Phenotypic and functional activation of alveolar macrophages, T lymphocytes and NK cells in patients with systemic sclerosis and primary Sjögren’s syndrome

Björn Gudbjörnsson, Roger Hällgren, Otto Nettelbladt, Rolf Gustafsson, Anna Mattsson, Eva af Geijerstam, Thomas H Tötterman

Abstract

Objectives—Attempts to differentiate between the pathogenesis of the severe pulmonary manifestations observed in systemic sclerosis (SSc) and the mild form in primary Sjögren’s syndrome (pSS) were performed by studying cell populations recovered during bronchoalveolar lavage (BAL).

Methods and results—Two-colour flow cytometric analysis of BAL fluid lymphocytes showed a similar degree of phenotypic activation (DR+) of CD4+ and CD8+ T lymphocyte subsets and CD16+ NK cells in patients with SSc (n = 13) and pSS (n = 11) groups and healthy controls (n = 11). Alveolar macrophages expressed the CD14 antigen at significantly increased densities in patients with SSc. Alveolar macrophage activation in SSc was also suggested by increased IL-6 concentrations in neat BAL fluid and increases in macrophage production of TNFα and EGF in vitro. SSc patients also had increased proportions of neutrophils and eosinophils in BAL fluid. No correlations were found between any cellular subsets or cytokine levels in BAL fluid and lung status at the time of lavage in SSc or pSS patients or the subsequent course of the pulmonary function in SSc patients.

Conclusion—It is concluded that the phenotypical activation of alveolar helper/inducer (DR+CD4+) and suppressor/cytotoxic (DR+CD8+) T lymphocytes and NK (DR+CD16+) cells is not a prerequisite for the development of lung fibrosis in SSc or bronchial hyper-responsiveness in pSS. Alveolar macrophage activation may contribute to the development of lung fibrosis in SSc.

(PATIENTS AND CONTROLS)

Thirteen patients, four females and nine males (mean age 55 years, range 39–69 years) with diffuse SSc according to the 1980 ARA preliminary diagnosis criteria§ and eleven female patients (mean age 51 years; range 40–61 years) fulfilling the Copenhagen criteria for pSS§ were included in this study. Four SSc patients were smokers, while all the pSS patients were non smokers. At the time of the investigation, all patients were admitted to the Rheumatology Section, University Hospital, Uppsala, Sweden.

The control group consisted of eleven non-smoking healthy individuals, seven men and
four women, mean age 26 years (range 20–31).

LUNG FUNCTION TESTS AND CHEST RADIOPHGRAP
All patients had a plain chest radiograph. The lung function tests included measurements of vital capacity (VC), FEV₁, FEF₂⁵ and diffusion capacity for carbon monoxide (DLco), measured with the transfer test (single breath test). The results obtained were compared with those of age and sex-matched healthy controls. The values were divided into categories based on the PD20 value: severe (<0-125 mg methacholine), moderate (0-125–1-2), mild (1-3–5-0) and slight (5-1–9-0).

BRONCHOALVEOLAR LAVAGE
Bronchoalveolar lavage (BAL) was performed under local anaesthesia with lidocaine hydrochloride, after premedication with 0-5 mg atropine combined with oral diazepam or with subcutaneous morphine. A wedged fiberoptic bronchoscope (Model BF 1T20D or BF 1T10, Olympus Corp. of America, NY, USA) was used. The bronchoscope was wedged in a segmental bronchus of the middle lobe and three boluses of 50 ml of sterile saline at 37°C were infused. The fluid was gently aspirated immediately after each instillation. The lavage fluid was kept on ice and filtered through a single layer gauze. The cells in the lavage fluid were collected by centrifugation at 400g for 15 minutes and were thereafter resuspended to a concentration of 10⁶ cells/ml excluding epithelial cells. The total number of cells in the lavage fluid was counted in a Bürker chamber. The cell differential was determined by using cytocentrifuge preparations and May-Grünwald-Giemsa staining. Lymphocytes were expressed as percentage of 200 cells (except epithelial cells) counted. Cell-free BAL fluid was freeze-stored at −70°C pending later analysis of soluble factors (see below).

CULTURE OF BAL CELLS
BAL alveolar macrophages were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum (Flow Laboratories, Glasgow, Scotland) and incubated at a concentration of 1 × 10⁶/ml in 2 ml plastic wells (Nunc, Denmark) for two hours at 37°C in a humidified 5% CO₂ atmosphere. Non-adherent cells were removed and adherent macrophages (1 × 10⁶/ml) were further cultured for 20 hours, after which supernatants were analysed for the presence of various cytokines (see below).

ANTIBODIES AND FACS ANALYSIS
Mononuclear cells were separated from heparinised blood by routine Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation. The cells were washed, suspended (10⁴/50 μl) in phosphate-buffered saline supplemented with 2% fetal calf serum (Flow Lab) and 0-1% sodium azide and incubated for 15 minutes at room temperature with combinations of optimally diluted fluorescein isothiocyanate-labelled monoclonal antibodies (Becton Dickinson, Mountain View, CA) raised against lymphocyte subsets or activation antigens (table 1). Irrelevant negative control antibodies of the same Ig isotypes (Simultest, Becton Dickinson) were used. To facilitate lymphocyte gating, samples were also stained with anti-CD45 (pan-leucocyte) plus anti-CD14 (monocyte) antibodies (Leukogate, Becton Dickinson). After washing, cells were suspended in the same medium supplemented with 1% paraformaldehyde (BDH, Poole, UK). Fluorescent cells were analysed in a FACStar (Becton Dickinson) analyser/sorter equipped with a 5W argon laser emitting at 488 nm and running at 0:2W. A total of 10⁵ lymphoid cells were gated and red (phycoerythrin) fluorescence was collected through a 585/42 nm filter and green (fluorescein isothiocyanate) fluorescence through a 530 nm filter. For enumeration of monocytes, scatter gates were set on lymphocytes and monocytes. Data were processed with a Hewlett-Packard 217 computer (H-P, Fort Collins, CO) with Consort 30 software (Becton Dickinson).

CYTOKINE ANALYSIS
Neat BAL fluid and supernatants of BAL macrophage cultures were analysed for the presence of the pro-inflammatory cytokines IL-1 (minimum detectable concentration (mdc): 5 pg/ml), IL-6 (mdc: 3-5 pg/ml) and TNFα (mdc: 5 pg/ml) using commercial ELISA kits (IRE Medgenix, Fleurus, Belgium). The levels of the growth factors EGF (mdc: 0-2 pg/ml) and PDGF (mdc: 8:4 pg/ml) were also determined using commercial ELISAs (British Biotechnology Products, Abingdon, UK).

STATISTICAL ANALYSIS
Values are given as means (SEM) (range). Non-parametric tests (Mann-Whitney U test and Spearman Rank Correlation coefficient) were used to analyse the data. A p-value of <0-05 was considered significant when comparing patient groups but a p-value of <0-01 was required, when comparing lymphocyte subsets and clinical or spirometric findings.

Results
CLINICAL FINDINGS
Patients with SSc had a mean disease duration of five years (range 0-5–12 years). All patients had various degrees of exertional dyspnoea. Fibrotic changes were visible in eight of 13 patients (63%) on chest radiograph. Pulmonary function tests demonstrated that the mean DLco for the SSc group was 51% of
the predicted value (range 5–81); 12 of 13 patients had an impaired Dlco. The mean VC was 78% of the predicted value (range 49–104); six of the 13 SSc patients had a reduced VC. Five (38%) of the SSc patients suffered from dry eyes and mouth and had pathological schirmer-I and salivation tests suggesting secondary SS. Four of 11 SSc patients demonstrated a significant progress in their pulmonary manifestations when they were followed up, on average 36 months (range 12–66) after the BAL evaluation.

 Patients with pSS had a mean disease duration of 10 years (range 2–17 years). Seven patients (64%) reported symptoms of xerotrachitis sicca and only one patient had history of exertional dyspnoea. Only one pSS patient had minimal diffuse interstitial densities in both lungs on chest radiograph. Their mean Dlco was 81% of the predicted value (range 63–111); five patients with pSS had a slightly impaired Dlco. The mean VC was 93% of the predicted value (range 65–125); only two patients had a reduced VC. The mean FEF25 was 74% of the predicted value (range 27–200); three patients had reduced FEF25, that is, <50%, and six patients had FEF25 <80% of predicted. Four of 10 tested pSS patients had mild or moderate BHR and two patients had slight BHR.

No patient was on corticosteroids or any disease modifying anti-rheumatic drugs at the time of the respiratory evaluation.

**CELL RECOVERY AND DIFFERENTIAL COUNT IN BAL FLUID**

No significant differences were observed in the amount of the re-covered BAL-fluid in the three investigated groups. The total mean (SEM) cell recovery in the lavage fluid was significantly increased in patients with SSc [3·2 (0·5) x 10^3 cells/ml; range 1·3–6·9] compared with controls (p < 0·01). No difference was observed between patients with pSS [2·8 (0·8) x 10^3 cells/ml; range 0·6–8·7] and controls [1·2 (0·3) x 10^3 cells/ml; range 0·2–2·8]. Patients with SSc had increased numbers of neutrophils and eosinophils in BAL fluid compared with both pSS patients (p < 0·05; p < 0·01) and controls (p < 0·01; p < 0·001). No difference was observed between pSS patients and healthy controls regarding total cells or cell differentials (fig 1, table 1). No correlation was observed between any of the differential cell types in BAL fluid and the spirometric findings in either patient group or the degree of BHR.

**T-CELL SUBSETS IN BAL FLUID AND PERIPHERAL BLOOD**

In all individuals studied, a significant (p < 0·0001) activation of helper/inducer T cells (T4+) (DR' out of CD4+) and suppressor/cytotoxic T cells (T8+) (DR' out of CD8+) and a decrease in naive, unprimed helper T cells (CD45RA+ out of CD4+) was observed in BAL fluid when compared with peripheral blood (fig 2). No significant differences were observed between the three groups when the absolute numbers of T cell subsets in BAL fluid were compared (table 2) and no correlation was found between these and the spirometric/chest radiographic findings.

The SSc patients had a significantly higher percentage of activated Tc cells in peripheral blood compared with pSS patients (p < 0·01) and controls (p < 0·001) and they also had a significantly increased percentage of activated T8c cells compared with the controls (p < 0·01) (fig 2).

**NK CELLS IN BAL FLUID AND PERIPHERAL BLOOD**

All individuals had a significantly increased phenotypic activation of NK cells (DR' out of CD16+) in BAL fluid compared with peripheral blood (p < 0·001). The BAL fluid of patients with SSc also contained significantly higher percentages of activated NK cells compared with patients with pSS (p < 0·05) (fig 2). When the absolute numbers of NK cells (CD16+) and activated NK cells were compared, no significant differences were observed between the groups (table 2) and no correlations were observed between these and the spirometric/chest radiographic findings.

Patients with pSS had a decreased mean (SEM) percentage of NK cells in peripheral blood [5·5 (1·0)] compared with controls [11·8 (2·3); p < 0·05].

**MONOCYTES IN BAL FLUID AND PERIPHERAL BLOOD**

Patients with pSS and SSc as well as healthy controls had similar percentages of monocytes/macrophages (CD14+) in BAL fluid and

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**Table 1 Monoclonal antibodies, their specificities and references**

<table>
<thead>
<tr>
<th>Mabs*</th>
<th>CD*</th>
<th>Cell population defined</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu 3</td>
<td>4</td>
<td>Helper/inducer T cells (T4+)</td>
<td>18</td>
</tr>
<tr>
<td>Leu 2</td>
<td>8</td>
<td>Suppressor/cytotoxic T cells (T8+)</td>
<td>18, 20</td>
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<tr>
<td>HLA-DR+Leu 3</td>
<td>ns + 4</td>
<td>Activated CD4+ cells</td>
<td>18, 21, 22</td>
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<tr>
<td>HLA-DR+Leu 2</td>
<td>ns</td>
<td>Activated CD8+ cells</td>
<td>18, 21, 22</td>
</tr>
<tr>
<td>Leu 18/Leu 3</td>
<td>45RA</td>
<td>Naive, unprimed CD4+ T cells</td>
<td>23</td>
</tr>
<tr>
<td>Leu 11</td>
<td>16</td>
<td>NK-like cells</td>
<td>24</td>
</tr>
<tr>
<td>HLA-DR+ Leu 11</td>
<td>ns + 16</td>
<td>Activated CD16+ cells</td>
<td>25</td>
</tr>
<tr>
<td>Leu M3</td>
<td>14</td>
<td>Monocytes</td>
<td>26</td>
</tr>
</tbody>
</table>

*MABS = monoclonal antibodies; CD = cluster of differentiation; ns = CD number not specified.

**Figure 1 Absolute numbers of lymphocytes, neutrophils and eosinophils in BAL fluid of patients with primary Sjögren’s syndrome (pSS) and systemic sclerosis (SSc).** Shaded areas indicate mean and 95% confidence interval for healthy controls. Significant difference with U-test: SSc v pSS: * = p < 0·05 and ** = p < 0·01. SSc v controls: † = p < 0·01 and ‡‡‡ = p < 0·001.
peripheral blood (table 3). On the other hand, the BAL macrophages of patients with SSc expressed the CD14 antigen molecule at a significantly higher density than those of controls (p < 0.05) (table 2). No correlations were observed between the percentage of BAL monocytes/macrophage or the intensity of BAL cytokines/macroage or the intensity of expression of the CD14 antigen and the spirometric/chest radiographic findings.

**CYTOKINES IN BAL**

The pro-inflammatory cytokines IL-1, IL-6 and TNFα as well as the growth factors EGF and PDGF were measured in neat BAL fluid and the supernatants of macrophage cultures (table 4). Increased concentrations of IL-6 (p < 0.01 vs controls) were found in patients with SSc. However, when indexed to BAL fluid albumin, this difference disappeared. Significant differences arise between patients with SSc and pSS as regards the concentrations of IL-1 in neat BAL fluid (p < 0.001).

The BAL macrophages synthesis of TNFα but not IL-6, was also increased in vitro in patients with SSc compared with pSS (p < 0.05). EGF was similar in neat BAL fluid in all three group, but was only detected in the macrophage cultures taken from patients with SSc. PDGF was not detectable either in the neat BAL fluid or the supernatants of BAL macrophage cultures in any of the three groups. Abnormal concentrations of measured cytokines were not related to spirometric/chest radiographic findings.

No correlation was found between any cellular subsets or cytokine levels in neat or cultured BAL fluid of the SSc patients and the subsequent course of their pulmonary function.

**Discussion**

The connective tissue diseases, systemic sclerosis (SSc) and primary Sjögren's syndrome (pSS), are both characterised by immunological abnormalities, including the presence of auto-antibodies and infiltration of activated T-cells in affected organs. However, the pronounced propensity of tissue fibrosis in SSc is lacking in pSS, indicating differences in their respective pathogenesis. Progressive pulmonary fibrosis is a major manifestation of SSc but quite rare in pSS. However, patients with pSS frequently suffer from small airways' disease and BHR. The present study of BAL fluid aimed to identify possible differences in

### Table 3: Monocytes/macrophages in BAL fluid and peripheral blood (PBL) from 11 patients with primary Sjögren's syndrome (pSS), 13 patients with systemic sclerosis (SSc) and 13 healthy non-smokers

<table>
<thead>
<tr>
<th>Monocytes/Macrophages (CD14+)</th>
<th>Relative CD14 intensity (linear channel no)</th>
<th>Controls</th>
</tr>
</thead>
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<tr>
<td>PBL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSc</td>
<td>7-5 (0-8)</td>
<td>246-8 (16-9)</td>
</tr>
<tr>
<td>pSS</td>
<td>7-1 (0-6)</td>
<td>233-2 (14-8)</td>
</tr>
<tr>
<td>Controls</td>
<td>8-5 (1-5)</td>
<td>213-0 (14-8)</td>
</tr>
<tr>
<td>BAL</td>
<td>42-1 (7-7)</td>
<td>840-8 (281-2)</td>
</tr>
<tr>
<td>SSc</td>
<td>44-8 (7-9)</td>
<td>396-0 (118-7)</td>
</tr>
<tr>
<td>pSS</td>
<td>56-2 (5-8)</td>
<td>306-0 (22-0)</td>
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Significant difference with Mann-Whitney U test: SSc vs controls: †p < 0.05.
the pathogenetic mechanisms involved in the pulmonary processes involved in these two CTDs. Comparison of the effector cells recovered by BAL to those present in open lung biopsies has shown that the cell population recovered by lavage accurately reflect the population of effector cells present in the alveolar structure. The cells involved in the parenchymal damage of systemic sclerosis are likely to originate from the alveoli. In contrast, BHR may be due to a bronchial inflammation, suggesting that the mononuclear effector cells are located in the bronchi themselves. However, in the presence of bronchial inflammation, it is impossible to tell whether the effector cells are airway or alveolar derived.

The major difference was the phenotypic activation of macrophages recovered from the BAL fluid of patients with SSc. Thus FACS analysis revealed a three fold increase in the expression of the CD14 molecules on alveolar macrophages in SSc. The expression of CD14, a selective marker for monocytes/macrophages and representing the receptor for bacterial lipopolysaccharides, is increased in the in vitro and in vivo activation of monocyte/macrophages. The cytokines IL-1, IL-6 and TNF-α are produced by several types of nucleated cells but activated monocytes/macrophages are major sources. Therefore a macrophage activation in the lung of SSc patients was also suggested by the finding of abnormally elevated IL-6 levels in their BAL fluid and increased production of TNF-α by their cultured alveolar macrophages. Other studies have also demonstrated signs of alveolar macrophage activation in SSc. Silver et al reported that alveolar macrophages in SSc produce fibronectin in vitro and Deguchi found that these cells have an increased in vitro production of transforming growth factor-β, a potent fibroblast mitogen. In our study, the growth factor EGF was only synthesised by SSc alveolar macrophages, while PDGF, another growth factor produced by activated macrophages and previously suggested to contribute to fibrotic lesions in SSc, was not detectable in near BAL fluid or macrophage culture supernatants from our SSc or pSS patients. However, the relevance of the increased activation of BAL macrophages to the clinical expression can be questioned since these cells were not correlated to the lung status at the time of the lavage or to the subsequent course of pulmonary function.

Two-colour FACS analysis was also used to quantify the phenotypic activation of CD4+ T helper cells, CD8+ T cytotoxic/suppressor cells and CD16+ NK cells in BAL fluid and peripheral blood. The naive, unprimed subset of T helper cells (CD45RA*CD4+) was also determined. A general feature emerged that was similar in both patients and healthy subjects—a profound local immune activation in the lower respiratory tract. Thus compared with circulating cells, BAL fluid T subset cells and NK cells expressed high levels of the activation marker HLA-DR, and the proportion of naive T helper cells was close to zero, indicating functional CD4+ T cell activation. Generally, however, no disease-specific or severity-related cell patterns were observed. Earlier studies have reported a marginally increased proportion of CD4+ and CD8+ T cells in the BAL fluid of patients with SSc and, normal, relative but increased absolute numbers of CD4+ and CD8+ T cells in patients with pSS. Subclinical BAL lymphocytosis has also been reported in pSS.

Our results of increased activated T and T helper cells in the peripheral blood of patients with SSc agree with previous reports and our study, like several other previous studies, fails to demonstrate significant abnormalities in the distribution of circulating lymphocyte subsets in patients with pSS.

Patients with pSS have only a minor propensity for pulmonary fibrosis. However, they frequently suffer from BHR and sputum abnormalities in small airways' disease. The mechanism behind the BHR in pSS is unknown but may be related to an inflammation in the lower respiratory tract. In other conditions, BHR has been suggested to be the result of an activation of various cell types, including mast cells, eosinophils and neutrophils. Furthermore, the release of cytokines may alter nerve and smooth muscle function, resulting in bronchial constriction. However, we did not find any correlation between the degree of BHR and the various cell subsets or cytokines analysed in BAL fluid of our pSS patients. In accordance with previous observations we found an increased numbers of neutrophils and eosinophils in the BAL fluid of patients with SSc.

In conclusion, the present study demonstrates local lymphocyte activation in the lower respiratory tract to a similar degree both in health and CTDs, supporting the view that local lymphocyte activation at the mucosal borders of the lungs reflects immune surveillance, rather than a pathogenetic process. On the contrary, we demonstrated an abnormally increased phenotypic and functional activation of alveolar macrophages in patients with SSc. This activation of alveolar macrophages may contribute to the endothelial damage and pulmonary fibrosis associated with this disease by means of a release of proinflammatory and mitogenic cytokines.


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