Leukocyte counts in hypertonic saline-induced sputum in subjects with occupational asthma


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We measured markers of eosinophilic inflammation in the blood and in the sputum induced by hypertonic saline (HS) inhalation of 24 subjects with occupational asthma who were still exposed to high molecular weight compounds (HMWCs, n=8) or to low molecular weight compounds (LMWCs, n=16); all subjects were symptomatic and showed bronchial hyperresponsiveness to methacholine at the time of study. Sputum cell counts were also measured in 14 normal subjects and in 24 subjects with non-occupational asthma with asthma severity similar to that of occupational asthmatics.

Both occupational and non-occupational asthmatic subjects showed higher neutrophil percentages in HS-induced sputum than normal subjects, asthmatics with LMWC-induced asthma showing the highest values. Eosinophil percentages in HS-induced sputum were higher in non-occupational asthmatics and in asthmatics with HMWC-induced asthma than in normal subjects and in subjects with occupational asthma due to LMWCs. No difference in bronchial responsiveness, peak expiratory flow variability and serum eosinophil cationic protein (ECP) levels were observed among the different asthma groups. Although sputum eosinophil percentages significantly correlated with blood eosinophil percentages, sputum allowed the detection of a higher number of subjects with eosinophilic inflammation than blood. Serum ECP levels were normal in most asthmatic subjects. A significant correlation between sputum eosinophil percentages and bronchial hyperresponsiveness to HS was observed.

Despite a similar degree of functional abnormalities, subjects with asthma due to LMWCs and still exposed to the occupational sensitizer showed a lower degree of eosinophilic inflammation and a higher degree of neutrophilic inflammation in the airways than subjects with occupational asthma due to HMWCs or non-occupational asthmatics. Furthermore, sputum eosinophil counts detect, better than blood indices, the degree of airway inflammation in both occupational and non-occupational asthma.

Introduction

Airway inflammation is a major factor in the pathophysiology of asthma (1). The cellular and molecular markers of this inflammation appear to correlate with the severity of asthma (2) and with its outcome according to the exposure to a specific sensitizer (3) and to pharmacological treatment (4). Until recently, the analysis of cells and mediators in the airway lining fluid and the bronchial mucosa from stable asthmatic subjects has been performed by means of bronchial lavage (BL) and biopsy (5,6), but discomfort, inconvenience and risks limit their applicability (7). Examination of sputum is a less invasive procedure (8), but most asthmatic subjects do not produce sputum spontaneously. The analysis of sputum induced by inhalation of hypertonic saline (HS) solution has shown that induced sputum from asthmatic subjects, as well as spontaneous sputum, contains a significantly higher proportion of eosinophils and metachromatic cells than sputum from healthy subjects and from smokers with chronic non-obstructive bronchitis (9,10). Recent studies have shown that induced sputum analysis is a reproducible and reliable method to assess airway inflammation (11,12) and to evaluate asthma outcome (13,14).

Some studies have investigated airway inflammation in subjects with occupational asthma. Bronchial biopsy and lavage have been performed in small groups of subjects with occupational asthma still at work or after removal from exposure to the offending agent (3,15–18) or during specific bronchial provocative test (19,20). Although the pathology of occupational asthma is similar to that of non-occupational asthma (21), the pathogenic mechanisms...
of occupational asthma may be heterogeneous depending on the chemical property of the specific sensitizer (allergens vs. simple chemicals). In particular, it is not completely understood whether the type and the severity of airway inflammation in subjects with occupational asthma are related to different pathogenic mechanisms (IgE- vs. non-IgE-mediated asthma) and whether it differs from non-occupational asthma.

The aim of this study was to measure markers of airway inflammation in induced sputum and in the blood from subjects with occupational asthma due to different sensitizers and to compare the results with the same markers obtained from normal subjects and from subjects with non-occupational asthma.

**Subjects and Methods**

**SUBJECTS**

We studied 24 subjects with occupational asthma induced by low molecular weight compounds [LMWCs: TDI, in 14 subjects, and methylene diphenyl-diisocyanate (MDI) in two subjects] and by high molecular weight compounds (HMWCs: flour in five subjects, tobacco or wood dust in three subjects) who were observed consecutively in our unit during a 2 yr period. All subjects were currently exposed to the sensitizing agent. The diagnosis had been previously confirmed by a positive bronchial challenge test with the appropriate sensitizing agent. At the time of the present study, all subjects were symptomatic and showed bronchial hyperresponsiveness to methacholine. The median duration of work exposure was 17 yr (range: 2-45 yr) and the median duration of illness was 5 yr (range: 1-23 yr). Thirteen patients were ex-smokers; they stopped smoking 2-20 yr before the beginning of the study. Six subjects were atopic as assessed by multiple skin prick tests to a panel of common allergens. All subjects had monitored the peak expiratory flow (PEF) for two working weeks before the treatment with regular inhaled beclomethasone dipropionate, 500-1000 μg daily (duration of previous treatment: 1-5 yr); however, all subjects withdrew pharmacological treatment in the last month, except inhaled β₂-agonists as needed.

For comparison, 24 non-occupational naive asthmatics (17 atopic and seven non-atopic subjects) with asthma severity of similar degree underwent the same protocol, and 14 normal subjects underwent sputum induction and analysis. There were two current smokers and nine ex-smokers (smoking cessation: 2-10 yr) in the non-occupational asthmatic group and three current smokers in the control group.

All subjects had been free of respiratory infections for at least 4 weeks. In each subject, asthma severity was assessed by frequency of symptoms and rescue medication, FEV₁ value and PEF variability, according to the international guidelines (1). The main clinical findings of normal and asthmatic subjects are reported in Table 1.

**STUDY PROTOCOL**

All subjects attended to our laboratory on two different days separated at most by 1 week. On day 1, they underwent spirometry and methacholine challenge tests. On day 2, a venous blood sample for eosinophil counts and serum eosinophil cationic protein (ECP) measurement was collected; then the subjects underwent sputum induction with HS.

The study had been approved by the local Ethics Committee of the University and informed consent was given by all subjects.

**METHACHOLINE CHALLENGE TEST**

Methacholine (Sigma, St. Louis, MO, U.S.A.) was delivered by a DeVilbiss 646 jet nebulizer using a procedure described elsewhere (22). Briefly, phosphate-buffered saline
method (ECP RIA Pharmacia, Uppsala, Sweden); this method has been proven as highly specific and sensitive. Whole blood (6 ml) was allowed to clot for 50-70 min at room temperature (20-22°C) and centrifuged at 1500g for 10 min, and the supernatant was again centrifuged to ensure complete cell removal. Serum samples were stored at -80°C until the assay.

ECP was measured by specific radioimmunological method (ECP RIA Pharmacia, Uppsala, Sweden); this method has been proven as highly specific and sensitive (detection limit: 2 μg l⁻¹) with high intra- and interassay reproducibility. The upper limit of the normal range is 16 μg l⁻¹ (24).

SERUM ECP

Whole blood (6 ml) was allowed to clot for 50-70 min at room temperature (20-22°C) and centrifuged at 1500g for 10 min, and the supernatant was again centrifuged to ensure complete cell removal. Serum samples were stored at -80°C until the assay.

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SPUTUM INDUCTION

Sputum was induced according to the method of Pin et al. (10), slightly modified (25). No β₂-agonist was administered as a pre-treatment, in order to evaluate also the airway response to the hypertonic stimulus. The HS was nebulized with an ultrasonic nebulizer (Sirius, Technomed, Firenze, Italy) with a 2.8 ml min⁻¹ output, and was inhaled for 5 min periods for up to 30 min. NaCl solution was increased at intervals of 10 min from 3% to 4% to 5%. FEV₁ and FVC values were measured before the inhalation test and every 5 min during HS inhalation. At 10 min after the start of nebulization and every 5 min thereafter subjects were asked to rinse their mouth and were encouraged to cough sputum into a clean container. To help control for salivary contamination of induced sputum, samples of saliva and sputum were collected separately and saliva was discharged. The nebulization was stopped after 30 min or when FEV₁ fell by 20% or more from baseline. Airway response to HS was expressed as total duration (minutes) of HS inhalation and as maximum FEV₁ (%) fall from baseline value.

SPUTUM PROCESSING

The entire collected sputum sample was used for analysis. Sputum samples were diluted with an equal volume of 0.1% dithiothreitol (Sputasol; Unipath Ltd, Basingstoke, U.K.). Samples were incubated in a shaking bath at 37°C for 20 min, then pipetted to dissolve mucus plugs further. An aliquot (150 μl) of sputum sample was cytocentrifuged (Cytospin; Shandon Scientific, Sewickley, PA, U.S.A.) and stained with Diff-Quik (Baxter Scientific Products, Miami, FL, U.S.A.). Two investigators, blinded to the subject's history, each first counted at least 500 cells on the slides (usually two or three slides) of each sputum sample to obtain the squamous cell percentage as an indicator of saliva contamination. Cytospin slides with an amount of squamous cells such that 500 non-squamous cells could not be counted were considered unsatisfactory and discarded. Thus, at least 500 non-squamous cells were counted on satisfactory slides. All cell percentages were averaged to give the final values reported here. Macrophage, lymphocyte, neutrophil, and eosinophil percentages were thus expressed as percentages of total inflammatory cells, excluding squamous cells.

The remainder of the sputum sample was centrifuged at 450 g for 10 min. The cell pellets were resuspended in normal saline for total cell counts with the Türk method in a haemocytometer. Total inflammatory cell counts in the whole sputum sample were derived taking into account the percentage of squamous cells and expressed as number of cells per unit of sputum volume.

In our laboratory, the reproducibility of differential cell percentages obtained with this method has been evaluated in 20 stable mild to moderate asthmatic patients in whom sputum was induced by HS inhalation on two different days separated by 1 week. Preliminary results show intraassay correlation coefficients (RI) (26) of +0.90 for macrophages, +0.88 for neutrophils, +0.23 for lymphocytes and +0.82 for eosinophils (27).

STATISTICAL ANALYSIS

Cell percentages in blood and in induced sputum, as well as serum ECP levels and PD₂₀ FEV₁ methacholine, were expressed as median (range). Baseline FEV₁, (percentage of predicted value), maximum percentage FEV₁ fall after HS inhalation and duration of FEV₁ fall after HS inhalation were expressed as mean ± SD.

Analysis of variance was used to compare maximum percentage FEV₁ fall and duration of hypertonic saline inhalation and total sputum cell counts among groups. The Kruskal-Wallis test was used to compare sputum and blood differential cell percentages, serum ECP levels and PD₂₀ FEV₁ methacholine between groups. The Spearman rank test was used to correlate markers of inflammation and bronchial hyperresponsiveness to methacholine or to HS inhalation (28).
Table 2. Inflammatory cell percentages in sputum induced by hypertonic saline inhalation in normal subjects and in subjects with occupational or non-occupational asthma (subjects with occupational asthma are divided according to the type of sensitizing agent; see Table 1)

<table>
<thead>
<tr>
<th></th>
<th>Occupational asthma</th>
<th>Non-occupational asthma</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LMWC</td>
<td>HMWC</td>
<td></td>
</tr>
<tr>
<td>Number of subjects</td>
<td>16</td>
<td>8</td>
<td>24</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>29 (11-86)*</td>
<td>39 (2-64)*</td>
<td>35 (13-75)*</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>0 (0-2 )*</td>
<td>0 (0-1)*</td>
<td>0 (0-2)*</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>68 (12-87)†</td>
<td>57 (30-87)*</td>
<td>46 (4-75)*</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>1 (0-14)††</td>
<td>6 (2-22)*</td>
<td>13 (0-49)*</td>
</tr>
</tbody>
</table>

*P<0.05 vs. normal.
†P<0.05 vs. non-occupational asthma.
‡P<0.05 vs. HMWC asthma.

Results

Although squamous cell contamination was relevant in some samples [median: 31% (1-4-86%)], all slides obtained from the examined subjects were considered satisfactory and used for differential cell counts, because a minimum of 500 non-squamous cells were counted for every subject.

Percentages of all inflammatory cells in HS-induced sputum were significantly different in asthmatic subjects compared with normal subjects (Table 2). Sputum neutrophil percentages were higher in asthmatic than in normal subjects (Fig. 1). This difference persisted even when only non-smokers were considered. Among asthmatics, subjects with asthma due to LMWCs had the highest sputum neutrophil percentages, and this difference reached statistical significance in the comparison with non-occupational asthmatics but not in the comparison with HMWC asthmatics. Sputum eosinophil percentages in subjects with occupational asthma due to LMWCs were significantly lower than in subjects with non-occupational asthma and in subjects with occupational asthma due to HMWCs (Fig. 2). Taking the highest sputum eosinophil percentages observed in normal subjects (i.e. 1%) as the cut-off value for normal eosinophil levels in induced sputum, eosinophil percentages in induced sputum were above normal in eight (100%) subjects exposed to HMWCs, in ten (63%) subjects exposed to LMWCs and in 22 (92%) subjects with non-occupational asthma. Sputum macrophage and lymphocyte percentages were significantly lower in asthmatic than in normal subjects, with no difference among the three groups of asthmatics. Sputum total inflammatory cell counts were not different among the four groups.

Blood eosinophil percentages in subjects with non-occupational asthma and in subjects with asthma due to HMWCs were significantly higher than in subjects with asthma due to LMWCs (Table 3). In particular, blood eosinophil percentages was greater than 5% (laboratory reference value) in one (12.5%) subject exposed to HMWCs, in one (6.2%) subject exposed to LMWCs and in ten (41.6%) subjects with non-occupational asthma. Serum
Table 3. Functional findings of normal subjects and subjects with occupational and non-occupational asthma (subjects with occupational asthma are divided according to the type of sensitizing agent; see Table 1)

<table>
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<tr>
<td></td>
<td>LMWC</td>
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</tr>
<tr>
<td>Number of subjects</td>
<td>16</td>
<td>8</td>
<td>24</td>
</tr>
<tr>
<td>FEV₁ (% predicted), mean ± SD</td>
<td>89.5 ± 14.3</td>
<td>88.9 ± 19.5</td>
<td>89.7 ± 14.1</td>
</tr>
<tr>
<td>PD₂₀ FEV₁ (mg), median (range)</td>
<td>0.274 (0.053-0.705)</td>
<td>0.264 (0.125-0.608)</td>
<td>0.153 (0.050-0.421)</td>
</tr>
<tr>
<td>Blood eosinophils (%), median (range)</td>
<td>0.08 (0-3-5-1)*</td>
<td>2.9 (1-2-9-4)</td>
<td>5.4 (1-3-12-6)</td>
</tr>
<tr>
<td>Serum ECP, median (range)</td>
<td>3.9 (2.2-15-3)</td>
<td>7.1 (2.6-55-7)</td>
<td>6.8 (2-25)</td>
</tr>
<tr>
<td>PEF MA (%), median (range)</td>
<td>13 (4-33)</td>
<td>10 (3-26)</td>
<td>10 (0-19)</td>
</tr>
<tr>
<td>Days with MA &gt;10% (%)</td>
<td>69</td>
<td>43</td>
<td>48</td>
</tr>
<tr>
<td>Duration of test (min), mean ± SD</td>
<td>16.9 ± 9.8‡</td>
<td>11.3 ± 9.2</td>
<td>10.8 ± 6.5</td>
</tr>
<tr>
<td>∆FEV₁ (%), HS, mean ± SD</td>
<td>19.6 ± 9.2‡</td>
<td>29.6 ± 15.9</td>
<td>31.4 ± 9.7</td>
</tr>
</tbody>
</table>

*P<0.05 vs. other groups.
†For statistical analysis it is considered to be 3.2 mg (maximal delivered dose of methacholine).
‡P<0.05 vs. non-occupational asthma.

ECP levels were higher than normal in four asthmatic subjects only, and on average they were not different among the groups of subjects studied.

Baseline FEV₁ and PD₂₀ FEV₁ were lower in asthmatic than in normal subjects, but they were similar in the different groups of asthmatic subjects, as were indices of PEF variability. Bronchial response to HS inhalation, expressed either by maximum percentage FEV₁ fall from baseline value or as duration of HS inhalation, was greater in subjects with asthma due to HMWCs and in subjects with non-occupational asthma than in subjects with asthma due to LMWCs. Normal subjects showed small responses to HS inhalation (Table 3).

A positive significant correlation between sputum and blood eosinophil percentages was observed when both all asthmatic subjects (Fig. 3) and single groups were considered, except the HMWC group. A positive correlation was also observed in all asthmatic subjects between sputum eosinophil percentages and the response to HS inhalation, expressed as duration of HS inhalation (p = 0.04, F = 0.03) but not between sputum eosinophil percentages and PD₂₀ FEV₁ metacholine (p = 0.09, F = 0.9).

Discussion

Our study shows that symptomatic subjects with occupational asthma, currently exposed to the sensitizing agent, have higher percentages of neutrophils and eosinophils in HS-induced sputum than normal subjects. To the best of our knowledge, this is the first report of induced sputum evaluation in subjects with occupational asthma, without an acute exacerbation of asthma and without specific bronchial challenge test. A previous study reported sputum cell composition in TDI asthmatic patients evaluated after bronchial challenge with diisocyanates (29). The comparison with subjects with non-occupational asthma (mostly atopics) but with asthma severity of similar degree, as assessed by symptoms, PEF variability and bronchial hyperresponsiveness to methacholine, showed that subjects with occupational asthma due to LMWCs had lower eosinophil percentages in sputum and in blood, and higher neutrophil percentages in sputum, than subjects with non-occupational asthma. This fact was not observed in subjects with occupational asthma due to HMWCs. Neutrophils seem to be common inhabitants of the large airways, and previous studies have shown increased percentages of neutrophils in BL in non-smoking normal subjects (30). Some studies had observed increased...
percentages of neutrophils in BL from subjects with occupational or non-occupational asthma (15,31), but in most studies neutrophil percentages were in the normal range (3–5). On the other hand, subjects with occupational asthma showed increased neutrophil counts in BL some hours after acute exposure to TDI or plicatic acid dust in a challenge chamber (19,20). An increase in sputum neutrophils has also been reported in subjects with non-occupational asthma during acute exacerbation of asthma (32). In the present study, the difference in sputum neutrophil percentages between normal and asthmatic subjects was not ascribed to smoking habit, because it still persisted when ex-smokers were excluded. This observation points out that neutrophils may have a role in airway inflammation in asthma. This fact seems particularly relevant in asthma due to simple chemicals, where sputum neutrophil percentages were higher than in other asthmatic groups. It has been hypothesized that LMWCs, like diisocyanates and plicatic acid, induce asthma by a non-IgE immunological mechanism, but they may also have other direct effects on the airways, like their environmental pollutants which induce significant oxidative damage on airway mucosa (33). Bronchial challenge with TDI induces a prominent neutrophilic acute airway inflammation as detected by BAL (19), and bronchial epithelial cells from animals exposed to TDI release some cytokines with potential neutrophil chemotactic activity on neutrophils (34).

Subjects with occupational asthma due to LMWCs were slightly older than the other asthmatic groups. However, there is no evidence that sputum neutrophilic inflammation increases with age. Our data on neutrophilic inflammation partly confirm previous results obtained by Boulet et al. in a group of subjects with occupational asthma due to either HMWCs or LMWCs, examined after recent cessation of exposure (18). In particular, subepithelial neutrophils were significantly increased in both asthmatic groups compared with normal subjects, although no difference was observed between HMWC and LMWC asthmatics. By contrast, BL analysis in the same subjects showed no difference in differential cell counts between normals and asthmatics. The discrepancy with the results of the present study, showing high eosinophil percentages in sputum of patients with HMWC asthma compared to patients with LMWC asthma, could be ascribed to the different technique used to evaluate airway inflammation (BL vs. sputum) or to the withdrawal of the occupational exposure in the subjects of the study by Boulet et al. (18). The discrepancy between induced sputum, BL and bronchial biopsies in the types of inflammatory cells can be explained by the different site of sampling (proximal vs. distal airways) and by the different activity of the inflammatory process (acute airway inflammation results in polymorphonuclear cells migrating in the airway lumen, chronic airway inflammation in mononuclear cell infiltration in the airway wall) (12).

Although asthmatic subjects had higher sputum eosinophil percentages than normal subjects, subjects with asthma due to LMWCs had significantly lower sputum and blood eosinophil percentages than subjects with non-occupational asthma and subjects with asthma due to HMWCs. This fact was not related to a different severity of asthma, as symptoms, bronchial responsiveness to methacholine and PEF variability were not significantly different among asthmatic groups; airway response to HS inhalation only was smaller in subjects with asthma due to LMWCs. The different percentages of sputum, and blood eosinophils in occupational asthmatics may also be related to a different degree of exposure to the sensitizer in the workplace: it is well known that, in the furniture and shoe-making industries, the use of TDI varnish or MDI foam is usually intermittent, while exposure to flour, tobacco and wood dust is usually more continuous. Thus, clinical findings may be less sensitive than inflammatory cell counts in blood and in sputum in assessing different degrees of asthma severity. Finally, we cannot exclude the possibility that the higher eosinophil percentages in sputum of subjects with HMWC asthma may be due to a different pathogenetic mechanism of HMWC asthma with respect to LMWC asthma.

Eight out of 24 occupational asthmatics had been previously treated with inhaled corticosteroids, which were withdrawn for at least 1 month before examination. In these subjects, stopping of regular medication could have induced an asthma exacerbation. However, when these subjects were excluded, the difference in the percentage of inflammatory cells in sputum and blood among groups was still present [e.g. sputum eosinophils: 1.5% (0–14%), 6.4% (2–22%) and 12.7% (0–49%) in LMWC, HMWC and non-occupational asthmatics respectively].

The groups of asthmatic subjects were different for sputum and blood eosinophil counts, but not for serum ECP levels. Also, most asthmatic subjects had sputum eosinophil percentages >1% (which is the highest value observed in normal subjects in our laboratory), while a small percentage of asthmatic subjects had blood eosinophil percentages and serum ECP levels higher than normal values. This confirms that induced sputum markers are more sensitive than blood or serum ECP markers in detecting the presence of airway inflammation.

Finally, we have observed a positive correlation between sputum eosinophil percentages and bronchial hyper-responsiveness to HS but not to methacholine inhalation, probably because the response to HS detects better than methacholine asthmatics patients with a higher severity of airway inflammation.

In conclusion, markers of airway inflammation can be observed in HS-induced sputum of subjects with occupational asthma currently exposed to the sensitizing agent. Although some difference in inflammatory cell percentages in subjects with different aetiologies of asthma were observed, depending on the severity of asthma and on the intermittent vs. continuous exposure to the sensitizer in the workplace, sputum eosinophil count was able to detect the presence of airway inflammation better that blood markers.

Acknowledgement

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References


