

PBMCs from both atopic asthmatic and nonatopic children show a T_H2 cytokine response to house dust mite allergen

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Background: The hypothesis that in atopic diseases the T-helper response is skewed toward a T_H2 -type cytokine response was based on studies with mitogen stimulation, T-cell clones, or both.

Objective: Using primary cultures, we investigated (1) whether atopic asthmatic patients have a T_H2 response and nonatopic subjects have a T_H1 response to allergen and (2) whether atopic patients have a decreased ability to mount T_H1 immune responses to mycobacterial antigens.

Methods: The responses of PBMCs to allergen (house dust mite [HDM]) or purified protein derivative of *Mycobacterium tuberculosis* (PPD) stimulation from 10 severely and 14 moderately asthmatic patients (all allergic to HDM) were compared with those of 17 nonatopic healthy black (Xhosa) children.

Results: HDM-stimulated proliferation, IL-5 release, and the IL-5/IFN- γ ratio were significantly increased in subjects with atopic asthma, whereas IFN- γ release was not significantly different. IL-4 levels were below the level of detection. PPD-stimulated proliferation, IL-5 release, IFN- γ release, and the IL-5/IFN- γ ratio were not significantly different among the groups. Each group had a significantly higher IL-5/IFN- γ ratio in response to HDM than to PPD (a T_H1 stimulus).

Conclusion: Our study, which used primary cultures to investigate the hypothesis that nonatopic subjects have a T_H1 response to allergens, indicates that HDM stimulates a T_H2 cytokine response in both atopic and nonatopic subjects but that the response is enhanced in atopic patients. Our results with PPD suggest that normal and atopic asthmatic subjects can have a T_H1 cytokine response to mycobacteria, but there is a subgroup of atopic subjects that have a T_H2 response. (J Allergy Clin Immunol 2000;106:84-91.)

Key words: Atopy, asthma, African, peripheral blood mononuclear cells, house dust mite, mycobacterial protein, IL-5, IFN- γ

Abbreviations used

δ dpm: Delta disintegrations per minute

HDM: House dust mite

PPD: Purified protein derivative of *Mycobacterium tuberculosis*

Allergic asthma is increasing dramatically worldwide.¹ The basic mechanisms underlying the development of asthma in childhood are still not known.² Atopic inflammation is primarily triggered by IgE-mediated release of proinflammatory mediators from mast cells³ and has been linked to excess production of the T_H2 cytokines IL-4 and IL-5 relative to the T_H1 cytokine IFN- γ .^{4,5} It is, however, the delayed, sustained, local inflammation, which is characterized by the accumulation of activated inflammatory cells, including monocytes, T cells, and eosinophils, that accounts for the severity of chronic allergic diseases, such as asthma.^{3,6} There is increasing evidence for the central role of allergen-specific T_H2 cytokine responses in both the initiation and the persistence of chronic allergic inflammation.⁶⁻⁸

The hypothesis that the T-helper response in allergy and asthma is skewed toward a T_H2 -type response was based on analysis of T-cell clones^{4,9} or mitogen stimulation of PBMCs.¹⁰ Allergen-specific T-cell responses are more relevant than mitogen-induced T-cell reactivity in investigating the immunology of allergic asthma.⁵ Studies on antigen-specific T-cell clones indicated that allergens, such as house dust mite (HDM), provoke a T_H2 -type response and that bacterial antigens stimulate a T_H1 -type response.¹¹ However, there is also evidence that T-cell clones from allergic patients respond to allergens with a predominantly T_H2 -type response, whereas those from normal subjects respond with a T_H1 -type response.¹² With freshly isolated PBMCs, it is controversial to what extent allergen-reactive T cells are present in nonatopic individuals.¹³⁻¹⁵

It has recently been suggested that there is an inverse relationship between allergy, which provokes a T_H2 response, and susceptibility to bacterial infections, such as tuberculosis.^{16,17} It is important to clarify whether allergic sensitization favors an immune response that is not protective against tuberculosis, especially in the Western Cape Province of South Africa, where tuberculosis is endemic, and where the incidence of allergies and asthma is increasing.¹⁰

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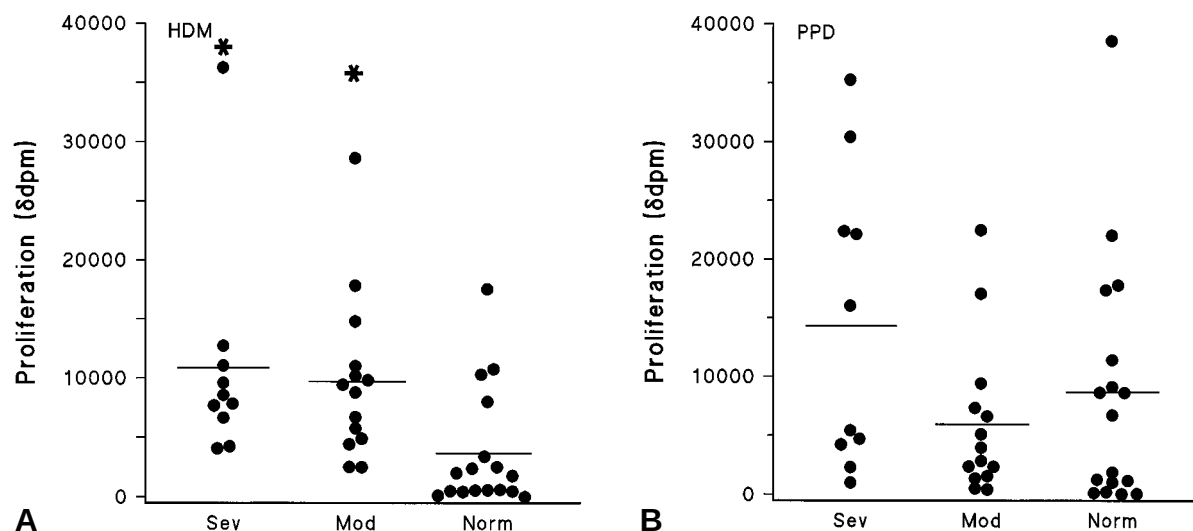


FIG 1. HDM-stimulated (**A**) and PPD-stimulated (**B**) proliferation of PBMCs from atopic children with severe (Sev; $n = 10$) or moderate (Mod; $n = 14$) asthma and age-matched, normal, nonatopic, control subjects (Norm; $n = 17$). Incorporated radioactivity is expressed in δ dpm (ie, mean of wells containing stimulant minus mean of control wells). Horizontal lines represent means. Asterisks indicate a P value of .01 relative to the normal group.

In this study freshly isolated PBMCs from allergic asthmatic and normal healthy black African (Xhosa) children were used. We characterized the proliferation and cytokine release stimulated by the major allergen HDM or the purified protein derivative of *Mycobacterium tuberculosis* (PPD). The T_H2 cytokines IL-4 and IL-5 and the signature T_H1 cytokine IFN- γ were measured. The aim of this study was to determine whether atopic asthmatic subjects have a T_H2 -type cytokine response to HDM and nonatopic subjects have a T_H1 -type cytokine response or whether HDM is a general T_H2 -type stimulus. Furthermore, we investigated whether atopic and nonatopic subjects differ in their cytokine responses to the mycobacterial antigen PPD.

METHODS

Subjects

Black (Xhosa) children with chronic asthma were selected on the basis of allergy to HDM, as indicated by a positive skin prick test response, or specific IgE to HDM (Der p 1) of greater than 9 kU/L, or both. The patients with severe asthma ($n = 10$; mean age, 8.7 years) and those with moderate asthma ($n = 14$; mean age, 8.9 years) had mean levels of HDM (Der p 1)-specific IgE of 87.7 ± 6.8 kU/L and 90.1 ± 6.8 kU/L, respectively.¹⁰ The healthy control subjects ($n = 17$) had no symptoms of allergy or asthma. Two of the normal subjects had measurable HDM-specific IgE levels of 2.4 and 2.1 kU/L, respectively. The characteristics of the subjects, as well as total serum IgE levels and specific IgE to common aeroallergens, food allergens, and *Ascaris lumbricoides*, have been reported previously.¹⁰

This study was approved by the Ethics and Research Committee of the University of Cape Town. Informed consent was obtained from subjects or their parents.

Antigens

Lyophilized spent HDM (*Dermatophagoides pteronyssinus*) culture was obtained from Professor Spieksma (University of Leiden,

The Netherlands). HDM extract was prepared by extracting 1 g of powder in 10 mL of PBS by stirring overnight at 4°C, followed by centrifugation and filter sterilization of the supernatant. The endotoxin content of the HDM extract was 0.01 EU/ μ g protein (Chromogenic Limulus Amebocyte Lysate test, BioWhittaker). Protein concentrations were determined by using a Pierce BCA Kit. A sterile solution of PPD (1 mg/mL in PBS) was prepared from freeze-dried preservative-free PPD 298 (Ministry of Agriculture Fisheries and Food, Central Veterinary Laboratory, Addlestone, UK).

Stimulation of PBMCs

PBMCs were isolated and cultured in complete RPMI medium supplemented with 10% human AB serum, as previously described.¹⁰ The PBMCs (2×10^5 cells/200 μ L/well in 96-well plates) were stimulated with HDM (100 μ g of protein/mL final) or PPD (3 μ g/mL final). These were the lowest concentrations to induced maximum proliferation, as measured at 7 days.

Proliferation was assessed by pulsing the cultures with tritiated thymidine (0.8 μ Ci/well; 1 Ci/mmol; ICN Biochemicals) after 6 days, and 20 hours later, the cells were harvested. Results from triplicate cultures were expressed as δ disintegrations per minute (δ dpm; ie, mean of wells containing stimulant minus mean of control wells).

Supernatants were collected from parallel cultures of unstimulated and antigen-stimulated cells after 6 days and stored at -80°C . Commercial ELISAs were used to quantitate IL-4 (Megenix) and IL-5 (R&D Systems). IFN- γ was measured by using a radioimmunoassay (Medgenix). The lower detection limits were 2 pg/mL for IL-4, 1 pg/mL for IL-5, and 0.2 IU/mL for IFN- γ .

Statistical analysis

Results are expressed as means $\pm$ SEM. ANOVA was used to compare proliferation of PBMCs and cytokine release among the 3 subject groups. The respective pairs of means contributing to a significant ANOVA were analyzed by the LSD planned comparison test. Two-tailed Student t tests were used to compare IL-5/IFN- γ ratios. Correlations were determined by using the Pearson coefficient. A STATISTICA program was used to analyze the data. A P value of less than .05 was considered significant.

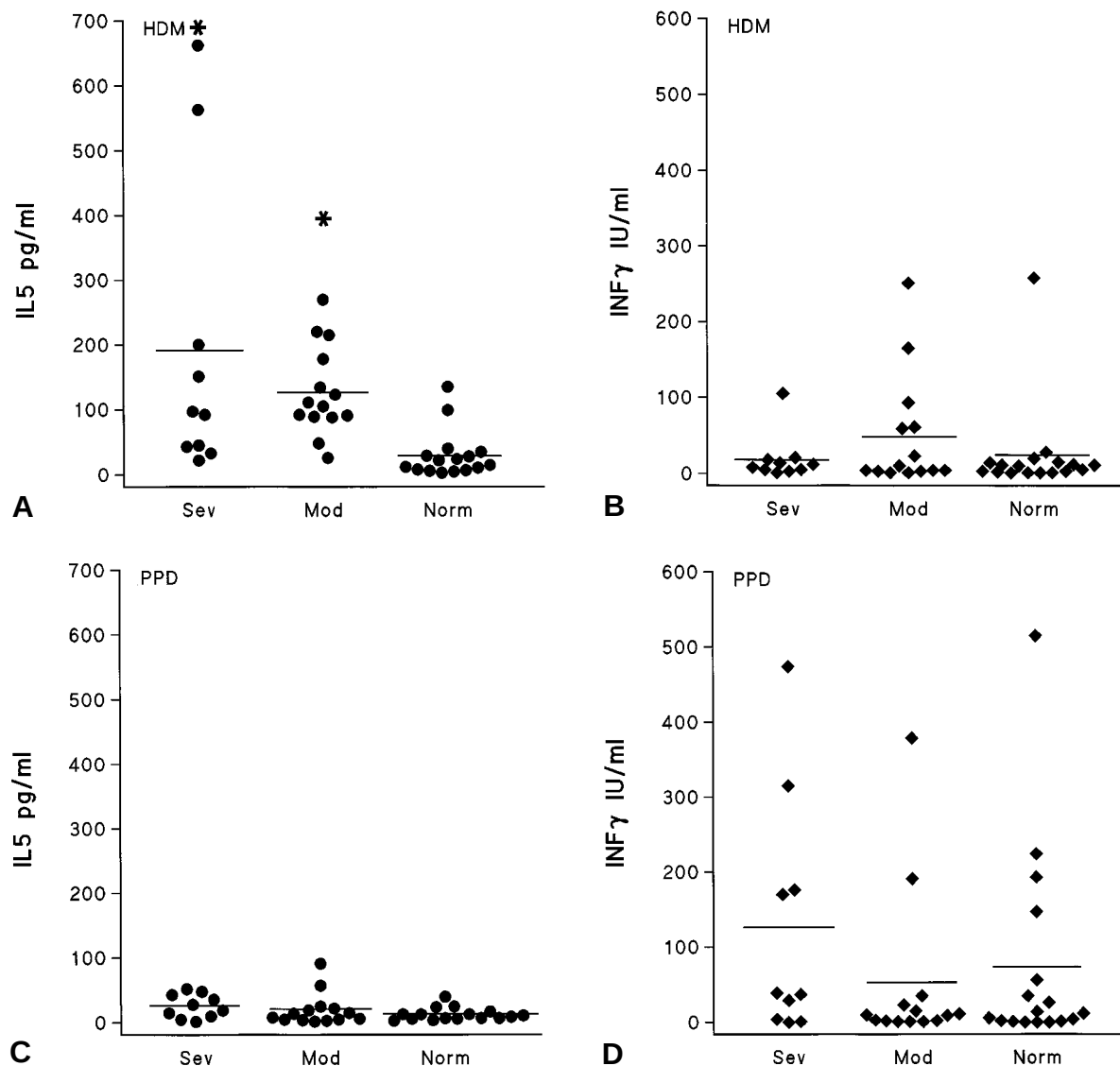


FIG 2. Release of IL-5 (circles) and IFN- γ (diamonds) by HDM-stimulated (A and B) or PPD-stimulated (C and D) PBMCs. Horizontal lines represent means. Asterisks indicate a *P* value of less than .04 relative to the normal group.

RESULTS

Proliferation

The HDM-stimulated proliferation of PBMCs was significantly increased in the group with severe asthma ($10,868 \pm 2948$ δ dpm, $P = .01$) or moderate asthma (9807 ± 1864 δ dpm, $P = .01$) compared with that found in the normal subjects (3592 ± 1219 δ dpm; Fig 1, A). There was no correlation between HDM-specific IgE and proliferation in the groups with atopic asthma (severe asthma: $r = -0.37$ and $P = .29$; moderate asthma: $r = -0.04$ and $P = .89$; data not shown). In the normal group there was a proliferative response of greater than 3500 δ dpm in only 4 of 17 subjects, each of whom had HDM-specific IgE levels of less than 0.35 kU/L.

The PPD-stimulated proliferation of PBMCs was not

significantly different among the 3 groups. Mean δ dpm values were $14,394 \pm 3975$ (severe asthma), 5966 ± 1741 (moderate asthma), and 8599 ± 2540 (normal; Fig 1, B).

Spontaneous proliferation was low and not significantly different among the severe (685 ± 210 dpm), moderate (532 ± 166 dpm), and normal groups (340 ± 218 dpm). Mean spontaneous proliferation for the whole cohort was 490 ± 118 dpm.

Release of IL-5, IL-4, and IFN- γ

Spontaneous release. Assay of unstimulated PBMC cultures (7-12 per subject group) showed that the spontaneous release of IL-5 and IFN- γ was either undetectable (the majority of cases) or very low compared with the corresponding antigen-stimulated culture.

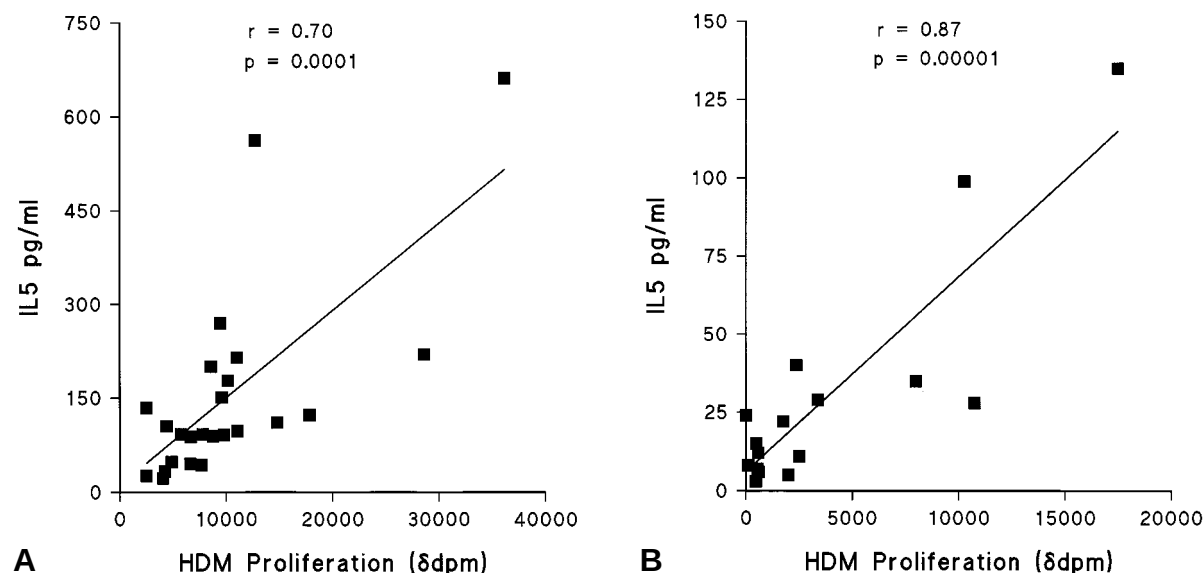


FIG 3. Significant correlation between the proliferation and the IL-5 released by HDM stimulation for the group with atopic asthma (**A**) and the normal group (**B**).

HDM-stimulated cytokine release. HDM-stimulated IL-5 release was significantly increased in the group with severe (191 ± 73 pg/mL, $P = .002$) or moderate asthma (128 ± 18 pg/mL, $P = .036$) compared with the normal group (30 ± 9 pg/mL; Fig 2, A). IL-4 release was below the limit of detection. HDM-stimulated IFN- γ release did not differ significantly among the 3 groups (Fig 2, B). The mean IFN- γ release was 19 ± 10 IU/mL (severe asthma), 49 ± 20 IU/mL (moderate asthma), and 23 ± 15 IU/mL (normal).

There was a strong significant positive correlation between proliferation and IL-5 release in HDM-stimulated PBMCs for both the group with atopic asthma (severe plus moderate: $r = 0.70$ and $P = .0001$; Fig 3, A) and the normal group ($r = 0.87$ and $P = .00001$; Fig 3, B). A significant correlation was found between proliferation and IFN- γ release for the normal group ($r = 0.75$ and $P = .0006$), whereas a weaker but significant correlation was found for the atopic asthmatic group ($r = 0.41$ and $P = .045$; data not shown).

PPD-stimulated cytokine release. Release of IL-5 and IFN- γ did not differ significantly among the 3 groups (Fig 2, C and D). Mean IL-5 levels were 26 ± 6 pg/mL (severe asthma), 20 ± 7 pg/mL (moderate asthma), and 13 ± 2 pg/mL (normal). Mean IFN- γ levels were 125 ± 51 IU/mL, 49 ± 29 IU/mL, and 73 ± 33 IU/mL for the severe, moderate, and normal groups, respectively. In PPD-stimulated PBMCs there was a significant positive correlation between the level of proliferation and IL-5 (atopic asthma: $r = 0.54$ and $P = .008$; normal: $r = 0.72$ and $P = .001$) or IFN- γ release (atopic asthma: $r = 0.75$ and $P = .00003$; normal: $r = 0.55$ and $P = .02$; data not shown).

T_H2/T_H1 cytokine index

Antigen-stimulated cytokine release was also analyzed as the ratio of the IL-5 (in picograms per milliliter)

to IFN- γ (in international units per milliliter) released in each culture that had a positive response to the stimulus as defined by a δ dpm value of greater than 1500 and measurable cytokine release. This ratio is expressed as a T_H2/T_H1 cytokine index in arbitrary units.

HDM stimulation of PBMCs resulted in positive responses in 10 of 10 subjects with severe asthma, 14 of 14 subjects with moderate asthma, and 8 of 17 normal subjects. PPD stimulation provoked positive responses in 7 of 10 subjects with severe asthma, 10 of 14 subjects with moderate asthma, and 9 of 17 normal subjects. In the normal group 3 subjects responded to HDM only, 4 responded to PPD only, and 5 responded to both HDM and PPD. There was no correlation between proliferation and the T_H2/T_H1 cytokine index for the atopic asthmatic or normal groups in either HDM-stimulated PBMCs (atopic asthma: $r = 0.02$ and $P = .94$; normal: $r = 0.14$ and $P = .75$) or PPD-stimulated PBMCs (atopic asthma: $r = -0.36$ and $P = .15$; normal: $r = -0.44$ and $P = .24$; data not shown).

The HDM-induced T_H2/T_H1 cytokine index was significantly increased in the group with severe asthma ($P = .04$; Fig 4, A) compared with the normal group. In the group with moderate asthma the T_H2/T_H1 cytokine index demonstrated a trend but was not statistically significant ($P = .06$). The mean T_H2/T_H1 index was 19 ± 6 AU (severe asthma), 26 ± 9 AU (moderate asthma), and 2 ± 1 AU (normal).

There was no significant difference in the PPD-stimulated T_H2/T_H1 index among the 3 groups. The mean T_H2/T_H1 index was 0.4 ± 0.2 (severe asthma), 2.4 ± 1.0 (moderate asthma), and 0.4 ± 0.1 (normal; Fig 4, B). This suggests that, in general, the patients with allergic asthma were not defective in response to the bacterial antigen PPD. There were, however, 4 moderate asthmatic patients with a PPD-stimulated T_H2/T_H1 index greater than 2.5 AU (ie, in the range of the HDM-stimulated indexes).

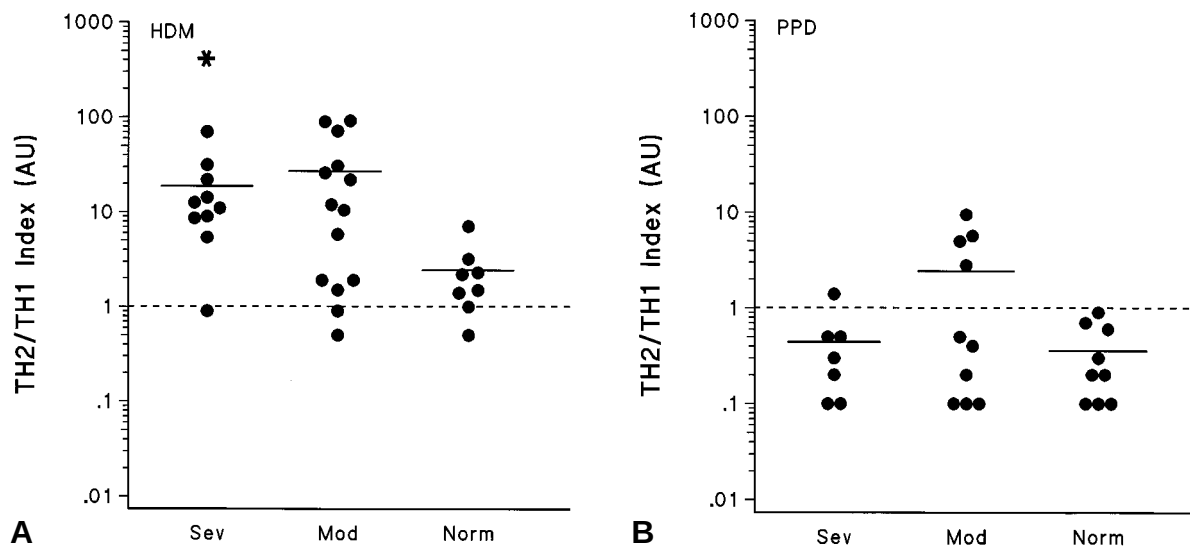


FIG 4. T_H2/T_H1 cytokine index for HDM-stimulated (**A**) and PPD-stimulated (**B**) PBMCs that responded to the stimulus with a δ dpm value of greater than 1500 and cytokine release. Asterisk indicates a *P* value of .04 relative to the normal group. HDM stimulation induced a significantly increased T_H2/T_H1 index compared with PPD stimulation for each group (Sev: *P* = .03; Mod: *P* = .04; Norm: *P* = .01).

Comparison of the subject groups' responses to HDM and PPD showed that HDM stimulation induced a significantly higher T_H2/T_H1 cytokine index compared with PPD stimulation in all 3 groups. The *P* values were .03 (severe asthma), .04 (moderate asthma), and .01 (normal).

DISCUSSION

It is an open question to what extent T cells that respond to allergens are present in nonatopic individuals. Although HDM-specific T-cell clones have been generated from the PBMCs of nonatopic subjects,¹² reports on the HDM-stimulated proliferation of freshly isolated PBMCs from normal individuals are inconsistent. HDM-specific T-cell proliferation has been reported to be virtually absent in nonatopic individuals,^{13,14,18-21} or alternately, to be of similar magnitude in patients with atopic diseases and healthy control subjects.^{15,22,23} One finding of similar HDM-specific T-cell proliferation in atopic and nonatopic subjects was considered to be due to the use of serum-free culture medium.¹⁵ However, similar results were also reported with medium supplemented with serum.^{22,23} We found that HDM-specific T-cell proliferation is more frequent and of significantly greater magnitude in the atopic asthmatic groups than in the nonatopic control group. This agrees with data from the majority of previous studies^{13,14,18-21,24,25} and supports suggestions that there are greater numbers of circulating HDM-specific T cells in atopic individuals caused by clonal expansion after sensitization.^{13,25-27}

There is increasing evidence that both the IgE-dependent early response and antigen-primed T_H2 cells are necessary for a persistent atopic inflammation character-

ized by the accumulation of activated inflammatory cells.^{6-8,28} The role of IL-4 in IgE production is well established.²⁹ We were unable to detect IL-4 in our PBMC cultures (6 days after antigen stimulation). This agrees with similar studies.^{26,30} IL-4 has, however, been measured in the femtogram per milliliter range in HDM-stimulated PBMCs at day 4.²⁴ This is consistent with time course studies on primary cultures, which indicated that peak IL-4 production occurs before that of IL-5 and IFN- γ and that IL-4 levels rapidly decline.^{31,32}

Recent studies have highlighted the role of IL-5 in the development of the chronic phase of atopic inflammation.^{25,28,33,34} IL-5 is the most specific cytokine for eosinophils^{20,28} and has been suggested to be the key cytokine in the expression of allergic diseases.^{19,25,33-35} We found that the HDM-stimulated release of IL-5 was significantly increased in patients with severe or moderate asthma relative to the control subjects. This is consistent with the evidence that eosinophils, activated T cells, and the T_H2 cytokines IL-4 and IL-5 are increased in the lungs of atopic asthmatic subjects.^{3,6,28,36} Our results also agree with those of other studies investigating PBMCs stimulated with HDM,^{13,19,25,34} cat,³⁷ cedar,³³ or birch pollen³⁸ allergens.

We found that the release of IL-5 and the proliferation induced by HDM was strongly positively correlated in both the normal and the atopic asthmatic groups. Similar results have been reported in children with atopic dermatitis.¹³ This implies that in vivo the stronger the T-cell proliferative response to an allergen, the more IL-5 will be released. Thus the significantly increased release of IL-5 in the allergic subjects could be linked to the increased proliferation of the PBMCs to HDM relative to

the control subjects. We found no correlation between serum HDM-specific IgE levels and the proliferation or IL-5 release stimulated by HDM in any of the 3 groups. This is in agreement with data from some but not all studies.^{13,25,26}

Studies on polyclonally stimulated PBMCs have implicated a reduction in IFN- γ release in the cause of atopic diseases.¹⁰ Investigations of allergen-stimulated release of IFN- γ from PBMCs have produced mixed results. Some studies found decreased IFN- γ release in subjects with atopic diseases relative to healthy control subjects,^{24,37} whereas other studies reported no difference.^{25,33,38} In this investigation there was no significant difference in HDM-stimulated IFN- γ release among the 3 groups (ie, severe asthma, moderate asthma, and normal). We did, however, find that the degree of correlation between the HDM-stimulated level of proliferation and IFN- γ release was lower in the atopic asthmatic subjects than in the control subjects. HDM-stimulated IFN- γ release has been reported to be closely correlated with proliferation in infants, but not in children, with atopic dermatitis.²⁴

There is no absolute definition of a T_H1- or T_H2-type cytokine response. Human T-helper subsets were based on the relative proportions of T_H1- and T_H2-type cytokines produced by T-cell clones.^{4,11,30} An excess of T_H2 cytokines relative to the T_H1 cytokine IFN- γ , rather than the absolute amounts of these cytokines, is implicated in the cause of atopy.^{4,39} Studies with PBMCs have also analyzed allergen-stimulated cytokine release as the amount of a characteristic T_H2 cytokine (IL-4³⁹ or IL-5^{21,25,38}) relative to the signature T_H1 cytokine IFN- γ . We analyzed antigen-stimulated cytokine release as the ratio of the IL-5 to IFN- γ released in each culture that had a positive response to the stimulus and expressed this ratio as a T_H2/T_H1 cytokine index. We designated this index as a cytokine index because PBMCs contain T_H1, T_H2, and T_H0 cells,⁴⁰ and, unlike with T-cell clones, cytokine release cannot be used to determine the phenotype of the cells. Stimulation in a milieu of a mixed population of T cells is closer to the situation in the tissues in vivo.⁴¹ We defined a positive response as a δ dpm value of greater than 1500 (ie, a 3-fold increase in proliferation over the mean spontaneous proliferation^{15,23,25}) together with measurable cytokine release. We also observed that this level of stimulated proliferation served to distinguish the cultures that released cytokines on stimulation from those that did not.

We found that for each subject group, including the normal group, the HDM-stimulated T_H2/T_H1 cytokine index was significantly higher than the T_H2/T_H1 cytokine index stimulated by PPD. This is consistent with the suggestions (based on the cytokine profiles of the great majority of allergen- or bacterial antigen-specific T-cell clones generated from PBMCs) that HDM is a T_H2-type stimulus, and PPD is a T_H1-type stimulus.^{11,30,42} However, the finding that the normal nonatopic group also had a significantly higher T_H2/T_H1 cytokine index in response to HDM than in response to PPD suggests that normal nonatopic individuals also have a T_H2-type cytokine

response to HDM. This conclusion is consistent with the previous finding that HDM-responsive T-cell lines from both atopic and nonatopic subjects resemble the T_H2 subset in requiring IL-4 for optimum proliferation.⁴³

The suggestion that nonatopic individuals have a T_H2-type cytokine response to allergen is at variance with what is generally accepted.^{8,35,44-46} Studies on T-cell clones from 3 donors suggested that T cells from normal subjects respond to HDM with a T_H1-type response, whereas allergic patients respond with a T_H2-type response.¹² This hypothesis has not previously been examined by using primary cell cultures. Our hypothesis is that allergens provoke a T_H2-type response in the T cells that specifically recognize the allergen but that there is a lower frequency of allergen-responsive T cells in nonatopic subjects than in atopic patients. Evidence for this hypothesis would focus research attention on mechanisms of T-cell recognition of, and activation by, allergens as opposed to the current emphasis on the cytokine milieu that possibly polarizes T cells to inappropriate T_H2 responses.

Although we concluded that both atopic and normal subjects had a T_H2-type cytokine response to HDM allergen, our evidence does suggest that patients with atopic asthma have an enhanced T_H2 cytokine response to HDM relative to healthy nonatopic control subjects. We found that both the HDM-induced IL-5 release and T_H2/T_H1 cytokine index were increased in patients with asthma compared with the normal group.

Exposure to infectious agents, such as *Mycobacterium tuberculosis*, which strongly promote a T_H1 immunological environment, could inhibit T_H2 immunity and the development of atopic disorders.^{5,17} Reciprocal inhibition of T_H1 immunity by excess T_H2 cytokines released in atopic individuals could also result in an increased susceptibility to infections, such as tuberculosis.¹⁶ We therefore investigated whether there was a difference between the responses of PBMCs from our highly atopic asthmatic and nonatopic healthy Xhosa children to mycobacterial PPD. This is a cohort with inherently high basal IgE levels⁴⁷ and who live where tuberculosis is endemic. We have, however, previously reported¹⁰ that mitogen-stimulated PBMCs from this group of Xhosa children have cytokine profiles that are similar to those documented for other cohorts.^{10,48}

We found that PPD stimulation of PBMCs did not result in any significant differences among the 3 groups (severe asthma, moderate asthma, and normal) with respect to proliferation, release of IL-5 or IFN- γ , or the T_H2/T_H1 cytokine index. This indicates that both healthy nonatopic and allergic asthmatic subjects can have a normal T_H1-type cytokine response to mycobacterial PPD. This agrees with results of Till et al²⁵ and suggests that in general atopic asthmatic children do not have a decreased ability to mount a T_H1 cytokine-driven cell-mediated immune response. However, we did observe that 4 patients with moderate asthma responded to PPD with a T_H2/T_H1 cytokine index in the range of the HDM-stimulated indexes. This suggests that there may be a subgroup of allergic subjects who have an inappropriate T_H2-type

cytokine response to PPD and who may have an increased risk of development of active tuberculosis.

The major findings of this study are as follows. First, PBMCs from patients with atopic asthma have increased proliferation to HDM allergen. This implies that there are greater numbers of circulating HDM-specific T cells in atopic subjects than in normal nonatopic individuals. Second, both normal and allergic asthmatic subjects have a T_H2 -type cytokine response to HDM. This is opposite to what is generally accepted; however, this hypothesis has not previously been examined with freshly prepared cells. Third, atopic asthmatic patients do have an enhanced T_H2 cytokine response to HDM compared with normal subjects. Fourth, both normal and atopic asthmatic subjects can have a T_H1 -type cytokine response to mycobacterial PPD. This suggests that, in general, atopic asthmatic children do not have a decreased ability to mount a T_H1 cytokine-driven cell-mediated immune response. However, there may be a subgroup of atopic subjects who have an inappropriate T_H2 -type cytokine response to PPD and who may be at risk of contracting active tuberculosis.

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