Expression of Activation Markers and Cytokine mRNA by Peripheral Blood CD4 and CD8 T Cells in Atopic and Nonatopic Childhood Asthma: Effect of Inhaled Glucocorticoid Therapy

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ABSTRACT. Hypothesis. Activated CD8 as well as CD4 T cells contribute to the production of asthma-relevant cytokines in both atopic and nonatopic childhood asthma.

Objectives. To measure the percentages of peripheral blood CD4 and CD8 T cells expressing naïve/memory (CD45RA/CD45RO) and activation (HLA-DR, CD25) markers, as well as mRNA-encoding interleukin-4 (IL-4) and interleukin-5 (IL-5) in atopic and nonatopic childhood asthmaics and in nonasthmatic controls matched for age and atopic status; and to study the effects of inhaled glucocorticoid therapy of the asthmatics on these measurements.

Methods. Peripheral blood mononuclear cells were isolated from 17 atopic and 8 nonatopic stable (not acutely ill) asthmatics aged 7 to 16 years with moderate-to-severe disease and from 15 nonasthmatic controls matched for age and atopic status. Activation markers on CD4 and CD8 T cells were measured by flow cytometry, and expression of cytokine mRNA by in situ hybridization with CD4 and CD8 T cells were isolated using magnetic beads. Measurements were repeated in 18 of the asthmatics 4 to 6 months after initiation or escalation of inhaled glucocorticoid therapy for inadequately controlled asthma.

Results. The percentages of CD4 T cells expressing CD45RO but not CD45RA were elevated in both asthma groups as compared with the relevant controls and were reduced in association with de novo or augmented inhaled glucocorticoid therapy. The percentages of CD8 T cells expressing both markers were not elevated in asthmatics as compared with controls, and significantly reduced in association with de novo or augmented inhaled glucocorticoid therapy. Elevated percentages of CD4 T cells expressing both markers were not elevated in asthmatics as compared with controls, and significantly reduced in association with de novo or augmented inhaled glucocorticoid therapy. Elevated percentages of CD4 T cells expressing both markers were not elevated in asthmatics as compared with controls, and significantly reduced in association with de novo or augmented inhaled glucocorticoid therapy. Elevated percentages of CD4 T cells expressing both markers were not elevated in asthmatics as compared with controls, and significantly reduced in association with de novo or augmented inhaled glucocorticoid therapy.

Conclusions. The data are consistent with the hypothesis that both activated CD4 and CD8 T cells are associated with child asthma, and that CD4 T cells make a greater contribution to IL-4 and IL-5 synthesis. Increased dosages of inhaled glucocorticoid resulted in clinical improvement in the asthmatics along with reduced T-cell activation and cytokine mRNA expression, suggesting a possible causal association.

ABBREVIATIONS. IL-5, interleukin 5; IL-4, interleukin 4; IgE, immunoglobulin E; FEV1, forced expiratory volume in 1 second; PEF, peak expiratory flow; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PBS, phosphate-buffered saline; NK, natural killer.

Asthma is a common and increasingly prevalent disease affecting 5% of adults and >10% of children.1–3 Although it is one of the most common diseases in childhood, its cellular and molecular pathology have not been studied frequently in children.

In adults, asthma is accompanied by chronic inflammation of the bronchial mucosa, in which selective eosinophil influx and local T-cell activation are characteristic (and almost universal) features.4–6 It is thought that toxic eosinophil products (lipid mediators and basic granule proteins) damage the bronchial mucosa in asthma, resulting ultimately in the clinical manifestations (variable airways obstruction and bronchial hyperresponsiveness). Evidence accumulated over the past 2 decades suggests that selective eosinophil accumulation and activation in the asthmatic bronchial mucosa is mediated, at least in part, by the actions of eosinophil-active cytokines derived predominantly from activated CD4 T cells, of which interleukin-5 (IL-5) is uniquely eosinophil-specific.4,7–9 These cells have a “Th2-type” profile of cytokine production.10 Interleukin-4 (IL-4) may also participate in selective eosinophil accumulation because it upregulates expression of vascular cell adhesion molecule on vascular endothelium, a ligand for very late antigen-4 expressed uniquely (among the granulocytes) on eosinophils.11 IL-4 is also 1 of...
only 2 cytokines causing immunoglobulin E (IgE) switching in B cells and is therefore implicated in the pathogenesis of inappropriate IgE synthesis, which characterizes atopy.12

Glucocorticoids are the most effective therapy for control of asthma symptoms and reversal of bronchial hyperresponsiveness. Previous evidence suggests that they do this at least partly by reducing T-cell activation and concomitant cytokine production.7,13,14

The relative lack of knowledge about the molecular immunopathology of asthma in children stems partly from the practical and ethical problems with obtaining access to the bronchial mucosa. Fortunately, the studies on the properties of peripheral blood T cells in adult asthma cited above suggest that the properties of these cells reflect those of cells in the bronchial mucosa, owing perhaps to a “spillover” or recirculation of these cells into the peripheral circulation.4–9,13,14 We have used this observation in previous studies to demonstrate that in atopic child asthmatics as compared with controls matched for age and atopic status, elevated percentages of both CD4 and CD8 peripheral blood T cells expressed activation markers,15 whereas elevated percentages of unfractionated peripheral blood mononuclear cells expressed mRNA encoding eosinophil-active cytokines including IL-5 and IL-4.16 First, we wanted to extend these studies to examine cytokine production by both CD4 and CD8 T cells in child asthmatics. We hypothesized that both CD4 and CD8 T cells show elevated expression of mRNA encoding IL-5 and IL-4, along with activation markers, in child asthmatics as compared with controls. Second, we wanted to address the question of whether or not there are fundamental differences in the molecular immunopathology of atopic and nonatopic asthma in children, particularly in terms of the expression of IL-4. Original studies addressing this question in adults17,18 suggested that, whereas T cells secreted excess IL-5 in both atopic and nonatopic asthmatics as compared with controls, excess IL-4 was produced only in atopic asthmatics. Subsequent studies have, however, suggested that elevated bronchial mucosal T cell expression of both IL-5 and IL-4 is a feature of nonatopic as well as atopic adult asthma.19,20 We hypothesized that elevated expression by peripheral blood T cells of mRNA encoding IL-5 and IL-4 is a feature of both atopic and nonatopic child asthma.

To address these hypotheses, we measured the expression of activation markers and mRNA encoding IL-5 and IL-4 in peripheral blood CD4 and CD8 T cells from groups of uncontrolled atopic and nonatopic child asthmatics and controls matched for age and atopic status. We also took the opportunity to examine changes in the expression of activation markers and cytokine mRNA in association with inhaled glucocorticoid therapy of the asthmatics.

**METHODS**

**Characteristics of the Patients and Nonasthmatic Controls**

We studied 25 children with asthma defined according to American Thoracic Society criteria,21 which was inadequately controlled according to European consensus guidelines22,23 as described previously.15,16 In brief, inhaled glucocorticoid therapy was initiated or increased if asthma severity was not considered mild. Mild asthma was defined as absence of exercise and nocturnal symptoms, no significant loss of school time, requirement for no more than 3 doses of inhaled β2-agonists weekly, and lung function test (FEV1) above 85% of the predicted values, as described previously.16 Children were recruited from the Department of Pediatrics of Ullevål University Hospital.

At entry to the study, 9 of the patients were taking inhaled glucocorticoids at dosages up to 400 μg/day, whereas an additional 3 were taking regular inhaled cromoglycate. All patients were taking inhaled β2-agonists “as required” (Table 1). The asthmatic children were characterized for atopic status (see below). In addition, 15 nonasthmatic children matched for age and atopic status were studied. Nonasthma was defined as a life-long absence of symptoms suggestive of asthma and spirometry (FEV1) within the normal range.

Atopy was defined as 1 or more positive skin-prick tests (Solumuprik, ALK, Horsholm, Denmark), and/or RAST tests (Phadi- atop, Pharmacia, Uppsala, Sweden), to a range of 8 common aeroallergens (Timothy grass pollen, birch tree pollen, house dust

| TABLE 1. Clinical Characteristics of the Study Participants* |
|----------------|-------------------|---------------|
| Variable       | Atopic            | P†            | Nonatopic     | P†            |
| Number         | 17                | 8             | 8             | 7             |
| Gender         | 11M + 6F          | 4M + 4F       | NA            | 8             |
| Age            | 10.9 (7–16) 2.7   | 8 (8–15) 3.0  | NS            | 12 (7–15) 2.5 |
| Therapy at     | 8/17 received IGC | NA            | 1/8 received IGC | NA            |
| commencement   | (100–400 μg)      |               | (400 μg)      |               |
| of study       | and 1/17 SCG (40 mg) |           | 2/8 SCG (40 mg) |               |
| FEV1 (% predicted) | 84 (70–105) 10  | 108 (94–119) 8.6 | <.001      | 86 (66–99) 10  |
| Total IgE (IU/mL) | 739 (29–5000) 1234 | 149 (15–297) 115 | NS           | 69 (11–214) 86.6 |
| Duration of disease (y) | 6.62 (1–15) 4.11 | NA           | 6.57 (1–11) 3.21 | NA            |
| PD20 metacholine (mg/mL) | 0.41 (0.04–1.6) 0.43 | NA           | 1.06 (0.04–2.4) 0.9 | NA            |

NA indicates nonapplicable; IGC, inhaled glucocorticoid; SCG, sodium cromoglycate; E+N, eye, nose drops; NS, nonsignificant statistical difference.

* Data where relevant are expressed as the mean (range) and standard deviation values. Of the patients receiving IGC, 8 were taking budesonide and 1 fluticasone. Doses are in μg/day. All patients were taking inhaled β2-agonists “as required.” Age, lung function data (FEV1), disease duration, and metacholine PD20 did not significantly differ in the atopic and nonatopic asthmatics at the commencement of the study (2-sample t test).

† Asthmatics versus controls.
mite, mugwort, Cladosporium mold, and cat, dog, and horse dander. A positive skin-prick test was defined as a wheal reaction at least as large as the positive histamine control in the presence of negative diluent control at 15 minutes. Nonatopy was defined as negative skin-prick tests to these 8 common aeroallergens.

None of the patients or the controls had clinical symptoms of acute lower or upper respiratory tract infections in the 4 weeks before the time of blood sampling. None of the children had used systemic glucocorticoids within the 3 months before commencement of the study. None of the atopic patients had ever received allergen immunotherapy and were suffering from no chronic illness other than asthma, rhinitis, and conjunctivitis.

Study Procedure

The study was conducted in accordance with the amended Declaration of Helsinki. Ethical approval was granted by the regional ethics committee. All patients and their parents received written information concerning the purpose of the study, and parents provided written consent.

At some centers children were assessed by a pediatric pulmonologist between 10 am and noon. They were asked not to take any medication 10 to 12 hours before their assessment in the clinic. Those children deemed uncontrolled following detailed history and medical examination, and those in need of new or increased preventive medication were included in the study. Of the 25 children recruited, 18 were given de novo or increased dosages of inhaled glucocorticoid therapy, while the remainder were commenced or continued on sodium cromoglycate. Only those 16 children receiving de novo or increased inhaled glucocorticoid therapy were followed longitudinally for the purpose of the present study.

At enrollment, all 25 asthmatic children underwent lung function testing in the clinic using flow volume curves and body plethysmography (Masterlab Body, Jaeger, Höchberg, Germany). The best of at least 3 independent curves was used to record FEV1. Bronchial hyperreactivity was measured by methacholine inhalation using a flow-controlled nebulizer system and tidal breathing (Jaeger) System after the modified method of Cockcroft. Results were expressed as the provocative concentration of methacholine causing a 20% fall in baseline FEV1 (after inhalation of normal saline). Skin-prick testing was performed where necessary. Before lung function testing, 10 to 15 mL of peripheral venous blood was collected into heparin for isolation of mononuclear cells and full blood count, and serum isolated for total (Unicap, Pharmacia) and specific IgE measurements. Severity of symptoms was graded as done previously, correlated to the method of Aas, which was shown in a previous study to correlate with eosinophil infiltration of the bronchial mucosa.

Children, assisted by their parents where necessary, recorded morning and evening peak expiratory flow (PEF) values (prebronchodilator) and completed daily diary cards documenting severity of day and night cough and wheeze (scale of 0–3), and total numbers of dosages of inhaled β2 agonist and glucocorticoid. Parents were asked to check these cards and compliance. Home PEF measurements were made with a “pocket peak flow” meter (Ferraris Medical, Orchard Park, NY), donated by GlaxoSmithKline. Again, parents were asked to monitor this procedure.

To ensure all children were currently taking inhaled glucocorticoid they were chosen where necessary to an equivalent dosage of fluticasone propionate (Discus dry powder), while all patients commencing inhaled glucocorticoid were given this same drug. Patients continued to use inhaled β2-agonist (salbutamol or terbutaline dry powder inhalers) as required.

Children were assessed in the clinic monthly for the first 2 to 3 months, and again at 4 to 6 months or with more frequent visits if clinically indicated. At each visit, completion of diary cards was verified and lung function was measured. Treatment was adjusted according to symptoms with the aim of rendering symptoms mild as recommended by international guidelines in our previous study.

Follow-up for the purpose of the present study was conducted for a minimum of 4 months and a maximum of 6 months. Thereafter follow-up was continued as clinically indicated. At the end of the study blood samples, lung function, metacholine PD20, detailed medical history, and examination were repeated.

Blood samples, skin-prick tests, lung function, detailed case history, and medical examination were performed only once on the controls.

Laboratory Investigations

Flow Cytometry

Flow cytometry was performed by dual fluorescent staining as previously described. 21 Viable cells that permeabilized T cells were stained directly in whole blood. Fluorochrome-IgG murine monoclonal antibodies against the following cell markers were used: fluorescein isothiocyanate (FITC)-labeled anti-CD4 and anti-CD8 (DAKO A/S, Glostrup, Denmark), phycoerythrin (PE)-labeled anti-CD25 (DAKO A/S, anti-HLA-DR (Becton Dickinson, San Jose, CA), anti-CD45RA-RA and anti-CD45RO (DAKO A/S,) and IgG1/IgG2a irrelevant control antibodies coupled to FITC and PE, respectively, (DAKO A5). Aliquots of 50 μL of heparinized whole blood were incubated with 5 to 10 μL of FITC and PE-labeled monoclonal antibodies for 20 minutes at room temperature. The erythrocytes were lysed for 10 minutes by adding 2 mL of cold lysing buffer (made in house, consisting of NH4Cl 8.32 g, NaHCO3 0.84 g, ethylene diamine tetracetic acid 0.5 g, and distilled water to 1000 mL). After 3 washes in phosphate-buffered saline (PBS), the cells were resuspended in 500 μL of 1% paraformaldehyde in PBS and maintained at 4°C until analyzed.

Flow cytometry was performed on a FACS Calibur flow cytometer (Becton Dickinson), calibrated by standard techniques. For each sample, 10 000 peripheral blood mononuclear cells were counted. After gating out cell debris, lymphocytes were identified on the basis of forward/side scatter characteristics, and CD4 and CD8 T cells on the basis of bright, green (FITC) fluorescence. Monocyte contamination of the CD4 population, as defined by CD14-positive staining, was routinely <1%. Natural killer (NK) cells, which stain relatively dimly with anti-CD3, were also excluded from analysis on the basis of weak staining, although it is impossible to rule out an insignificant degree of contamination. The percentages of CD4 and CD8 T cells staining positively for activation markers were corrected by subtracting the numbers of cells stained with PE-conjugated, isotype-matched control antibodies.15,16

Isolation of Subsets of Peripheral Blood T Cells

Phenotypic subsets of T cells were separated from whole peripheral venous blood using magnetisable microbeads coupled directly to phenotypically specific monoclonal antibodies. Heparinized peripheral venous blood (3.5 mL) was mixed with an equal volume of cold PBS immediately before the addition of 240 μL of prewashed magnetic beads coated with anti-CD4 or anti-CD8 antibody (Dynabeads, Dynal, Oslo, Norway).

All procedures were conducted at 4°C according to manufacturer’s instructions. The beads with adherent CD4 or CD8 T lymphocytes were then immobilized magnetically (Dynal), and non-adherent cells removed by gentle washing with PBS containing 2% v/v fetal bovine serum (Gibco, Uxbridge, England). After 3 washes, the CD4 or CD8 T cells were resuspended in Roswell Park Memorial Institute medium without serum. The Dynabeads were not subsequently removed from the cells. Using this technique, it was possible to obtain populations of cells of at least 1 × 106 cells/mL and >98% CD4 or CD8 positive. Most of these were likely T cells, although a small degree of contamination of CD4 population with monocytes, and the CD8 population with NK cells cannot be excluded. The viability, as assessed by trypan blue exclusion, was invariably >95%. These cells were analyzed for expression of cytokine mRNA, using in situ hybridization.

Cyto centrifuge slides (Polysine glass slides, nuclease-free, BDH, Pool, England) were made on a Shandon 2 cytospin (Shandon Southern Instruments, Runcorn, England), using aliquots (0.6 × 106 cells/mL) of the purified CD4 and CD8 T cells. Samples prepared on these slides were air-dried for 5 minutes, then fixed by immersion in 4% weight/volume paraformaldehyde in PBS for 30 minutes, followed by 2 changes of 15% weight/volume sucrose in PBS (1 hour each). Slides were rinsed quickly 3 times in PBS, drained, dried overnight at 37°C, then stored at −80°C pending in situ hybridization studies.

Probe Preparation

Radiolabelled (32P) antisense and sense (complementary RNA; identical to mRNA; cRNA) riboprobes specific for IL-4 and IL-5

http://www.pediatrics.org/cgi/content/full/109/2/e24
These data were derived from means of diary card data in the first and final weeks of the study, and 1 atopic patient lacks data for first IGC indicates inhaled glucocorticoid therapy; NS indicates nonsignificant statistical difference.

TABLE 2. Clinical Measurements and Symptoms in Atopic and Nonatopic Asthmatics Before and After Elevated Inhaled Glucocorticoid Therapy

<table>
<thead>
<tr>
<th>Variable</th>
<th>Atopic Asthmatics (n = 12) Duration of Follow-up (Months): 5.5 (5–6) 0.5 Difference in IGC Dosage (μg/day) From Start to End of Study Mean (Range) Standard Deviation and 95% Confidence Interval</th>
<th>Nonatopic Asthmatics (n = 6) Duration of Follow-up (Months): 5.7 (5–6) 0.5 Difference in IGC Dosage (μg/Day) From Start to End of Study Mean (Range) Standard Deviation and 95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before IGC Increase</td>
<td>After IGC Increase</td>
</tr>
<tr>
<td>FEV1 (%) predicted</td>
<td>82.7 (70–105) 10.8</td>
<td>97.2 (80–125) 13.6</td>
</tr>
<tr>
<td>Asthma score</td>
<td>33 (23–45) 6.36</td>
<td>16.6 (11–28) 5.6</td>
</tr>
<tr>
<td>PD20 metacholine mg/ml</td>
<td>0.43 (0.04–1.6) 0.46</td>
<td>1.76 (0.06–6.4) 1.76</td>
</tr>
<tr>
<td>PEF am (L/min) prebronchodilator†</td>
<td>267 (167–418) 78.9</td>
<td>338 (267–466) 63.3</td>
</tr>
<tr>
<td>PEF pm (L/min) prebronchodilator†</td>
<td>283 (196–421) 72.9</td>
<td>346 (266–469) 62.8</td>
</tr>
<tr>
<td>Daytime cough†</td>
<td>1.01 (0.1–1.7) 0.5</td>
<td>0.19 (0–1) 0.37</td>
</tr>
<tr>
<td>Daytime wheeze‡</td>
<td>1.01 (0.1–1.7) 0.5</td>
<td>0.19 (0–1) 0.37</td>
</tr>
<tr>
<td>Nighttime cough</td>
<td>0.72 (0–1.7) 0.57</td>
<td>0.07 (0–0.4) 0.14</td>
</tr>
<tr>
<td>Nighttime wheeze‡</td>
<td>0.8 (0–2.2) 0.75</td>
<td>0.14 (0–0.96) 0.26</td>
</tr>
<tr>
<td>Inhaled β2 agonists doses/4†</td>
<td>3.16 (2–5) 1.02</td>
<td>1 (0–4) 1.49</td>
</tr>
</tbody>
</table>

IgG indicates inhaled glucocorticoid therapy; NS indicates nonsignificant statistical difference.

* Data where relevant are expressed as the mean (range) and standard deviation values. P values by paired t test.
† These data were derived from means of diary card data in the first and final weeks of the study, and 1 atopic patient lacks data for first week.

RESULTS

Clinical Characteristics of Individuals

Children with atopic and nonatopic asthma were well-matched with their control groups in terms of age, gender, and total serum IgE concentrations (Table 1). χ² analysis showed that there were no significant differences in the proportions of the atopic asthmatics and atopic nonasthmatics sensitized to any of the panel of allergens used for skin-prick testing with the exception of dog dander, where sensitization was more prevalent in atopic asthmatics (P < .001; data not shown). There were no significant differences in disease severity (as assessed by lung function and metacholine PD20) and duration between the atopic and nonatopic asthmatics (Table 1).

Of the 25 asthmatics recruited because of inadequate disease control (ie, their disease was worse than “mild” according to international consensus definition), 22,23 7 were commenced or maintained by the pediatric pulmonologist on inhaled sodium cromoglycate. The remaining 18 patients (12 atopic and 6 nonatopic) required introduction or elevated dosages of inhaled glucocorticoid therapy. Those children commencing inhaled glucocorticoid therapy de novo were given fluticasone propionate Discus at a starting mean dosage of 360 μg/day. Only those 18 children commencing or increasing inhaled glucocorticoid therapy were followed up for the purposes of the present study, for a mean of 5.5 months (atopic asthmatics) or 5.7 months (nonatopic asthmatics). During this period, dosages of inhaled glucocorticoid were increased by a mean of 412.5 μg/day (95% confidence interval: 288–537) in the atopic asthmatics and 300 μg/day (95% confidence interval: 288–537).
137–463) in the nonatopic asthmatics with the aim of rendering the disease "mild"22,23 (Table 2). This was associated with significant improvements in FEV1, symptom scores, evening PEF, daytime cough and wheeze and nighttime cough, along with significant reduction in inhaled β2-agonist usage in both groups. In addition, morning PEF, metacholine responsiveness, and nighttime wheeze were significantly improved in the atopic asthmatics, whereas in the nonatopic asthmatics morning PEF was not significantly improved, whereas metacholine responsiveness and nighttime wheeze showed only a trend toward significant improvement (Table 2).

Expression of Activation Markers by Peripheral Blood CD4 and CD8 T Cells

At the commencement of the study, the mean absolute numbers of peripheral blood CD4 and CD8 T cells in both the atopic and the nonatopic asthmatics were not significantly different from those in the respective control groups, and there was not any significant difference in these numbers in the atopic and nonatopic asthmatics. Furthermore, the mean absolute numbers of circulating CD4 and CD8 T cells were not significantly altered after inhaled glucocorticoid therapy in both asthmatic groups (data not shown).

The percentages of CD4 T cells expressing the memory cell marker CD45RO were significantly elevated in both the atopic and the nonatopic asthmatics compared with their respective controls, whereas elevated percentages of CD8 T cells expressing the naive marker CD45RA were observed in the nonatopic asthmatics compared with controls (Table 3).

With regard to CD4 T cells, expression of both HLA-DR and CD25 was significantly elevated in both the atopic and nonatopic asthmatics compared with the appropriate controls. With regard to CD8 T cells, CD25 expression was also significantly elevated in both groups compared with the appropriate controls. In the case of HLA-DR expression on CD8 T cells, this difference was statistically significant only in atopic (Fig 1). In both groups of asthmatics, introduction or escalation of inhaled glucocorticoid therapy was associated with a significant reduction in the mean percentages of peripheral blood T cells expressing the activation markers HLA-DR and CD25 (Fig 2).

Expression of Cytokine mRNA by CD4 and CD8 Peripheral Blood T Cells

At the commencement of the study, comparing the asthmatics and controls as 2 single groups, elevated percentages of CD4 peripheral blood T cells expressed mRNA encoding both IL-4 and IL-5 (Fig 3). In the case of CD8 T cells, elevated percentages of these cells expressed mRNA encoding IL-5 but not IL-4 in the asthmatics compared with the controls.

The percentages of CD4 but not CD8 T cells expressing mRNA encoding IL-4 and IL-5 were reduced significantly in both asthma groups in association with introduction or escalation of inhaled glucocorticoid therapy (Fig 4).

**DISCUSSION**

This study is an extension of our previous studies15,16 on the properties of peripheral blood T cells in childhood asthma. In previous studies, we showed that elevated percentages of both CD4 and CD8 T cells express activation markers in child atopic asthmatics compared with controls, and that elevated percentages of unfractionated peripheral blood mononuclear cells from these patients express mRNA encoding asthma-relevant cytokines with a predominantly “Th2-type” expression profile (elevated expression of IL-3, IL-4, IL-5, and granulocyte-macrophage colony-stimulating factor but not IL-2 and IFN-γ). These studies support a role for activated “Th2-type” T cells and their eosinophil-active cytokine products in the pathogenesis of child asthma and have reinforced observations in adult asthmatics that the properties of peripheral blood T cells, at least in terms of expression of activation markers and cytokine mRNA, closely resemble those of T cells in the bronchial mucosa and lumens.5–9,13

One of our principal aims in the present study was to compare functional aspects of both CD4 and CD8 T cells in atopic and nonatopic child asthmatics. The rationale for this was based partly on our previous studies15,16 demonstrating that CD8, as well as CD4 T cells, show evidence of activation in child atopic asthma, and on recent studies in adults27 showing that both CD8 and CD4 T cells within the asthmatic bronchial mucosa are a source of mRNA encoding both IL-5 (the eosinophil-specific cytokine most strongly implicated in the regulation of asthma severity) and IL-4 (1 of only 2 cytokines shown to induce IgE synthesis in B cells and therefore implicated in the pathogenesis of atopy).

Because absolute numbers of CD4 and CD8 T cells did not differ in the asthmatics and the controls and did not change in the asthmatics after glucocorticoid therapy, we deemed it valid to compare percentages of these cells. We were able to show that elevated percentages of both CD4 and CD8 peripheral blood T

| TABLE 3. Percentages of Peripheral Blood CD4 and CD8 T Cells Expressing “Naive/Memory” Markers in Asthmatics and Controls at the Beginning of the Study* |
|------------------|------------------|-----------------|-----------------|-----------------|-----------------|
|                  | Atopic Asthma    | Atopic Controls | Nonatopic Asthma | Nonatopic Controls |                  |
| Percentages of   | n = 17           | n = 8           | n = 8           | n = 7            |                  |
| CD4 T-cells      |                  |                 |                 |                 |                  |
| CD45RA           | 63.5 (48–88)     | 62.25 (48–74)   | 61.25 (52–72)   | 61.1 (50–76)     |                  |
|                  | 9.93             | 8.5             | 6.8             | 8.75             |                  |
| CD45RO           | 63.7 (46–89)     | 38.8 (25.5–52)  | 61.9 (49–85)    | 45.3 (28–64)     |                  |
|                  | 12.8             | 10.7            | 12.7            | 12.1             | .023             |
| CD45RA           | 86.6 (76–93)     | 75.4 (50–100)   | 86.75 (71–94)   | 74.3 (64–82)     |                  |
|                  | 5.1              | 15.3            | 7.7             | 6.6              | .005             |
| CD45RO           | 35.5 (17–83)     | 29.7 (20–42)    | 41.4 (26–70)    | 30.4 (4–44)      |                  |
|                  | 17.7             | 7               | 14.5            | 9.3              |                  |

NS indicates nonsignificant statistical difference.

* Variables are presented as the mean (range) and standard deviation. Statistical comparisons between groups are calculated by t test.
cells expressed activation markers in both atopic and nonatopic child asthmatics compared with age- and atopy-matched controls (Fig 1). Furthermore, the percentages of both CD4 and CD8 T cells expressing these markers were significantly reduced after inhaled glucocorticoid therapy of the asthmatics (Fig

Fig 1. Percentages of peripheral blood CD4 and CD8 T cells expressing the activation markers HLA-DR and CD25 in the atopic asthmatics (AA), atopic controls (AC), nonatopic asthmatics (NAA), and nonatopic controls (NAC) at the commencement of the study. P values by 2-sample t test.

Fig 2. Percentages of peripheral blood CD4 and CD8 T cells expressing the activation markers HLA-DR and CD25 in the asthmatics at the commencement of the study (before) and after introduction or escalation of inhaled glucocorticoid therapy (after). P values by paired t test. Open circles = atopic asthmatics; closed circles = nonatopic asthmatics.

Fig 3. Percentages of peripheral blood CD4 and CD8 T cells expressing mRNA encoding IL-4 and IL-5 in the asthmatics (A) and the controls (C) at the commencement of the study. Open circles = atopic; closed circles = nonatopic. P values by 2-sample t test.

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which was associated with clinical improvement (Table 2). These observations are compatible with the hypothesis that activated CD8 as well as CD4 T cells play a role in the pathogenesis of atopic and nonatopic childhood asthma, and that inhaled glucocorticoid therapy reduces the extent of this activation concomitantly with clinical improvement.

Having said this, it was apparent that, concordant with our previous studies, elevated expression of the T cell "memory" marker CD45RO was a feature of CD4 but not CD8 T cells in both the atopic and nonatopic child asthmatics (Table 3). In the nonatopic asthmatics, the percentages of CD8 T cells expressing the "naïve" marker CD45RA were actually elevated compared with the controls (Table 3). The RA isoform of CD45 is expressed on newly formed T cells that have not yet encountered specific antigen; during activation by exposure to specific antigen, the RA isoform is stably modulated to RO, a process which involves differential, posttranscriptional processing of the CD45 mRNA.²⁸ Our data suggest, therefore, that although CD8 T cells show evidence of activation in childhood asthma, this may not be through encounter of these cells with specific antigen. One might speculate that CD8 T cell activation is a "bystander" effect of contact with cytokines from activated CD4 T cells, which do show evidence of ongoing antigenic activation. Notwithstanding these reservations, it remains plausible that CD8 T cells contribute to the production of asthma-relevant cytokines in child asthma as evidenced by the remainder of our data discussed below. The possible contamination of CD4 and CD8 cells with a very small number of monocytes and NK cells, respectively, cannot be completely ruled out, but would not have affected our data or conclusions.

With regard to cytokine production, clearly elevated percentages of CD4 T cells in the child asthmatics were a potential source of both IL-4 and IL-5 (Fig 3). This is in accord with previous studies, suggesting that although both IL-5 and IL-4 are "Th2-type" cytokines which might be expected to be upregulated together, expression of the cytokines may be dissociated in nonatopic asthma so that atopic asthma is characterized by elevated expression of both IL-5 and IL-4, whereas in nonatopic asthma only IL-5 is upregulated. Although at first sight an attractive explanation as to why nonatopic asthmatics do not show inappropriately elevated IgE synthesis, these studies were more recently refuted in a systematic comparison of bronchial biopsies from adult atopic and nonatopic asthmatics, in which it was shown that both IL-5 and IL-4 expression at the mRNA and protein levels was upregulated in both atopic and nonatopic asthmatics as compared with controls matched for atopic status. These observations sug-

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gest a possible role for IL-4 in asthma pathogenesis even in those subjects who do not manifest inappropriate IgE synthesis at least in the peripheral circulation, as assessed by serum total IgE concentrations and skin-prick testing. In the present study, interpretation of our data were somewhat impaired by the scarcity of nonatopic asthmatics in the age range of 6 to 16 years. Despite an extended recruitment period, we were able to identify only 8 nonatopic asthmatics meeting our inclusion criteria, of whom only 6 required introduction or escalation of inhaled glucocorticoid therapy for improved disease control. This scarcity of nonatopic child asthmatics mirrors clinical experience and epidemiologic studies and undoubtedly reduced the statistical power of our observations.

Despite these reservations, it is apparent from Fig 3 that IL-4 mRNA expression by both CD4 and CD8 peripheral blood T cells was elevated in a proportion of nonatopic child asthmatics as compared with the relevant control patients. On the other hand, it was undetectable in almost half of the subjects using our sensitive technique. At least 1 study has suggested that elevated IL-4 mRNA production by peripheral blood T cells is a feature of child atopic, but not nonatopic asthma albeit after stimulation in vitro.

Experiments with animal “models” of asthma typically involving ovalbumin sensitization and subsequent aerosol challenge of mice have also produced conflicting data on the possible roles of IL-4 and IL-5 in the pathogenesis of airway inflammation and hyperresponsiveness. In one such study, reconstitution of IL-4 “knockout” mice, which also failed to express IL-5, with exogenous IL-5 before challenge restored ovalbumin-induced airways inflammation and hyperresponsiveness in the absence of an ovalbumin-specific IgE response. On the other hand, in other experiments, concurrent deletion of both IL-4 and IL-5 inhibited ovalbumin-induced airways inflammation but not hyperresponsiveness, the latter being inhibited only by previous T cell depletion. In a third series of experiments, it was suggested that blocking of IL-13, but not IL-4, could inhibit ovalbumin-induced airways hyperresponsiveness in mice, again with no apparent effect on airways inflammation or ovalbumin-specific IgE production. The question of whether or not these studies are applicable to humans remains open, but they do raise the possibility that cytokines other than IL-5 and IL-4 may be involved in the regulation of asthma severity, and that cytokine-induced airways hyperresponsiveness may be dissociated, at least in part, from airway inflammation. Additional studies including greater number of patients, and preferably biopsies of the target organ would be needed to resolve this issue fully. Such observations may partly explain why we did not observe any correlation between the percentages of CD4 T cells expressing mRNA for IL-4 and IL-5 and metacholine PD20, as well as other measurements of disease severity in our asthmatic children at baseline (data not shown).

Despite these uncertainties, our data nevertheless suggest the possibility that elevated IL-4, and by extension elevated IgE synthesis perhaps locally within the target organ, may play a role in nonatopic as well as atopic asthma, as has been suggested in adults.

CONCLUSION

We have shown that both CD4 and CD8 T cells may contribute to the molecular immunopathology of childhood asthma (although, as in adults, CD4 cells would seem to be the most significant source of cytokines) and that both are susceptible to glucocorticoid inhibition. Our data raise additional questions about the role of IL-4, and by extension IgE synthesis in the pathogenesis of nonatopic asthma in children.

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REFERENCES


