

IL-5 secretion by allergen-stimulated CD4+ T cells in primary culture: Relationship to expression of allergic disease

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Background: IL-5-producing allergen-specific T cells are thought to play a prominent role in the pathogenesis of allergic inflammation. We hypothesized that T cell allergen-driven IL-5 synthesis is elevated in patients with atopic disease as compared with that in atopic patients free of disease and nonatopic control subjects.

Objectives: The purpose of this study was to compare IL-5 and interferon- γ (IFN- γ) secretion and proliferation by peripheral blood T cells from sensitized atopic patients with asthma, rhinitis, and no symptoms and from nonatopic control subjects in response to the allergen *Dermatophagoides pteronyssinus* (Der p) and the control recall antigen *Mycobacterium tuberculosis* purified protein derivative (PPD).

Methods: To measure allergen-induced IL-5 production and proliferation, we developed a short-term culture technique that required a single antigenic stimulation of freshly isolated peripheral blood mononuclear cells (PBMC). With this technique, we measured Der p- and PPD-induced IL-5 production and proliferation in PBMC from atopic patients with asthma who were allergic to Der p, atopic patients with rhinitis, atopic patients with no symptoms, and a group of nonatopic normal control subjects. In four experiments, CD4+ or CD8+ T cells were depleted from PBMC to confirm that IL-5 synthesis was T cell dependent.

Results: T cell IL-5 production, but not IFN- γ production, in response to Der p was elevated in atopic patients with asthma and atopic patients with rhinitis compared with findings in atopic patients with no symptoms or nonatopic control subjects. IL-5 production was abrogated by depletion of CD4+, but not CD8+, T cells. In subjects with asthma, allergen-driven IL-5 production correlated with bronchial hyperreactivity. Allergen-induced proliferation was also

higher in patients with asthma than in atopic subjects with no symptoms or nonatopic controls. T cell IL-5 and IFN- γ production and proliferation in response to PPD were similar regardless of atopic status or disease.

Conclusions: Elevated IL-5 production is a characteristic of allergen-specific peripheral blood CD4+ T cells from sensitized patients with atopic disease but not atopy per se. (J Allergy Clin Immunol 1997;99:563-9.)

Key words: Allergen, T cell, IL-5, asthma, rhinitis, atopy

Studies on allergen-specific T cell lines and clones have suggested that in atopic subjects these cells may represent a significant source of IL-5,¹⁻³ a cytokine that has been shown to have pleiotropic and specific proeosinophilic properties,⁴⁻⁶ in the context of a "Th2-like" cytokine profile. Thus local secretion of IL-5 by allergen-specific T cells may play a key role in the pathogenesis of allergic inflammation and asthma, in which selective eosinophil accumulation and activation are characteristic. A limitation of these studies, however, is that long-term culture of T cells, often with steps involving nonspecific mitogenic stimulation, is required. Such procedures might distort the allergen-specific T cell repertoire and unphysiologically augment cytokine synthesis as compared with conditions *ex vivo*. On the other hand, there have been a number of studies that showed elevated spontaneous^{7,8} or mitogen-induced⁹ IL-5 expression by freshly isolated peripheral blood T cells from subjects with asthma and elevated expression of IL-5 messenger ribonucleic acid in the bronchial mucosa and bronchoalveolar lavage fluid of subjects with asthma as compared with control subjects,^{10,11} but these have not allowed the question of whether the T cells are allergen specific to be addressed. Thus there is little or no evidence other than from T cell clones to suggest that the disease in atopic patients with asthma is characterized by elevated secretion of IL-5 by allergen-specific T cells. Furthermore, it is unclear whether such a phenomenon would be confined to subjects with asthma or would also be observed in atopic subjects who have allergic disease not involving the lungs (rhinitis) or who are entirely free of symptoms.

Allergen-driven T cell proliferation can be detected in the peripheral blood of certain nonatopic and atopic persons. If, therefore, elevated IL-5 secretion by allergen-specific T cells is characteristic only of atopic per-

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Supported by a Clinician Scientist Fellowship awarded (to CC) by the MRC (UK) and the National Asthma Campaign (UK).

Received for publication July 8, 1996; revised Oct. 24, 1996; accepted for publication Nov. 8, 1996.

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0091-6749/97 \$5.00 + 0 1/1/79225

Abbreviations used

INF- γ :	Interferon- γ
PBMC:	Peripheral blood mononuclear cells
PPD:	Purified protein derivative

sons, this would imply that T cell allergen-specific proliferation and IL-5 secretion may be dissociated. Such an observation, if confirmed, would have important implications for comparison of T cell reactivity to allergens in atopic and nonatopic persons.

We hypothesized that the development and severity of atopic disease in persons can be related to the amount of IL-5 synthesized by the peripheral blood allergen-specific CD4+ T cells and that this differential IL-5 synthesis is not observed with nonallergenic antigens. We also hypothesized that allergen-induced T cell IL-5 secretion can be dissociated from proliferation. To test these hypotheses, we developed and optimized a short-term culture method that involves direct ex vivo allergen and antigen stimulation of unfractionated peripheral blood mononuclear cells (PBMC) and selected subsets. We used this system to measure allergen-induced peripheral blood T cell IL-5 secretion and proliferation in subjects with atopic asthma, atopic rhinitis, and atopy without symptoms and in nonatopic subjects. To investigate the allergen specificity of the differential IL-5 synthesis, we compared allergen-induced with purified protein derivative (PPD)-induced IL-5 synthesis (PPD is a ubiquitous recall antigen to which the majority of subjects would be expected to be sensitized) in the same subjects. To investigate the cytokine specificity of differential IL-5 synthesis, we further compared IL-5 production with that of interferon- γ (IFN- γ) (a "Th1-like" cytokine not specifically implicated in allergen-specific T cell responses) in response to allergen and PPD in the same subjects.

METHODS**Subject populations**

Peripheral venous blood was obtained from 13 subjects with atopic asthma, from 9 subjects with atopic rhinitis, from 8 atopic subjects with no symptoms, and from 10 normal nonatopic control subjects (Table I). For the purposes of this study, atopy was defined as a ≥ 3 mm diameter wheal at 15 minutes after skin prick testing with *Dermatophagoides pteronyssinus* extract (Soluprick, ALK, Denmark). Patients with asthma were required to have a history of variable airways obstruction and evidence of bronchial hyperreactivity as defined by a 20% reduction in baseline FEV₁ after inhalation of ≤ 8 mg/ml aerosolized histamine (histamine PC₂₀). Patients with seasonal symptoms were excluded. Patients with rhinitis were required to have perennial symptoms of nasal blockage or discharge and to not have asthma (histamine PC₂₀ > 16 mg/ml). Patients who had received immunotherapy or who were receiving oral steroids were excluded. Atopic subjects with no symptoms were required to have a positive skin prick test result but a lifelong absence of any symptoms suggestive of allergic disease. Nonatopic subjects were required to be free of symptoms and to have negative skin

prick test results to extracts of a range of 12 common aeroallergens including *D. pteronyssinus*.

Preparation of cells

PBMC were isolated from heparinized blood samples by density gradient centrifugation over Ficoll-Hypaque solution (Pharmacia, Uppsala, Sweden), washed twice in HEPES-buffered RPMI medium (Chester Beatty Laboratories, London, United Kingdom), and resuspended in RPMI medium (Gibco, Paisley, Scotland) supplemented with 5% human AB serum (Sigma, Poole, United Kingdom), 100 IU/ml penicillin/streptomycin (Gibco), and 2 mmol/L L-glutamine (Gibco). CD4+ and CD8+ T lymphocytes were depleted from PBMC by twofold incubations with anti-CD4 or anti-CD8 monoclonal antibody-coated magnetic beads (Dynal, Wirral, United Kingdom) according to the manufacturer's instructions. Under these conditions >95% depletion was routinely achieved, as assessed by flow cytometry.

Cell cultures

In initial experiments, PBMC were resuspended at 10^6 , 2×10^6 , and 5×10^6 cells/ml and incubated in 200 μ l volumes (minimum of triplicate) in the presence of 0.025, 0.25, 2.5, and 25 μ g/ml concentrations of *D. pteronyssinus* (Aquagen extract, ALK, Hørsholm, Denmark). Control cultures were incubated with medium or 10 μ g/ml *Mycobacterium tuberculosis* PPD (Evans Medical Limited, United Kingdom) as a nonallergen control (amount established in preliminary experiments as optimal). Supernatants were harvested from wells on day 6 and stored at -20° C pending measurement of cytokine concentrations. Cellular proliferation was measured on day 7 by adding 0.5 μ Ci of tritiated methylthymidine (Amersham, United Kingdom) per well for the last 16 hours of culture and assaying label incorporation by liquid scintillation spectroscopy. In subsequent experiments, PBMC were cultured at 5×10^6 cells/ml with 2.5 μ g/ml Der p for cytokine production assays and 10^6 cells/ml and 25 μ g/ml Der p for proliferation assays. In depletion experiments, PBMC depleted (>95%) of CD4+ or CD8+ T cells were cultured in parallel with identical aliquots of undepleted PBMC so that the absolute concentrations of nondepleted cells were unchanged.

Cytokine assays

IL-5 concentrations in culture supernatants were measured with use of a specific sandwich ELISA sensitive above 6.5 pg/ml, as previously described.¹² A commercially available kit sensitive above 2 pg/ml was used for determination of IFN- γ (Endogen).

RESULTS

IL-5 secretion by Der p-stimulated PBMC originates from CD4+ T cells, is modulated by cell culture density, and can be dissociated from cellular proliferation

In initial experiments, IL-5 production and cellular proliferation were measured in PBMC from 12 atopic donors cultured in the presence of *D. pteronyssinus*. PBMC were incubated with varying concentrations of Der p and at three different cell densities (10^6 , 2×10^6 , and 5×10^6 cells/ml) in the same final volume. Allergen-induced cellular proliferation (expressed as tritiated thymidine uptake per unit cell number) increased in an allergen dose-dependent fashion (Fig. 1, a). The highest efficiency of proliferation (i.e., thymidine uptake per unit

cell number) was observed in cultures containing cells at the lowest density used, which suggests that proliferation was inhibited at higher cell densities. In the same cultures, the patterns of IL-5 secretion differed distinctly from the proliferative responses (Fig. 1, b). IL-5 supernatant concentrations were highest when cells were cultured at 5×10^6 cells/ml and the optimal allergen dose required to illicit this response appeared lower than that required for maximal proliferation. Measurement of IL-5 at days 3, 5, and 7 suggested that secretion of this cytokine reached a plateau at days 5 to 7 (data not shown). To confirm that IL-5 secretion originated from CD4+ T cells, CD4+ or CD8+ T lymphocytes were depleted from PBMC of four atopic donors before allergen stimulation. Depletion of CD4+, but not CD8+, T lymphocytes completely abrogated IL-5 production (Table II).

IL-5 secretion by Der p-specific peripheral blood T cells is elevated in atopic subjects with symptoms

Having established the most favorable conditions for allergen-induced peripheral blood T cell proliferation on the one hand and IL-5 secretion on the other, we next sought to determine the relationship between these variables and the expression of allergic disease.

The proliferative response of PBMC (10^6 cells/ml) cultured with Der p (25 μ g/ml) and a control recall antigen (PPD, 10 μ g/ml) was assessed in atopic subjects with asthma allergic to Der p, atopic subjects with rhinitis, and atopic subjects with no symptoms and in nonatopic normal control subjects (Table I). Culture with Der p induced detectable proliferation in PBMC from most subjects in each group, although this was statistically significantly elevated in the atopic subjects with asthma as compared with results in the atopic subjects with no symptoms and the nonatopic control subjects (Fig. 2). PBMC from all subjects in all groups showed a strong proliferative response to PPD with no significant differences between the groups.

IL-5 secretion by PBMC (5×10^6 cells/ml) stimulated with Der p (2.5 μ g/ml) and PPD (10 μ g/ml) was determined in the same subjects (Fig. 3). IL-5 secretion by PBMC from the symptomatic atopic subjects (those with asthma or rhinitis) was clearly elevated as compared with that by PBMC from the atopic subjects who had no symptoms and the nonatopic control subjects. Elevated IL-5 production was associated with allergen-specific T cells, because parallel control cultures done under identical conditions with PPD as the antigenic stimulus resulted in little or no IL-5 synthesis in all subject groups (Fig. 3). Furthermore, elevated IL-5 secretion by PBMC from the subjects with symptoms was not generalized for all cytokines, because when IFN- γ concentrations were measured in the same supernatants Der p-induced IFN- γ expression was not significantly variable according to atopic status or symptoms (Fig. 3). PPD induced strong IFN- γ secretion in PBMC from all patient groups. Finally, a correlation was observed between allergen-induced IL-5 secretion by PBMC from patients with

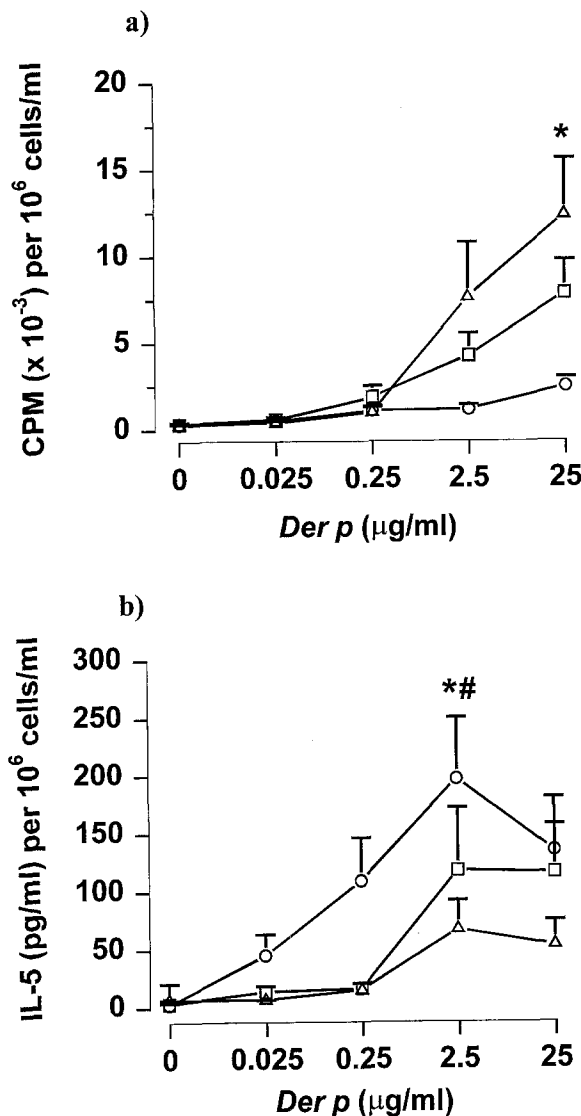


FIG. 1. Effect of allergen concentration and cell culture density (circles, 5×10^6 cells/ml; squares, 2×10^6 cells/ml; triangles, 10^6 cells/ml) on Der p-induced (a) PBMC proliferation and (b) IL-5 production. Supernatants were harvested after 6 days and proliferation was assayed after 7 days. Data are from 12 atopic subjects and are represented for clarity as median plus standard error. In each case, proliferation and IL-5 production are represented per 10^6 cells/ml. * $p < 0.05$, 5×10^6 cells/ml versus 10^6 cells/ml; # $p < 0.05$, 5×10^6 cells versus 2×10^6 cells/ml, by Mann-Whitney U test.

asthma and bronchial hyperreactivity to histamine (Fig. 4).

DISCUSSION

The association of the T cell Th2-type cytokine profile with responses to allergens and parasite antigens is established.^{1,2} This evidence has been provided principally by the study of antigen-specific T cell clones and lines, which requires prolonged propagation of T cells in vitro (weeks or months) in IL-2. Although these techniques have provided important information, the ex-

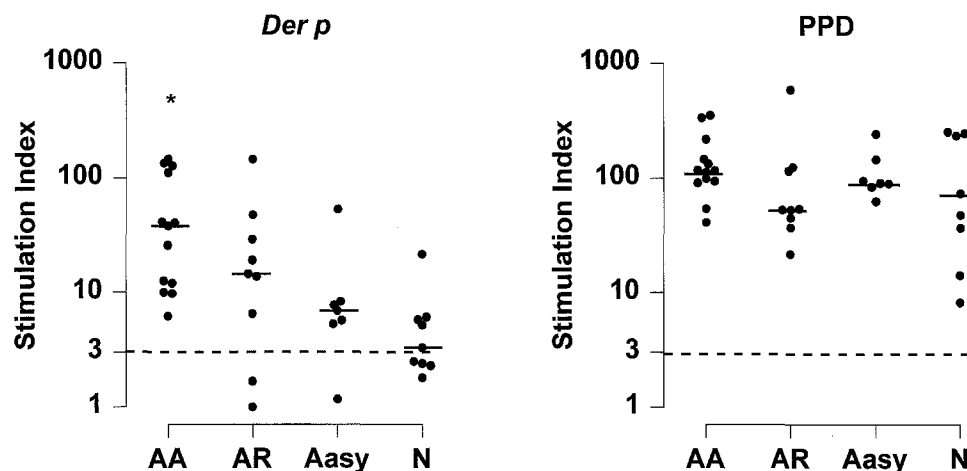


FIG. 2. PBMC proliferation (at 10^6 cells/ml) in response to Der p (25 μ g/ml) and PPD (10 μ g/ml) stimulation for 6 days. AA, Atopic subjects with asthma; AR, atopic subjects with rhinitis; Aasy, atopic control subjects with no symptoms; N, nonatopic control subjects; stimulation index, counts per minute in response to antigen divided by background (unstimulated) counts per minute. Stimulation index >3 is considered positive. *For Der p stimulation, atopic subjects with asthma versus atopic control subjects with no symptoms, $p = 0.009$; atopic subjects with asthma versus nonatopic control subjects, $p = 0.0004$, by Mann-Whitney U test.

TABLE I. Clinical characteristics of subjects

Subjects	Der p SPT	Der p RAST*	Histamine PC ₂₀
Subjects with allergic asthma ($n = 13$)	+	26.3 (13.1-100)	1.70 (0.02-3.66)
Subjects with perennial rhinitis ($n = 9$)	+	23.0 (0.37-100)	>16
Atopic subjects with no symptoms ($n = 8$)	+	1.99 (<0.35 -5.1)	>16
Normal control subjects ($n = 10$)	-	<0.35	>16

SPT, Skin prick test.

* $p = 0.0002$, subjects with allergic asthma versus atopic subjects with no symptoms.

TABLE II. Dependence of Der p-induced IL-5 production on CD4+ cells

	IL-5 concentrations (pg/ml)			
	Subject 1	Subject 2	Subject 3	Subject 4
Whole PBMC	2506	5253	5910	1879
CD8 depleted	1460	3515	2962	828
CD4 depleted	<6.5	15	<6.5	<6.5

tended nature of these cultures, involving probable selection and maturation of T cell subpopulations, raises questions as to the extent to which these cells functionally represent those found in vivo. In addition, they give no indication as to the relative amounts of cytokine synthesis by allergen-specific T cells in individual subjects.

In this study, we set out to determine the relationship between the presence and severity of atopic disease in a group of subjects allergic to Der p and in nonatopic control subjects and the amount of allergen-induced IL-5 secretion by the peripheral blood T cells of these subjects. To achieve this, and to minimize the problems

associated with establishing cell lines and clones, we used a bulk culture method that required a single ex vivo exposure of PBMC and no other stimulus. We believe that IL-5 secretion under the conditions we have described may better reflect both the numbers of cells responding to antigen and the propensity of individual cells to secrete IL-5 in the course of this response. The clear association between Der p-induced IL-5 production and allergic disease strongly suggests that this method is physiologically relevant. Furthermore, depletion experiments confirmed that CD4+ T cells represent the principal source, if not the only source, of IL-5 in culture supernatants under these conditions. Interest-

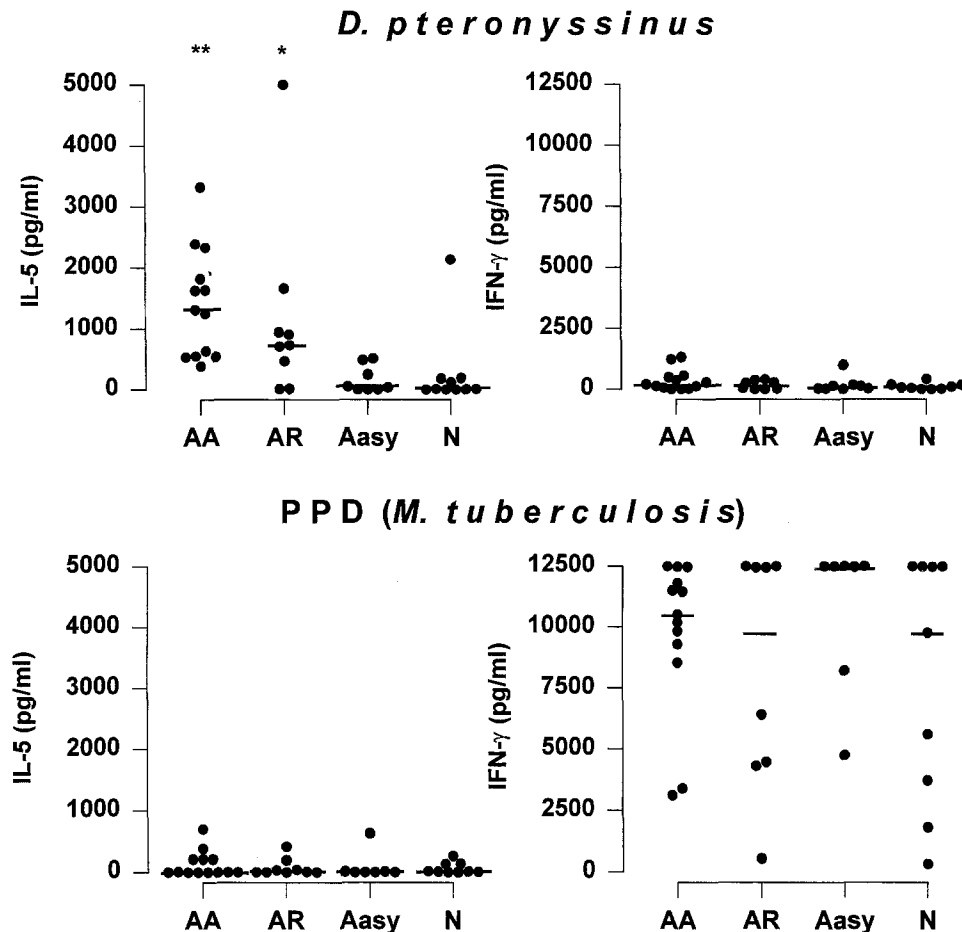


FIG. 3. IL-5 and IFN- γ production (at 5×10^6 cells/ml) in response to Der p (2.5 μ g/ml) and PPD (10 μ g/ml) stimulation for 6 days: values shown represent cytokine production in response to antigen minus concentrations in background (unstimulated) cultures. Abbreviations same as in Fig. 2. For IL-5 production in response to Der p stimulation, **atopic subjects with asthma versus atopic control subjects with no symptoms, $p = 0.0009$; **atopic subjects with asthma versus nonatopic control subjects, $p = 0.0007$; *atopic subjects with rhinitis versus atopic control subjects with no symptoms, $p = 0.024$; *atopic subjects with rhinitis versus nonatopic control subjects, $p = 0.013$, by Mann-Whitney U test.

ingly, depletion of CD8+ T cells in these cultures resulted in a reduction in Der p-induced IL-5 secretion even though the overall concentrations of CD4+ T cells remained the same (Table II). Although we cannot exclude the possibility that CD8+ T cells potentiate IL-5 synthesis by CD4+ T cells, the most logical explanation for this phenomenon would be the effects of lowering overall cell density, as illustrated in Fig. 1. Der p was chosen for this study because it is a ubiquitous, perennial allergen to which all subjects would be expected to be sensitized. We elected to focus on IL-5 secretion because this cytokine has been clearly related to disease severity in previous studies on allergic asthma^{13, 14} and, uniquely, has eosinophil-specific activities.⁴⁻⁶ In contrast, IL-4, although involved in the induction of IgE synthesis, is less clearly implicated in the pathogenesis of eosinophilic allergic inflammation.¹⁵

There have been a number of previous studies that involved analysis of cytokine expression after whole

culture with allergen extracts of PBMC from patients with rhinitis or asthma and control subjects. These studies have variously shown elevated expression of IL-4^{16, 17} or elevated production of eosinophil survival activity¹⁸ or eosinophil colony-forming activity.¹⁹ One study showed elevated IL-5 production by allergen-stimulated PBMC from subjects with asthma as compared with results from control subjects,⁹ but the concentrations of IL-5 measured in that study were considerably lower than we observed and were not correlated to asthma symptoms. In our study, PBMC from all subjects with asthma produced detectable IL-5 on stimulation with allergen, which likely reflects optimization of the culture conditions. A further weakness of these previous studies is that they compared responses in atopic subjects with disease conditions with those in nonatopic control subjects. Consequently, the question of whether elevated allergen-induced IL-5 synthesis is associated with asthma or rhinitis or just a

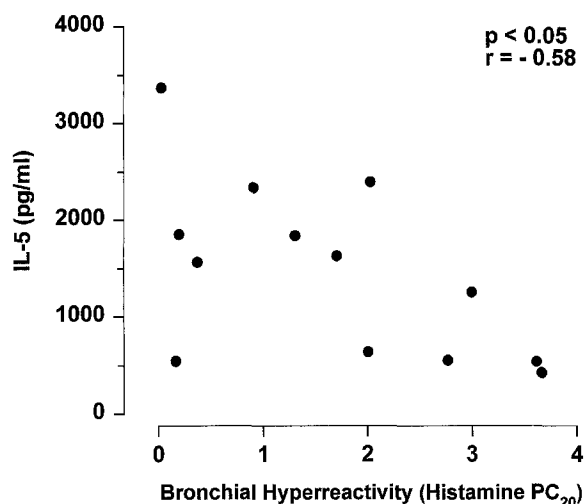


FIG. 4. Relationship among subjects with asthma between allergen-induced IL-5 synthesis and bronchial hyperreactivity (histamine PC₂₀). Analyzed by Spearman's rank correlation test.

positive skin prick test result to allergen has remained unanswered.

Cellular proliferation in response to Der p was significantly elevated in the patients with asthma as compared with results for atopic subjects with no symptoms and the normal control subjects, which is in line with previous studies^{20, 21} and suggests the possibility that patients with symptoms have greater numbers of circulating allergen-specific T cells. This is further supported by the fact that cellular proliferation induced by the recall, nonallergenic antigen PPD was similar in all four subject groups, which shows that elevated antigen-driven proliferation is not a generalized property of asthmatic PBMC. It is clear from our data and previous studies that IL-5 secretion by peripheral blood T cells, allergen specific or otherwise, cannot be inferred from the presence of a proliferative response. First, as shown in studies on T cell clones, different antigens differ in their propensities to induce the synthesis of particular cytokines,^{2, 22} as was the case in the present study for Der p and PPD. Second, as we have shown, IL-5 production by T cells was markedly enhanced by culture at relatively high cell densities, under which conditions cellular proliferation was markedly suboptimal. This clear dissociation suggests the presence of distinct regulatory processes in T cells governing proliferation and IL-5 secretion. A similar dissociation between T cell proliferation and IL-4 secretion in the presence of varying concentrations of allergen has also been previously demonstrated.²³

Our data suggest that allergen-induced IL-5 production by PBMC from sensitized atopic subjects with symptoms (asthma, rhinitis) is elevated as compared with that in atopic subjects with no symptoms and nonatopic normal control subjects. Thus elevated IL-5 synthesis appears not to be a feature of atopy per se, but is observed only in the presence of ongoing atopic disease. These observations are compatible with the

hypothesis that, in asthma and rhinitis, inappropriate IL-5 synthesis (reflecting increased numbers of allergen-specific CD4+ T cells, increased IL-5 secretion by individual allergen-specific T cells, or both) reflects a primary abnormality of the bronchial or nasal mucosal environment that allows these T cell abnormalities to develop. Although increased numbers of T cells that express IL-5, at least at the level of messenger ribonucleic acid, have been demonstrated in the relevant mucosal surface in patients with atopic asthma and rhinitis as compared with findings in control subjects,^{10, 11, 14} it is not known how many of these cells are allergen specific.

In contrast to findings with IL-5, secretion of IFN- γ by Der p-stimulated PBMC was generally low and statistically equivalent in the four subject groups. Moreover, the ratios of IL-5 to IFN- γ production were significantly higher in the asthmatic group than in atopic and nonatopic control subjects (data not shown), which suggests that inappropriate IL-5 secretion in response to Der p was disproportionate to that of IFN- γ . This observation is compatible with a Th2-type pattern of cytokine secretion by allergen-specific PBMC from atopic subjects, as has been inferred from work on T cell clones. One caveat to this observation is that our in vitro system was optimized for IL-5 and not IFN- γ secretion. Nevertheless, the uniformly low secretion of IFN- γ by allergen-stimulated, but not PPD-stimulated, PBMC from subjects in all four groups is consistent with a Th2-type profile of cytokine secretion by allergen-specific PBMC from atopic subjects obtained ex vivo. One previous study provided evidence for decreased IFN- γ production by allergen-stimulated PBMC from subjects with asthma relative to findings in normal nonatopic control subjects.¹⁶ Our experience in the present study was, however, that secretion of IFN- γ by allergen-stimulated PBMC even in nonatopic control subjects was so low that any further reduction in this secretion by PBMC from the subjects with asthma could not have been detected. In the present study, a correlation was observed among the patients with asthma between bronchial hyperreactivity to histamine and allergen-induced IL-5 synthesis. This was unexpected inasmuch as although the patients with asthma were required to have perennial symptoms, they were not necessarily uniquely allergic to Der p. Furthermore, elevated IL-5 production also was apparent in the patients with rhinitis, who had no evidence of bronchial hyperreactivity. Nevertheless, the data are consistent with the hypothesis that the development of atopic disease requires elevated IL-5 synthesis by allergen-specific CD4+ T cells and that this is necessary (though not sufficient) for the development of bronchial hyperreactivity. We also incidentally noted a correlation between the amount of peripheral blood T cell allergen-induced IL-5 secretion and serum concentrations of allergen-specific IgE (for all atopic subjects, $r = 0.79$, $p < 0.005$). This is in line with findings of a recent study that showed that atopic subjects with disease, as compared with those without disease, are more

likely to have elevated serum concentrations of relevant allergen-specific IgE.²⁴ Although the synthesis of allergen-specific IgE clearly does not inevitably result in the development of disease, this observation is again consistent with our speculation that atopic patients with disease conditions have an abnormality of the bronchial or nasal mucosal environment that allows exaggerated allergen-specific IgE and T cell responses to develop.

In summary, with use of simple but novel culture conditions, we have shown that allergen-specific T cells from patients with atopic disease can be induced to secrete high amounts of IL-5 but not IFN- γ , a pattern that is reminiscent of cytokine profiles observed in allergically inflamed human tissues. IL-5 expression under these circumstances is associated with symptoms of allergic disease, but not atopy per se, and correlates with bronchial hyperreactivity in patients with asthma. Our technique offers the opportunity for further analysis of the regulatory mechanisms controlling allergen-induced IL-5 synthesis by T cells in a physiologically relevant system.

We thank Dr. O. M. Kon and Dr. B. Haselden for their assistance in collecting blood samples. Aquagen extract was kindly provided by ALK, Hørsholm, Denmark.

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