Procoagulant and fibrinolytic activities in bronchoalveolar fluid of HIV-positive and HIV-negative patients


ABSTRACT: Imbalance between intra-alveolar procoagulant activity (PCA) and fibrinolytic activity may lead to fibrin deposition, as described in several pneumopathies, and may eventually contribute to fibrotic changes as observed in Pneumocystis carinii pneumonia (PCP). The aim of our study was to compare these activities in bronchoalveolar lavages of human immunodeficiency virus (HIV)-positive and HIV-negative patients.

The material comprised: a) controls (n=7); b) HIV-positive patients subdivided into PCP (n=11), bacterial pneumonia (n=8) and other pneumopathies (n=22); and c) HIV-negative patients with bacterial pneumonia (n=8).

PCA was significantly increased (p<0.05) in all patient groups compared to controls. The urokinase-type plasminogen activator (Tu-PA) antigen levels were highest during bacterial pneumonia. Regardless of the HIV status, in bacterial pneumonia there was a marked elevation of plasminogen activator inhibitor antigens with little residual fibrinolytic activity. In contrast, the fibrinolytic activity was not decreased in PCP. D-dimer were elevated during PCP compared to controls; the highest levels were found in HIV-negative bacterial pneumonia.

These data indicate that transient fibrotic changes seen in PCP may be favoured by increased PCA, but not by a depressed fibrinolytic activity. In bacterial pneumonia PCA is increased and fibrinolysis decreased independently of the HIV status.


Fibrin deposition is a pro-eminent feature of different pathological processes involving the lung. In order to understand the possible mechanisms leading to this intra-alveolar and interstitial deposition, several groups have demonstrated the importance of the equilibrium between procoagulant and fibrinolytic activities [1,2]. Procoagulant activity (PCA) of bronchoalveolar lavage is significantly increased in bacterial pneumonia, adult respiratory distress syndrome (ARDS) and interstitial lung disease compared to controls [3-8]. This PCA is due to the association of factor VII with tissue factor (TF) expressed principally by alveolar macrophages (AM) [9-12]. Concomitantly, AM possess an important fibrinolytic activity through the expression of urokinase-type plasminogen activator (u-PA), which is decreased in bacterial pneumonia, interstitial lung disease and ARDS [5,8,13,14]. Two histopathological studies [15,16] showed the possible link between fibrin deposition and subsequent lung fibrosis through the interaction of fibrin degradation products, inflammatory cells and fibroblasts. These cells are responsible for the collagen deposition which results in lung fibrosis [17].

Pneumocystis carinii pneumonia is a frequent pathology since the outbreak of human immunodeficiency virus (HIV) infection and may affect up to 75% of acquired immune deficiency syndrome (AIDS) patients [18]. PCP may lead to various degrees of interstitial lung fibrosis and to prolonged gas exchange perturbations even in the absence of ARDS [19,20]. These changes appear to be abolished when steroids are administered [21]. We were particularly interested to study the procoagulant and fibrinolytic balance in PCP in order to understand whether an eventual imbalance of this system could be responsible for the transient pulmonary fibrotic changes described. In order to compare the specificity of the results obtained we also analysed the PCA and fibrinolytic parameters in HIV infected and HIV non-infected patients with other pneumopathies.

Patients and methods

Patients

This study was approved by the Ethical Committee of the Department of Medicine, University of Geneva. Bronchoalveolar lavage (BAL) fluids from 56 patients (45 men and 11 women) were studied and divided into
three groups. Group I consisted of 7 control patients (5 men/2 women; mean age 62±3 yrs) undergoing bronchoscopy to investigate an isolated pulmonary nodule, who were also lavaged in the contralateral lung considered as normal. None of these patients had clinical and/or biological evidence of inflammatory disease. Group II represented 41 HIV-positive young patients (33 men/8 women, mean age 36±9 yrs); all except four (one in stage III and three in stage II) were classified as having AIDS. Bronchoscopy was performed in order to investigate dyspnoea. They were subdivided into a) PCP (n=11, 10 men/1 woman, mean age 35±8 yrs) defined by fever, dyspnoea or cough and Pneumocystis carinii recovered in BAL; b) bacterial pneumonia (n=8, 5 men/3 women, mean age 32±6 yrs) defined by a combination of clinical and laboratory features (e.g. fever, cough, new radiographic lung infiltrates, pleocytosis with leucocytosis, more than 10^4 bacteria recovered in BAL and a good response to conventional antibiotics); and c) other pneumopathies (n=22, 19 men/3 women, mean age 47±11 yrs). Tuberculosis was diagnosed in 4, bacterial pneumonia already antibiotic treated when BAL was performed in 10, Kaposi sarcoma in 1, lung fibrosis in 1 and no clear pathology was detected in 6. Group III consisted of 8 HIV-negative patients (7 men/1 woman, mean age 58±18 yrs), having clinically an infectious pneumopathy (defined by the same criteria), in whom a BAL was performed in order to identify the microorganism responsible for the infection. Surinfected atelectasis (E. coli) was diagnosed in 2 patients, bronchoaspiration pneumonia in 1, Pseudomonas aeruginosa in 1 and pneumococcal pneumonia in 1; in 1 pneumonia no microorganisms were recovered, and in 2 C. albicans only. No patient was mechanically ventilated at the time of BAL procedure.

Methods

Bronchoscopy was performed with a flexible bronchoscope that was introduced after local anaesthesia. BAL was performed by instilling 50 ml aliquots of sterile saline solution through the operating channel of the bronchoscope, up to a total of 200–250 ml [22]. The recovered fluid (between 80–150 ml) was filtered through gauze to remove mucus and each fraction was recovered for subsequent pooled or separate analysis. Total cell count was performed on the unconcentrated BAL fluid, by using a haemocytometer. Viability of recovered cells exceeded 85% (trypan blue exclusion test). Cell differential count was made after centrifugation and Wright-Giemsa staining. The cell-free supernatants were frozen in small aliquots. Each fraction was stored at -70°C and kept separately until analysed. Protein content was determined with the method described by BRADFORD [23], using bovine serum albumin (Sigma, St Louis, Missouri, USA) as a standard. Albumin concentrations were determined by laser nephelometry (analyzer and antiserum both from Behring, Marburg, Germany). Elastase-alpha-1-proteinase inhibitor complexes were measured by enzyme-linked immunosorbent assay (ELISA) (Merck, Darmstadt, Germany).

Coagulation time of a mixture of equal volumes of pooled plasma, BAL sample and CaCl_2 (25 mM) was determined by the normal tilt method, in duplicate, as described previously [24, 25]. Clotting times were translated into arbitrary procoagulant units (PC units) by reference to a curve obtained by dilutions of rabbit thromboplastin (Boehringer, Mannheim, Germany). To better define the factor(s) triggering the coagulation in BAL, five randomly selected samples were assayed with plasmas deficient in factor VII, factor VIII or factor X (Baxter, Düdingen, Switzerland).

Tissue plasminogen activator (t-PA) and plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2) antigens (ag) were measured with commercial ELISA kits (Tint Eliz® t-PA, Tint Eliz® PAI-1 and Tint Elize® PAI-2, respectively, Biopool, Umeå, Sweden). Urokinase type plasminogen activator (u-PA) antigen was determined with a commercial kit (Monozyme ApS, Virium, Denmark). Fibrinolytic activity determinations were kindly performed by E.K.O. Kruithof (Dept of Haematology, CHUV, Lausanne, Switzerland) by indirect chromogenic assay [26] using u-PA as standard. D-dimer levels were assayed by ELISA (Diagnostica Stago, Asnières, France).

Statistics

All data are expressed as means ± standard errors, median with range or in box-plot: 50% of values are represented by open boxes in which horizontal bar indicates the median, vertical bars outside the boxes the 10th and 90th percentile and circles outlying values. Between group comparisons were performed by Kruskall-Wallis analysis of variance and Mann-Whitney test. Significance was set at p<0.05. Correlations were tested using the Spearman’s rank-order correlation test and regression analysis.

Results

Analysis of BAL

Table 1 details the BAL cell and protein contents in controls and other groups. BAL samples from patient groups yielded a significantly greater amount of total cells than did samples from controls. Compared to controls, the cellular yield was particularly elevated in the lavages of HIV-positive (HIV+) patients with bacterial pneumonia (mean 280±132 x 10^6/ml cells, p=0.01). Polymorphonuclear percentage was highest in HIV-negative (HIV-) and HIV-positive bacterial pneumonia (69 and 53%, respectively, p=0.01). Relative lymphocytosis was particularly high in PCP groups (29%, p=0.03). Macrophage percentage was lower in all pathologies studied compared to controls; this was particularly marked in HIV-negative bacterial pneumonia (22%, p=0.01).
**PCA AND FIBRINOLYSIS IN HIV+ AND HIV- BAL**

### Table 1. - BAL cell differentials and protein contents

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Others</th>
<th>PCP</th>
<th>Bact.P. HIV+</th>
<th>Bact.P. HIV-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=7</td>
<td>n=22</td>
<td>n=11</td>
<td>n=8</td>
<td>n=8</td>
</tr>
<tr>
<td>Cell count *10^4* ml⁻¹</td>
<td>19±4</td>
<td>56±7</td>
<td>38±11</td>
<td>280±132</td>
<td>93±33</td>
</tr>
<tr>
<td>Macrophages %</td>
<td>85±2</td>
<td>71±6</td>
<td>51±7</td>
<td>34±6</td>
<td>22±4</td>
</tr>
<tr>
<td>Neutrophils %</td>
<td>3±2</td>
<td>4±1</td>
<td>17±5</td>
<td>3±3±9</td>
<td>6±9±4</td>
</tr>
<tr>
<td>Lymphocytes %</td>
<td>10±2</td>
<td>23±5</td>
<td>29±6</td>
<td>11±9</td>
<td>5±1</td>
</tr>
<tr>
<td>Protein µg·ml⁻¹</td>
<td>92±16</td>
<td>249±85</td>
<td>206±39</td>
<td>367±88</td>
<td>515±166</td>
</tr>
<tr>
<td>Albumin µg·ml⁻¹</td>
<td>37±6</td>
<td>132±26</td>
<td>70±13</td>
<td>215±69</td>
<td>184±74</td>
</tr>
<tr>
<td>Protein/albumin</td>
<td>2.8±0.5</td>
<td>4.2±1</td>
<td>2.9±3</td>
<td>2.5±0.5</td>
<td>3.8±0.6</td>
</tr>
<tr>
<td>Elastase ng·ml⁻¹</td>
<td>6±1</td>
<td>18±4</td>
<td>68.6±49</td>
<td>435±116</td>
<td>49±125</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. BAL: bronchoalveolar lavage; PCP: *Pneumocystis carinii* pneumonia; Bact.P: bacterial pneumonia; HIV+: human immunodeficiency virus HIV-positive patients; HIV-: HIV-negative patients; Elastase: elastase-alpha¹-proteinase inhibitor complex.

However, when absolute macrophage numbers were considered, no significant difference was observed between controls, PCP and HIV-negative bacterial pneumonia groups, whereas there was a significant increase in HIV-positive bacterial pneumonia (p<0.05) and in other pneumopathies (p<0.005).

Protein and albumin contents were also measured in order to evaluate the change in vascular permeability known to be associated with inflammation. There was a significant increase (p<0.05) in the alveolar protein and albumin content of all groups compared to controls. However, the means of individual protein/albumin ratios were not statistically significantly different between the groups. As indicated in table 1, elastase-alpha¹-proteinase inhibitor complex levels (a reflection of polymorphonuclear activation) were similarly increased in both groups with bacterial pneumonia (p<0.05). In PCP and other pneumopathies there was a less marked increase compared to controls (statistically nonsignificant). A good correlation was found between neutrophils (in percentage) and elastase-alpha¹-proteinase inhibitor complexes (p=0.001).

### Procoagulant activity

PCA was already detectable in controls; however, significantly higher PC units were observed in all groups compared to controls (p<0.05). Using factor deficient plasma this PCA was found to be completely dependent on factor X, independent of factor VIII and partially dependent on factor VII. PCA was first compared between controls and all HIV-positive patients and a significant increase (p<0.05) of PCA was observed in the HIV group (median 53, range 5–228).

We next subdivided the HIV-positive group to study whether PCA was differentially increased. Figure 1 shows that the three subgroups had significantly increased PCA (p<0.05) compared to controls. The highest values were found in bacterial pneumonia (median 84, range 27–164) but no statistical difference was found between bacterial pneumonia and PCP or different HIV pneumopathies.

The same pneumopathy (bacterial pneumonia) was compared between HIV-positive and HIV-negative patients. Figure 2 shows PCA of HIV-positive (median 88, range 54–494) and HIV-negative bacterial pneumonia (median 84, range 27–164): PCA was increased in both groups compared to controls but no statistical difference was found between them.
Table 2. - Fibrinolytic parameters in different groups studied

<table>
<thead>
<tr>
<th></th>
<th>Controls n=7</th>
<th>Others n=22</th>
<th>PCP n=11</th>
<th>Bact.P HIV+ n=8</th>
<th>Bact.P HIV- n=8</th>
</tr>
</thead>
<tbody>
<tr>
<td>u-PA ag ng·ml⁻¹</td>
<td>401±32</td>
<td>576±79</td>
<td>503±64</td>
<td>626±173</td>
<td>703±152</td>
</tr>
<tr>
<td>PAI-1 ag ng·ml⁻¹</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
<td>2.6</td>
<td>3.4</td>
</tr>
<tr>
<td>PAI-2 ag ng·ml⁻¹</td>
<td>0.2±0.1</td>
<td>0.2±0.1</td>
<td>0.1±0.1</td>
<td>2±1.3</td>
<td>3±1.4</td>
</tr>
<tr>
<td>u-PA mU·mP⁻¹</td>
<td>5±2</td>
<td>7±2</td>
<td>4±2</td>
<td>6±3</td>
<td>2±0.6</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SEM. u-PA ag: urokinase-type plasminogen activator antigen; PAI-1 and PAI-2 ag: plasminogen activator inhibitor 1 and 2 antigen; u-PA: urokinase plasminogen activator activity; NS: nonsignificant. For further abbreviations see legend to table 1.

**Fibrinolytic parameters**

Different fibrinolytic parameters of BAL were assessed, firstly with immunological assays: u-PA, t-PA and their inhibitors, i.e. PAI-1 and PAI-2. The results obtained for t-PA ag were not different between groups, in most samples t-PA ag being undetectable. Table 2 summarizes the results. There was a slight increase in u-PA ag in all groups as compared with controls. PAI-1 and PAI-2 ag, undetectable in controls, were increased in bacterial pneumonia, regardless of the HIV status. In order to evaluate the global fibrinolytic activity of BAL an indirect measure of the urokinase activity was also performed. The fibrinolytic activity of BAL was statistically decreased in HIV-negative bacterial pneumonia compared to controls. This was not true in HIV-positive bacterial pneumonia, since one patient had very elevated levels and changed the statistical analysis due to the small number of patients per group.

**D-dimer**

D-dimer are the end-products of the sequential action of coagulation and fibrinolytic systems: indeed once fibrin is cross-linked by factor XIIIa, it is degraded by the fibrinolytic system and its digestion results in fibrin degradation products containing D-dimer. Figure 3 shows that D-dimer in BAL were significantly higher in each group as compared to controls (p<0.05). The highest levels were observed in HIV-negative bacterial pneumonia (Bact.P) (296 fold increase of the median value for HIV-negative Bact.P., 46 fold increase for HIV-positive Bact.P., 20 fold for PCP and 9 fold for others). It was interesting to analyse whether PCA or fibrinolytic activity were correlated with D-dimer since they are the result of the activation of coagulation and fibrinolysis. For each activity (PCA and fibrinolytic activity) a significant correlation with D-dimer levels was found (p=0.001) when all groups were combined.

**Discussion**

BAL contains procoagulant and fibrinolytic activities, both coming mostly from AM [9-13]. Recently, alveolar epithelial cells have also been shown to be a source of a urokinase-like plasminogen activator [27, 28]. Concomitantly, AM can produce PAI-1 and PAI-2 [29-32]. There is now good evidence that an imbalance between PCA and fibrinolysis in BAL could explain the lung fibrin deposition observed in different pneumopathies, such as ARDS, interstitial lung disease and bacterial pneumonia [1-8]. The persistence of these deposits could modulate the intensity of inflammation through the attraction and immobilization of leucocytes. Fibrinopeptide B, as well as fibrin/fibronectin matrix, is a nidus for fibrogenesis and promotes the influx and attachment of fibroblasts [17, 33]. We investigated whether an abnormality of the haemostatic balance could be detected in HIV patients, especially in PCP, which could explain the fibrotic changes [19, 20] and long lasting exercise intolerance observed in this pathology [21].
We also analysed D-dimer, which are markers of both coagulation and fibrinolysis activation and have, to our knowledge, not yet been studied in BAL.

With regard to PCA, our results indicate that a significant increase is observed in PCP compared to controls. However, the rise in PCA was not restricted to PCP and was also found in bacterial pneumonia as well as in several other lung diseases in HIV-infected patients. The fact that PCA was detectable in controls does not indicate that they were inadequate, since it has already been observed that PCA is detected in BAL fluid of volunteers. Increases in HIV-positive patients with PCP and with other pneumopathies were less pronounced than those with bacterial pneumonia. When HIV-positive and HIV-negative patients with bacterial pneumonia were compared, no statistically significant difference was found. Since PCA in BAL has been shown to originate mostly from AM [9–12] our results could suggest that HIV, which infects and replicates in AM [34, 35], does not impair this particular macrophage function. This is in contrast with the results of others showing a decreased expression of tissue factor by monocytes in HIV patients [36]. The absence of correlation between the absolute number of AM and PCA suggests that PCA is produced not only by AM but also by other cells, such as alveolar epithelial cells [37], or that it is not the absolute number of macrophages but their state of activation induced by various stimuli [38–42] which should be taken into account. This is supported by numerous experimental studies which have demonstrated that several factors (especially bacterial lipopolysaccharide, immune complexes, lymphokines) could trigger the macrophage PCA [38–42].

Concerning fibrinolysis, PCP did not induce significant differences as compared to controls, suggesting that some transient fibrinolytic changes observed in this situation cannot be explained by a decreased fibrinolysis. In contrast, all except one HIV-positive and all HIV-negative bacterial pneumonia patients exhibited a moderate increase of u-PA ag, a strong increase in PAI-1 and a depressed global fibrinolytic activity. These data indicate that, as for PCA in bacterial pneumonia, alterations in fibrinolytic parameters in BAL are not specific for the HIV infection. These results are also consistent with other works suggesting that depressed levels of fibrinolytic activity in BAL could explain the marked fibrin deposition observed in the beginning of bacterial pneumonia [14]. It would be of interest to evaluate sequentially the fibrinolytic activity during the course of pneumonia, as has been done in some experimental and human studies [43–46].

PAI-1 ag is a well-known acute phase protein [31] and, therefore, is expected to increase in bacterial pneumonia with the movement of plasma protein into the alveoli. However, in 31% of Bact.P. patients this inhibitor was not detectable in BAL despite an increase of protein content. In PCP and other pneumopathies the figures were 72 and 81%, respectively. No PAI-1 ag was detectable in controls.

This might argue against a bulk movement of plasma constituents into the alveoli and suggest the presence of a local regulatory mechanism of fibrinolytic activity. Unfortunately, we could not measure PAI-1 ag levels in plasma and correlate it with its concentration in BAL. Nevertheless, some movement of plasma protein may occur, since, when all patients were pooled, a good correlation was found between PAI-1 ag and protein content (p<0.01).

The outcome of coagulation and fibrinolysis is reflected in D-dimer, which are specific markers of the fibrin breakdown. All patients exhibited elevated levels of D-dimer, attesting the importance of intra-alveolar haemostatic changes in human lung diseases. In the HIV-positive group, patients with bacterial pneumonia had higher D-dimer levels than PCP or patients with other pneumopathies. However, no statistically significant differences in D-dimer were found between HIV-positive and HIV-negative patients with bacterial pneumonia, which is in accordance with the lack of significant differences in PCA and fibrinolytic activity observed between these two groups.

Elastase-alpha, proteinase inhibitor complexes were dramatically increased in bacterial pneumonia regardless of the HIV status. This indicates an important activation of alveolar neutrophils in bacterial pneumonia which is also not impaired by the HIV status.

We conclude that important modifications of PCA and fibrinolytic activity can be detected in BAL. In PCP, PCA but not fibrinolytic activity was altered, whereas in bacterial pneumonia, an increase of PCA and a decrease of fibrinolysis were observed. The similar alterations observed in HIV-positive and HIV-negative patients with bacterial pneumonia indicate that these specific macrophage functions seem not to be impaired in HIV infection. To better evaluate the role of intra-alveolar PCA and fibrinolysis in fibrin deposition and possible fibrosis, a prospective study with sequential measures of these haemostatic parameters is required.

Acknowledgements: The authors thank F. El Habre and J-D. Graf for expert technical assistance.

References


