Exhaled nitric oxide levels in non-allergic and allergic mono- or polysensitised children with asthma

M Silvestri, F Sabatini, D Spallarossa, L Fregonese, E Battistini, M G Biraghi, G A Rossi

Abstract

Background—Increased fractional exhaled NO concentrations (FENO) and blood/tissue eosinophilia are frequently reported in allergic children with mild asthma and are thought to reflect the intensity of the inflammation characterising the disease. The aim of this study was to investigate possible differences in FENO levels or in the intensity of the blood eosinophilia in allergic and non-allergic asthmatic children.

Methods—112 children with stable, mild, intermittent asthma with a positive bronchial challenge to methacholine were consecutively enrolled in the study; 56 were skin prick test and RAST negative (non-sensitised) while 56 were sensitised to house dust mites (23 only to house dust mites (monosensitised) and 33 were sensitised to mites and at least another class of allergens (pollens, pet danders, or moulds)). Nineteen sex and age matched healthy children formed a control group.

Results—Compared with non-allergic patients, allergic children had a significantly higher rate of blood eosinophilia (p=0.0001) with no differences between mono- and polysensitised individuals. Forced expiratory in 1 second (FEV1), forced vital capacity (FVC), forced expiratory flow at 25–75% of vital capacity (FEF25–75%), and the degree of bronchial reactivity to methacholine were similar in non-atopic and atopic children, with no differences between mono- and polysensitised individuals. FENO levels measured by chemiluminescence analyser were higher in asthmatic children (15.9 (14.3) ppb) than in the control group (7.6 (1.6) ppb, p=0.04) and higher in allergic patients (23.9 (2.1) ppb) than in non-allergic patients (7.9 (0.8) ppb, p=0.0001), but there were no differences between mono- and polysensitised individuals (p>0.1). Significant correlations between blood eosinophilia and FENO levels were seen only in allergic (r=0.35, p<0.01) and in polysensitised individuals (r=0.45, p<0.05).

Conclusions—In children with mild asthma, a similar degree of functional disease severity may be associated with a higher inflammatory component in allergic than in non-allergic subjects.

Keywords: airway inflammation; bronchial hyperresponsiveness; atopy; asthma; exhaled nitric oxide Bronchial asthma, even in its mild form, is characterised by local infiltration and activation of a variety of inflammatory and immunoeffectector cells. The finding that eosinophils are toxic to human lung tissues and that their presence in the bronchial mucosa may correlate with morphological damage to the bronchial epithelium has strongly supported the hypothesis that these cells could play a role as major effector elements in the pathogenesis of asthma.

The overall hypothesis is that, at least in allergic asthma, eosinophil accumulation is mediated by products released by T cells and mast cells. In addition, although adult atopic and non-atopic asthmatic subjects may have distinct patterns of T cell activation and cytokine production, similar levels of eosinophilic inflammation in the airways have been described in allergic and non-allergic asthma.

The concept that airway inflammation may cause permanent airway remodelling and irreversible loss of pulmonary function has suggested that, even in mild asthma, monitoring of airway inflammation may be useful for gauging the severity of the disease and the efficacy of anti-inflammatory treatment, and also to identify individuals (children and/or adults) who may need a closer follow up and, possibly, anti-inflammatory medication.

Many attempts have been made to provide sensitive non-invasive markers to assess the presence and the intensity of airway inflammation in children. Measurements of several blood markers of inflammation have been proposed in the monitoring of asthma, but they are insufficiently sensitive since asthmatic inflammation is mainly confined to the airways. Assessment of airway inflammation can be obtained both invasively by bronchoalveolar lavage and bronchial biopsy and non-invasively by induced sputum; however, these methods are not easily applicable on a routine basis, particularly in young children. The measurement of nitric oxide (NO) concentrations in exhaled air has recently been proposed as a non-invasive, simple, well tolerated test to assess airway inflammation in asthma, even in children. NO is generated from L-arginine by various cells in the airway including airway and alveolar epithelial cells, vascular endothelial cells, smooth muscle cells, and alveolar macrophages. The lungs of healthy human subjects produce low but detectable levels of NO, whereas asthmatic patients have increased levels of exhaled NO, probably in response to inflammatory stimuli such as cytokines.
Fractional exhaled NO concentrations (FENO) are increased in allergic children with mild allergic asthma and correlate with the degree of blood and airway eosinophilia.17–19

The present study was designed to investigate possible differences in FENO levels and blood eosinophilia in allergic and non-allergic subjects with stable, mild, intermittent asthma. Among the allergic population, monosensitised children—that is, individuals sensitised only to one class of allergens—and polysensitised children—that is, those sensitised to more than one class of allergens—were separately evaluated.

Methods

SUBJECTS

The study was performed in 112 children (47 girls and 65 boys) of mean (SD) age 10.9 (0.3) years (range 4–18) referred to our outpatient clinic with a history of mild asthma.20 All subjects had a positive response to a methacholine inhalation challenge and were characterised as atopic or non-atopic according to skin prick test reactions to common allergens (see below). Participating subjects were in a stable clinical condition and had not taken inhaled steroids at least in the year preceding the study. None of the study participants had reported upper or lower respiratory infection in the 2 months preceding the study. Children sensitised to pollen allergens were evaluated out of the pollen season.

Nineteen sex and age matched healthy children of mean (SD) age 9.7 (1.3) years were evaluated as a control group. They had negative prick test reactions to the standardised skin test reactions to common allergens (see below). Parents or guardians of the children were informed of the scope of the study and of the procedures involved, and they gave their informed consent. The study protocol was approved by the hospital ethics committee. All the recruited children completed the study protocol.

SKIN PRICK TEST PROCEDURE

Sensitisation to the four most common classes of aeroallergens was evaluated by skin prick test.21 The allergen panels tested included: (a) house dust mite class (Dermatophagoides pteronyssinus 5000 PNU/ml and Dermatophagoides farinae 5000 PNU/ml); (b) pollen class (Parietaria officinalis 1000 UP/ml, mix of Grassinae 10 000 UP/ml, Compositae 10 000 UP/ml, Betulaceae 10 000 UP/ml, Oleaceae 10 000 UP/ml), (c) pet dander class (cat and dog skin scale allergen extracts 1:20), and (d) moulds (mix of Aspergillus 10 000 PNU/ml, Cladosporium 10 000 PNU/ml, Alternaria tenuis 10 000 PNU/ml; Bayopharm, Milan, Italy). A histamine solution in distilled water (10 mg/ml) and the glycerol buffer diluent of the allergen preparations were used as positive and negative controls, respectively. Each subject was skin tested in duplicate on the volar surface of the forearm using 1 mm prick lancets (Dome/Hollister-Stier, UK). On each visit skin prick tests were performed using the same panel of allergens. The tests were carried out by two specially trained nurses and the weak reaction was read by the same nurses under the supervision of a physician. The reactions were recorded within 15 minutes by evaluating the skin response rate to the inoculation of each allergen compared with the response in the negative control: a weal diameter 3 mm larger than the negative control was considered as a positive reaction.22 Antihistamines were stopped at least 3 weeks before skin testing.

BLOOD EOSINOPHIL COUNT EVALUATION

Eosinophil counts on peripheral blood samples were performed by Technicon H6000 (Technicon Instrument Corporation, Tarrytown, NY, USA), a system that automatically counts and differentiates leucocytes by an alkaline peroxidase method. Approximately 12 000 leucocytes were counted on each occasion. The coefficient of variation for eosinophil counts was 7.5%. Peripheral blood eosinophilia was evaluated both as the number and as percentage of cells, as previously described.17

PULMONARY FUNCTION AND BRONCHIAL HYPERRESPONSIVENESS

All children were able to perform forced expiratory manoeuvres. Forced vital capacity (FVC), forced expiratory volume in 1 second (FEV1), and forced expiratory flow at 25–75% of the vital capacity (FEF25–75%) were measured by spirometry (Med Graphics Pulmonary Function System 1070 series 2, Med Graphics Corporation, St Paul, MN, USA).17 On each occasion three forced expiratory manoeuvres were obtained and the best values were retained. All children had baseline FEV1 >80% of the predicted value.

Methacholine aerosols were delivered by an ampoule dosimeter device (MEFAR, Brescia, Italy); the same ampoule was used for each patient.23 Methacholine solution was made up freshly in 0.9% pyrogen-free saline solution on the day of use, and the methacholine challenge was started from a dose of 0.02 mg. The best of three FEV1 manoeuvres measured within 1 minute after inhalation of each methacholine dose was used to construct dose-response curves. The methacholine dose was doubled until FEV1 fell below 80% of the control value (inhalation of saline) or up to a maximal dose of 5 mg. The dose causing a 20% fall in FEV1 (PD20) was calculated by interpolation of the dose-response curves.23

DETECTION OF EXHALED NO

A chemiluminescence analyser (Logan LR 2000 System, Kent, UK) sensitive to NO concentrations from 2 to 5000 parts per billion (ppb, by volume) was used. The system was adapted for online measurement of NO and therefore did not require collection of exhaled air, a potential source of variable loss of reactive NO.14 Certified NO mixtures (100 ppb) in nitrogen (BOC Gases, Guildford, UK) were used for daily calibration. Environmental NO was measured before and after each study and never exceeded 15 ppb. After flushing the analyser with NO-free compressed air, the subjects were asked to perform a slow expiratory vital
capacity manoeuvre over 10–15 seconds through a wide bore Teflon tube against a positive pressure of 6–8 cm H₂O. During this manoeuvre the oropharyngeal pressure increases enough to cause closure of the soft palate, thereby minimising nasal NO contamination. Expiratory flow was maintained at 50 ml/s with the aid of visual feedback. Typically, the NO concentration peaks early during expiration, probably as a result of the contribution of nasal NO. This peak is followed by a plateau which is believed to represent NO from the lower respiratory tract. Mean plateau values were calculated for each exhalation. The highest value from three successive reproducible recordings obtained at 2 minute intervals was retained for statistical analysis. All measurements were made by two independent observers who were unaware of the patients’ state of health. The repeatability of NO measurements in orally exhaled air was evaluated as proposed by Bland and Altman. For this purpose, two measurements taken at an interval of >1 hour on the same day between 08.30 and 10.00 hours in 12 children were compared. The mean difference in the NO concentration in air exhaled from the lungs between the two measurements was 0.38 (0.27) ppb (p<0.1). The coefficient of repeatability was 2.03 ppb.

### Table 1 Blood eosinophilia in allergic and non-allergic children

<table>
<thead>
<tr>
<th></th>
<th>Allergic children</th>
<th>Monosensitised children</th>
<th>Polysensitised children</th>
<th>Non-allergic children</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosinophil number</td>
<td>500.0 (370.0–855.0)</td>
<td>500.0 (370.0–892.5)</td>
<td>500.0 (262.5–750.0)</td>
<td>125.0 (100.0–300.0)</td>
</tr>
</tbody>
</table>

Data are expressed as median with lower and upper quartiles (indicated in brackets), whereas all the other data are expressed as mean (SD). The Mann-Whitney U test was used when appropriate. Correlations were determined using Spearman’s rank correlation coefficient. R(r) indicates the r value showing correlations between eosinophil numbers and FENO levels, while r'(%) indicates the r value showing correlations between eosinophil percentages and FENO levels. The χ² test was used to compare the rates. The level of statistical significance was set at p<0.05.

### Results

#### ALLERGIC SENSITISATION IN ALLERGIC AND NON-ALLERGIC CHILDREN

Of the 112 children enrolled in the study, 56 were non-allergic while 56 were sensitised at least to house dust mites. Twenty three allergic children (41.1%) were monosensitised—that is, sensitised only to house dust mites—while 33 (58.9%) were polysensitised—that is, sensitised to house dust mites and at least another class of allergens (pollens, pet danders, moulds).

Compared with non-allergic children, allergic children had a significantly higher degree of blood eosinophilia, both as a percentage (median difference 4.6%, 95% CI 3.2 to 5.9, p=0.0001) and as absolute numbers (median difference 375.0 cells/mm³, 95% CI 237.9 to 512.1, p=0.0001, table 1). No difference in blood eosinophilia was observed between mono- and polysensitised children (p>0.1).

#### PULMONARY FUNCTION AND FENO LEVELS IN ALLERGIC AND NON-ALLERGIC CHILDREN

All the enrolled children were able to make satisfactory recordings of pulmonary function parameters and FENO. There were no significant differences in FEV₁, FVC, and FEF₁₋₂₅% values between non-allergic and allergic asthmatics, or between mono- and polysensitised individuals (p>0.1 for each comparison, table 2). Similarly, the degree of bronchial reactivity...
to methacholine was similar in non-atopic and in atopic children, with no differences between mono- and polysensitised individuals (p>0.1 for each comparison).

NO measurements showed that patients with asthma had higher F ENO values than normal non-atopic children of comparable age (15.9 (14.3) ppb v 7.6 (1.6) ppb; mean difference 8.3 ppb, 95% CI 5.5 to 11.1, p=0.040; not shown). Higher FENO levels were found in allergic patients (23.8 (2.1) ppb) than in non-allergic patients (7.9 (0.8) ppb; mean difference 15.9 ppb, 95% CI 11.5 to 20.3, p=0.0001; fig 1). In addition, 46 of the 56 allergic children (82.1%) had raised FENO levels (higher than 10.8 ppb, that is, >2 standard deviations of the mean in healthy subjects), whereas only 12 of the 56 non-allergic children (21.4%) had increased FENO concentrations ($\chi^2 = 38.94$, 95% CI 6.06 to 48.65, p<0.0001).

No significant difference in FENO levels was found between monosensitised and polysensitised children (25.8 (2.9) ppb and 24.3 (3.3) ppb, respectively, p=0.74; fig 1).

**CORRELATION BETWEEN FENO, BLOOD EOSINOPHILIA AND PULMONARY FUNCTION**

When asthmatic children were analysed as a whole, a significant correlation was found between FENO levels and the percentage or number of blood eosinophils ($r(\%)=0.50$, p=0.0001; $r(n)=0.47$, p=0.0001; respectively, fig 2). A high proportion (50.9%) of asthmatic children had increased FENO levels ($>8.8$ ppb) associated with an increased percentage of blood eosinophils ($>3$% white blood cells), whereas only 11.3% of asthmatic children had increased FENO levels ($>8.8$ ppb) and low levels of blood eosinophilia ($<3$% white blood cells), ($\chi^2 = 15.67$, 95% CI 2.31 to 16.37, p=0.00008). Similarly, 43.4% of asthmatic children had increased FENO levels ($>8.8$ ppb) associated with an increased number of blood eosinophils ($>300$ cells/mm$^3$), whereas only 18.9% of asthmatic children had increased FENO levels ($>8.8$ ppb) and low levels of blood eosinophilia ($<300$ cells/mm$^3$), ($\chi^2 = 9.29$, 95% CI 1.55 to 9.59, p=0.0023). Evaluating the allergic and non-allergic populations separately, a significant correlation was found between blood eosinophilia and FENO levels in the allergic group ($r(\%)=0.36$, p=0.007; $r(n)=0.35$, p=0.010; fig 3) but not in the non-allergic group ($r(\%)=-0.20$, p=0.157; $r(n)=-0.21$, p=0.152; not shown). Blood eosinophilia correlated with FENO levels in polysensitised ($r(\%)=0.43$, p=0.016; $r(n)=0.45$, p=0.011) but not in monosensitised children ($r(\%)=0.22$,

![Figure 2 Relationship between fractional exhaled NO (FENO) levels and blood eosinophilia in the whole asthmatic population (allergic and non-allergic children) expressed as (A) percentage of white blood cells and (B) the number of eosinophils/mm$^3$.](http://www.thoraxjnl.com)

![Figure 3 Relationship between fractional exhaled NO (FENO) levels and blood eosinophilia in allergic children expressed as (A) percentage of white blood cells and (B) the number of eosinophils/mm$^3$.](http://www.thoraxjnl.com)
Exhaled nitric oxide in allergic and non-allergic asthma in childhood

The two constitutive isoenzymes, eNOS and nNOS, are basally expressed in many cells in the airways of normal individuals including airway epithelial cells, vascular endothelial cells, alveolar macrophages, and inflammatory cells. The correlation between FENO levels and blood eosinophilia in asthmatic patients could, at least in part, result from a fall in airway pH. Indeed, lowering of airway pH not only produces bronchospasm and causes the release of bronchoconstrictor and proinflammatory substances from eosinophils, but also causes the conversion of endogenous nitrogen dioxide into nitric oxide. No data exist on the possibility that the two types of asthma may differ in airway pH values. It is very unlikely that the pharmacological treatment of asthma in our patients interfered with the FENO levels recorded. The patients were taking only inhaled β2 agonists on an as required basis and these were discontinued at least 12 hours before the study. In any case, FENO concentrations are not affected by β2 adrenoceptor agonists.

We found no differences between mono- and polysensitised individuals in the degree of bronchial reactivity to methacholine, blood eosinophilia, or FENO levels. This result is in agreement with a previous observation in school children sensitised to house dust mite in which similar blood eosinophil counts and degree of allergen induced mononuclear cell proliferation were detected in monosensitised and polysensitised individuals. Since only a few individuals were sensitised to pet dander or to moulds, and since this study was performed out of the pollen season, most of the cellular inflammatory response in the blood or in the airways of our patients could be related to subclinical allergen exposure to house dust mite, irrespective of the number of allergens to which the subjects were sensitised.

The observation of a significant correlation between FENO levels and blood eosinophilia in allergic individuals with stable, mild, intermittent asthma is in agreement with previous observations and further supports the concept that FENO may indeed be related, at least in part, to the intensity of airway inflammation in these individuals. As for other markers of inflammation in asthma, their relationships may be lost after allergen exposure or steroid treatment, possibly because of the different temporal kinetics of the various parameters.

The correlation between FENO levels and blood eosinophilia was lost when monosensitised children were evaluated alone, possibly...
because of the small number of individuals. Indeed, this sample (n=23) provides 10% power for the relationship between blood eosinophilia expressed as a number and FENO levels and 17% power for the relationship between blood eosinophilia expressed as a percentage and FENO levels. To achieve 80% power, 350 and 150 patients, respectively, would be needed.

Finally, we did not find any correlation between FENO levels and pulmonary function parameters or bronchial reactivity to methacholine. The data reported here are similar to those reported in some of our previous studies and further support the hypothesis that airway inflammation in patients with stable mild allergic asthma is not strictly related to the reduction in lung volumes, the degree of airflow limitation, or the intensity of bronchial hyperreactivity. Further studies are needed to elucidate the molecular mechanisms underlying NO production in the lower airways of atopic and non-atopic subjects with asthma.

The authors thank Dr Sabrina Zanardi and Dr Barbara Biasotti (Department of Health Science, Biostatistic Section, University of Genova, Italy) who provided statistical support.