Persistence of sputum eosinophilia in children with controlled asthma when compared with healthy children


ABSTRACT: We aimed to describe induced sputum cell counts in healthy nonasthmatic children, and to compare these to children with controlled and uncontrolled asthma.

Following clinical assessment and spirometry, ultrasonically nebulized hypertonic saline was used to induce sputum from children with asthma (n=50) and without asthma (n=72). Sputum was dispersed and cell counts performed to yield total and differential cell counts. Specific stains were used for eosinophil and mast cell counts. All of the children with asthma were receiving inhaled and/or oral corticosteroids. Current asthma control was assessed in terms of symptoms and lung function. Children were classified as controlled on inhaled corticosteroids (no current symptoms, normal lung function n=15), current symptomatic asthma (n=16) and asthma exacerbation (n=11).


It was found that eosinophils comprised a median 0.3% (interquartile range (IQR): 0, 1.05) of cells in sputum from healthy children. Sputum eosinophils (4.3% (IQR: 1.5, 14.1) p=0.0005) and epithelial cells (14% (IQR: 6, 19.4) p=0.0005) were significantly higher in children with asthma than in nonasthmatic children. Children whose asthma was controlled, as well as those with symptoms, had more sputum eosinophils and epithelial cells than the nonasthmatics. Mast cells were found in the sputum of only four of the 42 children with asthma.

This study demonstrates that eosinophilic airway inflammation and epithelial damage can occur in children with asthma. Airway inflammation persists even in those children who are receiving inhaled corticosteroids, have normal lung function and good symptomatic control of their disease.

Methods

Assessment of normal controls

Healthy nonasthmatic children were identified from an epidemiological study. A cohort of 263 healthy full-term infants were recruited for a longitudinal study of the development of mucosal immunity and the occurrence of allergy and respiratory disease, especially asthma [6, 7]. The clinical features of these children have been followed since birth and they were re-evaluated between the ages 8–14 yrs. A total of 170 children attended the John Hunter Hospital as part of a follow-up survey which involved: 1) completing a questionnaire containing validated items including the presence of respiratory symptoms, asthma as diagnosed by a doctor, asthma therapy and a family background of respiratory and allergic diseases [7]; 2) allergy skin-prick testing; 3) spirometry; 4) hypertonic saline challenge; 5) sputum induction; and 6) nasal smears. The 72 children from this study who fulfilled each of the following criteria were regarded as normal controls:

1) Never wheezed; based upon negative response to the question: "has your child ever wheezed? (Wheeze is a whistling noise which comes from the chest)".
2) Never diagnosed as having asthma; based upon a negative response to the question "has your child ever been diagnosed as having asthma by a doctor or hospital?".
3) A fall in forced expiratory volume in one second (FEV1) of <15% of predicted value after hypertonic saline challenge [8].
4) Baseline FEV1 80% pred [9].

A child was considered atopic if a weal diameter 3 mm or greater developed 15 minutes after skin prick testing for any of the allergens (house-dust mite, mould mix, mixed grass, cat fur and cockroach).

Hypertonic saline challenge. Antihistamines were withheld for 48 h prior to testing. After spirometry, children proceeded to the hypertonic saline challenge [8] and sputum induction. Saline (4.5%) was inhaled for doubling time exceeded to the hypertonic saline challenge [8] and sputum for 48 h prior to testing. After spirometry, children proceeded to the hypertonic saline challenge [8] and sputum induction. Saline (4.5%) was inhaled for doubling time exceeded to the hypertonic saline challenge [8].

Assessment of children with asthma

Subjects. A total of 50 children 6–18 yrs of age who were attending the Paediatric Respiratory Clinic for regular follow-up visits or who were attending the Children's Respiratory Laboratory for lung function tests were recruited into this study. The children performed spirometry before and after β-agonist administration, followed by sputum induction. Sputum induction was unsuccessful in eight (16%) subjects who were excluded from further analysis. A questionnaire was administered by either a doctor or a nurse and sought details of asthma symptoms and treatment over the previous 2 weeks, as detailed in table 1.

Based upon the results of these questions, children were classified into three groups: asthma controlled on inhaled corticosteroids, symptomatic uncontrolled asthma, and current exacerbation of uncontrolled asthma. Asthma controlled on ICS referred to children who had none of the symptoms described in table 1, whose peak expiratory flow (PEF) at its best, who were not requiring β2-agonist therapy for acute symptom relief and whose baseline FEV1 was 80% pred. A child with one or more positive

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controlled (n=15)</th>
<th>Uncontrolled (n=27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV1 % pred</td>
<td>107.5±3.8</td>
<td>89.2±3.1</td>
</tr>
<tr>
<td>Baseline FEV1 &lt;80% pred</td>
<td>0 (0)</td>
<td>6 (22%)</td>
</tr>
<tr>
<td>FEF25–75% % pred</td>
<td>100.5±6.3</td>
<td>74.2±5.7+</td>
</tr>
<tr>
<td>BDR % baseline FEV1</td>
<td>8.1±2.6</td>
<td>11.3±2.7</td>
</tr>
<tr>
<td>Treatment, past 2 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC n (%)</td>
<td>15 (100)</td>
<td>26 (96)</td>
</tr>
<tr>
<td>dose µg</td>
<td>947±95</td>
<td>848±84</td>
</tr>
<tr>
<td>OC n (%)</td>
<td>0 (0)</td>
<td>9 (33)</td>
</tr>
<tr>
<td>β2 n (%)</td>
<td>15 (100)</td>
<td>25 (93)</td>
</tr>
<tr>
<td>times daily+</td>
<td>1.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>2 (13)</td>
<td>9 (33)</td>
</tr>
<tr>
<td>Theophylline</td>
<td>2 (13)</td>
<td>5 (17)</td>
</tr>
<tr>
<td>Cromoglycate</td>
<td>0 (0)</td>
<td>1 (3.7)</td>
</tr>
<tr>
<td>Ipratropium</td>
<td>1 (6.6)</td>
<td>2 (7.4)</td>
</tr>
<tr>
<td>Asthma control, past 2 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Missed school/decreased activity</td>
<td>0 (0)</td>
<td>6 (22)</td>
</tr>
<tr>
<td>Lower PEF than best</td>
<td>0 (0)</td>
<td>6 (22)</td>
</tr>
<tr>
<td>Wheeze</td>
<td>0 (0)</td>
<td>14 (51)</td>
</tr>
<tr>
<td>Night waking with asthma</td>
<td>0 (0)</td>
<td>13 (48)</td>
</tr>
<tr>
<td>Morning waking with asthma</td>
<td>0 (0)</td>
<td>10 (33)</td>
</tr>
<tr>
<td>Exacerbation of asthma</td>
<td>0 (0)</td>
<td>11 (37)</td>
</tr>
</tbody>
</table>

Values are presented as mean±SD or absolute number with percentage in parenthesis. Inhaled corticosteroid (IC) is expressed as mean daily dose per child. Three out of 15 children in the controlled group received bronchodilators daily, others used β-agonists less often, and prescribed only when needed. Twenty of the 27 children in the uncontrolled group received bronchodilators more than twice daily, others only when needed. *; p=0.0007, p=0.004, using two sample t-test. FEV1: forced expiratory volume in one second; FEF25–75%: forced mid-expiratory flow; BDR: bronchodilator response; OC: oral corticosteroid; PEF: peak expiratory flow.

Nasal cytology. Nasal cytology was performed using saline moistened, cotton tipped swabs inserted into the nares beneath the inferior turbinate. The swab was placed against the nasal mucosa and withdrawn in a spiral fashion. It was then spread thinly over glass slides prior to fixation and staining. Smears were stained with May-Grunwald-Giemsa (MGG) for examination of the eosinophils (one slide) and with toluidine blue after Carrey’s fixation for metachromatic cells [10, 11].
answers to these questions was classified as having symptomatic asthma. Symptomatic asthma together with a positive response to question 7, "have you had an exacerbation of asthma in the past 2 weeks?" was defined as having exacerbation of asthma.

**Lung function.** Each child performed three forced expiratory manoeuvres on a computerized spirometry system (Medgraphic, Pulmonary Function System 1070, Series 2, St. Paul, Minnesota, USA). Values were expressed as a percentage of the predicted value [9]. The best forced expiratory manoeuvre was used as the baseline value. Each child then inhaled 5 mg of salbutamol delivered via Aerflo (Waite & Co, Sydney, Australia) jet nebulizer and face mask. Ten minutes after nebulized salbutamol, spirometry was repeated and the best of three readings was taken as the postbronchodilator value. Bronchodilator response was expressed as the change in FEV1 as a percentage of the baseline value. Sputum induction was attempted if the FEV1 after bronchodilator response was at least 60% pred.

**Sputum induction.** Induced sputum samples were obtained by inhaling 4.5% hypertonic saline via an Omron NE-U06 ultrasonic nebulizer (Omron Corp, Tokyo, Japan) with an output of 3 mL·min⁻¹ and mouthpiece. After measurement of the post bronchodilator FEV1, each child rinsed his/her mouth with water in order to remove the saliva. Hypertonic saline was inhaled for double time periods (30 s, 1, 2, 4 min) and continued for a cumulative time of 20 min or until adequate sputum (i.e., at least three plugs) was obtained. FEV1 was measured for 1 min following each dose. Two puffs (100 µg each) of salbutamol (Ventolin; Allen & Hanburys, Melbourne, Australia) from a pressurized metered dose inhaler via a Volumatic spacer (Allen & Hanburys) were administered if the FEV1 dropped by >10% from baseline. If salbutamol was administered, the child was observed until lung function improved.

**Sputum analysis.** Sputum was processed as described [5, 12] with modification. Briefly, the sputum volume was recorded, and the sample was poured into a petri dish and examined against a black background. The macroscopic characteristics were recorded and a 300 µL aliquot of sputum plug was aspirated from the petri dish using a positive displacement pipette. The aliquot was added to 2,700 µL of dithiothreitol 1.10 (Sputolysin; Calbiochem, La Jolla, CA, USA), mixed by rotating for 30 min at room temperature and stained with 0.5% toluidine blue in 0.7 N hydrochloric acid at pH 0.1. 1,500 nucleated cells were counted on each of two slides and the mean of these two values reported as the mast cell count. A differential cell count was obtained from 200 cells counted on MGG stained slides. The quality of induced sputum samples was assessed based upon a slide quality assessment procedure which evaluated the presence of three parameters: 1) an adequate number of cells for enumeration; 2) the presence of pulmonary macrophages on the slide; and 3) the proportion of squamous epithelial cells. Cell number was scored as 0 if <200 cells, 1 if 200–399 cells, and 2 if >400 cells were present. Pulmonary macrophages were scored as absent (1) or present (2). The proportions of squamous epithelial cells was scored 0 if <50%, and 1 if >51%. This gave a quality score ranging 0 (poor quality) to 6 (good quality sample).

**Ethics**

Written informed consent was obtained from parents and children. The study was approved by the Hunter Area Research Ethics Committee and the University of Newcastle Ethics Committee.

**Statistics**

Cell counts were expressed as the median and interquartile range (IQR). Mean values with 95% confidence intervals (95% CI) were also reported in healthy subjects. There was a large proportion of zero counts and positive counts tended to be skewed. Eosinophils and mast cell counts were, therefore, categorized as the proportion of zero counts and nonzero counts for the purposes of comparison. Comparison of the demographic details between groups was performed using the Chi-squared test and Fisher's exact test. The Mann-Whitney test was used to compare continuous data which was distributed in a non-normal fashion. The Kruskal-Wallis test was used to compare multiple groups (n>2) and comparison between these groups was performed according to Steel [13]. A p-value <0.05 was considered statistically significant.

**Results**

**Normal controls**

The healthy nonasthmatic children included 29 males, and had a mean (SEM) age of 10.5(0.2) yrs. The mean (SEM) FEV1 was 97.6(1.1)% pred and 32 (44%) children were atopic. Cell counts from induced sputum and nasal smears are shown in table 2. Normal children had a median sputum total cell count of 1.5 × 10⁶·mL⁻¹, with 0.3% of cells being eosinophils. The total cell count was significantly higher in the nonatopic group than the atopic group (p<0.05, Mann-Whitney test). The proportion of children with eosinophils observed in their sputum was higher in the atopic group than in the nonatopic normal group (45 versus 7.9%, p<0.001, Chi-squared test). There were no differences between the proportion of atopic children and nonatopic children with sputum mast cells (9.7 versus 7.9%, NS), nasal eosinophils (34 versus 21%, NS), and nasal mast cells (12.5 versus 7.7%, NS).

**Asthma.** The clinical characteristics of the children with asthma are shown in table 1. The children were 12 (SEM: 0.4) yrs of age and 28 were male. There were 15 children with asthma controlled on inhaled corticosteroid and 27 with uncontrolled asthma, of whom 11 were experiencing an exacerbation. The children with uncontrolled asthma (symptomatic and exacerbation) had lower lung function, with six of the 27 (22%) having an FEV1 <80% pred. The children with asthma controlled on inhaled corticosteroid reported no current asthma symptoms and all had an FEV1...
>80% pred. Both groups were using moderately high doses of inhaled corticosteroid therapy and were prescribed short-acting β-agonists. Sputum eosinophils (p<0.0001, Mann-Whitney test) and epithelial cells (p<0.00005, Mann-Whitney test) were significantly higher in children with asthma than with normal children (table 3). Each of the asthmatic groups had elevated sputum eosinophils (p=0.0005, Kruskal Wallis test), and epithelial cells (p=0.0005, Kruskal Wallis test), when compared with the normal control children. There was a nonsignificant trend to increased sputum eosinophils and epithelial cells in the children with an exacerbation of asthma (table 4). There were no significant differences in total cell counts between normal subjects, and children with asthma controlled on inhaled corticosteroid, symptomatic asthma or an exacerbation of asthma (p>0.05, Kruskal Wallis test). Children with controlled asthma had higher eosinophils (p=0.012) and epithelial cells (p=0.015) than atopic asymptomatic children (tables 2 and 4). Sputum eosinophils were negatively correlated with FEV1% pred (r=-0.41, p=0.006), whereas there was a weaker positive association between neutrophils and lung function (r=0.3, p=0.03).

**Discussion**

This study reports values for induced sputum cell counts from healthy nonasthmatic children, and compares these to results from children with asthma that was controlled with inhaled corticosteroid, and to those with symptomatic asthma. The criteria for normality in this study were rigorous. All of the children had been followed from birth and had no history of wheeze, no diagnosed asthma, normal lung function and had normal airway responsiveness to hypertonic saline. Induced sputum from these normal children contained predominantly macrophages and neutrophils, reflecting the dominant cells in the airway lumen. By contrast, children with asthma had elevated numbers of eosinophils and desquamated bronchial epithelial cells. These features of asthmatic airway inflammation persisted after control of asthma with inhaled corticosteroid therapy, albeit at a lower level than in children with symptomatic asthma.

The finding of elevated sputum eosinophils in asthmatic patients extends the published data from adults [14] to childhood asthma. The eosinophil is believed to be one of the main effector cells in asthma and accumulates within normal tissue or an exacerbation of asthma (p>0.05, Kruskal Wallis test). Children with controlled asthma had higher eosinophils (p=0.012) and epithelial cells (p=0.015) than atopic asymptomatic children (tables 2 and 4). Sputum eosinophils were negatively correlated with FEV1% pred (r=-0.41, p=0.006), whereas there was a weaker positive association between neutrophils and lung function (r=0.3, p=0.03).

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the airway lumen in response to cytokines such as interleukin (IL)-5 produced by type 2 T-helper cell (Th2)-lymphocytes and releases granule-associated proteins to cause airway mucosal damage, including epithelial detachment. The increase in airway eosinophils is one of the most characteristic findings in asthma.

We also found that significant epithelial shedding occurred together with eosinophilic airway inflammation. The median epithelial cell count in sputum from asthmatic children was several fold higher (14%) than from normal subjects (1.5%). Although this has been well-documented in adults with asthma [15, 16], and some epithelial damage has been reported in children with chronic cough [17], the marked epithelial shedding reported in this study is a novel finding in childhood asthma. This study clearly demonstrates the degree of epithelial damage which can occur in children with asthma, even when this is controlled. Hypertonic saline inhalation is unlikely to be contributory since both groups (normal and asthma) inhaled hypertonic saline and prior work has found no effect of hypertonic saline on epithelial loss in asthma [18]. Both groups of children had sputum induced after inhalation of hypertonic saline. This is a reliable technique which is not known to alter sputum eosinophil or epithelial cell counts [18–20] and the results from different ultrasonic nebulizers are comparable [18, 20]. The asthmatic children inhaled β₂-agonist prior to sputum induction. This was done for safety purposes, in order to prevent airway obstruction [5]. It does not alter sputum cellularity [20].

The airway epithelium is now seen as playing a pivotal role in the inflammatory response in asthma [21]. When activated, epithelial cells can be a source of eosinophil-attracting cytokines such as regulated on activation, normal T-cell expressed and secreted factors (RANTES) [22], and eotaxin [23]. Epithelial loss may contribute to the genesis of airway hyperresponsiveness, and to microvascular leakage [24] in asthma. The data from this study identify that epithelial loss is an integral part of asthma in children, and that it occurs in association with eosinophilic inflammation.

This study also examined the relationship between asthma control and airway inflammation. The children with controlled asthma had minimal symptoms and normal lung function. By contrast, those children with uncontrolled asthma reported symptoms, increased use of bronchodilators and reduced lung function. We found that both sputum eosinophils and epithelial cells were at significantly higher levels in uncontrolled asthma compared with levels in normal children. Interestingly, we found that the numbers of eosinophils and epithelial cells in sputum were also higher in controlled asthma than in normal children, including atopic asymptomatic children. This suggests that eosinophilic inflammation occurs even when the clinical features of asthma are suppressed by high-dose inhaled corticosteroid therapy. Corticosteroid therapy reduces airway inflammation [2] and epithelial damage [3] when measured using bronchial biopsy [2, 3], and sputum [25]. Although some studies in adults suggest that airway eosinophilia is abolished by corticosteroid therapy [25], this is not a uniform finding [2, 3]. Our results indicate that in children, just as in adults [2], there may be a component of asthmatic airway inflammation that is relatively resistant to corticosteroid therapy. It will be important to establish this using prospective studies of treatment in children with asthma.

We found that relatively few children had mast cells in their sputum. Mast cells were found in only four of the 42 asthmatic children. This contrasts with adult asthma where mast cells are frequently seen [5, 26]. Possible explanations are that the mast cells were present in the airway but degranulated, or restricted to the airway wall only. GIBSON et al. [27] demonstrated increased mast cells in the bronchial epithelium of adults with asthma, but not in bronchoalveolar lavage (BAL) fluid. Other studies support the epithelial accumulation of mast cells in asthma [28], and find no difference in lamina propria mast cells [29]. A third possibility is that corticosteroid therapy may erode mast cells from the airway in asthma. The literature, however, reports a reduction but not a complete clearance of mast cells from the airway with corticosteroid therapy [2, 25]. At the present time the significance of airway mast cells in childhood asthma requires further study.

This study also provides interesting comparative data on sputum cell counts in atopic and nonatopic children without asthma. In the nonatopic normal group, the sputum total cell count was higher than in the asthmatic normal group. This was an unexpected finding and remains unexplained. We found that atopic normal children were more likely to have sputum eosinophils than nonatopic normal children. Eosinophils were observed in the sputum of 45% of the atopic children compared with 7.9% of the nonatopic children. This is consistent with the finding by DRIKANOFF et al. [30], who found higher numbers of airway eosinophils in bronchial biopsies from atopic non-asthmatic adults than a nonatopic normals. These data indicate that the atopic state can be associated with mild airway inflammation without any evidence of airway hyperreactivity or clinical asthma. This finding may be of relevance to both the mechanisms of symptom generation in asthma, and to the future development of asthma. Since atopic children have a mild degree of airway eosinophilia but no asthma symptoms, this suggests that there may be a threshold in the intensity of airway inflammation that is required before symptoms become apparent. Alternative possibilities are that either additional cell types are required (e.g. mast cells), or additional features such as airway hyperreactivity are necessary for symptoms to develop. Allergic rhinitis and atopy are both risk factors for the subsequent development of asthma. The finding of mild airway inflammation in some atopic nonasthmatic children raises the possibility that this may predispose them to future symptomatic asthma, an hypothesis which could be tested in a prospective study.

In our study, we did not find a significant difference in nasal eosinophils and mast cells between atopic and nonatopic groups. IGAISBE et al. [31] reported that nasal biopsies from allergic subjects contained significantly more eosinophils than did biopsies from normal subjects. However, the difference in the number of mast cells between the two groups did not reach significance. The difference in nasal eosinophils between their study and ours may relate to subject characteristics. All of their allergic subjects had a history of seasonal rhinitis, while in our study, only eight of the 32 atopic normal subjects reported a history of rhinitis. In support of this, we found a nonsignificant trend for increased nasal eosinophils in the children with allergic rhinitis (data not shown).
In conclusion, sputum induction using hypertonic saline provides opportunities for increasing our understanding of airway inflammation in asthma. Sputum from children without asthma contains airway neutrophils and macrophages. Airway inflammation was found in children with or without asthma contains airway neutrophils and macrophages. However, unlike in adult asthma, mast cells were seldom seen. Further work is required to determine whether airway inflammation is a marker of asthma, controlled or un-controlled, and whether deteriorating asthma control is associated with an increase in airway inflammation.

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