

In childhood asthma the degree of allergen-induced T-lymphocyte proliferation is related to serum IgE levels and to blood eosinophilia

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Objective: To investigate whether the state of activation of circulating T-cells in childhood asthma could be related to serum IgE levels and/or to blood eosinophilia.

Methods: Seventeen atopic asthmatic children, sensitized to *Dermatophagoides pteronyssinus* (Der p), in stable condition at the time of the study and 15 sex-matched and age-matched controls were studied. The expression of activation surface markers (HLA-DR and CD25) on peripheral blood mononuclear cells (PBMCs) was tested by monoclonal antibodies and FACS analysis, while the PBMC proliferative response to Der p antigens was measured by tritiated thymidine ($^3\text{HTdR}$) incorporation.

Results: As compared to controls, atopic children showed higher eosinophil counts ($P < .01$), similar lymphocyte counts ($P > .1$, each comparison) but higher proportion of HLA-DR+ and CD25+ T-lymphocytes ($P < .05$, each comparison). A significant Der p allergen-induced PBMC proliferation was observed in atopic children ($P < .01$) but not in controls ($P > .1$). Both in controls and in atopic children, no correlations were found between lymphocyte counts and eosinophil counts or total or allergen-specific IgE levels ($P > .1$, each comparison). In contrast, weak correlations were detected between the degree of allergen-induced PBMC proliferation and: a) allergen-specific IgE levels in serum ($P < .05$) and b) eosinophil counts ($P < .05$).

Conclusion: These data support the concept that the degree of activation of allergen-specific T-lymphocytes in blood may reflect the intensity of allergic sensitization in childhood asthma.

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INTRODUCTION

Asthma is a chronic disorder of the airways characterized by bronchial hyperresponsiveness to a variety of stimuli, associated with inflammatory changes in the bronchial mucosa.¹ Several studies have provided convincing evidence that T-lymphocytes and eosinophils are important components of the inflammatory reaction that characterizes allergic asthma.^{2–4}

The most obvious function of T-lymphocytes in allergic reactions is the modulation of IgE production, and in vitro and in vivo studies have indeed demonstrated that these cells are intimately involved in the regulation of the synthesis and release of this type of immunoglobulins by B-lymphocytes.^{5,6} More recently it has been shown that activated T-lymphocytes synthesize other signal proteins (lymphokines) able to orchestrate the inflammatory responses through the regulation of the activity of other cells, including eosinophils, mast cells, and basophils.⁷ In asthmatic subjects, allergen exposure increases the numbers of activated T-cells expressing cytokine mRNA for GM-CSF, IL-3, IL-4, and IL-5.⁸ Granulocyte/macrophage col-

ony stimulating factor, IL-3, and IL-5 are signal proteins stimulating maturation, recruitment and activation of eosinophils,^{9,10} while IL-3 and IL-4 appear to be involved in mast cell proliferation¹¹ and basophil degranulation.¹² In addition to T-lymphocytes, increased numbers of activated eosinophils are present in the blood and in the airways of asthmatics.^{13,14} A great deal of evidence suggests that secretory products released by eosinophils are responsible for mucosal damage which leads to bronchial obstruction and irritability in asthma.^{15,16} These include the demonstration that eosinophils themselves are toxic to human lung tissues^{17,18} and that their presence in the bronchial mucosa may correlate with morphologic damage to the bronchial epithelium.¹⁹

Although most studies have focused on the presence of inflammatory and immunoeffector cells in the bronchial tissue or in the airway lumen or secretions, it has been shown that cellular changes in blood may reflect disease activity or severity.

Relationships between the extent of blood eosinophilia and the severity of asthma or the degree of bronchial hyperreactivity have been demonstrated.^{20–22} In addition, in allergic asthmatic patients an abnormally elevated frequency of allergen-specific T-cells has been described, correlating with the severity of the bronchial response to allergen.²³

With this background the present study was designed to evaluate whether, in allergic children with asthma, the state of activation of circulating T-lymphocytes could be re-

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Table 1a. Characteristics of Allergic Asthmatic Patients

Name	Age (yr)	Allergen-Specific Sensitization	PRIST (KU/L)	RAST (class)	Blood Eosinophilia	
					No. (× 1000/microL)	%
1	2	house dust mites	81	4	0,6	3,3
2	5	house dust mites	287	5	0,7	9,6
3	5	house dust mites	253	6	0,4	4,4
4	6	house dust mites	198	1	0,3	3,6
5	7	house dust mites	17	2	0,1	1,7
6	8	house dust mites	1674	6	0,46	6
7	11	house dust mites	102	4	0,3	3,6
8	12	house dust mites	843	6	0,6	10,7
9	5	house dust mites, pollens	102	1	0,16	1,9
10	6	house dust mites, pollens	201	4	0,4	4,1
11	8	house dust mites, pollens	2828	6	0,8	8,6
12	11	house dust mites, pollens	129	5	0,38	
13	12	house dust mites, pollens	980	6	0,7	10,9
14	5	house dust mites, pollens, cat	288	3	1	14
15	9	house dust mites, cat	446	2	0,41	5,6
16	11	house dust mites, cat	245	5	1	6,8
17	8	house dust mites, cat, molds	366	5	0,7	10,9
Mean ± SD	7.59 ± 2.95		531.77 ± 724.99	4.05 ± 2.02	0.36 ± 0.05	4.16 ± 3.78

Table 1b. Characteristics of Control Subjects

Name	Age (yr)	Allergen-Specific Sensitization	PRIST (KU/L)	RAST (class)	Blood Eosinophilia	
					No. (× 1000/microL)	%
1	7	none	<2	0	0,1	1,4
2	8	none	<2	0	0,1	0,8
3	2	none	<2	0	0,1	1,1
4	6	none	<2	0	0,1	1,2
5	11	none	<2	0	0,1	1,6
6	12	none	<2	0	0,2	2
7	5	none	<2	0	0	0,7
8	12	none	<2	0	0,9	1,5
9	4	none	<2	0	0,1	1,3
10	6	none	<2	0	0,1	0,9
11	6	none	<2	0	0,1	1
12	5	none	<2	0	0,1	1,4
13	9	none	<2	0	0,2	2
14	8	none	<2	0	0,1	2
15	11	none	<2	0	0,1	1,5
Mean ± SD	7.48 ± 0.78				0.16 ± 0.05	1.36 ± 0.81

lated to serum levels of allergen-specific IgE and/or to the intensity of blood eosinophilia.

MATERIALS AND METHODS

Patient Population

Seventeen atopic asthmatic children (8 females and 9 males, aged from 5 to 12 years old, mean age: 7.7 ± 0.7 years old) sensitized to house dust mites were studied out of the pollen season. Eight patients were sensitized only to house dust mites (monosensitized)

while the remaining 10, in addition to house dust mites, were sensitized also to other classes of allergens (polysensitized) (Table 1a). None of the children demonstrated sensitization to birch pollen. At the time of the study, patients were in stable condition with no asthma exacerbations or respiratory tract infections during the month preceding the study. None of the patients had received inhaled or systemic corticosteroids or antihistaminics in the 4-week period before the evaluation.

Inhalant therapy with β_2 -adrenoceptor agonists was withheld at least 48 hours before patients entered the study. Asthma was defined according to the criteria of the American Thoracic Society.²⁴ Allergic sensitization was determined by skin prick test and by evaluation of the serum levels of allergen-specific IgE against *Dermatophagoides pteronyssinus*.

As a control group, 15 sex-matched and age-matched children were evaluated. They were nonatopic as demon-

strated by negative prick test to common inhalants, normal total IgE serum levels and no demonstration of allergen-specific IgE against common inhalants (Table 1b). In addition they never had asthma and none had any respiratory tract symptoms and/or were treated with any drug in the month preceding evaluation.

Analysis of Allergic Sensitization

Blood eosinophil and lymphocyte counts were evaluated both as number and as percentage of cells. Allergic sensitization was determined by skin prick test performing with a panel of ten allergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Parietaria officinalis*, mixed-grass pollen, birch pollen, cat fur allergen extract, dog dander allergen extract, *Alternaria*, *Aspergillus*, and *Hormodendrum*). A histamine solution in distilled water (10 mg/mL) was used as positive control and the glycerol-buffer diluent of the allergen preparations as negative control. Each subject was skin tested on the volar surface of the forearm using 1-mm prick lancets (Dome/Hollister-Stier, Great Britain). The reactions were recorded within 15 minutes by evaluating the skin response rate to each allergen inoculation, in comparison to the wheal given by the negative control. A wheal diameter 4 mm larger than the negative control was considered as a positive reaction. Total serum IgE levels were determined by PRIST kits (Pharmacia) and shown in Tables 1a and 1b. In addition, specific IgE against *Dermatophagoides pteronyssinus* were measured using Phadebas RAST (Pharmacia, Sweden) and the sensitization graded as follows: (1) very low positive, if the concentration of specific IgE was between 0.35 and 0.7 kU/L (class 1); (2) low positive, if it was between 0.7 and 3.5 kU/L (class 2); (3) moderate positive, if it was between 3.5 and 17.5 kU/L (class 3); (4) highly positive, if it was between 17.5 and 50.0 kU/L (class 4); and (5) strongly positive, if it was between 50.0 and 100.0 kU/L (class 5) and (e)

very strongly positive, if it was higher than 100.0 kU/L (class 6).

Identification of Lymphocyte Subpopulations and Evaluation of Surface Antigen Expression by PBMCs

Specific antibody staining and flow-cytometry analysis were used to characterize helper/inducer (CD4+) and suppressor/cytotoxic (CD8+) T-lymphocyte subpopulations and to evaluate the expression of "activation" surface molecules, ie, IL-2R (CD25) and HLA-DR (MHC class II antigens). Blood mononuclear cells were isolated by centrifugation on a discontinuous gradient of Ficoll-Paque from asthmatic patients and control subjects. The partially purified PBMCs were placed into U-bottom microtiter 96 wells/plates (2×10^5 cells/well) and resuspended in 200 μ L of phosphate buffered saline (Flow ICN) supplemented with 1% fetal calf serum and 0.2% sodium azide. The cells were then stained with 5 μ L of FITC-conjugated mabs recognizing CD4 or CD8 surface antigens (Becton Dickinson, Mountain View, CA) or double-stained with 5 μ L of the anti-CD25 FITC-conjugated mab (Becton Dickinson), recognizing an antigen that is the human low-affinity IL-2R or of the L-243 anti-HLA-DR FITC-conjugated mab (Becton Dickinson), recognizing a non-polymorphic determinant on HLA-DR molecules (Becton Dickinson). In addition, control staining with isotype MslgG2a or MslgG1 FITC-conjugated mabs was performed. After a 30-minute incubation at 4°C, cells were washed three times with cold supplemented phosphate buffered saline, fixed with 0.5% paraformaldehyde and analyzed by flow cytometry (FACS scan, Becton Dickinson).²⁵ The data were collected and stored in list-mode files, analyzed with Lysis II software (Becton Dickinson). The expression of IL-2R and of HLA-DR molecules was expressed as percentage of positive cells. To compare the fluorescence intensity of different samples from the same experiments, the analyses were performed with identical set-

tings of the logarithmic amplifier and listmode files analyzed with Lysis II software (Becton Dickinson), as previously described.²⁶ Briefly, after conversion to linear fluorescence intensity units, to obtain a linear function of fluorescence intensity over a wide range,²⁷ the average background linear fluorescence obtained with the control antibody level could be subtracted from the average fluorescence intensity of the specifically stained cells. The possible differences in cell size in the different experimental conditions were controlled for by means of the forward light scatter signal of the flow cytometer, proportional to cell size, and algebraically adjusting for increases in cell surface area so that the intensity in relative linear fluorescence units could be obtained. This correction permits one to correlate directly the average fluorescence intensity with the cell surface density of the stained antigens. All experiments were performed three times.

Dermatophagoides pteronyssinus and Birch Allergen Extract

The allergen extracts used in this study were a purified Der p and a birch powder preparations (Lopharma s.r.l., Milan, Italy) containing multiple allergenic determinants, as identified by polyacrilamide gel electrophoresis. The choice to use it, instead of a more purified allergen extract containing single determinants, was due to the consideration that the repertoire of allergen-specific T-lymphocytes of each atopic patient includes different cell clones likely specific to different allergen determinants.²³ Preliminary data established that the optimal T-lymphocyte proliferative response to Der p allergen extract and to birch extract was obtained at a concentration of 5 μ g/mL. The *Limulus amoebocytes* gelification test, used to assess the presence of bacterial endotoxin, demonstrated that the allergen extract yielded 0.02 Endotoxin Units/mL, not different from the amount found in the medium alone (data not shown).²³

Table 2. Counts, Phenotypic Characterization and Expression on Activation Marker on Blood T-Lymphocytes

		Control Subjects	Asthmatic Patients	P Value
Lymphocyte counts	No ($\times 1000/\mu\text{mL}$)	2.8 (2.6–4.0)	2.8 (2.1–3.2)	$>.1$
	% cells	41.9 (33.4–52.2)	35.4 (25.3–40.3)	$>.1$
Phenotypic characterization	CD4/CD8 ratio	2.0 ± 0.5	1.9 ± 0.3	$>.1$
Activation marker expression	CD25+ (%)	0.1 ± 0.1	0.9 ± 0.1	$<.05$
	DR+ (%)	5.0 ± 5.0	14.4 ± 0.9	$<.05$

PBMC Proliferation Assay

Peripheral blood mononuclear cells proliferation assay was performed as previously described.²⁷ Briefly, PBMCs were isolated by centrifugation on a discontinuous gradient of Ficoll-Paque from asthmatic patients and control subjects, washed and resuspended at $2 \times 10^6/\text{mL}$ in complete medium (RPMI1640). Peripheral blood mononuclear cells were cultured without allergenic stimulus (unstimulated cultures) or with $5 \mu\text{g}/\text{mL}$ of Der p allergen extract in 96-well round-bottom plates. Cell cultures were performed in quadruplicate in a humidified atmosphere that contained 5% CO_2 . Negative control cultures with purified birch allergen extract ($5 \mu\text{g}/\text{mL}$) were also performed. DNA synthesis was measured by adding $0.5 \mu\text{Ci}$ of tritiated thymidine ($^3\text{HTdR}$), specific activity $54 \mu\text{Ci}/\text{mmol/L}$ (Amersham, England) per well on day 5. Cells were harvested (Skatron cell harvester, Lier, Norway) after 18-hour pulsing onto glass fiber filters. $^3\text{HTdR}$ incorporation was measured in a liquid scintillation β -counter (LKB, Finland) and expressed as the mean cpm.

Data and Statistical Analysis

All values were expressed as median and lower and upper quartiles were indicated in brackets. Comparisons between the groups were made by unpaired t test or by Mann Whitney U test. Spearman's rank correlation test was used to examine the relationship between the various parameters: the $r_{(n)}$ means the r value referred to correlations between cell numbers while, the $r_{(\%)}$ means the r value referred to correlations between cell percentages. Statistical significance was assumed at P value lower than .05.

RESULTS

Total or Allergen-Specific IgE Levels and Blood Eosinophil or Lymphocyte Counts in Control Subjects and in Allergic Patients

Serum total IgE levels were respectively $<2 \text{ kU/L}$ in controls and 253 kU/L (17 to 2828) in allergic patients ($P < .001$), while median RAST values to Der p were respectively: a) class 0, in control subjects and class 5 (1 to 6), in allergic patients (Tables 1a and b).

As compared with control subjects, allergic patients showed a higher proportion of eosinophils. This was true expressing blood eosinophilia both as cell number [controls: 0.1 (0.1 to 0.1), allergic patients: 0.5 (0.4 to 0.7)] and as cell percentage [controls: 1.4 (1.0 to 1.6), allergic patients: 5.9 (3.6 to 9.9)] ($P < .001$, each comparison). In contrast, no statistically significant differences in the number or percentage of blood lymphocytes were found between controls and allergic patients ($P > .1$, each comparison) (Table 2).

Phenotypic characterization of T-cell subpopulations demonstrated that the ratio of CD4+/CD8+ T-lymphocytes was similar in control subjects and in asthmatic children ($P > .1$) (Table 2). As compared with controls, however, asthmatic children show a slightly higher percentage of PBMC expressing IL-2R and HLA-DR ($P < .05$, each comparison) (Table 2). Total serum IgE levels, median RAST values to Der p, and blood cell counts were similar in monosensitized and polysensitized patients ($P > .05$, each comparison) (not shown).

No correlations were observed between blood lymphocyte and eosinophil counts, expressing data both as absolute number or as percentage, in

controls [$r_{(n)} = 0.14$ and $r_{(\%)} = 0.13$, respectively; $P > .1$] and in allergic patients [$r_{(n)} = 0.22$ and $r_{(\%)} = 0.32$, respectively; $P > .1$], (not shown). Similarly, in controls and in asthmatics, no correlations were demonstrated between CD4+, CD8+, CD25+ or HLA-DR+ T-cell counts and blood eosinophilia ($P > .05$, each correlation) (not shown).

PBMC Proliferative Response to Allergens

No proliferative response to Der p extract was seen in cell cultures from control subjects [unstimulated cultures = $686.2 \pm 107.1 \text{ cpm}$; Der p-stimulated cell cultures = $663.5 \pm 126.8 \text{ cpm}$ ($P > .05$)] (not shown). In contrast, in all cell cultures from allergic patients, Der p extract efficiently induced PBMC proliferation, [unstimulated cultures = $766.7 \pm 115.0 \text{ cpm}$; Der p-stimulated cell cultures = $19463.9 \pm 2424.5 \text{ cpm}$ ($P < .001$)]. In allergic patients, not sensitized to birch pollen, the proliferative response to the purified birch allergen extract ($2039.2 \pm 952.3 \text{ cpm}$) was significantly lower, as compared with the proliferative response to the Der p ($P < .01$) and similar to the unstimulated cultures ($P > .05$, each comparison) (Fig 1). Data were similar in monosensitized and polysensitized patients ($P > .05$, each comparison) (not shown).

Correlation between PBMC Proliferative Response to Allergens, Blood Eosinophil or Lymphocyte Counts, and Total or Allergen-Specific IgE Levels

In asthmatic children, a slight positive correlation was found between PBMC

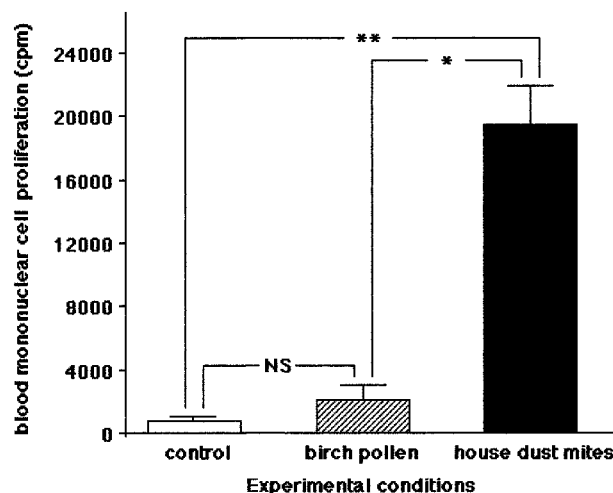


Figure 1. Blood mononuclear cell proliferation in unstimulated (control) and in stimulated cultures (birch pollen and house dust mites). On the ordinate, cell proliferation is expressed as count per minute (cpm); on the abscissa, the different experimental conditions are shown. * $P < .01$, ** $P < .001$, and NS = not statistically significant.

proliferative response to Der p allergen extract and eosinophil counts [$r_{(n)} = 0.575$ and $r_{(p)} = 0.475$, respectively; $P < .05$, each correlation] (Figs 2A and B). Similarly, in asthmatic children the degree of allergen-induced [^3H]thymidine incorporation slightly correlated with log PRIST ($r = 0.490$, $P < .05$) (Fig 2C) and with RAST classes ($r = 0.530$, $P < .05$) (Fig 2D). No correlations were observed between the PBMC proliferative response to Der p allergen extract and total lymphocyte counts or T-lymphocyte subpopulations ($P > .1$, each correlation) (not shown).

DISCUSSION

In this study we have found that the degree of activation of blood T-lymphocytes, as measured by surface marker expression and proliferative response to allergen, was increased in atopic children with asthma as compared with control subjects. In addition, in asthmatic children the intensity of allergen-induced blood mononuclear cell proliferation showed a slight, although significant, positive correlation with both the allergen-specific serum IgE levels and the degree of blood eosinophilia.

T-lymphocytes play a primary regulatory role in the cascade of events

associated with allergic asthma including modulation of IgE synthesis and recruitment and activation of cells bearing high or low affinity receptors for IgE. These cells that include eosinophils, mast cells, basophils, and macrophage/monocytes are all involved in the inflammatory response that follows allergen exposure.²⁸

The production of allergen-specific IgE in allergic individuals likely depends on direct lymphokine-mediated cooperation between T- and B-lymphocytes. The high levels of serum IgE observed in atopy has been attributed to quantitative and/or qualitative differences in the allergen-specific T-cell compartment.^{29,30} Indeed it was shown that T-cell derived cytokines may have different effects on IgE production and that the amount of IgE synthesis is related to the amount of IL-4 secreted.³¹ Other cytokines, including low molecular weight B-cell growth factor (BCGF) and IL-2 can further enhance IgE production, probably through their nonspecific enhancement of B-cell proliferation.³² Other lymphokines, such as IL-6, may provide additional signals to enhance ongoing IgE synthesis.³³

The production of elevated amounts of allergen-specific IgE in atopy may also be related to the expansion of al-

lergen-specific T-cell clones. Indeed, we have here demonstrated an increase in the proportion of blood lymphocytes expressing activation surface markers and proliferation in response to the sensitizing allergen in asthmatic children. In agreement with the results here presented, Rawle et al showed that peripheral blood lymphocytes from patients sensitized to house dust mites on in vitro allergen challenge, proliferate more than cells from control subjects.³⁴ Similarly, we have previously demonstrated in adults that allergen-induced T-lymphocyte proliferation and the frequency of allergen-specific T-cells were significantly higher in asthmatic patients than in control subjects.²³ In addition, in the same study, allergen-specific T-cell frequency in blood positively correlated with the dose of allergen necessary to produce a significant bronchospastic response in vivo.²³

The relative role of the number and/or of the functional characteristics of B-cells committed to produce allergen-specific IgE in modulating in vivo the response to allergens has not been extensively studied. Using Epstein-Barr virus transformation and limiting dilution microcultures, it was found that the frequency of B-cells committed to produce allergen-specific IgE is not different in patients with atopic asthma. This was in contrast with healthy subjects, and among patients with and without a late asthmatic response to allergen inhalation challenge.²³

T-lymphocytes are also able to produce pro-inflammatory lymphokines, able to attract and activate inflammatory cells, including eosinophils.^{35,36} There are several lines of evidence strongly suggesting a key role of eosinophils in the pathogenesis of asthma: (1) eosinophils and their products are present in the airways and in the blood of asthmatics; (2) eosinophil-derived products have the ability to produce many of the pathologic and physiologic features of asthma; and (3) correlations between the intensity of blood or of airway eosinophilia and asthma severity have been demonstrat-

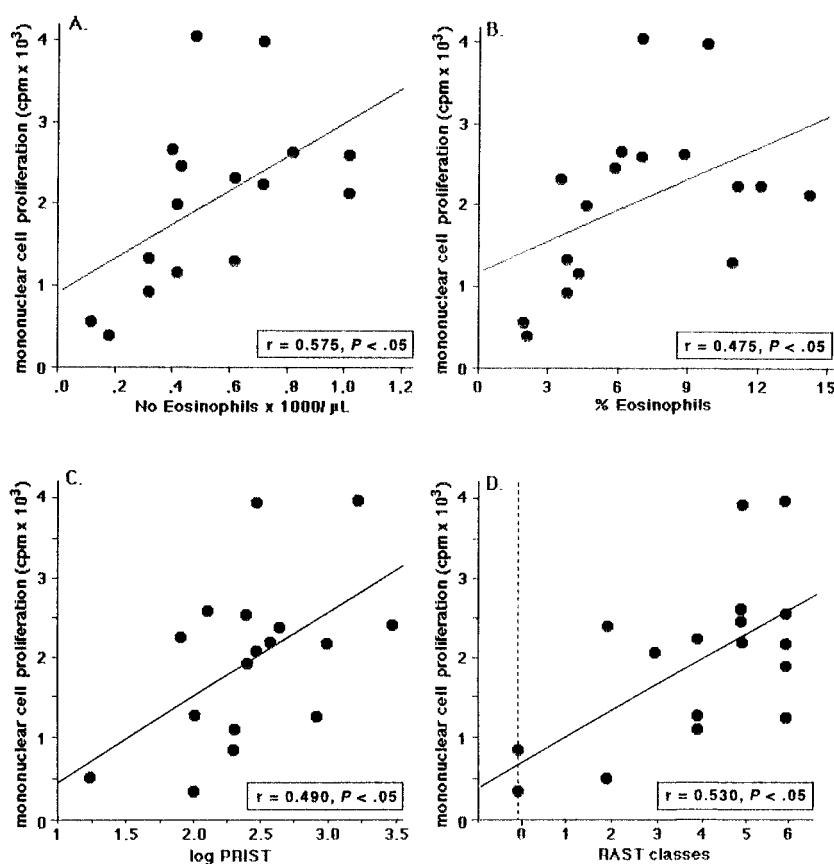


Figure 2. Correlations between allergen-induced blood mononuclear cell proliferation and eosinophil counts (A, B), total IgE (C) or specific IgE (D) levels in allergic asthmatics. On the ordinate, cell proliferation is expressed as count per minute (cpm); on the abscissa, data are expressed as (A) numbers of cells $\times 10^3/\mu\text{L}$, (B) cell percentage, (C) log PRIST, and (D) RAST classes.

ed.^{21,22,37-40} Although the number of eosinophils in blood may be normal in patients with asthma,⁴¹ several investigations have found an increased number of eosinophils in the peripheral blood of asthmatic patients compared with healthy controls.^{21,22,38,40} Indeed, links between blood eosinophilia and asthma severity,³⁹ pulmonary function impairment,^{38,42} nonspecific bronchial hyperactivity^{43,44} and degree of allergic sensitization⁴⁵ have been demonstrated both in adults and in children.

The production of eosinophils in the bone marrow, their maturation, recruitment, and activation appear to be controlled, at least in part, by soluble products released by T-lymphocytes that include IL-3, IL-5, and GM-CSF.^{8,9,10} Indeed it has been shown in vitro and

in vivo that experimental allergen challenges induce the expression mRNA encoding IL-5 and GM-CSF by activated T-cells⁸ and the release of the corresponding cytokines.¹⁰

The demonstration here presented that of a positive correlation between the intensity of allergen-induced blood mononuclear cell proliferation and allergen-specific IgE and degree of blood eosinophilia. This finding further supports a link between immuno effector cells (T- and B- lymphocytes) and inflammatory cells (eosinophils) that can be detected in childhood asthma.

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