Number and activity of inflammatory cells in bronchoalveolar lavage fluid in asthma and their relation to airway responsiveness

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ABSTRACT Bronchial responsiveness to inhaled methacholine was measured four to six days before fibreoptic bronchoscopy in 22 asthmatic patients (10 smokers) and 20 control subjects (12 smokers). The asthmatic patients had a baseline FEV₁ greater than 60% predicted and a PD₁₀₀FEV₁ (provocative dose of methacholine causing a 20% fall in FEV₁) of 0.006-3.7 mg. The 20 control subjects had normal pulmonary function and a PD₁₀₀FEV₁ above the maximum cumulative dose of methacholine of 6-4 mg. Bronchoalveolar lavage of a middle lobe segment (lingula in four subjects) was performed with three sequential 60 ml aliquots of sterile saline. Cellular metabolic activity was stimulated with latex in aliquots of resuspended cells, and measured by means of luminol enhanced chemiluminescence to assess neutrophil activity and lucigenin enhanced chemiluminescence to assess macrophage activity. Mean absolute total cell counts were similar in the asthmatic and control groups but there were differences in differential cell counts, with a significant increase in eosinophil (p < 0.05) and lymphocyte (p < 0.05) counts in asthma. PD₁₀₀FEV₁ was negatively correlated with percentage neutrophil counts (p < 0.005). Luminol enhanced chemiluminescence/1000 neutrophils was increased about twofold in asthmatic subjects (p < 0.001), but was not correlated with PD₁₀₀FEV₁. Lucigenin enhanced chemiluminescence/1000 macrophages was increased nearly fourfold in asthmatic patients (p < 0.001) and showed a negative correlation with PD₁₀₀FEV₁ (p < 0.01). The macrophage count was increased twofold in current smokers in both groups, but other cell numbers were not altered significantly. Smoking did not affect cellular metabolic activity in either group. This study supports the idea that an inflammatory process is present in the airways of those with asthma and suggests a relation between bronchial responsiveness and both neutrophil numbers and macrophage activity.

Introduction

Inflammatory change within the airways, recognised as a histopathological feature of asthma for many years,¹ has more recently been proposed as an important factor in the development of bronchial hyperresponsiveness in man.² ³ Studies of the development of cellular inflammation in animal airways after exposure to ozone⁴ and specific allergen⁵ have shown an association between the induction of transient airway hyperresponsiveness and neutrophil influx. When the cellular inflammatory response was prevented by prior neutrophil depletion the development of bronchial hyperresponsiveness was inhibited.⁶ ⁷ The importance of an inflammatory cell influx in the development of increased airway responsiveness has, however, been disputed in other animal models.¹

Lavage has now been performed in patients with asthma in several centres, and has been generally well tolerated.⁹ Increased numbers of eosinophils have been recovered in lavage fluid from patients with allergic asthma⁹,¹² and during allergen induced late asthmatic reactions.¹³ ¹⁴ Increase in neutrophils have also been found in lavage fluid from patients with asthma before¹⁴ and after allergen challenge¹² ¹⁴ and after inhalation of ozone¹⁰ and toluene diisocyanate,¹⁵ and mast cell numbers are increased in lavage fluid in atopic patients¹⁶-¹⁸ and may be related to bronchial responsiveness.¹⁷ ¹⁸

Circulating neutrophils from asthmatic patients may be activated after challenge with allergen,¹⁹ ²⁰ and...
this increase in activation may be inhibited by steroids. The degree of activation of pulmonary neutrophils has not been investigated, but increasing in vivo and in vitro evidence suggests that alveolar macrophage function may be enhanced in asthma. There is little published evidence, however, of a relation between the activity of inflammatory cells in the airway and airway hyperresponsiveness, a feature related to the patient's symptoms and treatment requirements. Luminol and lucigenin enhanced chemiluminescence have been used as markers of reactive oxygen species released during the metabolic burst from neutrophils and macrophages respectively and have been adopted in this study as measures of cell function.

The aims of this study were (1) to compare the cell profile of lavage fluid and the metabolic activity of pulmonary neutrophils and macrophages in 22 patients with stable asthma showing a wide range of bronchial responsiveness and in 20 non-asthmatic subjects; (2) to relate the numbers and activity of inflammatory cells obtained from lavage fluid in the patients with asthma to airway responsiveness measured as the provocative dose of methacholine causing a 20% fall in FEV₁ (PD₂₀FEV₁).

**Methods**

**Subjects**

We studied 22 patients with stable asthma and a wide range of PD₂₀FEV₁ values in response to inhaled methacholine and 20 control subjects with no evidence of airflow obstruction. The mean age of the asthmatic patients was 47 (range 17–73) years and of the controls 53 (range 21–71) years. Ten asthmatic and 12 control subjects were smokers. Ethical approval for the study was obtained from the Newcastle Area Health Authority's ethical committee, with the proviso that all subjects must have a clinical indication for bronchoscopy. The nature of the investigation and details of the procedure were fully explained to each individual, and written consent was obtained. Indications for bronchoscopy among the asthmatic patients included streaking haemoptysis (13), undue cough (8), and possible stridor (1). No asthmatic patient had evidence, before or after bronchoscopy, of a neoplasm, infection, or infarction, and none had recognised bronchiectasis. The control subjects had either a peripheral radiographic abnormality (9), unexplained haemoptysis (6), or cough (5). Four of this group proved to have lung cancer, always on the side opposite to the one that was lavaged. The study followed internationally agreed guidelines and all subjects fulfilled the following criteria: (1) age 16–75 years, with an FEV₁ over 60% of the predicted value; (2) no oral corticosteroids for at least the previous three months; (3) no evidence of a chest or upper respiratory tract infection within eight weeks.

Medication in the asthmatic patients consisted of regular inhaled bronchodilators alone in nine; seven others had low dose (< 400 µg/day) inhaled steroids in addition. Six were receiving no regular treatment. Airway responsiveness to methacholine to a maximum cumulative dose of 6·4 mg was measured in all subjects four to six days before bronchoscopy by a standardised inhalation challenge technique with microprocessor controlled dosimeter. A logarithmic dose-response curve was constructed and the PD₂₀FEV₁ obtained by linear interpolation. Medication was withheld for 12 hours before this test.

Atopy was assessed by performing skin prick tests with house dust mite, grass pollens, and Aspergillus species, and any other antigen for which the subject gave a history suggesting sensitisation. A positive result was recorded if any reaction was greater than that induced by the histamine control.

Spirometry was performed before and 30 minutes after an inhaled dose of 400 µg of salbutamol, and single breath transfer factor for carbon monoxide (TLCO) was measured in all current or former tobacco smokers. All the asthmatic patients had an increase in FEV₁ of over 15% after inhaling salbutamol, a normal or raised TLCO value, and a PD₂₀ methacholine value of less than 6·4 mg. Thirteen of the 22 asthmatic patients were atopic. None of the control subjects had a measurable PD₂₀FEV₁ with 6·4 mg inhaled methacholine, and all had FEV₁ and TLCO values over 90% of the values predicted for age and height. Four subjects were atopic.

**Bronchoalveolar Lavage**

Subjects took their usual medication two to three hours before bronchoscopy, which was performed with an Olympus OT10 bronchoscope. No additional bronchodilator was given. Salbutamol was available for inhalation throughout the procedure, but was required after bronchoscopy by only one subject. Asthmatic patients were admitted overnight after lavage for observation. Premedication with intramuscular atropine 0·6 mg and papaveretum 10 mg was given 30 minutes before the procedure and 4 ml 1·5% (isotonic) lignocaine was given via a Porta-neb (Medic-aid Ltd) nebuliser compressor unit for 10 minutes before bronchoscopy. Further 2 ml aliquots of 1·5% lignocaine were administered throughout the bronchoscopy as required. The mean total volume of lignocaine given was 12 (range 8–16) ml. After routine endobronchial examination, and before any specimens were obtained for cytological or histological examination, the bronchoscope was usually wedged in a segment of the middle lobe, but in three control subjects with right sided neoplasia a segment of the
Table 1  Mean (SD) percentage and absolute cell counts in bronchoalveolar lavage fluid from 22 asthmatic and 20 control subjects

| Subjects | Total cells | Macrophages | Lymphocytes | Neutrophils | Eosinophils | Epithelial cells 
|----------|-------------|-------------|-------------|-------------|-------------|----------------
| Percentage counts | | | | | | |
| Control | 83-4 (10-9) | 12-4 (9-9) | 3-0 (2-4) | 0-2 (0-5) | | 1-0 (2-0) |
| Asthmatic | 71-8* (15-4) | 20-8* (15-1) | 4-4 (3-3) | 1-0* (1-2) | | 2-0 (2-2) |
| Absolute counts (× 10^6/l) | | | | | | |
| Controls | 1-88 (1-2) | 1-61 (1-0) | 0-19 (0-14) | 0-06 (0-05) | 0-003 (0-01) | 0-022** (0-03) |
| Asthmatics | 1-90 (1-6) | 1-42 (1-2) | 0-34* (0-34) | 0-08 (0-06) | 0-022** (0-03) | 0-04 (0-04) |

*p < 0-05, **p < 0-001 in the comparison between asthmatic and control subjects (analysis of variance).

LINGULA was used. Lavage was performed with three 60 ml aliquots of sterile phosphate buffered saline at 37°C. The fluid was aspirated immediately into siliconised glassware by vacuum suction (50–100 mm Hg) and kept at 4°C until analysis. Supplementary oxygen (4 litres/min) was given throughout the procedure and continued for at least 30 minutes after its completion.

MORPHOLOGICAL STUDIES
Analysis was completed within two hours of lavage. The specimens were filtered through 200 μm sterile steel mesh to remove mucus. Total cell counts were then performed on each aspirate by two experienced observers using a Neubauer counting chamber and counting a total of 300 cells. The results were expressed as mean values of cells × 10^6/l. As the data were skewed values were logarithmically transformed. The geometric mean ratio between the cells counts for each observer was 0-99 (95% CL = 0-94–1-05). The aspirates were centrifuged at 1200 rev/min for five minutes and the cell pellets resuspended in cell medium 199 at a concentration of 5 × 10^6/l. Cell viability was assessed by trypan blue exclusion, and cytospin preparations were made with a Shandon II Cytospin (Shandon Southern Instruments, Sewickley, PA) using 100 μl of cell suspension. The slides were air dried and stained with May-Grünwald-Giemsa and 300 cells were counted by scanning several fields, moving diagonally across the cell preparation to minimise possible artefacts induced by centrifugation. The results were expressed as mean values in absolute (× 10^6/l) and percentage terms. Monoclonal antibodies to cytokeratin were used to stain several slides at random and confirmed the accuracy with which epithelial cells were being counted in Giemsa stained preparations.

MEASUREMENT OF CHEMILUMINESCENCE
Five hundred microlitres of cell suspension, containing a calculated 250 000 cells, were added to 900 μl of 10^-4 M solutions of either luminol or lucigenin and warmed to 37°C. After the addition of 100 μl of 5% unopsonised latex particles (Sigma Chemicals), chemiluminescence was measured with an LKB 1250 luminometer. Assays were performed in triplicate, and the mean peak height was recorded in mv. This was expressed both as total chemiluminescence and as chemiluminescence per 1000 neutrophils for luminol and chemiluminescence per 1000 macrophages for lucigenin to exclude variations due to differing cell profiles. Lucigenin enhanced chemiluminescence has previously been used to represent alveolar macrophage function, and recent work has shown that, cell for cell, alveolar macrophages produce almost twice as much lucigenin enhanced chemiluminescence as polymorphs. In our mixed cell populations, which consisted largely of macrophages, lucigenin enhanced chemiluminescence will predominantly reflect macrophage activity. Luminol enhanced chemiluminescence has often been used to assess neutrophil function, and is not produced in substantial amounts by alveolar macrophages. The within subject variance for assay was 2% for lucigenin enhanced chemiluminescence and 3% for luminol enhanced chemiluminescence.

Analysis of variance was used to compare data from asthmatic and control subjects, and for analysis of the

Table 2  Mean (SD) luminol and lucigenin chemiluminescence (CL) in cells from bronchoalveolar lavage fluid in 22 asthmatic and 20 control subjects

<table>
<thead>
<tr>
<th>Total luminol CL (mv)†</th>
<th>Total lucigenin CL (mv)†</th>
<th>Luminol CL/1000 neutrophils</th>
<th>Lucigenin CL/1000 macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthmatics</td>
<td>Controls</td>
<td>Asthmatics</td>
<td>Controls</td>
</tr>
<tr>
<td>6:77 (6-2)*</td>
<td>2:04 (2-2)</td>
<td>14:93 (14-2)**</td>
<td>4:39 (4-7)</td>
</tr>
</tbody>
</table>

*p < 0-005, **p < 0-001 in the comparison between asthmatic and control subjects (analysis of variance).
†Mixed aliquots of 250 000 cells.
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Percentages of neutrophil count

![Graph showing percentage neutrophil count against \(\log \text{PD}_{20}\)]

- For smokers, \(r = 0.60, p < 0.005\)
- For non-smokers, \(r = 0.52, p < 0.05\)

Lavage cell profiles

The total cell counts and the absolute numbers of macrophages, neutrophils and epithelial cells did not differ significantly between the two groups (table 1). The absolute and percentage eosinophil counts,

Percentage lymphocyte count

![Graph showing percentage lymphocyte count against \(\log \text{PD}_{20}\)]

- For smokers, \(r = 0.43, p < 0.05\)

Results

Bronchoalveolar lavage was well tolerated and none of the subjects developed wheezing. The median total volume aspirated at lavage was similar for the asthmatic (86 (range 11–128) ml) and control subjects (89 (range 32–130) ml). Cell viability was over 92% in all specimens.

Luminol CL (mv/1000 neutrophils)

![Graph showing luminol chemiluminescence (CL) against \(\log \text{PD}_{20}\)]

- For smokers, \(r = 0.17\), NS

Effects of smoking. All data were logarithmically transformed to reduce skewness. Linear regressions were computed by least squares analysis, after logarithmic transformation of \(\text{PD}_{20}\) FEV1 values.
however, were significantly greater in the asthmatic patients than in the control subjects (p < 0.05), as were absolute and percentage lymphocyte counts (p < 0.05). Percentage macrophage counts were lower in the asthmatic patients than in the control subjects (p < 0.05), reflecting the other changes.

**METABOLIC CELLULAR ACTIVITY**

Mean total luminol enhanced chemiluminescence was greater in the cells obtained from asthmatic patients than in those from control subjects. Mean lucigenin enhanced chemiluminescence was also greater in cells from asthmatic patients than in cells from control subjects. These differences are also highly significant when expressed as chemiluminescence per 1000 neutrophils and per 1000 macrophages respectively (table 2).

**RELATION BETWEEN CELL NUMBERS, FEV₁, AND BRONCHIAL RESPONSIVENESS**

Initial FEV₁ was not correlated significantly with PD₂₀FEV₁, cell counts, or indices of cellular activity in either group. In the asthmatic patients PD₂₀FEV₁ was correlated negatively with percentage neutrophil counts (fig 1) but not with absolute neutrophil counts (r = −0.17). There was a significant positive correlation between PD₂₀FEV₁ and percentage lymphocyte counts (fig 2) but no significant correlations between PD₂₀FEV₁ and macrophage (r = 0-24), eosinophil (r = 0-30), or epithelial cell numbers (r = 0-03).

**EFFECT OF SMOKING ON CELL NUMBERS**

Smoking was associated with an increased total cell count in each group (table 3; p < 0.001). This was almost entirely due to an increased number of macrophages (p < 0.001). There were more neutrophils and eosinophils in smokers than in non-smokers, but these differences did not reach statistical significance.

**EFFECT OF INHALED STEROIDS ON CELL NUMBERS AND ACTIVITY**

The asthmatic patients who regularly inhaled steroids did not differ in PD₂₀FEV₁ values, cell numbers, or chemiluminescence values from the asthmatic group as a whole (fig 1–4).

**DISCUSSION**

In this study we have shown a significant increase in...
the numbers of eosinophils and lymphocytes obtained in lavage fluid from patients with stable asthma; the increase in lavage fluid neutrophils was non-significant, though the absolute numbers of neutrophils were much greater than the numbers of eosinophils. The metabolic activity of both neutrophils and macrophages obtained at lavage, assessed by stimulated chemiluminescence, was increased in our asthmatic patients.

An increase in eosinophils in lavage fluid has been described in patients with stable asthma by Godard et al.9 and Tomioka et al.,10 and by others in response to a chemotactic or allergic stimulus.11,12 A correlation between eosinophil numbers and airway responsiveness has also been reported in a small group of individuals with atopic asthma.13 Our subjects were generally older than those in the study by Kirby et al.,14 (47 v 31 years). In our study eosinophils were no more numerous in atopic than in non-atopic asthmatic patients and their numbers in lavage fluid did not correlate significantly with bronchial responsiveness. We did, however, find a significant correlation between percentage neutrophil count and bronchial responsiveness in the asthmatic patients, a relationship not previously reported,15 although both Fabbri et al.16 and Seltzer et al.,17 using isocyanate and ozone challenges, have described large influxes of neutrophils into the airways in association with the induction of bronchial hyperresponsiveness. Both eosinophils and neutrophils are capable of secreting widely varying inflammatory mediators, including leukotriene B4, prostaglandins, and platelet activating factor; but it is not yet clear which has the predominant role in inducing hyperresponsiveness in the inflammatory reaction found in the airways in man.

Previous studies9,18 have reported an increase in lymphocyte numbers in stable asthma, Godard et al.9 finding an almost twofold increase in the percentage of lymphocytes in lavage fluid in patients who were similar to ours in mean age and in the amount of lavage fluid recovered. We found a significant positive correlation between the numbers of lymphocytes recovered in lavage fluid and PD_{25}FEV_{1}. Circulating lymphocytes have been reported to be activated in asthma19 and pulmonary lymphocytes are now thought to play some part in mast cell recruitment.20 Recently the amount of histamine releasing factor produced by lymphocytes has been shown to be correlated with bronchial responsiveness.21 Diaz et al.22 have shown changes in lymphocyte subsets after allergen challenge,23 suggesting a possible immune modulating effect. Further studies of lymphocyte markers and function in asthma may clarify their role in this condition.

Like most other workers, we were unable to show any differences in absolute macrophage numbers between asthmatic and control subjects. Godard et al.9 like us, reported a decrease in percentage macrophage counts in asthmatic patients, but his absolute numbers did not differ significantly from control values.

We did not count the numbers of mast cells present in our cytospin preparations as estimates of mast cell numbers from slides not specifically prepared for this purpose might have been inaccurate. Several different techniques have been used to fix and stain mast cells, and the numbers recorded in lavage fluid from patients with stable asthma has varied considerably (from 0.25%16 to 3%17). Although mast cells probably play an important part in the development of the early asthmatic reaction, and their numbers in lavage fluid may be correlated with bronchial responsiveness,17,18 their role in chronic asthma remains to be established.24

Seven of our asthmatic patients were regularly inhaling corticosteroids. We could detect no differences in airway responsiveness or lavage results between these patients and the asthmatic subjects as a whole. We are currently investigating their effects on the numbers and activation of cells obtained from the airways of asthmatic patients in more detail.

We have previously shown that filtration of lavage fluid to remove mucus can also reduce cell counts, largely through modest loss of macrophages.25 Accurate cell counts and functional studies are difficult to perform in the presence of mucus, and the small resulting change in cell counts was considered a necessary expedient. A chemically inert steel mesh was used rather than the more frequently used cotton gauze to avoid the risk of cell activation. As lavage fluid from all subjects was treated in an identical manner, routine filtration is unlikely to have introduced major inaccuracies.

The production of superoxide radicals by macrophages is an important microbiocidal mechanism30 that has been shown to occur after sensitisation with serum from asthmatic patients.31 Release of superoxide radicals contributes to inflammation31 and they are thought to be predominantly responsible for lucigenin enhanced chemiluminescence,32 which has now been used to assess alveolar macrophage activity in several conditions.29,30 Lucigenin enhanced chemiluminescence is abolished by superoxide dismutase (unpublished observation). The present results indicate that alveolar macrophage activity is directly related to the degree of airway responsiveness, and suggest a pivotal role for this cell in the pathophysiology of asthma. Evidence that alveolar macrophages have a role in asthma has previously been suggested by the finding of increased levels of β glucuronidase33 and platelet activating factor22 in lavage fluid after local antigen challenge. These
mediators are thought to be released after macrophage activation via IgE receptors, which have been found in greater numbers on the surface of the alveolar macrophages of asthmatic patients than of control subjects.23

Luminol enhanced chemiluminescence is myeloperoxidase dependent, and greatly depressed in patients with myeloperoxidase deficient leukocytes.42 A recent study in which luminol enhanced chemiluminescence was increased in lavage fluid cells from asthmatic patients was interpreted as indicating increased macrophage activity.43 Granulocyte numbers, however, were closely correlated with luminol enhanced chemiluminescence, suggesting that activity of these cells, and not macrophages, was being measured. This would be in keeping with our findings40 and those of Williams and Cole.25

We also found luminol enhanced chemiluminescence to be increased in patients with asthma, and interpret this as indicating neutrophil activation. There is already some evidence suggesting activation of neutrophils in asthma. Neutrophil complement rosettes were increased during both early and late asthmatic reactions in patients challenged with allergen.19 20 These changes were immediately preceded by a rise in circulating neutrophil chemotactic factor in both studies, and could be reversed by corticosteroid treatment,21 which also caused a decrease in leukotriene release by stimulated neutrophils.44 Our data suggest that neutrophil numbers but not activity are directly related to the degree of bronchial responsiveness. Neutrophils appear to be uniformly active over a wide range of disease activity.

It has been suggested that cellular activity may be depressed in the presence of neoplasia, so the possibility that chemiluminescence was reduced in our four control subjects who had lung cancer must be considered, though the contralateral lung was lavaged in each case. The chemiluminescence results for these subjects were similar to the mean chemiluminescence values for the control group, so neoplasia in these four subjects is unlikely to have affected the group comparisons.

Cigarette smoking is known to cause a twofold to fourfold increase in absolute lavage fluid cell counts, mainly owing to an increase in macrophages.45 In the present study there was a twofold increase in macrophage numbers in smokers in both the control and the asthmatic group. There were more neutrophils in smokers than in non-smokers, and among non-smokers the neutrophil count was higher in the asthmatic than the control subjects, though in both cases the changes failed to reach statistical significance. A smoking related increase in neutrophils in lavage fluid has been described previously and may be relevant to the development of flow limitation in smokers.46

Smoking did not alter luminol or lucigenin enhanced chemiluminescence per 1000 neutrophils, but contributed to total chemiluminescence in asthmatic subjects by increasing cell numbers. Others have found in vivo exposure to cigarette smoke to have profound effects on the numbers and function of both pulmonary macrophages and circulating neutrophils47 48 and, although alveolar macrophages from smokers appeared to be generally activated in vivo, certain functional characteristics are depressed by cigarette smoke.49 Alveolar macrophages from smokers have been reported to have increased glucose consumption.50 Smoking had no effect on glucose oxidation in another study,51 however, and, although the production of superoxide appeared to be greater in macrophages obtained from the lungs of smokers, there was no concomitant increase in oxygen consumption per cell. More recent work has shown a significant increase in superoxide production from smokers' macrophages only after specific chemical stimulation.52 Our data suggest that smoking has no effect on superoxide generation from macrophages stimulated with 5% latex.

This study has shown correlations between the degree of airway responsiveness and the numbers and activity of certain inflammatory cells in lavage fluid from a group of patients with asthma. Although the function of inflammatory cells in asthmatic airways is likely to be complex, our findings are consistent with a primary effector role for alveolar macrophages in asthma. These have the potential to induce an influx of neutrophils into the airways by the release of chemotactic factors.53 Once the neutrophil reaches the airway it appears to be activated to a similar extent across a wide range of airway responsiveness.

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References

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37 Joseph M, Tonnel A-B, Capron A, Voisin C. Enzyme rise and superoxide anion production by human alveolar


45 Reynolds HH, Newball HH. Analysis of protein and respiratory cells obtained from human lungs by bronchial lavage. J Lab Clin Med 1974;84:559–73.


