Blood eosinophils, leukotriene C₄ generation, and bronchial hyperreactivity in formerly preterm infants

U Schauer, Sonja Alelsen, Ruth Jäger, F Riedel, C H L Rieger

Abstract

Infants born prematurely are known to display longstanding bronchial hyperreactivity. The mechanism responsible for this is still unclear. Eosinophils are thought to play a central part in the development of bronchial hyperreactivity in asthma. It was the aim of this study to assess the relation of bronchial hyperreactivity to potential markers of eosinophilic inflammation in peripheral blood.

Eosinophil count, the concentration of serum eosinophilic cationic protein, the capacity of purified eosinophils to generate leukotriene C₄, and bronchial reactivity were studied in 24 non-atopic children born prematurely, 12 healthy controls, and 12 children with asthma aged 6 to 9 years. There was no difference in serum concentrations on eosinophil cationic protein and eosinophil counts. However, eosinophils from the formerly preterm infants with significant bronchial hyperreactivity generated significantly higher amounts of leukotriene C₄ than normal controls and prematurely born children without bronchial hyperreactivity. Levels of leukotriene C₄ in this group were comparable with those obtained with eosinophils from patients with asthma. In contrast with cells from the other groups, eosinophils from the children with bronchial hyperreactivity born prematurely show no enhancement of leukotriene C₄ generation on prestimulation with platelet activating factor.

It is concluded that bronchial hyperreactivity of children born prematurely is accompanied by the prestimulation of eosinophils.

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Methods

PATIENTS

Formerly premature children

Twenty four children who had been admitted to the neonatal intensive care unit during the period between 1983 and 1985 and who had a documented delivery at the 30th to 36th week of gestation were studied. All children were non-atopic as defined by negative skin prick test and normal IgE. Fifteen children showed bronchial hyperreactivity (determined as the concentration of inhaled histamine that caused a decrease of 60% in the specific conductance (PC⁰₀₆₉Gaw) <6·0 mg/ml histamine), whereas nine children showed normal bronchial reactivity (PC⁰₆₉Gaw >6·0 mg/ml histamine) (table 1). Patients with atopy related diseases were excluded from this group.

The records of these patients were reviewed and the birth weight, gestational age, duration of oxygen treatment, oxygen concentrations, the time of assisted ventilation, and complications such as respiratory distress syndrome and patent ductus arteriosus were recorded. The oxygen score was defined as proposed by Coates et al.¹⁺ one point for each hour of exposure to fractional inspired oxygen (FIO₂) between 0·21 and 0·39; two points for each hour in an FIO₂ between 0·40 and 0·60; three points for each hour in an FIO₂ between 0·61 and 0·80; and four points for each hour when FIO₂ exceeds 0·80.
**Blood eosinophils, leukotriene C4 generation, and bronchial hyperreactivity in formerly preterm infants**

507

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**Table 1** Characterisation of the study groups; values are median (range)

<table>
<thead>
<tr>
<th></th>
<th>Formerly premature infants</th>
<th>n</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hyperactive (n=15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (F:M)</td>
<td>6:9</td>
<td>3-6</td>
<td></td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>33 (29-35)</td>
<td>33 (29-36)</td>
<td>39-5 (38-41)</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>1590 (1130–2790)</td>
<td>2050 (1350–2400)</td>
<td>3420 (2640–4130)</td>
</tr>
<tr>
<td>Age at follow up (years)</td>
<td>8-5 (6-9)</td>
<td>0-6 (6-9)</td>
<td>0-6 (6-9)</td>
</tr>
<tr>
<td>PC_{60sGaw}</td>
<td>2-1 (0-9-5-5)</td>
<td>8-0 (6-5-8-0)</td>
<td>8-0 (6-5-8-0)</td>
</tr>
<tr>
<td>IgE (U/mL)</td>
<td>17 (2-96)</td>
<td>28 (2-87)</td>
<td>490 (186-2780)</td>
</tr>
<tr>
<td>Mean number of positive skin tests</td>
<td>0-5</td>
<td>0-5</td>
<td>0-5</td>
</tr>
<tr>
<td>Eosinophilic cationic protein (ng/ml)</td>
<td>45 (0-240)</td>
<td>126 (0-215)</td>
<td>385 (32-960)</td>
</tr>
<tr>
<td>Eosinophilic cationic protein (ng/ml)</td>
<td>4 (1-8)</td>
<td>4-5 (2-7)</td>
<td>10-5 (4-73)</td>
</tr>
</tbody>
</table>

*Concentration of inhaled histamine that caused a decrease of 60% in the specific conductance.

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**Controls**

Twelve volunteers aged 6–9 years with no previous history of asthma, skin disease, or other allergy related diseases, and normal deliveries were examined. The children had negative personal and family histories of atopy and a normal concentration of total serum IgE. In addition, no specific IgE to inhaled allergens could be detected by skin prick test (table 1).

**Patients with asthma**

Twelve children (age 6–9 years) were studied in whom asthma has been diagnosed on the basis of a history of episodic wheezing and impaired lung function tests. These patients were free of lesions from atopic dermatitis (table 1).

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**INFORMED CONSENT**

The study was approved by the ethical committee of the Philippus University. Parents and children gave informed consent.

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**CLINICAL EVALUATION**

Assessment of all patients entering the study was performed in the afternoon to avoid circadian variations in bronchial hyperreactivity and eosinophil functions. In the group with asthma, drugs were withheld for at least 12 hours before venous puncture and pulmonary function testing.

The patients and their parents were asked about the severity of respiratory symptoms and the patients underwent a detailed physical examination and a skin test with extracts of nine common allergens (Dermatophagoides pteronyssinus, timothy, birch, mugwort, cat, dog, alternaria, cladosporium, aspergillus). A weal of >2 mm was considered as positive. Age at onset of asthmatic symptoms and atopic dermatitis, drugs taken, and number of positive skin tests were recorded. Blood was drawn for the determination of serum IgE and eosinophil function, and pulmonary function tests including histamine challenge were performed.

In addition, the parents completed a questionnaire about socioeconomic status, number of siblings, pets, parental smoking, house cooking with gas stove, and the frequency of wheeze and cough in the last three months.

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**SERUM IGE**

Serum IgE of each child was detected using a commercial ELISA kit (Behringwerke).

**PULMONARY FUNCTION TEST**

A maximum flow expiratory volume curve was recorded to determine the volume expired at the first second of the forced expiration (FEV₁) and the maximum expiratory flow at 25% of remaining vital capacity (MEF₂₅). For this measurements mouth flow was integrated to obtain volume. The values of FEV₁ and MEF₂₅ are given as percentages of the predicted value according to height and weight of the patient.

The degree of bronchial hyperreactivity was determined by unspecific provocation testing with inhaled histamine using a modified version of the protocol described by Cockcroft et al. Histamine dehydrochloride in buffered saline was nebulised in a 10 litre reservoir by pressure nebuliser (Pari Proventis, particle size 0.5–5 µm) with increasing concentrations (0.5, 1, 2, 4, and 8 mg/ml). To fill the reservoir, 0.75 ml histamine solution and 120 seconds of nebulisation were necessary. When the reservoir was full, the nebulised aerosol was inhaled by tidal inhalation through a one way valve. One minute after inhalation the thoracic gas volume (TGV) and airway resistance (Raw) were measured in a pressure corrected flow plethysmograph with the patient in the sitting position. The specific conductance (sGaw) was calculated from the reciprocal Raw/TGV. Bronchial hyperreactivity was determined as the concentration of inhaled histamine that caused a decrease of 60% in the specific conductance (PC_{60sGaw}).

Duplicate reactivity tests within one week displayed a difference between two measurements within 1.1 doubling steps of histamine.

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**CELL PREPARATION**

Preparation of eosinophils and neutrophils was accomplished according to the method described by Kloprogge et al. Briefly, 3–10 ml blood were drawn directly into ethylenediaminetetra-acetic acid (EDTA). Anticoagulated blood was sedimented in 0.5 volume of 3% gelatine in saline solution for 45 minutes at 37°C. The plasma leucocyte suspension was washed twice in Ca²⁺ free Tyrode buffer at pH 7.0 that contained 2 mmol/l EDTA. After resuspension in washing buffer,
the cells (2×10⁶/ml) were layered onto a discontinuous isotonic Percoll (Pharmacia) gradient with densities of 1·070 and 1·085 g/ml and centrifuged for 30 minutes (1200 g, 25°C). The eosinophilic leucocytes at the bottom of the tubes were resuspended in washing buffer, counted, assessed for their purity, and, if the purity was lower than 75%, layered onto a second discontinuous isotonic Percoll gradient with densities of 1·095, 1·105 g/ml in the ratio 1:0·5 respectively. The cells were separated by centrifugation for 20 minutes (1200 g, 25°C). The recovery of eosinophils collected from the fraction 1·105 g/ml Percoll was >90% in all subjects studied. Incubations were performed with or without ionophore A 23187 (0·2 μg/ml) for 30 minutes at 37°C. Catalase (Sigma) at a concentration of 3000 U/ml was included in all incubations. In some experiments, a parallel culture was set up after preincubation with platelet activating factor (Sigma) at a concentration of 0·12 μmol for 10 minutes at 37°C. Platelet activating factor itself did not induce leukotriene C₄ generation in the absence of the ionophore A 23187. At the end of incubation, the test tubes were centrifuged and the supernatants were collected and analysed for leukotrienes.

**Figure 1** Leukotriene C₄ generation by purified eosinophils (ng/10⁶ eosinophils) measured in 15 prematurely born children with bronchial hyperreactivity, nine prematurely born children without bronchial hyperreactivity, 12 healthy controls, and 12 children with asthma. The median values are represented by bars. Levels of significance are shown.

**Figure 2** Relation between bronchial responsiveness and enhancement of leukotriene C₄ generation by eosinophils induced by platelet activating factor. Eosinophils (2·5×10⁶/ml) were isolated from the peripheral blood of 15 prematurely born children with bronchial hyperreactivity, nine prematurely born children without bronchial hyperreactivity, 12 healthy controls, and 12 children with asthma. Eosinophils were preincubated with 0·12 μmol platelet activating factor for 10 minutes and subsequently stimulated with 2 μg/ml ionophore A23187 for 30 minutes. Results are expressed as percentage enhancement of leukotriene C₄ generation.

**Quantitation of Leukotrienes**

The radioimmunoassay¹¹ for leukotriene C₄ was performed in 300 μl volumes that contained TRIS Isogel buffer (0·1 M TRIS-HCl, 0·14 M NaCl, 1·5 mM CaCl₂²⁻, 5 mM MgCl₂²⁻, pH 7·4), culture supernatant or synthetic leukotriene C₄ labelled with tritium (specific activity 34 Ci/mmol; New England Nuclear) and a rabbit antiserum to leukotriene C₄ (also specific for leukotriene D₄ and leukotriene E₅) in 1·5 ml polypropylene test tubes. One hundred microlitres of goat antirabbit IgG serum were added at slight antibody excess and incubation was continued for 18 hours at 4°C. The immune precipitate was sedimented by centrifugation at 2500 g for 25 minutes at 4°C dissolved in 500 μl of 0·1 M NaOH, mixed with 4 ml Readyseve (Beckman), and assessed for radioactivity in a liquid scintillation counter. A calibration graph was established with synthetic leukotriene C₄ in the range 0·4–10 ng. The data are expressed as the mean of duplicate samples. The method has been shown to be suitable for the detection of leukotriene C₄ in supernatants of ionophore A 23187 stimulated cells.¹² The percentage enhancement of leukotriene C₄ (LTC₄) formation by platelet activating factor (PAF) was calculated as

\[
\text{LTC}_4 \text{ with PAF} - \text{LTC}_4 \text{ without PAF} \times 100
\]

**Assay for Eosinophil Cationic Protein**

For the measurement of eosinophil cationic protein serum was collected after 30 minutes of clotting time. Eosinophil cationic protein was measured by a radioimmunosorbent assay using a radioimmunoassay kit (Pharmacia). The range of the standard curve was 2–200 μg/ml. All assays were run in duplicate.

**Statistical Methods**

The Mann-Whitney U test and paired Wilcoxon’s signed rank test were used as appropriate. All statistic calculations were performed on a personal computer.

**Results**

Eosinophils isolated from the peripheral blood of 15 prematurely born children with bronchial hyperresponsiveness (PC₂₀₂Gaw <6·0 mg/ml histamine), nine prematurely born children with normal bronchial hyperresponsiveness (PC₂₀₂Gaw >6·0 mg/ml histamine), 12 children with asthma, and 12 healthy children were compared for their ability to produce leukotriene C₄ after a challenge with ionophore A 23187 in vitro. Figure 1 shows that eosinophils isolated from the peripheral blood of prematurely born children with bronchial hyperresponsiveness produced significantly more leukotriene C₄ than hypodense eosinophils from prematurely born children without bronchial hyperresponsiveness or healthy children. In this regard the hyperreactive group was comparable with patients with asthma.
Blood eosinophils, leukotriene C4, generation, and bronchial hyperreactivity in formerly preterm infants

When eosinophils were preincubated with platelet activating factor at a concentration which in itself does not induce leukotriene C4 generation, however, cells from all other groups but not from the hyperreactive formerly premature group showed an enhancement of ionophore induced leukotriene C4 generation (fig 2). There was no difference in eosinophil number or the concentration of IgE eosinophilic cationic protein (table 1).

To assess whether the activity of isolated eosinophils is the only difference between hyperreactive and normoreactive formerly premature children we analysed a number of confounding factors known to influence bronchial hyperreactivity in childhood. There was no difference in gestational age, age at follow up, or atopic status. The hyperreactive group had a lower birth weight, but the difference did not reach statistical significance (p=0.26) (table 1). There was also no significant difference in the perinatal history, although there were higher numbers of patients with patent ductus arteriosus and respiratory distress syndrome in the hyperreactive group (table 2).

Families with hyperresponsive children tend to have more pets, but again this difference does not reach statistical significance (table 3). The current respiratory status including basic lung function was comparable in the two groups (table 4).

**Discussion**

In this study we show that eosinophils from prematurely born children with bronchial hyperreactivity generate significantly more leukotriene C4 than eosinophils from normal control children or from prematurely born children with normal bronchial reactivity. In this regard eosinophils from the hyperreactive group behave like eosinophils isolated from the peripheral blood of children with asthma.

As other risk factors for the development of bronchial hyperreactivity are evenly distributed between the hyperreactive and the normoreactive group of prematurely born children, the difference in leukotriene C4 generation may be indicative of pathophysiological mechanisms responsible for bronchial hyperreactivity. Eosinophils are thought to play an essential part in the pathophysiology of bronchial hyperresponsiveness in asthma. Activated eosinophils which form a major component of the cellular infiltrate during the late asthmatic response significantly contribute to tissue injury by generating high energy oxygen species and releasing mast cell protamine.

There are two important differences between children with asthma and hyperreactive prematurely born children, however. Firstly, prestimulation of the eosinophils from the hyperreactive prematurely born children did not result in an enhancement of leukotriene C4 generation. Secondly, the formerly preterm infants chosen for our study did not show symptoms of bronchial hyperreactivity such as cough or wheezing.

It appears that prestimulation has occurred in vivo in the hyperreactive prematurely born children. The reason for this presumed prestimulation remains a matter of speculation. There are basically two types of factors which enhance eosinophil functions: haematopoietic factors, such as interleukin-5, interleukin-3, or granulocyte-macrophage colony stimulating factor, which have been found in correlation with increased eosinophil numbers in patients with asthma and chemotactic factors such as the lipid mediator platelet activating factor or the chemotactic peptide RANTES. As eosinophil numbers were not increased in the prematurely born children, it seems that not haematopoietic but chemotactic factors are responsible for the observed in vivo prestimulation of eosinophils. Furthermore, the lack of haematopoietic factors might be responsible for the lack of symptoms found in our patients. In vivo and in vitro studies show that marked eosinophil chemotaxis and hence eosinophil inflammation is induced only when haematopoietic and chemotactic factors are present. A candidate for a chemotactic factor is platelet activating factor, which has been shown to induce bronchial hyperreactivity on inhalation. In addition, platelet activating factor has been implicated in the pathophysiology of bronchopulmonary dysplasia as well as premature labour. This is interesting because Nickerson

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**Table 2** Perinatal history in prematurely born patients; values are median (range)

<table>
<thead>
<tr>
<th></th>
<th>Hyperreactive (n=15)</th>
<th>Normoreactive (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No with Apgar score &lt;5 at five minutes</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>IPPB (hours)*</td>
<td>12 (0-180)</td>
<td>17 (0-348)</td>
</tr>
<tr>
<td>CPAP (hours)*</td>
<td>36 (0-264)</td>
<td>48 (0-244)</td>
</tr>
<tr>
<td>Oxygen (hours)</td>
<td>72 (0-1272)</td>
<td>96 (0-564)</td>
</tr>
<tr>
<td>Oxygen score</td>
<td>150 (6-6582)</td>
<td>237 (24-1956)</td>
</tr>
<tr>
<td>Respiratory distress syndrome</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Patent ductus arteriosus</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

*IPPB=intermittent positive pressure breathing; CPAP=continuous positive airway pressure.

**Table 3** Environmental factors in the development of prematurity born patients

<table>
<thead>
<tr>
<th></th>
<th>Hyperreactive (n=15)</th>
<th>Normoreactive (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low socioeconomic status*</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>No of siblings</td>
<td>1.5 (0-3)</td>
<td>1.0 (0-5)</td>
</tr>
<tr>
<td>Pets</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Parental smoking</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Home cooking with gas stove</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

*Parents who were either unskilled or unemployed.

**Table 4** Current respiratory status; values are median (range)

<table>
<thead>
<tr>
<th></th>
<th>Hyperreactive (n=15)</th>
<th>Normoreactive (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptoms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheeze</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Cough</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Lung function*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FVC (l/min)</td>
<td>86 (63-103)</td>
<td>88 (71-105)</td>
</tr>
<tr>
<td>TGV</td>
<td>85 (72-133)</td>
<td>94 (72-120)</td>
</tr>
<tr>
<td>FEV1 (l)</td>
<td>90 (60-106)</td>
<td>99 (72-117)</td>
</tr>
<tr>
<td>sGaw (l/sec×Kpa)</td>
<td>1.18 (0.68-1.71)</td>
<td>1.33 (0.59-1.79)</td>
</tr>
<tr>
<td>MEF25 (l/sec×Kpa)</td>
<td>82 (47-133)</td>
<td>79 (63-117)</td>
</tr>
</tbody>
</table>

*Percentage of the reference values.

FVC=forced vital capacity; TGV=thoracic gas volume; FEV1=volume expired in first second of force expiration; MEF25=maximum expiratory flow at 25% of remaining vital capacity; sGaw=specific conductance.
and Taussig has postulated a common genetic defect leading to bronchial hyperreactivity, bronchopulmonary dysplasia, and premature labour.5

These considerations are highly speculative, however, and are beyond the scope of the present report. Nevertheless, the presented work might be the basis of further research to clarify the association between eosinophil stimulation, bronchial hyperreactivity, and premature birth.

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