Special neutrophil elastase inhibitory activity in BAL fluid from patients with silicosis and asbestosis


Pneumoconiosis is defined as the disease resulting from a chronic exposure to different inorganic dusts. In order to assess the lung defence against the effects of dust exposure, we studied the bronchoalveolar lavage (BAL) fluids from 30 silicotic patients (9 of them having a diagnosis of progressive massive fibrosis (PMF)) and 8 subjects with a diagnosis of asbestosis. Total protein content, N-acetyl-β-D-glucosaminidase activity, free elastase-like activity, immunoreactive α₁-protease inhibitor (α₁PI) and neutrophil elastase inhibitory capacity (NEIC) were determined, and the values obtained were compared to those of 14 control BAL fluids. In all of the patients, our data showed a significant increase of total protein content and free elastase-like activity. In contrast, N-acetyl-β-D-glucosaminidase activities did not reach statistical significance. Values concerning immunoreactive α₁PI and NEIC were significantly raised only in patients with PMF and with asbestosis. When the ratio NEIC/immunoreactive α₁PI was calculated, a significant difference was noticed in the asbestosis group; on the other hand, this ratio was significantly reduced in the group of PMF patients. After neutrophil elastase addition, an electrophoretic study by SDS-PAGE and immunoblotting was carried out; it showed more proteolyzed α₁PI in the BAL fluids having a lowered NEIC/α₁PI ratio. These facts could be explained by the presence of inhibitors of neutrophil elastase different from α₁PI.

Material and methods

Patient and control populations

The patient population was composed of two groups of patients suffering from occupational lung diseases. Since cigarette smoking causes inflammation of the lower respiratory tract, inhibits asbesos clearane [9] and therefore worsens asbestosis [10], we evaluated only nonsmoking subjects.

The first group was composed of 30 patients with silicosis (27 men who were coal workers and three women working with abrasive powders). The subjects with
silicosis were divided into two sub-groups: 21 patients with simple silicosis and the 9 patients with a diagnosis of massive fibrosis (PMF). The diagnosis was based on radiological findings according to the Bureau International du Travail.

The second group consisted of eight subjects having a diagnosis of asbestosis (all male). The diagnosis was based on the history of exposure, on radiological findings and the characterization of the fibres.

The control population was composed of 14 nonsmoking subjects, four of them were healthy volunteers, the others underwent routine fibroptic bronchoscopy. All the controls had normal pulmonary function tests and chest X-rays.

Informed consent was obtained from all subjects.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed in Calmette Hospital, Lille, using 250 ml of sterile saline solution in aliquots 5x50 ml [11], with immediate gentle vacuum aspirations after each aliquot. The fluid recovered from the first aspiration was discarded because it is thought to be representative of the bronchial level [12, 13]. The fluids from the other aspirations were pooled and immediately centrifuged at 800 g for 10 min. The supernatant fluid was frozen in aliquots for later assays.

Cell analysis

The cell pellet obtained after centrifugation of the bronchoalveolar lavage was washed twice with Hank's medium. The total cells were counted in a haemocytometer chamber and the differential count on smears stained by the May-Grünewald method. The results of the cell count were expressed as the total number of cells for the recovered fluid. The relative amount of each type of cell was expressed as a percentage of cellularity.

Biochemical analysis

The determinations were all performed on native uncentrifugated fluid in duplicate. The results were expressed per ml of recovered BAL fluid.

Protein content was measured by the Coomassie Blue method [14] using human serum albumin as the standard. The results were expressed as µg·ml⁻¹.

N-acetyl-β-D-glucosaminidase activity was determined using 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide as described previously [4]. Enzymatic activity was expressed as nmol of substrate released·min⁻¹·ml⁻¹ (arbitrary units).

Free elastase-like activity (amidolytic activity) was quantitated using the synthetic substrate succinyl-(trialanyl)-paranitroanilide (SLAPN, Biosys) after a 2 h incubation at 37°C with the BAL fluids [6]. In the same way, the activity of dilutions of a solution of neutrophil elastase was evaluated and compared to those of BAL fluids. The neutrophil elastase was purified from purulent sputum according to MARTODAM et al. [15] and found to be more than 90% active by active site titration using published kinetic constants [16]. Therefore, the free elastase-like activity was expressed as neutrophil elastase equivalent (10⁻⁵ mol·ml⁻¹).

Immunoreactive α₁-protease inhibitor (α₁PI) was determined by immunonephelometry-laser as previously described [17]. A Hyland-laser nephelometer PDQ (Hyland Laboratories) was used with procedures recommended by the manufacturers (Hyland α₁PI-antibody and Hyland-test standard A). This method was shown to give accurate results even if BAL α₁PI is in a complexed and/or a proteolyzed form [17].

The use of some commercial standards for α₁PI evaluation was recognized as yielding overestimated values by radial immunodiffusion [18]. Since an international standard is not yet available, we standardized the Hyland α₁PI standard with serum α₁PI prepared in the laboratory [19], the purity of which was checked by immunoelectrophoresis and analysis in SDS-PAGE. The preparation was quantified by the Lowry method. Dilutions of the α₁PI solution were prepared with the working buffer (10 mM sodium phosphate pH 7.4, containing 0.14 M NaCl, 2 g% TWEEN 20 and 40 g% polyethyleneglycol Mr 6,000) with 0.050 g% human serum albumin in order to take into account the protein composition of the BAL fluids. The concentration of α₁PI solution was calculated from the nephelometric measurements of α₁PI dilutions: it was 10% lower than that determined by the Lowry method. Taking into account the variation coefficients of the nephelometric method [17] (within-day reproducibility and day-to-day precision), we considered the BAL measured α₁PI values as the true values; they were converted in mol·ml⁻¹ using a Mr equal to 52,000.

The neutrophil elastase inhibitory capacity (NEIC) was measured by hydrolysis of L-arginine-β-nitroanilide (S 2484, Kabi diagnostica) as previously described [20]. Briefly a constant amount of neutrophil elastase (0.05 µM) was incubated for 10 min at 25°C with increasing amounts of BAL fluid in saline buffer (sodium phosphate 10 mM pH 7.4, NaCl 0.3 M). After addition of the substrate (final concentration 0.45 mM) the residual activity was measured at 410 nm. The NEIC was calculated from the point of functional equivalence (determined by linear regression) and expressed as moles of elastase inhibited·ml⁻¹ BAL fluid.

The electrophoretic studies were carried out according to LAEMMLI [21] in a 5–25% polyacrylamide gradient slab gel in the presence of SDS (SDS-PAGE). The dimensions of the gels were 150 x 150 x 1 mm. Before being electrophoresed, the BAL fluids were analysed for protein and α₁PI contents. Volume samples were calculated in order to separate 20 µg protein and loaded by refill if necessary. Neutrophil elastase was added to the BAL fluids, in order to have about a 1.5 fold excess over α₁PI content (mol·ml⁻¹). The mixtures were incubated for 15 min at room temperature before the beginning of the electrophoresis procedure. Proteins separated by SDS-PAGE were transferred electrically to nitrocellulose paper using an LKB Multiphor II Novablot, with the
discontinuous buffer system recommended by the manufacturers (anode solution pH 10.4: Tris 0.3 M, 20% methanol, cathode solution pH 7.6: 6-amino-n-hexanoic acid 40 mM, 20% methanol).

The nitrocellulose sheets were stained by an immunoperoxidase method [22] using 4-chloro-1-naphthol as the developer [23]. The antibodies against α1PI were from Dako. The antiserum against neutrophil elastase was obtained by immunizing rabbits with neutrophil elastase purified from purulent sputum according to Martodam et al. [15].

Statistics

The results were expressed as mean±standard deviation. Significance of differences between groups was determined by Student's t-test. When correlations between two variables were found, a linear regression was calculated. Significance was determined at p<0.05.

Results

General characteristics of BAL

The amount of fluid obtained in subjects with silicosis was decreased as compared to the controls, but was not significantly different in asbestosis patients (table 1).

Table 1. - General characteristics of bronchoalveolar lavage

<table>
<thead>
<tr>
<th></th>
<th>Recovered fluid (ml)</th>
<th>Number of cells·mL⁻¹·x10⁶</th>
<th>Differential cell count %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>157±27</td>
<td>6.5±2.4</td>
<td>87±7</td>
</tr>
<tr>
<td></td>
<td>n=14</td>
<td>n=10</td>
<td>n=6</td>
</tr>
<tr>
<td>Silicosis</td>
<td>118±37</td>
<td>24±21</td>
<td>85±12</td>
</tr>
<tr>
<td></td>
<td>n=30</td>
<td>n=23</td>
<td>n=20</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.001</td>
<td>p&lt;0.005</td>
<td>NS</td>
</tr>
<tr>
<td>Asbestosis</td>
<td>111±52</td>
<td>15±6</td>
<td>81±16</td>
</tr>
<tr>
<td></td>
<td>n=8</td>
<td>n=8</td>
<td>n=6</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

ns: non significant. Statistical evaluations were calculated from patients versus controls.

The number of total cells was significantly higher in the patients with silicosis when compared to controls (table 1), while no differences were found in the asbestosis patients. The percentages of macrophages and lymphocytes in each disorder were similar to that of controls. Even if the mean percentage of neutrophils was elevated in both groups of patients, the differences between patients and controls did not reach statistical significance.

Protein content

Total protein content was significantly elevated for all of the patients (table 2). No differences were noted between the different groups of patients.

For N-acetyl-β-D-glucosaminidase, the values obtained for all patients were higher than those of control subjects (table 2). However, the differences were not significant. It should be noted that the values corresponding to PMF patients did not significantly differ from those of the other silicotic patients.

Free elastase-like activity

Free elastase-like activity was detected in 3 out of the 14 controls, in 14 out of 21 patients with simple silicosis, in 4 out of 9 patients with PMF, and in 6 of the 8 patients with asbestosis. Under our experimental conditions (2 h incubation), all the values obtained were very low (10⁻¹³ mol·ml⁻¹) in controls as well as in patient fluids. However, the differences between patients and controls were significant (table 2).

Immunoreactive α1PI

In order to establish the mean values for immunoreactive α1PI, we did not use results <1.5x10⁻¹¹ mol·ml⁻¹ since under our experimental conditions, this value represents the lower detection limit of the method. A concentration <1.5x10⁻¹¹ mol·ml⁻¹ was observed in the BAL of six control subjects and one patient with simple silicosis. In patients with silicosis with and without PMF, the mean value of immunoreactive α1PI was higher than in controls (table 2). There was no significant difference for patients with simple silicosis, while for the group of PMF patients the difference was significant. A significant difference was also observed in the patients with asbestosis. A positive and significant correlation was found between total protein content and immunoreactive α1PI in the patients with simple silicosis (p<0.02) and in the patients with PMF (p<0.02). On the contrary no correlation was found for these two parameters in the patients with asbestosis.
Table 2. - Biochemical analyses of bronchoalveolar lavage fluids

<table>
<thead>
<tr>
<th></th>
<th>Total protein</th>
<th>N-acetyl-β-D-glucosaminidase</th>
<th>Free elastase-like activity</th>
<th>Immunoactive α₁,PI</th>
<th>Neutrophil elastase inhibitory capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg·mL⁻¹</td>
<td>units</td>
<td>10⁻¹³ mol·mL⁻¹</td>
<td>10⁻¹¹ mol·mL⁻¹</td>
<td>10⁻¹¹ mol·mL⁻¹</td>
</tr>
<tr>
<td>Controls</td>
<td>48±24</td>
<td>1±0.7</td>
<td>4±6</td>
<td>&lt;1.5</td>
<td>1.7±0.5</td>
</tr>
<tr>
<td>n=14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silicosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simple</td>
<td>81±52</td>
<td>2±2</td>
<td>22±25</td>
<td>&lt;1.5</td>
<td>2.6±2</td>
</tr>
<tr>
<td>n=21</td>
<td>p&lt;0.02</td>
<td>ns</td>
<td>p&lt;0.02</td>
<td>3.3±2.9</td>
<td>ns</td>
</tr>
<tr>
<td>PMF</td>
<td>118±57</td>
<td>1.8±1.2</td>
<td>15±18</td>
<td>5±3.7</td>
<td>3.3±2.3</td>
</tr>
<tr>
<td>n=9</td>
<td>p&lt;0.001</td>
<td>ns</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Asbestosis</td>
<td>132±95</td>
<td>1.8±1.7</td>
<td>29±31</td>
<td>3.3±1.4</td>
<td>4±1.3</td>
</tr>
<tr>
<td>n=8</td>
<td>p&lt;0.005</td>
<td>ns</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

NS: non significant; α₁,PI: α₁-proteinase inhibitor; PMF: progressive massive fibrosis. All the statistical evaluations were calculated from patients versus controls.

**Neutrophil elastase inhibitory capacity**

The values obtained in the group of patients with simple silicosis were not significantly different from controls, while PMF patients as a group showed significantly elevated values when compared to controls (p<0.05). NEIC values in patients with asbestosis were also significantly higher than controls (p<0.0005). In order to assess the part taken by α₁,PI in the NEIC, we calculated the ratio NEIC/α₁,PI (mol neutrophil elastase inhibited·mL⁻¹/mol immunoactive α₁,PI·mL⁻¹) in controls and patients having an α₁,PI>1.5±10⁻¹¹ mol·mL⁻¹ (six controls, twenty simple silicosis, nine PMF patients and eight asbestosis). The results are graphically expressed in figure 1. On average, no difference was seen between controls and patients with simple silicosis as a group. This fact must be due to a very high value (2.90) obtained in one patient.

In contrast, the PMF values were lower than control values (p<0.05), while in the asbestosis group, they were significantly higher than controls (p<0.05). These values were also significantly high when compared to the simple silicosis group (p<0.02) and the PMF group (p<0.001).

**Electrophoretic study**

The electrophoretic study was first carried out to investigate the state of α₁,PI in the BAL fluids, since α₁,PI could be found either unaltered or pre-co-dosed or complexed to some proteases [24]; these latter species have a modified Mr and are therefore easily located after SDS-PAGE followed by immunoblotting. Secondly, the same techniques allowed us to explore the α₁,PI ability to inhibit added neutrophil elastase: the presence on the immunoblots of a newly formed complex between α₁,PI and elastase would give information about the α₁,PI inhibitory capacity.

![Fig 1](image_url) - Graphic representation of the ratio NEIC/α₁,PI expressed as mol. neutrophil elastase inhibited per mL/mol immunoactive α₁,PI per mL in BAL fluids from controls, patients with simple silicosis (●) PMF patients (○) and patients with asbestosis. The horizontal bars represented the mean values (Controls: 0.9±0.3; simple silicosis: 0.85±0.58; PMF: 0.57±0.23; asbestosis: 1.32±0.35).
Figure 2A shows the results obtained when analysing a BAL fluid in which $\alpha_1$PI was thought to be largely inactive (NEIC/$\alpha_1$PI ratio equal to 0.6). Before addition of neutrophil elastase, a major component (b) was seen, identified to native $\alpha_1$PI by its Mr; after addition of neutrophil elastase in excess, formation of a complex between $\alpha_1$PI and elastase was demonstrated by the presence of a new band (a) revealed not only by anti-$\alpha_1$PI antibodies but also by anti-elastase antibodies. Many degraded products revealed by anti-$\alpha_1$PI antibodies were seen, one of them being the proteolyzed form of $\alpha_1$PI (c); $\alpha_1$PI had not been prevented from degradation by the presence of other active inhibitors. In contrast, BAL fluid analysed in figure 2B, obtained from an asbestosis patient, had an NEIC/$\alpha_1$PI ratio equal to 1.18. After addition of neutrophil elastase in excess, the complex between $\alpha_1$PI and elastase was easily seen; the only $\alpha_1$PI degraded product observed was the proteolysed form (c), which is normally formed when inhibition of neutrophil elastase by $\alpha_1$PI occurs [25]. This observation brought new evidence of the presence of some other neutrophil elastase inhibitor(s) different from $\alpha_1$PI.

Discussion

The present study demonstrated that in BAL fluids from patients with asbestosis and silicosis, the same modifications were obtained for protein content, N-acetyl-$\beta$-D-glucosaminidase, and free elastase-like activity. In contrast, $\alpha_1$PI and NEIC seemed to vary specifically with the different diagnoses.

The protein content of BAL fluids of patients is increased, this may just be an indicator of the altered pulmonary epithelium [26]. Local synthesis may also be involved [27, 28].

Glycosidases possess biological activities against many of the structural components of pulmonary tissue [29]; therefore, they may be of importance in the pathogenesis of lung diseases, such as pneumoconiosis. Our data show a non-significant increase of N-acetyl-$\beta$-D-glucosaminidase activity in the BAL fluids of all the silicotic patients. However, we showed in a previous work that glycosidases were significantly increased in BAL fluids from nonsmoking pneumoconiotic coal-workers ($p<0.05$) [4]. Similar results were also obtained by others in asbestosis [7]. These discrepancies could be explained by the low values found in seven BAL fluids from the silicotic group and two BAL fluids from the PMF group.

The free elastase-like activity we detected in the BAL fluids of patients was significantly higher than in controls, but the values were very low when compared to the NEIC of these BAL fluids. Despite major methodological differences in lavage procedures, as well as the manner of determining the enzyme activity, our results are in good agreement with other papers [30–32], i.e. the elastase-like activity is very low in BAL fluids from healthy nonsmokers, but it is impossible to compare our values with those of other authors. This elastase-like activity probably originates from neutrophils. However, it was significantly increased in all patient BAL fluids, whereas the neutrophil counts were not significantly modified. This activity may also derive from macrophages [31] and be representative of the activation state of these cells. In fact, silica and asbestos fibres induce a perpetual recruitment of macrophages in the lung and newly arrived macrophages differ from other subpopulations in their biological properties, such as secretion of enzymes [33]. The separation of macrophage subpopulations followed by the study of their biological activities will be of interest when studying pneumoconiosis.

Immunoreactive $\alpha_1$PI measurements gave a wide range of values and significant differences were obtained only for the group of PMF patients and for the patients with asbestosis. In fact, the immunoreactive $\alpha_1$PI was on average lower in asbestosis than in the silicotic patients having PMF; $\alpha_1$PI concentration is dependent on the plasma concentration and the degree of pulmonary inflammation leading to an increased transudation [34]. In all the patients with silicosis, immunoreactive $\alpha_1$PI content was related to total protein content, in favor of an increased transudation. In contrast, in asbestosis, protein and immunoreactive $\alpha_1$PI contents were not related; this fact may be the consequence of local synthesis of other proteins such as immunoglobulins [28].
The values obtained for NEIC in silicotic patients with PMF, and patients with asbestosis were significantly higher than in controls. NEIC is representative of all the inhibitors present in BAL fluid. α-PI was reported to be the major anti-neutrophil-elastase found at the alveolar level in normal subjects [35]. However, recently several supposedly distinct inhibitors have been described in healthy subjects [36] as well as in bronchitic [37] and α-PI deficient patients [38].

Even in nonsmoker control BAL fluids, α-PI was shown to be partly inactive against neutrophil elastase [39, 40]. Therefore, the values of the molar ratio NEIC/α-PI are normally lower than 1. In fact, BOUDEIR et al. [36] recently demonstrated a heterogeneity in the composition of lung anti-elastases, by evaluation of the ratios NEIC/α-PI: in some subjects, NEIC/α-PI ratios were higher than unity, in others they were equal or lower than unity. Our data showed the same kind of results. For the controls, the value of the ratio was in good agreement with that described by BOUDEIR et al. [36] and very close to that given by ARFORD et al. [41], although our technical conditions differed from theirs; according to MORRISON et al. [42] they should lead to an underestimation of the NEIC values that we obtained. This ratio was significantly lower in PMF subjects in contrast to those with simple silicosis. On the other hand, the mean value of this ratio in the patients with asbestosis was higher than in controls. The results in figure 1 showed that the eight patients with asbestosis had a ratio higher than the mean value in controls, while in PMF patients, all but one had a value lower than the mean control value.

Therefore, it seemed that patients with asbestosis had a high protection against neutrophil elastase at the alveolar level, not totally due to α-PI: the electrophoretic study that we carried out demonstrated a functional activity of only a part of immunoreactive α-PI. Therefore, there is no doubt that other inhibitors of neutrophil elastase are present at the alveolar level, one of them being the human mucus proteinase inhibitor (or "bronchial inhibitor") but it represents only 14% of α-PI molar concentration [36]. Up to now, the other inhibitors are not well-defined; their presence at different concentrations would explain why the defence against neutrophil elastase injury is high only in some controls, some patients with simple silicosis and patients with asbestosis. In asbestosis when emphysematous lesions are seen, they are from tracional origin; the elastic framework remains intact [43], and this may occur in part because in this disease anti-neutrophil elastase defence is high.

Acknowledgements: The authors greatly thank Prof. Voisin and his team for providing them with the BAL fluids and the information concerning the patients. The writers also thank M.P. Decoeur and B. Van Brussel for their excellent technical assistance.

References


**RÉSUMÉ:** L'inhalation chronique de poussières inorganiques est responsable du déclenchement de la maladie pneumoconique. Afin d'évaluer le potentiel de défense, au niveau alvéolaire, nous avons étudié les liquidités de lavages bronchoalvéolaires (LLBA) de 30 patients atteints de silicose, de 9 d'entre eux présentaient une fibrose massive progressive (FMP) et de 8 malades avec un diagnostic d'asbestose. Sur ces LLBA, on a déterminé: teneur en protéines, activité de N-acétyl-β-D-glucoosaminidase, activité estérase de type élastase, teneur en α1-antiprotéase, PI immunoréactive et capacité inhibitrice vis-à-vis de l'élastase leucocytaire (CIEL). Les valeurs obtenues ont été comparées à celles de 14 LLBA provenant de sujets témoins. Pour tous les malades, on peut constater une augmentation statistiquement significative de la teneur en protéines et de l'activité de type élastase. Par contre, les valeurs obtenues pour l'activité de N-acétyl-β-D-glucoosaminidase ne sont pas significatives. En ce qui concerne l'α1-PI et la CIEL, les valeurs ne sont significativement augmentées que pour les malades atteints de FMP et d'asbestose. Le rapport CIEL/α1-PI immunoréactive est significativement élevé chez les sujets atteints d'asbestose alors qu'il est abaissé chez les malades atteints de FMP. Lorsque le rapport CIEL/α1-PI est largement inférieur à 1, une étude électrophorétique a montré la prédominance des formes protéolytiques de l'α1-PI obtenues après addition d'élastase leucocytaire en excès.