Platelet-activating factor in bronchoalveolar lavage fluid from asthmatic subjects


ABSTRACT: Bronchoalveolar lavage (BAL) was performed on 28 asthma patients, 7 patients with emphysema and 11 control subjects. Total and differential cell counts were performed and cellular metabolic activity was assessed using luminol and lucigenin amplified chemiluminescence. BAL supernatants were assayed for platelet-activating factor (PAF) and lyso-PAF using a sensitive guinea-pig blood assay. Eight of the asthma patients but none of the emphysema patients or control subjects had PAF in their BAL fluid. Lyso-PAF was measurable in BAL fluid in most subjects and no differences were detected between groups. Among the asthma patients, the presence of PAF in BAL supernatant was significantly associated with a combination of low neutrophil and high lymphocyte counts (p<0.05) and with macrophage metabolic activity as assessed by lucigenin chemiluminescence (p<0.05).

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The phospholipid platelet-activating factor (PAF) has recently attracted attention as a pro-inflammatory mediator in a variety of disease states including asthma. PAF can induce smooth muscle contraction and oedema [1] and is chemotactic, particularly for eosinophils [1, 2] which have been implicated in the inflammatory reactions associated with asthma [3]. In addition to acute bronchoconstriction, prolonged increases in bronchial responsiveness after the inhalation of PAF have been described in animals [4] and in humans [5, 6].

PAF itself is rapidly metabolised in vivo, having a half-life of less than one minute in blood [7] but its major metabolite lyso-PAF is more stable [8]. It might be expected therefore that either PAF or lyso-PAF should be detectable in human airways in asthma if PAF is important in its pathophysiology, and that a correlation should be found between asthmatic activity and the levels of PAF. PAF had previously been detected in sputum from asthmatic subjects [9]. Alveolar macrophages from asthmatic subjects have been shown to be capable of synthesising PAF [10] and PAF release during antigen induced bronchoconstriction has been suggested by the subsequently reduced responsiveness of circulating platelets to PAF [11]. While PAF has been detected in animal lungs [12], it has not previously been isolated directly from human airways.

The technique of bronchoalveolar lavage (BAL) is now widely used to obtain cells and mediators from human airways in a variety of conditions including asthma [13].

Pro-inflammatory mediators including histamine [14], prostaglandin D\textsubscript{2} (PG D\textsubscript{2}) [15] and leukotrienes D\textsubscript{4} and E\textsubscript{4} [16] have been detected in this way and PGD\textsubscript{2} levels in the airways of asthmatic subjects have been shown to increase following allergen challenge using this technique [15]. In this study, we have looked for the presence of PAF and lyso-PAF in BAL samples from patients with chronic stable asthma, non-asthmatic control subjects and patients with irreversible airflow obstruction due to emphysema. We have attempted to correlate the presence of PAF in BAL fluid from asthma patients with a variety of indices of asthmatic activity and airway inflammation.

Methods

Subjects

Twenty eight asthma patients (16 male; median age 43 yrs; range 16-66 yrs) underwent fiberoptic bronchoscopy between December 1985 and June 1987. Twenty-one were undergoing routine diagnostic bronchoscopy for clinical reasons: a "suspicous" chest radiograph (9), undue cough (3), streaking haemoptysis (7) and suspicion of malignancy or tracheal compression. None of these subjects proved to have a bronchial carcinoma. Seven were volunteers with no clinical indication for bronchoscopy. The diagnosis of asthma was based on a typical
clinical history accompanied by documentation of at least 15% variability in forced expiratory volume in one second (FEV₁) either spontaneously or in response to bronchodilators. All were in a stable clinical state at the time of bronchoscopy: all were taking inhaled bronchodilators and 14 were taking inhaled corticosteroids (beclomethasone dipropionate, dose range 200-2,000 \( \mu g \), median dose 400 \( \mu g \) daily). Eight of these subjects had been taking inhaled corticosteroids for less than 6 months. No subject was taking theophylline and caffeine containing beverages were avoided for 6 h prior to bronchoscopy. Seven subjects were cigarette smokers.

In parallel, BAL was performed on 11 “control” subjects (8 male; median age 56 yrs; range 29-73 yrs) without airflow obstruction and 7 subjects (4 male; median age 62 yrs; range 47-75 yrs) with airflow obstruction due to emphysema (airflow obstruction with no response to 400 \( \mu g \) salbutamol and a reduced gas transfer factor). These subjects were also undergoing routine diagnostic bronchoscopy. A final diagnosis of bronchial carcinoma was made in 2 of the emphysema patients and 2 of the control subjects. Six of the emphysema patients and 3 of the control subjects were smokers. Six emphysema subjects were taking inhaled bronchodilators and 3 were taking inhaled corticosteroids. No control subject was taking regular medication. All subjects gave consent for BAL and the study was approved by the local Ethics Committee.

Prior to bronchoscopy, all subjects underwent inhalation provocation testing with methacholine using a dosimeter technique [17] to determine non-specific bronchial responsiveness expressed as provocative dose producing a 20% fall in forced expiratory volume in one second (PD₂₀_FEV₁). Atopic status was quantified by skin prick testing to common allergens; house dust, \( D. \) pteronyssinus, \( A. \) fumigatus and mixed grass pollen (Bencard, England). Subjects with at least one weal diameter greater than that caused by 0.1% histamine as control were judged to be atopic [18].

**Bronchoalveolar lavage**

Subjects received intramuscular premedication with atropine 0.6 mg and papaveretum 10 mg, 30 min prior to bronchoscopy. No intravenous sedation was given and anaesthesia was with topical 1.5% isotonic lignocaine. Asthma patients took their usual inhaled medication on the morning of bronchoscopy, and no additional bronchodilators were given. BAL was performed in a sub-segment of the middle lobe (or the lingula in 4 subjects where there was a suspicion of local pathology in the right lung) using 3x60 ml aliquots of phosphate buffered saline prewarmed to 37°C. Aspirated fluid was immediately cooled to 4°C and mucus removed by straining through a stainless steel gauze with a 200 \( \mu m \) pore diameter. Cells were removed by centrifugation at 500 g for 10 min and the supernatant removed and stored at -20°C for later analysis.

Prior to centrifugation, aliquots were removed for determination of total cell count using a modified Neubauer Counting Chamber and differential cell count (Giemsa stain), 300 cells being counted independently by two experienced observers and the results meanned. Cellular metabolic activity was measured using the technique of latex stimulated chemiluminescence (CL) [19]. Luminol amplified CL was used to assess granulocyte metabolic activity [20] and lucigenin amplified CL to assess macrophage activity [21]. 100 \( \mu l \) of a 5% latex suspension (1.09 \( \mu m \) mean diameter, Sigma) was added to 250,000 cells in the presence of either luminol (0.1 mM, Sigma) or lucigenin (0.1 mM, Sigma). Peak chemiluminescence was measured using an LKB 1250 (LKB-Wallac) or Lumac M 2010 (Sonco Ltd) luminometer, the results being expressed as mV-1000 cells. All chemiluminescence assays were performed within 3 h of BAL.

**PAF assay**

The stored BAL supernatant was thawed and PAF was extracted by adding 5.4-21.6 ml acetone (BDH, Aristar) at -20°C, mixing for 30 s and incubating at 4°C for 10 min before centrifuging at 1,560 g for 5 min. The supernatant was added to an equal volume of chloroform (BDH, Analar) at -20°C, mixed for 30 s and centrifuged at 1,560 g for 5 min. The organic layer was divided into 2 aliquots and evaporated to dryness. Using this technique, the extraction efficiency for PAF suspended in Tris-bovine serum albumin (Tris-BSA) was 98.2±0.6% and for lyso-PAF was 97.6±0.8%.

Aliquot 1 was re-suspended in 0.2 ml Tris-BSA (0.25% w/v) and PAF was assayed using a guinea-pig platelet-rich plasma bioassay [22]. Platelet aggregation was measured after the addition of 35 \( \mu l \) of the test sample to 450 \( \mu l \) of platelet rich plasma (300x10⁶ platelets-ml⁻¹) in a single channel Coulter aggrego-meter (Model 335). Assays were performed in the presence of indomethacin 10 \( \mu M \), creatine phosphokinase 22.5 \( U/\text{ml} \), phosphocreatine 11.3 \( \text{mM} \) and hirudin 2 \( \text{U/ml} \). In all samples where platelet aggregating activity was found, the presence of PAF was confirmed by its co-migration with PAF (Sigma) on thin layer chromatography using a chloroform: methanol: water (65:35:6) solvent system. The lower limit of detection of the assay was 180 pmol-35 \( \mu l \) which was equivalent to a PAF concentration in BAL fluid of 0.1 nM.

**Table 1.** - The presence of PAF and lyso-PAF in BAL fluid from patients with asthma and emphysema and from control subjects

<table>
<thead>
<tr>
<th></th>
<th>Asthma</th>
<th>Emphysema</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>28</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>No. with PAF</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Median PAF concn</td>
<td>2.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>range ( \text{nM} )</td>
<td>(0.5-7.1)</td>
<td>(0.5-7.1)</td>
<td>(0.5-7.1)</td>
</tr>
<tr>
<td>No. with Lyso-PAF</td>
<td>22</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Median Lyso-PAF concn</td>
<td>2.8</td>
<td>3.3</td>
<td>2.4</td>
</tr>
<tr>
<td>range ( \text{nM} )</td>
<td>(0.3-85.4)</td>
<td>(2.8-11.8)</td>
<td>(1.6-2.8)</td>
</tr>
</tbody>
</table>

Medians and ranges are for those subjects whose BAL fluid contained PAF/Lyso-PAF only.
Aliquot 2 was acetylated by continuous agitation with dry pyridine (400 µl) and acetic anhydride (400 µl) at 25°C for 16 h to convert lyso-PAF to PAF and then evaporated to dryness. The residue was washed twice with chloroform, resuspended in Tris-BSA and assayed for PAF as above. The efficiency of the conversion process was 39.1% (range 37.8-41.4%). A lyso-PAF (Sigma) standard was incubated at the time of each assay and the measured lyso-PAF concentrations corrected to allow for the conversion efficiency.

Statistical methods

Data for asthmatics were compared with those for non-asthmatics using Chi-square tests and analysis of variance using the statistical package Minitab (CLE.COM, Birmingham). Associations between PAF and other parameters in BAL were tested by linear logistic analysis using the statistical package GLIM (NAG, Oxford).

Results

The median baseline FEV₁ in the 3 groups studied were: asthma patients 2.7 l (range 1.1-4.1 l; median % predicted 85%); emphysema patients 1.3 l (0.7-2.6 l; median % predicted 64%); and control subjects 2.6 l (2.0-4.8 l; median % predicted 105%). Sixteen asthma patients, one control subject but no emphysema patients, were atopic. Geometric mean PD₂₀FEV₁ was 169 µg methacholine for the asthmatics (range 5-3,800 µg) and 163 µg for the emphysema patients (range 23 to >4000 µg). PD₂₀FEV₁ was greater than 6,400 µg methacholine (the maximum cumulative dose given) for all control subjects. PAF was detected in BAL fluid from 8 of the 28 asthmatic patients but from none of the non-asthmatic subjects (χ²=6.3; p<0.05; table 1). Lyso-PAF was detected in 22 of the 28 asthma patients and 14 of the 18 non-asthmatic subjects (χ²=0.1; p>0.5; table 1). The distribution of PAF and lyso-PAF levels among the asthmatic subjects is shown in figure 1. There was no association between the levels of PAF and lyso-PAF (χ²=0.5; p>0.5).

Table 2. - Comparison of asthmatic subjects with and without PAF in BAL fluid

<table>
<thead>
<tr>
<th></th>
<th>Asthmatics with PAF</th>
<th>Asthmatics without PAF</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>(range)</td>
<td>(19-64)</td>
<td>(16-66)</td>
<td>&gt;0.9</td>
</tr>
<tr>
<td>Sex M/F</td>
<td>2/6</td>
<td>13/7</td>
<td></td>
</tr>
<tr>
<td>Atopic</td>
<td>2</td>
<td>9</td>
<td>&gt;0.9</td>
</tr>
<tr>
<td>Smokers</td>
<td>3</td>
<td>4</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Inhaled steroids</td>
<td>5</td>
<td>9</td>
<td>&gt;0.9</td>
</tr>
<tr>
<td>PD₂₀FEV₁ (µg methacholine)</td>
<td>223</td>
<td>146</td>
<td>&gt;0.1</td>
</tr>
</tbody>
</table>

Asthmatic subjects whose BAL fluid contained PAF were compared to those without. They did not differ in terms of age, sex, atopy, cigarette smoking or use of inhaled corticosteroids (table 2). Two asthma patients without PAF had high neutrophil counts in their BAL fluid, presumably due to unsuspected bacterial infection at the time of BAL and their data excluded from the linear logistic analysis of cell counts and function. However, inclusion of this data does not alter any of the conclusions. There were no associations between the presence of PAF and total cell counts, macrophage or eosinophil counts (table 3). However, it appeared to be weak associations between the presence of PAF and low neutrophil (p=0.1) and high lymphocyte counts (p<0.01) and the combination of low lymphocyte and high neutrophil counts together was significantly associated with PAF (p<0.01; fig. 2).

Polymorphonuclear cell metabolic activity (luminol amplified CL) showed no association with PAF whereas macrophage metabolic activity (lucigenin amplified CL) was significantly associated with PAF (p<0.05; table 3).
The levels of lyso-PAF showed no association with PD_{20} FEV_{1}, cell counts or cellular metabolic activity, or any of the other parameters above.

Table 3. - Cell counts and cellular metabolic activity for asthmatics with and without PAF

<table>
<thead>
<tr>
<th></th>
<th>Asthmatics with PAF</th>
<th>Asthmatics without PAF</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell count</td>
<td>175 (60-330)</td>
<td>155 (50-450)</td>
<td>&gt;0.7</td>
</tr>
<tr>
<td>Macrophages</td>
<td>144 (53-216)</td>
<td>90 (32-432)</td>
<td>&gt;0.9</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>2.9 (0.9-9.9)</td>
<td>6.8 (0-24.0)</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>35 (1-89)</td>
<td>23 (3-61)</td>
<td>0.1&gt;p&gt;0.05</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>1.3 (0.03-0.19)</td>
<td>2.7 (0.01-0.18)</td>
<td>&gt;0.1</td>
</tr>
</tbody>
</table>

**Metabolic activity**

<table>
<thead>
<tr>
<th>Luminol amplified</th>
<th>Chemiluminescence</th>
<th>Lucigenin</th>
<th>Chemiluminescence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mV·10^{3} cells^{-1}</td>
<td>(0.05-0.9)</td>
<td>(0.01-0.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.085</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.03-0.19)</td>
<td>(0.01-0.18)</td>
</tr>
</tbody>
</table>

Data given as mean and range in parentheses. Values exclude 2 extremes with high neutrophil counts. PAF: platelet-activating factor.

![Figure 2](image.png)

**Discussion**

There is a considerable amount of evidence implicating PAF as an important mediator of inflammatory reactions [1]. The demonstration of its presence in BAL fluid in some asthmatics together with our failure to detect it in BAL fluid from normal subjects and in subjects with emphysema might further support the suggestion that it has a pathophysiological role in this condition. However, some caution must be exercised before accepting this interpretation as we were unable to detect PAF in the majority of our asthma patients and its presence was not related to asthmatic activity as measured by PD_{20} FEV_{1}.

Currently, a bioassay is the only widely available technique which is sensitive enough to detect PAF in biological fluids [23] and our own assay appeared to be adequate for this purpose. It is possible, however, that levels of PAF below the lower limits of detection were in fact present in the BAL fluid from the majority of asthmatic subjects, particularly in view of the relatively large dilutions involved in BAL.

A number of technical factors may have reduced our ability to measure PAF. PAF is readily adherent to glass and plastic surfaces in vitro [24], but our own data show that over 30% of exogenous PAF added to BAL fluid can be recovered using handling and storage techniques similar to those used in the present study [25]. PAF can also be rapidly taken up and metabolised by neutrophils [26] but this process is slow at 4°C the temperature at which the BAL fluid was stored prior to cell separation. We did, however, note a trend towards higher PAF levels where neutrophil numbers were low, but this only reached statistical significance in association with high lymphocyte numbers. Endogenous PAF antagonists have been described [27] and it is possible that the presence of such substances could have interfered with some samples when using a bioassay technique. Intracellular PAF levels were not measured.

The effects of medication may have further affected our ability to measure PAF and to demonstrate an association with asthmatic activity. Corticosteroids in particular have been shown to reduce the ability of macrophages from asthmatic subjects to synthesize PAF [28]. However, a large number of our subjects were not taking corticosteroids and there was no association between the demonstration of PAF activity and use of corticosteroids.

It is less likely that PAF was generated during the early stages of processing of the BAL fluid. Although pulmonary macrophages from asthmatic subjects are capable of synthesizing PAF in vitro when appropriately stimulated [10], in general, cell damage does not cause the release of substantial amounts of PAF [29]. The pulmonary macrophages were significantly more active metabolically, as judged by lucigenin amplified chemiluminescence, in asthmatic subjects whose BAL fluid contained PAF. However, as PAF is capable of stimulating oxidative metabolic activity in macrophages [30], this enhanced chemiluminescence may have been the consequence of prior exposure of the macrophages to PAF in vivo rather than a source of the PAF.
Airway inflammation can be assessed by BAL in terms of cell counts and cellular metabolic activity [31]. In this study, PAF levels did not correlate with either macrophage or eosinophil counts although there was a correlation with a combination of high lymphocyte and low neutrophil counts. Relatively little attention has focused on the lymphocyte in asthma but recently increases in lymphocyte numbers and activity have been described both in chronic and acute severe asthma [32, 33]. PAF is a potent eosinophil chemotaxin and the lack of any association with eosinophil numbers in this study appears to be paradoxical. However, we were unable to study eosinophil activation or density which might better reflect the function of the eosinophils in the airways. We were primarily concerned with a chronic stable clinical situation and it may be that under these circumstances the well-documented tachyphylaxis to PAF [6] had prevented active cellular recruitment to the airways. However, this could potentially be overcome in situations of more acute deterioration. The significance of the relationships between PAF and cell profiles found in this study remains to be explained.

Lyso-PAF, as well as being the major metabolite of PAF is also its major precursor [24]. It can be formed through the action of phospholipase A₂, in cleaving a fatty-acid from the 2 position of a membrane phospholipid. PAF is formed by acetylation of lyso-PAF in the 2 position and this is the rate-limiting step in its biosynthesis. Thus, although lyso-PAF was frequently found in BAL fluid from both asthmatic and non-asthmatic subjects, this cannot be taken as marker of the presence of PAF in vivo, as suggested by the poor correlation between the levels of PAF and lyso-PAF. Lyso-PAF levels on the other hand, may reflect phospholipase A₂ activity and therefore, serve as a marker of arachidonic acid release and possibly prostaglandin and leukotriene synthesis, although this has not been closely studied. Lyso-PAF itself appears to be devoid of biological activity [1].

We have demonstrated that PAF can be measured in BAL fluid but only from a minority of asthmatic subjects. A number of technical problems need to be overcome if an assay of this type is to be useful in furthering the role of PAF and our conclusions must remain tentative. However, the lack of any correlation between PAF levels and PD₂₀ FEV₁ suggests that PAF may not have been the only factor responsible for maintaining bronchial hyperresponsiveness in this group of chronic asthmatic subjects.

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References

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RÉSUMÉ: Le lavage broncho-alvéolaire (BAL) a été réalisé chez 28 patients asthmatiques, 7 patients emphysématiques et 11 sujets contrôle. Les décomptes totaux et différentiels des cellules ont été réalisés, et l’activité cellulaire métabolique a été appréciée en utilisant la chimiluminescence amplifiée par lumi- nol et lucigenine. Les surnageants du BAL ont été testés pour PAF et lyso-PAF au moyen d’un essai biologique sensible chez le cobaye. Huit des patients asthmatiques, mais aucun des emphysématiques ou des sujets contrôle, avaient du PAF dans le liquide broncho-alvéolaire. Le lyso-PAF était mesurable dans le liquide broncho-alvéolaire chez la plupart des sujets, sans que des différences soient constatées entre les groupes. Chez les sujets asthmatiques, la présence de PAF dans le surnageant du BAL est en association significative avec la coexistence de décomptes abaissés de neutrophiles et augmentés de lymphocytes (p<0.05), ainsi qu’avec une activité métabolique macrophagique appréciée par la chimiluminescence à la lucigenine (p<0.05).

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