensure that all possible antigen specificities could be identified. Very few lymphocytes expressing the surface phenotype expected for "naive" cells were identified in this study, supporting the conclusion that alveolar space lymphocytes are largely restricted to cells recognising antigens previously encountered by the individual.

Our results on the expression of Leu-8, CD45RA, and CD29 on blood lymphocytes are similar to those of others. 26-30 Semenzato et al 26 found that most CD4⁺ lavage T lymphocytes were Leu-8⁺ as determined by immunofluorescence microscopy, but differences in Leu-8 expression between normal subjects and patients with sarcoidosis, as in their study, were
Expression of surface antigens distinguishing "naive" and previously activated lymphocytes in bronchoalveolar lavage fluid

not found in ours. Gerli et al,39 studying CD4+ lymphocytes, and Saltini et al,38 studying CD3+ lymphocytes,28 found that most lymphocytes in lavage fluid were CD45RA+ and CD29+. Although both these studies indicate that CD45RA+ lymphocytes are present in the lung, the distribution of the intensity of fluorescence was not described, so it is not clear whether the "CD45RA+" cells reported in these studies correspond to the weakly stained CD45RA+cells we observed. Munro et al39 reported that CD4+ and CD8+ lymphocytes present within sarcoid granulomas were Leu-8- and CD45RA+, as we observed in our study of alveolar space cells.

Few Leu-8+ or CD45RA+ cells were observed in lavage fluid, as would be expected for previously activated cells. Similarly, few CD29+ cells were recovered by lavage from most of the normal subjects and patients with sarcoidosis, though appreciable numbers (up to 16%) of CD4+ or CD8+ lavage fluid T lymphocytes from some individuals expressed the CD29+ phenotype expected for "naive" lymphocytes. The kinetics of change in the expression of Leu-8, CD45RA, and CD29 surface antigens after lymphocyte activation in vitro has been studied. Leu-8 expression is rapidly lost after activation,29 whereas loss of expression of CD45RA and the increase in expression of CD29 begin more than three days later, maximal change occurring after more than 10 days.27,28 Comparative studies suggest that after activation the virtual disappearance of cells with the CD45RA+ phenotype as defined in this study precedes the appearance of CD29+ cells.27 Thus the small population of CD29+ cells recovered by lavage from some individuals may represent cells activated more recently. In support of this idea, activation of CD4+ T lymphocytes is associated with transient down regulation of the expression of the CD4 antigen,39 and lavage CD4+ T lymphocytes reacted more strongly with the CD4 antibody than did blood CD4+ T lymphocytes. In contrast, activation of CD8+ T lymphocytes does not modulate CD8 expression, and lung and blood CD8+ T lymphocytes expressed similar amounts of CD8 antigen.

The expression of the Leu-8, CD45RA, and CD29 surface antigens on CD4+ T lymphocytes from normal subjects and patients with sarcoidosis did not differ significantly in this small study, though blood T8+ lymphocytes from the patients were more likely to be CD4+. Further studies are needed to determine whether expression of these antigens on blood T lymphocytes is related to disease activity in sarcoidosis.

Several limitations of the present study should be considered. We studied only cells recovered from the alveolar compartment and the airways. Possibly lymphocytes in other lung compartments are not activated, and could respond in situ to previously unencountered antigens. This study does not indicate where the alveolar surface lymphocytes were initially activated or what proportion of lavage fluid lymphocytes arrived by recruitment. Lung T lymphocytes can proliferate in the presence of specific antigen and clonal expansion locally could greatly increase the number of lymphocytes present on the alveolar surface without changing the expression of Leu-8, CD45RA, or CD29, as T lymphocyte clones proliferating in vitro usually have the Leu-8-CD45RA-CD29+ phenotype.27,29

This study suggests that essentially all lymphocytes present on the alveolar surface have been previously activated. Other studies are required to determine whether "naive" T lymphocytes are present in other lung compartments.


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