Antiproteases are increased in bronchoalveolar lavage in interstitial lung disease

Y. Sibille, J.B. Martinot, P. Staquet, L. Delaunois, B. Chatelain, D.L. Delacroix

ABSTRACT: The present study evaluates different cellular and soluble components in the bronchoalveolar lavage (BAL) from patients with interstitial lung disease. We observed an increased $T_4/T_8$ lymphocyte ratio in BAL but not in blood from 24 patients with active pulmonary sarcoidosis compared to sixteen normal individuals and to eleven patients with inactive pulmonary sarcoidosis. Seven patients with hypersensitivity pneumonitis had a normal $T_4/T_8$ ratio. In the active sarcoidosis and hypersensitivity pneumonitis groups, $\alpha_1$-Protease Inhibitor ($\alpha_1$PI) in BAL is significantly higher than in the normal group and a significant correlation between the two antiproteases ($\alpha_1$-macroglobulin and $\alpha_1$PI) is observed. These data demonstrate that antiprotease levels ($\alpha_1$PI and $\alpha_2$M) are increased in the lower respiratory tract of patients with interstitial lung disease and that among cellular and soluble components of BAL, $\alpha_2$M represents a sensitive marker of the alveolitis.

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The interstitial lung diseases include disorders of unknown and known aetiology, and even distinguishing among the diseases of known cause is sometimes tedious. In addition to the problem of diagnosing specific causes of interstitial lung disease, the assessment of disease activity, especially in pulmonary sarcoidosis, remains an important issue. In recent years three techniques have been proposed to assess disease activity in sarcoidosis: $^67$Gallium scan, Angiotensin Converting Enzyme (ACE) and Bronchoalveolar Lavage (BAL) [1–4]. In the past ten years BAL has been widely used and may be a useful technique in the management of interstitial lung diseases [5, 6]. The lymphocytosis of the BAL has been proposed as an index of disease activity in pulmonary sarcoidosis: a high percentage of lymphocytes in the BAL corresponding to a "high intensity alveolitis" [7, 8]. Furthermore, the pre-treatment BAL lymphocytosis was recently reported to predict steroid responsiveness in sarcoidosis [9].

Other groups have, however, failed to demonstrate a correlation between BAL lymphocytosis and disease activity and consider an increased T helper/T suppressor ratio ($T_4/T_8$) in the BAL as a better index for active disease [10, 11]. This BAL $T_4/T_8$ has also been shown to drop to normal values with regression of the disease. In contrast with these cellular studies, the usefulness of soluble components in BAL has not yet been demonstrated [6].

We recently reported that BAL $\alpha_2$-macroglobulin ($\alpha_2$M) is increased in BAL from patients with active interstitial lung diseases [12]. These studies confirm and extend our previous observations and include data for $\alpha_1$-protease inhibitor ($\alpha_1$PI) which is the predominant antiprotease in the serum, whose fate in interstitial lung disease has not yet been explored. The purpose of this study is to evaluate in BAL different cellular parameters (including lymphocyte subpopulations) and soluble components (including the two major antiproteases $\alpha_1$PI and $\alpha_2$M) during the course of interstitial lung disease.

Material and methods

Study populations

1. Single lavage group. After agreement of the local Ethics Committee, the following non-smoking individuals were investigated: 16 normal volunteers (group I) and 35 consecutive patients with biopsy proven sarcoidosis (22 newly diagnosed and 13 with disease known for 1–5 years before referral to us) further subdivided into group II and group III.

Group II consisted of 24 untreated patients defined as 'active' sarcoidosis based on BAL lymphocytosis >15%. These included 10 patients with radiological stage I (hilar adenopathy), 7 with stage II (hilar adenopathy and lung infiltrates) and 7 with stage III (lung infiltrates alone). Group III included 11 patients with pulmonary sarcoidosis considered as 'inactive' based on BAL lymphocytosis <15% (3 stage I, 3 stage II, 5 stage III) including 3 patients treated with steroids. Group IV consisted of 7 untreated patients with typical hypersensitivity pneumonitis. Three patients were pigeon breeders, two fancy bird breeders and two had farmer's lung.

2. Serial lavages group. Twelve patients with
pulmonary sarcoidosis were sequentially lavaged (one, two or three times) after the initial BAL. This group included two patients under steroid treatment at the time they were referred to us.

**Bronchoalveolar lavage**

Bronchoalveolar lavage was performed as described [5, 12, 13] through the fibre-optic bronchoscope using 200 ml sterile 0.9% saline solution in 50 ml aliquots instilled and gently aspirated. The first aliquot (bronchial lavage) was discarded and the studies were performed on the three following aliquots. The recovered fluid was filtered through a single layer of gauze to remove gross mucus and an aliquot was saved for a total cell count, using a Coulter® cell counter, and for cell differential. The lavage was then centrifuged and the cell pellet was used in lymphocyte subpopulation studies, while the supernatant was kept at -20 °C for protein analysis.

**Cellular studies**

Cell differentials were performed on cytospin preparations, using the same cytocentrifuge (Cytospin I) at the same centrifuge speed to minimize artificial variations between the different samples [14]. Lymphocyte subpopulations in blood (obtained on the day of the lavage) and BAL were determined using the fluorescent monoclonal antibodies OKT11 (T lymphocytes), OKT4 (helper) and OKT8 (suppressor) according to Reinherz et al. [15]. The cell bound fluorescence in the lymphocyte population was determined using an Epics C Coulter® flow cytometer. Data are expressed as percentage of positive (OKT11, OKT4, OKT8) cells in the total lymphocyte population.

**Proteins assays**

Serum levels of albumin, IgG, IgM and α1PI were determined by immunonephelometry [16]. The immunoradiometric assay (IRMA) was used for measurement of α2M in the serum and for all proteins in BAL. This assay, previously described in detail [12, 17], does not require concentration of the BAL fluid. Results are expressed as previously in coefficient of excretion relative to albumin (RCE), to correct for both serum concentration of the different proteins and variable dilution of BAL [12].

\[
RCE = \frac{\text{BAL protein}}{\text{serum protein}} \times \frac{\text{BAL albumin}}{\text{serum albumin}}
\]

**Statistical analysis**

Values in the different groups were tested for significance using an unpaired t-test and correlations between variables were evaluated by linear regression.

**Results**

**Cell differentials and lymphocyte subpopulations**

BAL volumes recovered, total cell counts and cell differentials in each group are given in table I. The lavage fluid from patients with active sarcoidosis and hypersensitivity pneumonitis contained more cells than the BAL from normal individuals and from patients with inactive sarcoidosis. Patients with active sarcoidosis also demonstrated a significantly higher percentage of lymphocytes than normals and patients with inactive sarcoidosis. Patients with hypersensitivity pneumonitis had higher percentages of both lymphocytes and polymorphonuclear neutrophils.

As illustrated in figure 1, the group of patients with active sarcoidosis had a significantly higher T4/T8 ratio in the BAL than the normal group or the groups of patients with inactive sarcoidosis or hypersensitivity pneumonitis. This increased T4/T8 ratio in the BAL from the active sarcoidosis group reflects a combined increase of BAL T4 subpopulations and a decrease of BAL T8 subpopulations. No significant difference was observed in the blood T4/T8 ratio between the different groups (data not shown).

<table>
<thead>
<tr>
<th>Table 1. - Cellular components of BAL</th>
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<tr>
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<tr>
<td><strong>Bal recovery</strong></td>
</tr>
<tr>
<td><strong>Cell count</strong></td>
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<tr>
<td>% macrophages</td>
</tr>
<tr>
<td>Normal n=16</td>
</tr>
<tr>
<td>Sarcoidosis n=35</td>
</tr>
<tr>
<td>Active sarcoidosis n=24</td>
</tr>
<tr>
<td>Inactive sarcoidosis n=11</td>
</tr>
<tr>
<td>Hypersensitivity pneumonitis n=7</td>
</tr>
</tbody>
</table>

*Mean±SD. *p<0.05 when compared to normal group values. PMN: polymorphonuclear neutrophils
Table 2. - BAL immunoglobulins G and M (in RCE)

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Active sarcoidosis n=24</th>
<th>Inactive sarcoidosis n=11</th>
<th>Hypersensitivity pneumonitis n=7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IgG</strong></td>
<td>0.74±0.26*</td>
<td>1.90±0.90**</td>
<td>1.08±0.50*</td>
<td>2.28±1.02*</td>
</tr>
<tr>
<td></td>
<td>(0.20–1.38)*</td>
<td>(0.64–3.62)</td>
<td>(0.60–1.80)</td>
<td>(1.42–4.00)</td>
</tr>
<tr>
<td><strong>IgM</strong></td>
<td>0.08±0.07</td>
<td>0.51±0.43**</td>
<td>0.28±0.20*</td>
<td>1.54±1.21**</td>
</tr>
<tr>
<td></td>
<td>(0.01–0.16)</td>
<td>(0.08–1.65)</td>
<td>(0.12–0.49)</td>
<td>(0.40–4.75)</td>
</tr>
</tbody>
</table>

*mean±sd. * range in brackets, *p<0.05, **p<0.01 when compared to normals, p<0.05 when compared to inactive sarcoidosis; RCE: relative coefficient of excretion.

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Fig. 1. The T4/T8 lymphocyte ratio in BAL from active sarcoidosis patients (SA) (n=24) is significantly increased when compared to normals (N) (n=16) and non-active sarcoidosis patients (SNA) (n=11). No significant difference was observed between hypersensitivity pneumonitis patients (HP) (n=7) and normals. Columns represent means and bars standard deviations. Significant differences were observed between SA and N, between SNA and N, between HP and N and between SA and SNA. Same symbols (*) as in figure 1. ** p<0.01 when compared to normals.

**Immunoglobulins G and M and α2-macroglobulin in BAL**

A significant increase in RCE of α2M (fig. 2) and IgG (table II) is observed in the active sarcoidosis
group (mean values respectively 0.39 and 1.90) compared to normals (respectively 0.05 and 0.74) (p<0.01). RCE of IgM for the active sarcoidosis patients (mean = 0.51) was also higher than that of the normals group (mean = 0.08) (p > 0.05). Patients with hypersensitivity pneumonitis also demonstrated a significant increase in RCE of α2M (0.69), IgG (2.28) as well as IgM (1.54) compared to the normal group (fig. 2, table II).

α1-protease inhibitor levels in BAL

The RCE mean value of α1 PI in BAL from the normal group was 1.24, significantly lower than the corresponding RCE value in the active sarcoidosis group (2.04) (p<0.01) or the hypersensitivity pneumonitis group (1.83) (p<0.05). The mean RCE value in the non-active sarcoidosis group (1.08) was not significantly different from the normal value (fig. 3).

In the hypersensitivity pneumonitis group and in the inactive sarcoidosis groups, a significant correlation between α1 PI RCE and α2M RCE is observed (respectively r = 0.86 and r = 0.57, p<0.05). In the active sarcoidosis group, there is no significant correlation between the two antiproteases RCE (r = 0.34). However, in the group of 17 patients with radiographic stage I and II disease, a significant correlation is again demonstrated (r = 0.67, p<0.05). In contrast, no correlation between α1 PI RCE and α2M RCE is observed in the normal group. No other correlation between protein and cellular data of the BAL was significant in the patient groups.

Follow-up studies

In the group of 12 patients with sarcoidosis, who were lavaged at least twice, eight were considered initially as 'active' and remained untreated. These patients, except one, had initially high α2M RCE and BAL T4/T8 ratios (table III). In subsequent lavages, both α2M RCE values and BAL T4/T8 values remained above the mean range of the corresponding normal values except for one patient who initially suffered stage I disease with erythema nodosum and after eight months was considered free of disease.

Two other patients had initially 'active' disease and high α2M RCE; their α2M RCE dropped to the normal range under treatment (fig. 4).

Finally, two patients were under steroid treatment before being referred to us. Parallel to the progressive withdrawal of the steroids, we observed a gradual increase in RCE of α1 PI in BAL from patients with active sarcoidosis (D) or with hypersensitivity pneumonitis (E) is increased when compared to normals (C) or to patients with inactive sarcoidosis (F). Same symbols (*) and (▲) as figure 1.

Table 3. - BAL data from sequential lavages in "active" untreated sarcoidosis patients

<table>
<thead>
<tr>
<th></th>
<th>Total cell count x10⁶ cells/100 ml</th>
<th>Lymphocytes %</th>
<th>T₄/T₈</th>
<th>α₂M RCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial BAL</td>
<td>36.5*</td>
<td>27.4</td>
<td>9.7</td>
<td>0.37</td>
</tr>
<tr>
<td>n=8</td>
<td></td>
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<tr>
<td>Second BAL after 3-4 months</td>
<td>32.4</td>
<td>28.2</td>
<td>13.8</td>
<td>0.45</td>
</tr>
<tr>
<td>n=8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Third BAL after 6-9 months</td>
<td>26.3</td>
<td>14.7</td>
<td>10.6</td>
<td>0.32</td>
</tr>
<tr>
<td>n=4</td>
<td></td>
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* mean values; RCE: relative coefficient of excretion.
Discussion

Much promise has been expected from the development of the BAL. For example, BAL lymphocytosis or BAL lymphocyte subpopulation studies were proposed as sensitive markers of sarcoidosis alveolitis [6, 8, 10]. Since previous reports suggested that a high lymphocytosis in the BAL reflects a 'high intensity alveolitis', we arbitrarily divided our patients with sarcoidosis into two groups ('active' and 'inactive') using 15% lymphocytes in the total BAL cell population as the discriminating criterion [7]. However, this criterion is not uniformly accepted and more sensitive criteria may be required [18, 19]. The study of lymphocyte subpopulations has been proposed by different groups to better assess the alveolitis in sarcoidosis [10, 20, 21]. As reported by others, we observed in the present study a significant increase of the BAL $T_4/T_8$ ratio in the active sarcoidosis group, without any significant change in the blood $T$ lymphocyte subpopulations when compared to the normal and non-active sarcoidosis groups [10, 22]. No significant change is noticed in the blood or in the BAL lymphocyte subpopulations from patients with inactive sarcoidosis. Different groups reported an increased proportion of OKT$_8$ (suppressor) lymphocytes in BAL from hypersensitivity pneumonitis patients [23–25]. We observed that the mean value of the BAL $T_4/T_8$ in the hypersensitivity group was not significantly different from the normal group. This observation may be related to the delay between the last exposure to the allergen and the lavage procedure as previously reported [26], more than to methodological problems. Patients with active pulmonary sarcoidosis have a higher RCE for $\alpha_2M$ with little overlap with the normal group values. The group of patients with sarcoidosis considered as inactive (based on BAL lymphocytosis) expressed a significantly higher mean value of RCE for $\alpha_2M$ when compared to the normal group. However, ten out of the eleven patients in this group have a RCE for $\alpha_2M$ within the normal range and no symptoms; the $\alpha_2M$ RCE of 0.78 was observed in a patient with 7% lymphocytes in the BAL. However, the patient was symptomatic (fatigue and dyspnoea) and had a dramatic increase in BAL $T_4/T_8$ ratio of 9.3. This supports further the...
concept that the BAL $T_4/ T_8$ ratio provides a more sensitive cellular marker of the alveolitis than the BAL total lymphocytosis.

Although the follow-up studies concerned a limited number of patients over a limited period of time, we observed that patients with active disease kept $\alpha_2M$ RCE values above the normal range when untreated, while the values dropped to the normal range under steroid treatment. Patients with normal $\alpha_2M$ RCE under steroids demonstrated a rise of $\alpha_2M$ RCE back to abnormal values when steroid treatment was discontinued. Furthermore, in these patients, it appears that $\alpha_2M$ RCE changes occur prior to both the BAL lymphocytosis and $T_4/ T_8$ ratio suggesting that $\alpha_2M$ better reflects early changes of the intensity of the alveolitis. As described for $\alpha_2M$, the levels of the major antiprotease ($\alpha_1PI$) were significantly increased in BAL from patients with active pulmonary sarcoidosis or hypersensitivity pneumonitis. Moreover, in the hypersensitivity pneumonitis and inactive sarcoidosis groups (but not in normal volunteers) we observed a linear correlation between the concentrations of these antiproteases after correction of the values for their serum concentration and dilution of the BAL. This occurred despite the large difference in their molecular weight (53,000 for $\alpha_1PI$ and 82,000 for $\alpha_2M$). This correlation was also present in the 17 patients of the active sarcoidosis group with stage I and stage II disease. Whether the remaining 7 patients (stage III) with high $\alpha_2M$ RCE associated with relatively low $\alpha_1PI$ RCE represent a subgroup of patients, or have a different prognosis, remains unknown at this point.

Although our method of immunoassay does not allow us to distinguish between native and complexed antiproteases, or to estimate the antiprotease activity, it still demonstrates a local increase of the two major antiprotease levels in diseases where proteolytic activity is likely to be enhanced.

In conclusion, the measurement of soluble components in BAL in addition to cellular studies may help the clinician in the management of patients with interstitial lung disease. Moreover, three lines of evidence suggest that $\alpha_2M$ may be a better index of the alveolitis than the BAL lymphocytosis or $T_4/ T_8$ ratio: a) the overlap of BAL $T_4/ T_8$ ratios between normals and patients with active sarcoidosis is larger than the overlap of $\alpha_2M$ RCE values; b) in sarcoidosis, the changes of $\alpha_2M$ occur earlier than the changes of BAL $T_4/ T_8$ ratios during the course of the disease; c) the RCE of $\alpha_2M$ is elevated in the acute phase of hypersensitivity pneumonitis while the $T_4/ T_8$ ratios remain normal or decreased. Finally, the correlated increase of the two major antiproteases ($\alpha_1PI$ and $\alpha_2M$) in BAL from patients with interstitial lung disease may be at least part of the defence mechanism against the potential proteolytic activity responsible for the fibrosis occurring in advanced sarcoidosis or hypersensitivity pneumonitis. However, definitive analysis of these data awaits long term studies.

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References


RÉSUMÉ: Il s'agit d'une évaluation de différents composants cellulaires et solubles du lavage broncho-alvéolaire de patients atteints d'une maladie interstitielle pulmonaire. Nous observons une augmentation du rapport des lymphocytes T⁴/T⁸ dans le lavage broncho-alvéolaire, et non dans le sang, chez 24 patients atteints d'une sarcoidose pulmonaire active, par comparaison avec 16 individus normaux, et avec 11 patients avec une sarcoidose pulmonaire inactive. Sept patients atteints de pneumopathie d'hypersensibilité, ont un rapport T⁴/T⁸ normal. Dans la sarcoidose active et dans le groupe de pneumopathie d'hypersensibilité, l'alpha I anti-protéase (alpha I PI) du lavage broncho-alvéolaire est significativement plus marquée que dans le groupe normal, et l'on observe une corrélation significative entre les deux anti-protéases (alpha 2-macroglobuline et alpha 1 PI). Ces données démontrent que les niveaux d'anti-protéases (alpha 1 PI et alpha 2 M) sont augmentées dans le tractus respiratoire inférieur des patients avec maladie pulmonaire interstitielle et que, parmi les composants cellulaires et solubles du lavage broncho-alvéolaire, l'alpaha 2-macroglobuline est un marqueur sensible de l'alvéolite.