Relationship between the inflammatory infiltrate in bronchial biopsy specimens and clinical severity of asthma in patients treated with inhaled steroids

Jacob K Sont, J Han J M van Krieken, Christine E Evertse, Ria Hooijer, Luuk N A Willems, Peter J Sterk

Abstract

Background – Current guidelines on the management of asthma advocate the use of anti-inflammatory treatment in all but mild disease. They define disease control in terms of clinical criteria such as lung function and symptoms. However, the relationship between the clinical control of the disease and inflammation of the airways is not clear. A cross sectional study was therefore undertaken to investigate the relationship between airways inflammation and measures of clinical control and bronchial hyperresponsiveness in asthmatic patients treated with inhaled steroids.

Methods – Twenty six atopic adults (19–45 years) with mild to moderate asthma (baseline forced expiratory volume in one second (FEV₁) ≥ 50% predicted, concentration of histamine causing a 20% fall in FEV₁, (PC₂₀) 0–02–7–6 mg/ml) on regular treatment with inhaled steroids entered the study. Diary card recordings during the two weeks before a methacholine challenge test and bronchoscopic examination were used to determine peak flow variability, symptom scores, and use of β₂ agonists. Biopsy specimens were taken by fibreoptic bronchoscopy from the carina of the right lower and middle lobes, and from the main carina. Immunohistochemical staining was performed on cryostat sections with monoclonal antibodies against: eosinophil cationic protein (EG₁, EG₂), mast cell tryptase (AA1), CD45, CD22, CD3, CD4, CD8, CD25, and CD45RO. The number of positively stained cells in the lamina propria was counted twice by using an interactive display system.

Results – There were no differences in cell numbers between the three sites from which biopsy specimens were taken. The PC₂₀ for methacholine was inversely related to the average number of total leukocytes, EG₁+, and EG₂+ cells, mast cells, CD8+, and CD45RO+ cells in the lamina propria. These relationships were similar for each of the biopsy sites. Symptom scores, β₂ agonist usage, FEV₁, and peak flow variability were not related to any of the cell counts.

Conclusions – Infiltration of inflammatory cells in the lamina propria of the airways seems to persist in asthmatic outpatients despite regular treatment with inhaled steroids. The number of infiltrating leucocytes such as mast cells, (activated) eosinophils, CD8+, and CD45RO+ cells in bronchial biopsy specimens from these patients appears to be reflected by airway hyperresponsiveness to methacholine, but not by symptoms or lung function. These findings may have implications for the adjustment of anti-inflammatory treatment of patients with asthma.

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Keywords: atopic asthma, bronchial hyperresponsiveness, inflammatory infiltrate.

Asthma is a chronic inflammatory disorder of the airways with a characteristic inflammatory infiltrate of mast cells, lymphocytes, and eosinophils in the bronchial epithelium and lamina propria. The symptoms of wheezing, chest tightness, difficult breathing, and coughing develop after exposure to bronchoconstrictor stimuli and are associated with variable airways obstruction which can be provoked in the laboratory, thereby demonstrating airway hyperresponsiveness. The disease can therefore be assessed at different levels: the severity of symptoms, the level of obstruction and degree of airway hyperresponsiveness, and the extent of airway disease.

To date there is no “gold standard” parameter that ultimately reflects asthma severity towards which therapeutic interventions should be aimed. The present international consensus on the diagnosis and treatment of asthma is based on the working hypothesis that it is desirable to reverse or prevent airways inflammation. However, at present the treatment level is solely guided by clinical indices such as symptoms and lung function and the current therapeutic approach is based on the assumption that there is a relationship between the clinical indices of asthma severity and airways inflammation.

When using the scoring system of Aas to assess symptoms over a one year period, it has been shown that the severity of the symptoms is related to the number and activity of eosinophils in the airways epithelium and in the bronchoalveolar lavage (BAL) fluid of patients.
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The forced expiratory volume in one second (FEV₁), expressed as a percentage of the predicted value,⁶-⁸ and airway hyperresponsiveness to inhaled methacholine⁹-¹² are positively related to the number and activity of eosinophils and activated CD4 + T cells in BAL fluid from subjects with atopic asthma. Furthermore, the provocative concentration of methacholine causing a 20% fall in FEV₁ (PCₒ₀) has also been shown to be related to the number of activated eosinophils in bronchial biopsy specimens.⁷,¹³,¹⁴ Hence, there is evidence that some of the clinical indices of asthma are associated with airways inflammation, at least in patients not on treatment with anti-inflammatory medications.

In order to adjust treatment in patients using anti-inflammatory drugs, the international guidelines rely on the following clinical characteristics: symptom severity, β₂ agonist usage, peak flow variability, and spirometric parameters.¹⁵ To date it has not been systematically investigated which of these indices is most closely related to the level of airways inflammation in asthmatics using inhaled corticosteroids. In addition, it is still unclear whether measures of airways responsiveness can provide complementary information to symptoms and lung function on the disease state of the airways in these patients.

In the present study we have investigated whether clinical asthma severity, expressed according to present guidelines, is related to inflammatory activity in the airways of patients already using regular inhaled steroids. If so, such a relationship justifies the usage of clinical indices to adjust anti-inflammatory therapy. To that end we have assessed the relationship of symptom scores, β₂ agonist usage, peak flow variability, FEV₁, and airways responsiveness to methacholine with mast cell number and number and activity of eosinophils and (sub) populations of lymphocytes in bronchial biopsy specimens from patients with atopic asthma who were on regular treatment with inhaled corticosteroids.

Methods

Subjects

Twenty-six non-smoking atopic adults (17 men, six ex-smokers) of mean age 29 years (range 19–45) with a wide range in asthma severity were asked to participate in the study (table 1) at the outpatient clinic of the University Hospital Leiden. All patients had a history of episodic chest tightness and wheezing in the previous year and were on regular treatment with inhaled corticosteroids for a mean (SD) of 21 (26) months. Atopy was indicated by a positive skin prick test (>3 mm weal) to one or more common airborne allergen extracts (Soluprick, ALK, Denmark). Pre-bronchodilator FEV₁ was more than 50% of predicted¹⁶ and >1.5 l, whilst post-bronchodilator FEV₁ was within the normal range (>80% predicted). The PC₂₀ for histamine at the start of the study was 0.02–7.3 mg/ml (geometric mean 0.42 mg/ml). Symptoms were additionally controlled by on demand usage of short acting β₂ agonists that were withheld for at least eight hours before each challenge test. All subjects gave their informed consent and the study was approved by the local ethical committee.

Study Design

The study was of a cross sectional design. At entry to the study the subjects were equipped with diary cards and a mini-Wright peak flow meter and were asked to record symptoms, peak flow rates (PEF), and bronchodilator usage on the diary cards during the two weeks before the methacholine challenge test. Fibre-optic bronchoscopy was carried out 3–6 days later.

Diary Cards

The symptom score parameter was calculated from the diary card scores for night time asthma, morning tightness of the chest, daytime asthma, and daytime cough.¹⁷ Each item could range from 0 to 4 on each day. The highest score for each day among these four items was then added up over a period of 14 days.

Bronchodilator usage was recorded as the total number of puffs of salbutamol or ipratropium bromide during 14 days registered on the diary cards and was expressed per week.¹⁵ PEF values were obtained from twice daily measurements, on waking and before bedtime, and from additional measurements when a bronchodilator had been used. Diurnal variability in PEF was expressed as the mean value of 14 days calculated from the highest minus the lowest daily value, divided by the highest.¹⁵

Inhalation Challenge Test

Methacholine inhalation challenge tests were performed according to a standardised tidal breathing method.¹⁶ The level of baseline lung function was determined as the mean of three reproducible values (within 5%) of the FEV₁, and was expressed as a percentage of the predicted value (FEV₁/%pred). Doubling concentrations of methacholine in normal saline (0.03–256 mg/ml; Janssen Pharmaceutica, Beerse, Belgium) were aerosolised at room temperature by a DeVilbiss 646 nebuliser (output 130 mg/min; DeVilbiss Co, Somerset, Pennsylvania, USA) operated by oxygen and connected to the central chamber of an inspiratory and expiratory valve box with an expiratory aerosol filter (Pall Ultipor BB50T). The aerosols were inhaled by tidal breathing for two minutes at five minute intervals through the mouth with the nose clipped. The response was measured as FEV₁ recorded by a dry rolling seal spirometer (Morgan Spiroflow, Rainham, UK) 90 seconds after each dose. Bronchodilators were administered after completion of the test.

The response of FEV₁ was expressed as the percentage fall from mean baseline value and was plotted against the log concentration of methacholine. The dose-response curves were characterised by their position, which was expressed as PC₂₀.¹⁸

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Table 1  Patient characteristics, clinical indices of asthma severity and airways hyperresponsiveness to methacholine

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Inhaled steroids</th>
<th>Mean symptom score</th>
<th>p(2) agonist usage (puff/week)</th>
<th>FEV1 (Pred)</th>
<th>PEF variability (%)</th>
<th>PCCO2* (mg/ml)</th>
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<tr>
<td>Mean (SD)</td>
<td>29 (7-4)</td>
<td></td>
<td>21 (26)</td>
<td>3.2 (2.3)</td>
<td>8.0 (11.5)</td>
<td>90.2 (16.9)</td>
<td>10.3 (6.8)</td>
<td>0.73 (2.1)</td>
</tr>
</tbody>
</table>

FEV1 = forced expiratory volume in one second; PEF = peak expiratory flow; PC20 = concentration of methacholine provoking a 20% fall in FEV1; BDP = beclometasone dipropionate; Bud = budesonide.

* Geometric mean (SD) in doubling dose.

BRONCHOSCOPIC EXAMINATION
Fibreoptic bronchoscopy was carried out by an experienced investigator (LNAW) using a standardised protocol according to recent recommendations. In brief, the subjects were premedicated with atropine 0.5 mg subcutaneously, codeine 20 mg orally, and salbutamol 400 μg by metered dose inhaler, and the nasal passages and the oropharyngeal space were sprayed with 10% lignocaine 30 minutes before the procedure. The lower airways were anaesthetised by nebulising a solution containing 2% lignocaine. Fibreoptic bronchoscopy was performed using a Pentax bronchoscope (outer diameter 6 mm; Pentax Optical Co, Japan). Three bronchial biopsies were taken at (sub)segmental level from the right lower lobe, the middle lobe, and the main carina using a pair of cup forceps (Olympus FB-22C, Tokyo, Japan). Throughout the procedure 100% oxygen was delivered via a nasal cannula (4 l/min) and oxygenation was monitored positively in all subjects using an oximeter (N-180, Nellcor Inc, Hayward, USA) with a finger probe.

PROCESSING OF ENDOBRONCHIAL BIOPSY SPECIMENS
Biopsy samples were immediately embedded in OCT medium (Miles Inc, Diagnostics Division, Elkhart, USA), and snap frozen in isopentane cooled by iced CO2. Samples were stored at −70°C pending further processing. Six micrometer cryostat sections were air-dried for one hour and fixed in a mixture of equal parts of acetone and methanol for two minutes. Immunohistochemical staining was performed using an indirect immunoperoxidase technique with monoclonal antibodies against: eosinophil staining for eosinophil cationic protein (EG2) or the secreted form of eosinophil cationic protein (EG3) (both Pharmacia, Sweden), mast cell tryptase (AA1, donated by Dr R Djukanovic, Southampton University General Hospital, Southampton, UK), total leucocytes (CD45), B cells (CD22), T lymphocytes (CD3), T helper cells (CD4), T suppressor cells (CD8), activated T cells (CD25) (all Becton Dickinson, Mountain View, California, USA), and T cells CD45RO (UCHL1) (DAKO, Glostrup, Denmark).

QUANTITATIVE ANALYSIS OF BIOPSY SPECIMENS
All biopsy specimens were coded and sections were examined twice at 200× magnification by one investigator (JKS) in a blind fashion by means of a video interactive display analysis system (VIDAS II, Kontron Elektronik GmbH, Munich, Germany) at least one week apart. A total area of 0.123 mm2 was randomly chosen and presented on a video screen. The area of lamina propria was subsequently determined by delineating the widest possible 125 μm deep zone beneath the epithelial basement membrane (at least 10 000 μm2), excluding damaged tissue and BALT. The number of positively stained cells was counted within this area and expressed as the number of cells/mm2 calculated on pooled data of the two occasions.

STATISTICAL ANALYSIS
The differences in mean cell numbers between the three sites were tested by multiple analysis of variance (MANOVA). Spearman’s rank correlation coefficients were used to assess the relationships of the following clinical indices of asthma severity as independent variables: symptom scores, β2 agonist usage, FEV1,pred, peak flow variability, and the methacholine PC20 with the following dependent variables: average cell number/mm2 for the three sites staining for CD45, EG1, EG2, CD3, CD4, CD8, CD25, CD22, CD45RO, and the CD4/...
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Table 2  Mean (SD) number of stained cells/mm² in the lamina propria at the (sub) segmental and central level in 26 atopic asthma subjects using inhaled corticosteroids

<table>
<thead>
<tr>
<th>Cells</th>
<th>Right lower lobe (n=22)</th>
<th>Middle lobe (n=24)</th>
<th>Main carina (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45+</td>
<td>1354 (705)</td>
<td>1626 (861)</td>
<td>1471 (686)</td>
</tr>
<tr>
<td>EG+</td>
<td>110 (89)</td>
<td>105 (124)</td>
<td>93 (81)</td>
</tr>
<tr>
<td>EG+</td>
<td>118 (123)</td>
<td>86 (105)</td>
<td>81 (116)</td>
</tr>
<tr>
<td>AA1+</td>
<td>175 (105)</td>
<td>157 (86)</td>
<td>182 (111)</td>
</tr>
<tr>
<td>CD3+</td>
<td>1111 (715)</td>
<td>1446 (878)</td>
<td>1311 (667)</td>
</tr>
<tr>
<td>CD4+</td>
<td>558 (298)</td>
<td>800 (550)</td>
<td>651 (456)</td>
</tr>
<tr>
<td>CD8+</td>
<td>583 (399)</td>
<td>750 (590)</td>
<td>766 (425)</td>
</tr>
<tr>
<td>CD22+</td>
<td>10 (22)</td>
<td>32 (89)</td>
<td>6 (16)</td>
</tr>
<tr>
<td>CD45RO+</td>
<td>1514 (1023)</td>
<td>1792 (851)</td>
<td>1614 (778)</td>
</tr>
<tr>
<td>CD4+/CD8+</td>
<td>1-09 (0-83)</td>
<td>1-33 (1-29)</td>
<td>0-94 (0-51)</td>
</tr>
</tbody>
</table>

CD8 ratio. MANOVA was used on the rank numbers to assess whether a significant relationship between the average number of a particular cell and clinical index was different among the three biopsy sites. The interaction term of the biopsy site and the clinical index was tested against the between subjects error term.21 Results were considered significant when the chance of a type I error was <5% (p<0-05). All analyses were performed using the SPSS-PC +.

Results

Results of the diary card recordings and lung function measurements are presented in table 1. The average symptom score was 3-2, ranging from 0 to 9 (16 = maximum possible) and the average β2 agonist usage was 8-0 puffs/week (range 0–51). The mean FEV1 before the methacholine challenge was 90% of predicted (range 51–120% predicted). The mean PEF variability was 10-3% (range 2-1–27-1) and the geometric mean methacholine PC20 was 0-73 mg/ml (range 0-02–18-2 mg/ml).

The mean (SD) numbers of (sub) populations of leucocytes per area (cells/mm²) in the lamina propria of biopsy specimens at the subsegmental level of the right lower lobe, the segmental level of the middle lobe, and the central level of the main carina are shown in table 2. No significant differences in cell numbers were seen between the three sites. CD25+ cells were only found in one subject and were therefore excluded from statistical analysis. The relationships between the clinical indices of asthma severity and the average cell counts at the three sites are shown in table 3. The methacholine PC20 was inversely related to the mean number of total leucocytes (r = -0-43, p = 0-05), EG+ (r = -0-56, p<0-005) and EG+ + mast cells (r = -0-58, p<0-005), mast cells (r = -0-41, p = 0-05), CD8+ (r = -0-47, p<0-05), and CD45RO+ cells (r = -0-43, p<0-05) (figs 1 and 2). There was a trend towards a negative relationship between the methacholine PC20 and CD3+ cells (r = -0-33, fig 2). There were no significant differ-

![Figure 1](http://thorax.bmj.com/)  Relationship between airways responsiveness to methacholine and the number of (A) eosinophils (EG+,), (B) activated eosinophils (EG+ +), (C) mast cells (AA1+,), and (D) total leucocytes in the bronchial lamina propria averaged for three sites. Spearman’s rank correlation coefficients are indicated.
ences in the relationships between methacholine $PC_{20}$ and the cell counts among the three biopsy sites.

Symptom scores, $\beta_2$ agonist usage, FEV$_1$, and peak flow variability were not significantly related to any of the cells (table 3). However, there was a trend towards a negative relationship between FEV$_1$,%pred and CD8$^+$ cells ($r = -0.34$) and between peak flow variability and the number of mast cells ($r = 0.38$).

**Discussion**

This study shows that infiltration of inflammatory cells in the lamina propria of the airways seems to persist despite regular treatment with inhaled steroids in asthmatic outpatients. In addition, it appears that, among the clinical indices of asthma severity, the degree of airway hyperresponsiveness to methacholine is most closely related to the number of leukocytes, (activated) eosinophils, mast cells, CD8$^+$, and CD45RO$^+$ lymphocytes in bronchial biopsy specimens from atopic asthmatic subjects who are receiving treatment with inhaled steroids. This inverse relationship is a consistent one and is observed at the (sub)segmental level in the right lower lobe, the middle lobe, and the main carina. It is remarkable that the most commonly used clinical indices such as symptom severity, $\beta_2$ agonist usage, peak flow variability, and FEV$_1$, are not significantly correlated with inflammatory cell counts in the lamina propria of the airways. These findings may have implications for the adjustment of anti-inflammatory treatment in asthma.

There have been a number of conflicting reports on the relationship between mast cell or eosinophil number in bronchial biopsy specimens and the clinical indices in atopic asthma. It has been reported that symptom severity over a one year period is related to the number and activity of eosinophils in the airway epithelium and in the BAL fluid in patients not on treatment with anti-inflammatory medications.

However, in our group of atopic asthmatic subjects using inhaled corticosteroids we could not establish a positive relationship between symptom scores and (activated) eosinophils. Our results confirm and extend those of Djukanovic et al$^{13}$ who could not demonstrate a correlation between symptom scores, $\beta_2$ agonist consumption, or peak flow variability and submucosal AA1 + mast cells or EG2 + eosinophils in subjects with asthma who were not treated with inhaled steroids. Our findings therefore further confirm that the currently recommended clinical indices of asthma severity do not reflect the extent of airways inflammation in this disease.

In contrast to symptoms and lung function, the degree of airways hyperresponsiveness appeared to be associated with the histopathology in our patients. Again, this has been a matter of controversy. An inverse relationship between the $PC_{20}$ for methacholine and the number of activated eosinophils and mast cells in bronchial biopsy specimens has been reported previously in asthmatic patients without steroid treatment.$^{14,22}$ However, other investigators were unable to confirm this.$^{13,25}$ This failure might have been due to a small sample size, or to a relatively narrow range in airways hyperresponsiveness compared with our study. The relatively weak relationship between $PC_{20}$ and CD45RO$^+$ cells in our study confirms previous findings in a group of atopic asthmatic
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subjects who were not using inhaled corticosteroids. Thus, a relationship between airways hyperresponsiveness and histopathology may only be revealed by using a sufficiently large group of atopic asthmatics characterised by a wide range of airways hyperresponsiveness as in the present study.

The absence of a relationship between clinical indices of asthma severity and histopathology of the airways does not seem to be caused by patient selection, study design, statistical power, or the processing and analysis of bronchial biopsy specimens for the following reasons. Firstly, in order to ensure that time related variability in the measurements did not obscure potential associations, the bronchoscopy was carried out within six days of the FEV₁ and PC20 measurements and last diary card registrations. Secondly, the patients in this study were carefully selected to be non-smokers, having atopic asthma of mild to moderate severity with a wide range in FEV₁ between 51% and 120% of the predicted value. Thirdly, with the present number of subjects a correlation coefficient of ≥0.40 or ≤−0.40 yields a p value <0.05. Therefore, it seems unlikely that the absence of a significant correlation of modest strength can be explained by a problem with the power of the study. It can be argued that the use of inhaled steroids by these patients might have masked a potential relationship between clinical indices and cell counts. Due to the method of patient selection we cannot exclude such a possibility, because this study was designed to assess which parameter among the clinical indices best reflected the degree of airways inflammation in patients already using anti-inflammatory treatment. The range and mean numbers of infiltrating inflammatory cells in the bronchial biopsy specimens in our study were slightly higher than those in other studies of asthmatic patients. In those studies ≤1 µm sections of specimens embedded in plastics were used, whereas our cell numbers were similar to those reported by Trigg et al who used 5 µm cryostat sections. The present histopathological methods are further validated by the highly consistent findings between the three sites in the trachea and bronchial tree. We therefore believe that our results are unlikely to be explained by methodological errors.

How can the present results be interpreted? The association between eosinophils and mast cells in the lamina propria and methacholine responsiveness fits in with their role in the development of airways hyperresponsiveness. This includes secretion of a variety of inflammatory mediators capable of inducing epithelial desquamation, plasma extravasation, and hypersecretion, thereby increasing the thickness of the airway wall. The complexity of the pathophysiology of asthma is well illustrated by our present findings. In four subjects who reported symptoms we were unable to detect an infiltrate of eosinophils in the lamina propria. In contrast, some other subjects had clear evidence of airway inflammation in spite of the absence of symptoms and with lung function parameters within the normal range.

The latter finding extends observations in acute severe asthma indicative of a dissociation between clinical recovery and bronchial hyperresponsiveness and is in keeping with a persistent increase in airways responsiveness in subjects who are clinically considered to be in remission of asthma. Our findings seem to imply that the presence of an inflammatory infiltrate does not in itself lead to symptoms of asthma or airways obstruction, but might point to long term disease activity.

According to the current international recommendations for the treatment of asthma the level of anti-inflammatory treatment is chosen to minimise symptoms, optimise lung function, and to prevent exacerbations. The dosage of regular treatment should be kept to the minimum level that fulfils these objectives. The question is whether this approach leads to the optimal long term outcome of asthma. A number of studies have shown that treatment with inhaled steroids up to four months is very effective, particularly in reducing the number of mast cells and eosinophils in the airway wall which is often paralleled with improvements in symptoms, lung function, and airways hyperresponsiveness to methacholine. Long term studies on the effect of inhaled steroids on the histopathology of the airways have not yet been reported. However, the observation that airways hyperresponsiveness continues to improve even after six months of treatment, when no further increase in FEV₁ occurs, suggests that it may take relatively longer to resolve the airway histopathology.

It can be argued that maintenance of an inflammatory state of the airways may result in anatomical changes and functional disorders. This might be reflected by abnormal PC20 levels since two recent population based prospective studies have shown that airways hyperresponsiveness is a significant predictor of subsequent accelerated decline in pulmonary function. We therefore postulate that, in addition to the current guidelines, the treatment of asthma should also be directed towards reducing airways hyperresponsiveness. This might benefit the long term goal of asthma management as formulated in the international guidelines – that is, the prevention of the development of irreversible airways obstruction. Clearly, such a treatment regimen needs to be tested in long term prospective studies.

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