Bronchoalveolar lavage fibronectin in patients with sarcoidosis: correlation to hyaluronan and disease activity

L. Bjermer*, A. Eklund**, E. Blaschke***

ABSTRACT: Bronchoalveolar lavage was performed in 51 patients with sarcoidosis and in 21 healthy nonsmokers. The concentration of fibronectin was significantly higher (p<0.001) in lavage fluid from sarcoid patients (median 267 μg·l⁻¹) than in that of controls (46 μg·l⁻¹). Furthermore, a significantly higher concentration of fibronectin was found in patients with active disease than in those with inactive disease (p<0.001). In a six month follow-up prospective, patients with a progressive disease course had significantly higher levels of fibronectin than those who had a stable or regressive disorder (p<0.01). Correspondingly, lavage hyaluronan was higher (p<0.001) in sarcoid patients (35 μg·l⁻¹) than in controls (9 μg·l⁻¹) and higher (p<0.01) in those with active than in those with inactive disease. Patients with progressive disease had higher (p<0.01) concentrations of hyaluronan than those in whom the disease was stable. A significant correlation was found between lavage fibronectin levels and hyaluronan (r=0.81, p<0.001). The percentage of mast cells was also higher in patients with active than in those with inactive disease (p<0.01) and higher in progressive than in stable sarcoidosis (p<0.001). Ten out of 10 patients with progressive disease had mast cells ≥0.5%, hyaluronan ≥50 μg·l⁻¹ and fibronectin ≥50 μg·l⁻¹ compared to eight out of 41 patients with stable or regressive disease.

We conclude that high concentrations of fibronectin and hyaluronan in BAL fluid, especially when combined with a concomitant elevation of BAL mast cells, in sarcoidosis on a short-term basis seem to reflect disease activity as well as disease progression. However, prognostic parameters have to be evaluated with caution, as patients with inactive and stable disease also usually have higher concentrations of these substances than do normal subjects.

The outcome of sarcoidosis in the lungs is usually favourable, but in a minor proportion of patients the inflammatory reaction progresses to fibrosis with functional impairment. Determining markers which could predict the course of the disease has for a long time been a challenge. However, most of the reported prognostic factors have later been shown to be of a limited value. During the last decade the bronchoalveolar lavage technique (BAL) has been used to discover early signs of an on-going fibrosing process. Enhanced proportions of various alveolar cells and soluble components in the BAL fluid have been reported to be associated with active and possibly progressive disease [1–3]. Special interest has been paid to constituents such as fibronectin, hyaluronan (hyaluronic acid) and procollagen III peptide, known to reflect macrophage and fibroblast activity [4–6]. However, it is still not clear whether increases in these substances adequately reflect fibrosing processes in the lung. Increased concentrations of these substances might also reflect enhanced activity of the inflammatory cells in the bronchoalveolar space rather than a fibrogenic process.

In the present study, hyaluronan and fibronectin were measured in the BAL fluid from sarcoid patients whose clinical course was evaluated six months before and after the BAL. Both constituents were significantly higher in patients with active disease than in those with inactive disease, as well as in progressive versus stable disease.

**Subjects and methods**

**Subjects**

Fifty one patients with histopathologically verified sarcoidosis were included in the study (27 women, median age 57 yrs; range 29–71 yrs; and 24 men, median age 44 yrs, range 30–71 yrs). None of the patients had been...
treated with steroids in the past six months. Twenty one healthy nonsmoking volunteers served as controls (median age 31 yrs; range 21–57 yrs). All patients and controls gave their informed consent and the study was approved by the local Ethics Committee.

Clinical evaluation

The chest radiographic findings were classified as follows: stage 0 = normal; stage I = bilateral hilar lymphoma (BHL); stage II = BHL with parenchymal infiltrates; and stage III = parenchymal infiltrates without BHL (table 1). Lung volumes (vital capacity (VC) and forced expiratory volume in one second (FEV₁)) were determined by using a Bernstein spirometer and the diffusion capacity was measured according to the single-breath carbon monoxide technique (DLCo). The results are presented as a percentage of predicted normal values [7].

Table 1. - Chest X-ray stage from 54 patients with sarcoidosis at time of bronchoalveolar lavage investigation

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td>14</td>
<td>10</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>n=51</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inactive disease</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>n=13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active disease</td>
<td>7</td>
<td>9</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>n=38</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stable/regressive</td>
<td>14</td>
<td>8</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>n=41</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progressive</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>n=10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

0 = normal; I = bilateral hilar lymphadenopathy (BHL); II = BHL + lung infiltrates; III = lung infiltrates without BHL. Patients were also subdivided according to the course during a 6 month follow-up period, with stable or regressive disease compared to progressive disease.

The patients were classified according to the activity of the disease. The classification was based on the chest radiograph and lung function data six months prior to and at the time of the BAL. Patients without clinical symptoms, with an unchanged radiograph and unaltered VC, FEV₁, and DLco during the past 6 months were regarded as having inactive sarcoidosis. Important clinical symptoms reflecting clinical activity were: long-standing irritating cough, periods of low-grade fever, increasing dyspnoea on effort, tiredness previously absent, and recent weight loss >3 kg. In order to avoid analysis bias, the activity criteria were primarily based on clinical data read blindly without prior knowledge about BAL data. The patients were also classified according to the clinical course during the 6 month follow-up period after the lavage. Patients with increased pathological radiographic findings and/or a deteriorating lung function with >10% fall in VC and FEV₁ or >15% decline in DLco during the observation period were regarded as having progressive disease.

Bronchoalveolar lavage (BAL)

Before bronchoscopy patients and controls were given atropine or scopolamine combined with morphine or pethidine chloride, subcutaneously. The upper respiratory tract was anaesthetized with lignocaine hydrochloride. A fiberoptic bronchoscope (Olympus BF IT or BF 4B2, Tokyo, Japan) was wedged in the anterior part of the middle lobe and 240 ml sterile Krebs' Ringer phosphate buffer (pH 7.3) at 37°C was infused in boluses of 60 ml. The fluid was gently aspirated immediately after each instillation. The recovery of fluid instilled was 47±10% with no statistical differences between patients and controls. The lavage fluid was kept on ice and filtered through a nylon filter with a pore diameter of 100 μm (Syntab Product AB, Malmö, Sweden). The cells were centrifuged at 400 x g for 15 min and then resuspended in balanced salt solution to a concentration of 10⁶ cells·ml⁻¹, excluding epithelial cells. The total number of cells was counted in a Bürker chamber. Cytological smears were made in a cytospin centrifuge, 1,000 rpm for 5 min (Cytospin Shandon, Southern Frod. Ltd, Runcorn, England) with 5x10⁴ non-epithelial cells·slide⁻¹.

Cell analysis

The cytocentrifuge preparations were stained according to May-Grünwald-Giemsa for cellular differential counting. Mast cells were stained with acid toluidine blue and counter-stained with Mayer's acid haematoxylin [8]. Ten visual fields with 16x magnification were counted. All cell counts were expressed as a percentage of total cells recovered. Albumin was determined by a nephelometric technique, and results are given in mg·l⁻¹.

Fibronectin

Fibronectin (FN) was analysed by a double sandwich enzyme-linked immunoassorbent assay (ELISA) developed at the department of clinical chemistry, Karolinska Hospital, Stockholm and described elsewhere [9]. Briefly, microtitre plates (NUNC, Denmark) were coated with rabbit antihuman fibronectin antibodies (Dakopatts, Denmark) in phosphate-saline buffer, pH 7.2. After addition of unconcentrated BAL fluid samples the plates were incubated at room temperature for 2 h. Horseradish peroxidase-labelled antihuman fibronectin (Dakopatts) was added as second antibody and the plates were incubated for 1 h. The amount of bound peroxidase, which is proportional to the amount of fibronectin in the
sample, was measured by analysing its enzymatic activity on orthophenyldiamine. Serum fibronectin of nephelometric quality from Behring-Hoechst (Frankfurt am Main, FRG) was used as standard. Concentrations of FN were expressed in $\mu$g·l$^{-1}$. The detection limit was 10 $\mu$g·l$^{-1}$. Intra- and interassay coefficients of variation were 3.7 and 6.4%, respectively.

**Hyaluronan (hyaluronic acid)**

Hyaluronan (HA) was analysed in duplicate in unconcentrated lavage fluid by a radiometric assay in principle according to Engström-Laurent [10] using the Pharmacia HA-test kit (Pharmacia, Uppsala, Sweden). Concentrations were expressed in $\mu$g·l$^{-1}$ and the detection limit was 5 $\mu$g·l$^{-1}$. The intra- and interassay coefficients of variation were 4 and 6.4%, respectively.

**Statistical analyses**

Because the values observed in patients with sarcoidosis were not normally distributed, results are given as medians and interquartile ranges (i.q.). They were analysed using Wilcoxon's non-parametric rank sum test and Spearman's rank correlation coefficient. In figures 1 and 2 data are presented as multiple box and whisker plots showing median value, upper and lower quartile and range. Extreme values are plotted separately.

In order to estimate the predictive value of different BAL parameters, a multiple analysis of variance test (MANOVA) was used (SPSS/PC+, SPSS inc. Chicago). The numbers of mast cells and the concentrations of HA and FN were close to log-normal distributed according to the Kolmogorov-Smirnov one sample test for goodness-of-fit and, therefore, the log-values of these parameters were used in the MANOVA test.

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**Fig. 1.** Bronchoalveolar lavage cell data on the percentage of macrophages, lymphocytes and mast cells from 51 sarcoidosis patients divided according to estimated disease activity and to 6 month follow-up prognosis. Inactive (IAD), active (AD), stable/regressive and progressive disease. Data are presented as multiple box and whisker plots and statistical comparison were made with Wilcoxon's non-parametric rank sum test. For further details, see materials and methods.

**Fig. 2.** Bronchoalveolar lavage fluid concentrations of hyaluronan, fibronectin and albumin. The concentration data were found to be log normal distributed and are therefore presented with a logged y-axis. For abbreviations and details of presentation see legend to figure 1.
**Lung function data**

At the time of the BAL investigation the vital capacity, forced expiratory volume and diffusion capacity did not differ among the groups of patients with inactive or active disease and stable or progressive disease, respectively, (table 2).

**Cellular BAL findings in patients vs controls**

In the patients with sarcoidosis the median cell concentration was not different from the controls, 7.0 \times 10^7 (i.q. 5.6–8.8) cells \cdot l^{-1} and 6.4 \times 10^7 (5.0–8.0) cells \cdot l^{-1}, respectively, (table 3). The proportions of macrophages 62% (38–80%), lymphocytes 31% (17–47%) and mast cells 0.43% (0.13–0.95%) differed significantly (p<0.001 for all) from the controls, 92% (82–95%), 6% (4–14%) and 0.0% (0.0–0.1%), respectively.

**Table 2. - Vital capacity (VC) forced expiratory volume (FEV\textsubscript{1}) and single-breath diffusion capacity (DLco) presented as % of predicted normal, from 51 patients with sarcoidosis, at time of bronchoalveolar lavage investigation**

<table>
<thead>
<tr>
<th></th>
<th>VC</th>
<th>FEV\textsubscript{1}</th>
<th>DLco</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients n=51</td>
<td>93±10</td>
<td>97±14</td>
<td>76±17</td>
</tr>
<tr>
<td>Inactive n=13</td>
<td>93±9</td>
<td>96±13</td>
<td>79±15</td>
</tr>
<tr>
<td>Active n=38</td>
<td>93±10</td>
<td>98±15</td>
<td>74±16</td>
</tr>
<tr>
<td>Stable/regressive n=41</td>
<td>93±9</td>
<td>97±15</td>
<td>78±15</td>
</tr>
<tr>
<td>Progressive n=10</td>
<td>91±12</td>
<td>98±12</td>
<td>67±20</td>
</tr>
</tbody>
</table>

Patients were also subdivided according to the course during a 6 month follow-up period, with stable or regressive disease compared to progressive disease. Data are presented as mean±sd.

**Soluble BAL components in patients vs controls**

As presented in table 4 the concentrations of lavage albumin, fibronectin and hyaluronan all differed significantly (p<0.001) from those of the controls. The concentrations of albumin were about twice as high in sarcoidosis patients as in the controls. Correspondingly, the concentrations of fibronectin and hyaluronan were about ten times higher in the patients with sarcoidosis than in the controls.

**Cellular and soluble components of BAL fluid in patients with active vs inactive sarcoidosis**

Thirty nine patients were regarded as having active disease but their total cell numbers did not differ from those of patients with inactive disease (fig. 1). They had a significantly (p<0.001) higher percentage of lymphocytes, 38% (20–66%), than those with inactive disease, 14% (5–18%). Consequently the percentage of macrophages was lower (p<0.001) in active disease, 58% (22–68%), than in inactive disease, 83% (77–92%). Active disorder patients had a higher (p<0.01) percentage of mast cells than those with inactive sarcoidosis, 0.60% (0.25–1.00%) and 0.13% (0.13–0.38%), respectively.

The concentrations of all soluble components measured were higher in active than in inactive disease (fig. 2). The differences were most pronounced for albumin, 145 (90–209) mg \cdot l^{-1} and 62 (42–72) mg \cdot l^{-1} (p<0.001), and for fibronectin, 564 (208–1,245) \mu g \cdot l^{-1} and 92 (69–172) \mu g \cdot l^{-1} (p<0.001).

A highly significant correlation was found between HA and FN (r=0.81, p<0.001, fig. 3). A weaker correlation was found between the total amounts of lymphocytes and lavage concentration of FN (r=0.63, p<0.001) and HA (r=0.50, p<0.001), respectively. For the total numbers of mast cells versus HA and FN the correlations were (0.45, p<0.002) and (0.47, p<0.001), respectively.

The upper normal values (mean±2sd) for HA and FN were 20 \mu g \cdot l^{-1} and 85 \mu g \cdot l^{-1}. Forty seven of the 51 patients had HA concentrations above this level, and 42 had increased FN levels. Eight out of 12 in the inactive disease group had both parameters elevated compared to 34 out of 38 in the active disease group.
Table 4. - Bronchoalveolar biochemical data from patients versus controls.

<table>
<thead>
<tr>
<th></th>
<th>Albumin</th>
<th>Fibronectin</th>
<th>Hyaluronan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>103</td>
<td>267</td>
<td>55</td>
</tr>
<tr>
<td>n=51</td>
<td>62-168</td>
<td>98-742</td>
<td>31-87</td>
</tr>
<tr>
<td>Controls</td>
<td>45</td>
<td>46</td>
<td>9</td>
</tr>
<tr>
<td>n=21</td>
<td>26-60</td>
<td>38-72</td>
<td>5-14</td>
</tr>
</tbody>
</table>

p <0.001 

Data are presented as median values with upper and lower quartiles. Statistical comparison between the groups were performed with Wilcoxon's non-parametric rank sum test.

Fig. 3. - Bronchoalveolar lavage fluid concentrations of hyaluronan and fibronectin from 51 patients with sarcoidosis. *: The correlation was estimated with Spearman's rank correlation test.

Table 5. - Predictive value of different bronchoalveolar lavage parameters regarding the course of the disease after 6 months follow-up.

<table>
<thead>
<tr>
<th>BAL parameter</th>
<th>Progr.</th>
<th>Stat.</th>
<th>Regr.</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Le &gt;35%</td>
<td>6/9</td>
<td>9/17</td>
<td>7/24</td>
<td>67</td>
<td>72</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>b) PMN &gt;15%</td>
<td>0/9</td>
<td>3/17</td>
<td>0/24</td>
<td>0</td>
<td>0</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>c) Mast ≤0.5%</td>
<td>10/10</td>
<td>8/17</td>
<td>6/24</td>
<td>100</td>
<td>82</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>d) HA ≥50 µg·l⁻¹</td>
<td>10/10</td>
<td>10/17</td>
<td>9/24</td>
<td>100</td>
<td>76</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>e) FN ≥350 µg·l⁻¹</td>
<td>10/10</td>
<td>9/17</td>
<td>7/24</td>
<td>100</td>
<td>79</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>f) c+d+e</td>
<td>10/10</td>
<td>6/17</td>
<td>2/24</td>
<td>100</td>
<td>90</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The sensitivity and specificity for prediction of prognosis is calculated. *: Chi² test; Le: lymphocytes; PMN: polymorphonuclear neutrophils; Mast: mast cells; HA: hyaluronan; FN: fibronectin; BAL: bronchoalveolar lavage.
Discussion

The present findings of increased proportions of alveolar lymphocytes and mast cells in patients with sarcoidosis, especially in those with active disease is in agreement with earlier reports [11, 12]. Similarly, the present findings of increased concentrations of albumin, fibronectin (FN) and hyaluronan (HA) found in the BAL fluid of sarcoid patients, and of significantly higher concentrations of these components in active than in inactive disease, confirm previous reports [13]. A clearly significant correlation was found between elevated numbers of lymphocytes and increased concentrations of HA and FN, respectively. The increase of HA and FN also correlated, however less strongly, with the increased numbers of mast cells. The presence of concomitant elevated levels of these markers, however, indicates that they are linked to a common pathophysiological pathway. The accumulation of cells in the alveolar space in sarcoidosis reflects an increased recruitment of cells due to enhanced chemotactic activity [14].

While the role of lymphocytes in sarcoidosis alveolitis has been investigated frequently [15, 16], reports on mast cells are few. It is reasonable to assume that the mast cell increase may represent a later stage of the inflammatory processes in sarcoidosis, i.e. being more closely related to the development of tissue damage and fibrosis. In the present study, increase of mast cells was the single most important predictor of a progressive course of the disease. This finding is also in agreement with an earlier study reporting that an increase in the proportion of mast cells, and to a much lesser degree increase of lymphocytes, was found to be especially high in those with active disease [12]. Similarly, the present findings of increased proportions of lymphocytes and increased concentrations of albumin, fibronectin and hyaluronan in the BAL fluid of sarcoid patients, and of significantly higher concentrations of these components in active than in inactive disease, confirm previous reports [13]. Possibly lymphocytes release mediators capable of stimulating the macrophages to secrete fibronectin. The outcome of sarcoidosis is generally favourable but a minor proportion of subjects may develop fibrosis. The variable course of the disease makes it difficult to predict the outcome. As the fibrogenic process probably proceeds in different parts of the lungs at various stages [14], it may be hazardous to consider the concentration of a marker at one site as representative of changes in the whole lung. Prognostic indicators have, therefore, to be evaluated with great caution. Keeping this in mind we would nevertheless, like to point out that concomitantly high concentrations of hyaluronan and fibronectin in lavage fluid in sarcoidosis seem to reflect disease activity and, for the timespan of months, even disease progression. However, patients with inactive disease and stable disease also have concentrations that are higher than those of the controls.

The possibility of increased BAL fluid levels of HA and FN due to increased permeability must be considered. Related to albumin, however, the concentration of HA was on average more than 500 times higher and of FN more than 100 times higher than the serum values. Beside this, no correlation was found between the BAL concentrations of FN and the concentrations found in serum (data not shown). Therefore, it seems highly unlikely that the increased concentrations of HA or FN to any substantial degree could be due to passive leakage from blood to alveoli.

In conclusion, the concentrations of hyaluronan and fibronectin in the BAL fluid from patients with sarcoidosis were found to be especially high in those with active and progressive disease. These patients also showed an increased proportion of mast cells in their BAL fluid.

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References


La fibronectine du lavage broncho-alvéolaire chez les patients atteints de sarcoïdose; corrélation avec l’hyaluronan et l’activité de la maladie. L. Bjerner, A. Ekblud, E. Blaschke.

RÉSUMÉ : Un lavage broncho-alvéolaire a été exécuté chez 51 patients atteints de sarcoïdose et chez 21 non fumeurs bien portants. La concentration de fibronectine est significativement plus élevée (p<0.001) dans le liquide de lavage des patients atteints de sarcoïdose (médiane 267 µg/l) que chez les contrôles (46 µg/l). D'autre part, une concentration significativement plus élevée de fibronectine a été décelée chez les patients dont la maladie est active par rapport à ceux où elle est inactive (p<0.001). Dans la perspective d'un follow-up de six mois, les patients dont la maladie a un décours progressif ont des niveaux de fibronectine significativement plus élevés que ceux qui ont une maladie stable ou régressive (p<0.01). Parallèlement, l’hyaluronan du lavage est plus modéré (p<0.001) chez les patients atteints de sarcoïdose (55 µg/l) que chez les contrôles (9 µg/l) et plus modéré (p<0.01) chez ceux dont la maladie est inactive que chez ceux où elle est inactive. Les patients avec maladie progressive ont des concentrations plus élevées d’hyaluronan (p<0.01) que ceux dont la maladie est stable. Une corrélation significative a été décelée entre les niveaux de fibronectine dans le lavage et l’hyaluronan (r=0.51, p<0.001). Le pourcentage de mastocytes est également plus élevé chez les patients dont la maladie est active que chez ceux où elle est inactive (p<0.01) et plus élevé dans les sarcoïdoses progressives que dans les sarcoïdoses stabilisées (p<0.001). Dix patients sur dix dont la maladie est progressive ont des taux de mastocytes ≥5,5% d’un hyaluronan ≥50 µg/l, et une fibronectine ≥350 µg/l, par comparaison avec dix seulement de 41 patients dont la maladie est stable ou régressive. Nous concluons que des concentrations élevées de fibronectine et d’hyaluronan dans le liquide de lavage alvéolaire, particulièrement en combinaison avec une élévation concomitante des mastocytes du BAL dans la sarcoïdose, semblent refléter à court terme l’activité de la maladie aussi bien que la progression de celle-ci. Toutefois, les paramètres de pronostic doivent être évalués avec prudence, puisque les patients atteints de maladie inactive ou stabilisée ont habituellement des concentrations de ces substances supérieures à celles des sujets normaux.