

Original article

“Bystander” amplification of PBMC cytokine responses to seasonal allergen in polysensitized atopic children

Background: Atopic children show increased expression and production of the Th2-associated cytokines IL-4, IL-5, IL-13, and IL-9 from PBMCs after stimulation with allergen, but it has previously not been clearly determined whether the Th2-cytokine production is restricted to the inhalant allergen the child is sensitized to, and whether perennial or seasonal allergens induce different cytokine responses. Our purpose was to determine whether *in vitro* Th2 cytokine production is specific to the sensitizing allergen, and to compare the cytokine responses to a perennial and a seasonal allergen in monosensitized and polysensitized children.

Methods: Using semiquantitative RT-PCR, we analyzed the expression of the cytokines IL-4, IL-5, IL-13, IL-9, IL-10, and IFN- γ after stimulation of PBMCs with house-dust-mite (HDM) or ryegrass allergen. The cells were sampled from groups of 6-year-old children sensitized to either HDM ($n=20$) or ryegrass ($n=24$), or to both allergens ($n=20$), as well as from a nonatopic group ($n=20$).

Results: After stimulation with HDM allergen, PBMCs from children sensitized only to HDM expressed increased mRNA levels of the Th2 cytokines, but not of IL-10 and IFN- γ , whereas ryegrass stimulation did not result in increased cytokine expression. PBMCs from children sensitized to HDM and ryegrass expressed increased Th2 cytokines after stimulation with either of the two allergens. In contrast, PBMCs from children sensitized only to ryegrass did not express increased levels after stimulation with either of the allergens.

Conclusions: The expression of Th2 cytokines after *in vitro* stimulation of PBMCs from atopic children is specific to the sensitizing allergen, indicating that atopic status per se does not affect the type of T-cell response. In addition, T cells specific to seasonal allergens circulate in the blood out of season only if the child is concomitantly sensitized to a perennial allergen.

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Sensitization to allergens with production of specific IgE and development of a positive skin prick test (SPT) is associated with increased expression and production of Th2 cytokines, such as IL-4, IL-5, IL-13 and IL-9 (1–3). IFN- γ is generally reported to be produced in lower (4, 5) or similar (3, 6) amounts in sensitized relative to un-sensitized individuals. However, we recently described that, at least in children, peripheral blood mononuclear cells (PBMCs) from atopics can produce higher levels of IFN- γ in response to allergen stimulation than those of nonatopics (7). The cytokine pattern has been elucidated by analyzing the expression of mRNA or production of cytokine protein from PBMCs, as well as from cells in the target organ, such as the lung or the gut (3, 7–9). IL-4 and IL-13 induce IgE production by B cells, whereas IL-5 is the main factor in regulating eosinophils (10, 11). IFN- γ is an inhibiting factor in IgE production, and IL-10 inhibits antigen-specific proliferation and cytokine production in both Th1 and Th2 cells (12, 13).

The cytokine profile of T cells from atopic individuals has been determined primarily when PBMCs have been stimulated *ex vivo* with the allergen that the subject is sensitized to, but rarely with allergens to which they are not sensitized. Consequently, it has not been clearly established, particularly in children, whether the *in vitro* production of inhalant allergen-induced Th2-type cytokines is specifically associated with the *in vivo* markers of sensitization, or is a general characteristic of the atopic status. Accordingly, in the present study, we examined whether allergen-induced Th2 cytokine production in children was restricted to individuals sensitized to the same allergen, or whether it was associated with the presence of a positive SPT for any allergen; i.e., the presence of atopy. The second aim of the study was to determine whether the *in vitro* cytokine response to a perennial (house-dust-mite [HDM]) and a seasonal (ryegrass) allergen is similar in subjects monosensitized and polysensitized to these allergens. To this end, we

measured the expression of IL-4, IL-5, IL-13, IL-9, IL-10, and IFN- γ in children sensitized to either HDM allergen only (HDM+) or ryegrass allergen only (Rye+), or both allergens (HDM+ Rye+), and compared the results with the expression in nonatopic children (SPT-).

Material and methods

Subjects

This study, which was approved by the Princess Margaret Hospital Ethics Committee, was conducted in 84 children who were within 2½ months of their 6th birthday. The samples were selected from a large ongoing cohort study according to SPT reactivity to a panel of allergens, comprising HDM (*Dermatophagoides pteronyssinus*), 30 000 AU/ml, ryegrass (*Lolium perenne*), 1:20 w/v, mold mix (1:10 w/v), and cat (cat pelt, acetone precipitated, 10 000 BAU/ml), all from Bayer Corporation, Elkhart, USA. A wheal diameter of ≥ 2 mm was used as the criterion to select the SPT+ groups. Twenty children were SPT+ for HDM only, 24 were SPT+ for ryegrass only, 20 were SPT+ for HDM and ryegrass, and 20 were SPT- for all allergens tested. We also measured the presence of IgE antibody in serum to HDM and ryegrass allergens by radioallergosorbent test (RAST) using the CAP System (Pharmacia, Australia).

Cell culture

PBMCs that had been isolated from heparinized blood samples by density gradient centrifugation over Ficoll-Hypaque (Lymphoprep, Nycomed, Norway) were cryopreserved at collection to be thawed and resuspended at 1×10^6 viable cells/ml in mercaptoethanol-supplemented AIM-V medium (Gibco Life Technologies, Australia) on the day of culture. Aliquots of 1 ml from each sample were cultured for 24 h at 37°C with 5% CO₂ as follows: with medium alone, with medium containing HDM allergen (aqueous extract of lyophilized Der p, supplied by CSL Limited, Australia), with medium containing ryegrass allergen (collected in Western Australia, aqueous extract of lyophilized Lol p, supplied by Greer Laboratories, USA), or with phytohemagglutinin (PHA; HA16, Murex, Australia). All stimulants were added at a final concentration of 10 µg/ml. This dose was optimal both for HDM and ryegrass when different doses were tested in cultures for capacity to induce proliferation. Using polymyxin B (an antibiotic that binds LPS and therefore inhibits the binding of LPS to LPS-receptors on cells), we demonstrated that T-cell responses to the allergens are not influenced by LPS contamination of the allergen preparation (not shown). Furthermore, we have previously ensured that culturing cells from children with allergens for 24 h best distinguishes between atopics and nonatopics with respect to HDM mRNA responses (3, 14). After the culture, the cells were collected by centrifugation and used immediately for RNA extraction.

Analysis of allergen-specific cytokine response

Due to the variable frequency of positive PBMC cytokine responses to seasonal allergens such as ryegrass when subjects are sampled out of season, we chose to employ more sensitive methods involving determination of cytokine-specific mRNA, as detailed below.

Extraction of mRNA and semiquantitative RT-PCR. Total RNA from the cell pellets was obtained with 250 µl of RNeasy Lysis Buffer (Qiagen, Crawley, Australia) and 25 µl of chloroform. First-strand synthesis of cDNA was performed in a total volume of 25 µl at 42°C for 65 min using 125 ng oligo-(dT)₁₅ (Biotech International, Bentley, Australia) and 1.25 U of avian

myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA) in the presence of 6.25 U of ribonuclease inhibitor (Biotech). For the PCR reactions, the reaction mixture contained 1 µl of cDNA, 50 ng of the specific primers (Gibco/Life Technologies, Melbourne, Australia), 1xPCR buffer (Biotech), 0.2 mmol/l each dNTPs (Biotech), 1.3 mmol/l MgCl₂, and 0.5 U of Taq platinum DNA polymerase enzyme, in a total volume of 12.5 µl overlaid with mineral oil. The PCR was run as follows: an initial denaturation step of 94°C for 5 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. All reactions were performed in a programmable thermocycler (Perkin-Elmer, Melbourne, Australia). Stimulated and unstimulated samples from individual children were always tested together.

The following number of cycles and primers were used: β -actin (28 cycles), IL-4 (38 cycles), and IL-5 (35 cycles), as described previously (14); IL-13 (35 cycles), IL-9 (36 cycles), and IL-10 (32 cycles), as described previously (3); and IFN- γ (36 cycles): expected product size, 230 bp (5' TTT GGG TTC TCT TGG CTG TT 3' and 5' TGT CTT CCT TGA TGG TCT CC 3'). Cycle analyses were performed with each primer set, as previously described, to ensure that we had reached detection levels and that the reaction remained in the linear phase (14). Briefly, for each set of samples we analyzed the product (all cytokines and β -actin) after running the PCR with various cycle numbers, and determined after which number of cycles the product was in the linear phase.

Identification of PCR product. After denaturation with sodium hydroxide, the PCR were blotted onto nylon membranes (Hybond N+, Amersham, UK) with a slot-blotter (Hoefer Scientific Instruments, San Francisco, CA, USA). The membranes were hybridized overnight with double-stranded probes produced by PCR reactions of cDNA obtained from adult PBMCs stimulated with PHA using biotin-16-dUTP (Boehringer Mannheim, Perth, Australia) at a ratio of 5:1 (dTTP:dUTP). The binding was visualized by chemiluminescence with a commercial kit (ECL, Amersham) and exposure to hyperfilm (Amersham). The intensity of each slot was determined by a densitometer (Scan Analysis 2.02, Biosoft, Cambridge, UK), and the results were expressed as a ratio of cytokine to β -actin density. The final values for each cytokine were obtained by subtracting the ratio of the cells cultured in medium from the ratio of the allergen-stimulated culture for each individual and expressing the final results as means $\pm$ SEM. There was no significant difference in the β -actin levels of the stimulated and the nonstimulated cells. Fig. 1 shows an example of typical IL-4 and β -actin blots derived from HDM and ryegrass stimulation of cells from one child of each group.

Statistical analysis

We used nonparametric tests to analyze the statistical significance. We compared the difference in cytokine responses between the four groups HDM+, HDM+ Rye+, Rye+, and SPT- by the Kruskal-Wallis test. We then compared each of the SPT+ groups with the SPT- group by the Mann-Whitney U-test. Correlations were calculated by the Spearman rank test. All *P* values were corrected for ties, and the results were considered significant if *P* was <0.05 . The statistical package Statview for Windows was used for all analyses.

Results

Wheal diameter and IgE production

The cells were selected according to the SPT reactivity for HDM and ryegrass in the donor child, and the individuals selected were matched for the size of the

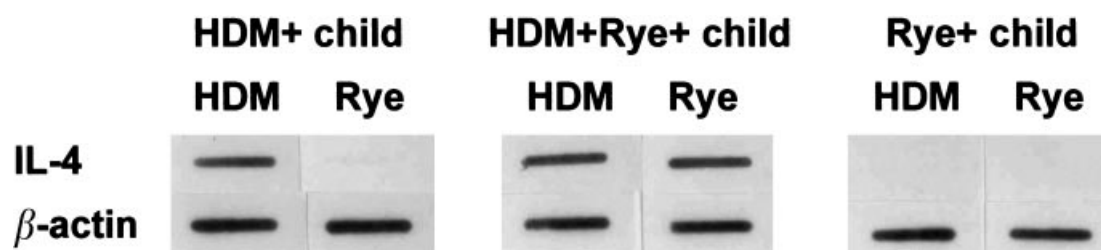


Figure 1. Slot-blots of IL-4 (upper row) and β -actin (lower row) RT-PCR products after stimulation of PBMCs with HDM and ryegrass allergen. Cells were derived from children sensitized to either HDM only, HDM and ryegrass, or ryegrass only.

wheat in the SPT (Table 1). We also measured the levels of specific IgE for HDM and ryegrass in the selected individuals and found that the median values of IgE were also similar except for the IgE against ryegrass in the group sensitized to ryegrass only, which was lower (Table 2). As many as six out of 20 individuals in the group SPT+ for ryegrass were RAST-negative for ryegrass. The overlap between the two tests was 80–100% for the other groups. To ensure that this discrepancy did not affect the conclusions, we regrouped the subjects according to their production of specific IgE to HDM and ryegrass and re-evaluated the cytokine expression. The cytokine levels in these IgE-defined groups were similar to those based on the SPT results (not shown).

Cytokine responses to polyclonal stimulation

All samples were able to respond to polyclonal stimulation. The levels of each cytokine were similar in all PHA-stimulated cultures of the SPT+ groups, showing that there was no difference in the ability of the cells from the different groups to respond to polyclonal stimulation (Fig. 2). However, the levels of IL-4, IL-5, and IL-13 were significantly lower in the SPT– group than in each of the SPT+ groups, whereas no significant difference was seen for IL-9, IL-10, and IFN- γ . The higher levels of Th2 cytokines after PHA stimulation in atopic children than nonatopic children agree with the results from a previous study, in which higher levels of IL-4 were found after PHA stimulation of cells from atopic children (15).

Cytokine responses to HDM

The Th2-associated cytokine mRNA expression in

Table 1. Wheal diameter (mm) of skin prick test (SPT) for house-dust-mite allergen (HDM) and ryegrass allergen in groups of atopic children

Groups ^a	HDM		Ryegrass	
	Mean	SD	Mean	SD
HDM +	6.2	2.2	0.0	0.0
HDM + Rye +	6.1	2.9	6.9	3.5
Rye +	0.0	0.0	6.0	2.7

^a Children with positive SPT for either HDM or ryegrass, or for both allergens.

cultures stimulated for 24 h with the perennial HDM allergen is illustrated in Fig. 3. IL-4, IL-5, IL-13, and IL-9 were expressed at a significantly higher level in the group sensitized to HDM only, and IL-4, IL-5, and IL-13 were expressed to a higher degree in the group sensitized to HDM and ryegrass than in the SPT-negative children. In contrast, no significant cytokine increases were seen after HDM stimulation of cells from children sensitized to ryegrass only. However, there were no significant differences in the IFN- γ levels or the IL-10 levels among any of the groups stimulated with HDM (Fig. 4). These results show that only Th2 cytokines are expressed in increased amounts in atopic individuals, and that the expression is seen only after stimulation with the allergen the child is sensitized to.

Cytokine responses to ryegrass

The Th2-associated cytokine expression in cultures stimulated with the seasonal ryegrass allergen is illustrated in Fig. 5. Only the group sensitized to both HDM and ryegrass expressed increased levels of Th2 cytokine mRNA, and the difference relative to the SPT negative group was significant for IL-5 and IL-13. Neither the groups sensitized to HDM only nor the group sensitized to ryegrass only had increased expression of Th2 cytokine mRNA. None of the groups showed increased expression of IFN- γ or IL-10 compared with the nonatopic controls (Fig. 6).

Cytokine production correlated to SPT reactivity and IgE production

We calculated the correlation of cytokine expression to wheal diameter and to level of specific IgE, respectively, in HDM-stimulated cells from HDM-sensitized indi-

Table 2. IgE levels (RAST kU/l) for house-dust mite allergen (HDM) and ryegrass allergen in groups of atopic children

Groups ^a	HDM		Ryegrass	
	Median	Interquartile ranges	Median	Interquartile ranges
HDM +	5.0	0.54–27.8	0.0	0.00–0.00
HDM + Rye +	4.1	0.41–71.6	5.6	2.50–11.4
Rye +	0.0	0.00–0.00	1.3	0.35–3.81

^a Children with positive SPT for either HDM or ryegrass, or for both allergens.

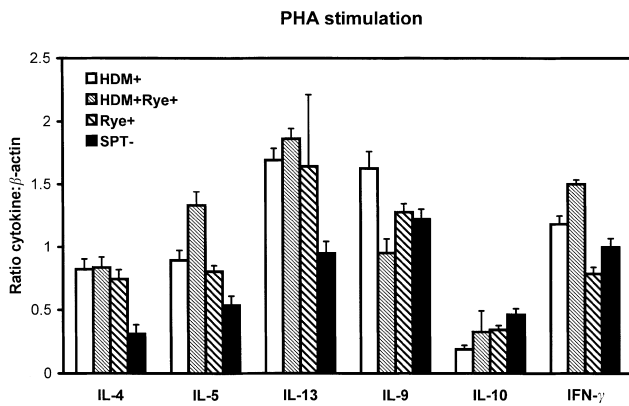


Figure 2. IL-4, IL-5, IL-13, IL-9, IL-10, and IFN- γ responses to PHA stimulation in PBMCs from groups of 6-year-old children sensitized to HDM allergen, ryegrass allergen, or both, and nonatopic controls (SPT-). Cytokine-specific mRNA relative to mRNA specific for housekeeping gene β -actin was determined by semiquantitative RT-PCR. Data shown are group means with SEM bars. When each atopic group was compared to nonatopic group by Mann-Whitney *U*-test, following significant *P* values were obtained: HDM + group IL-4 ($P=0.004$), IL-5 ($P=0.013$), IL-13 ($P=0.0003$); HDM + Rye + group IL-4 ($P=0.001$), IL-5 ($P=0.0001$), IL-13 ($P<0.0001$); Rye + group IL-4 ($P=0.0012$), IL-5 ($P=0.009$), IL-13 ($P=0.027$).

duals. Only expression of IL-4 correlated significantly to wheal diameter and to the level of specific IgE (Fig. 7A). Neither a significant positive nor a negative correlation was obtained for any of the other cytokines tested, as illustrated by the correlation for IL-5 (Fig. 7B). We also analyzed the correlation between the expression of the various combinations of IL-4, IL-5, and IL-13 in the HDM-sensitized group. IL-5 and IL-13 expression correlated significantly ($r=0.53$, $P=0.022$), whereas IL-4 expression correlated neither with IL-5 nor with IL-13. These results indicate that the expression of IL-4 in a short-term *in vitro* culture with allergens reflects the level of specific IgE production in the child, reinforcing the evidence that our experimental system is relevant to the immune function *in vivo*.

Discussion

It has not previously been determined in detail whether T cells from atopic children have a general propensity to respond to allergens via Th2 cytokine expression, or whether this reaction is confined to stimulation with the allergen to which the child is sensitized. One previous study of IL-4-producing cells in birch- or cat-allergic adults indicates that birch-allergic subjects reacted with IL-4 production only to birch, and not to cat (16). We extended these results by analyzing several additional cytokines, studying children, and using different allergens. Our results clearly show that lymphocytes from preschool children sensitized to an allergen express the Th2-associated cytokines IL-4, IL-5, IL-9, and IL-13 only after stimulation with that particular allergen, and

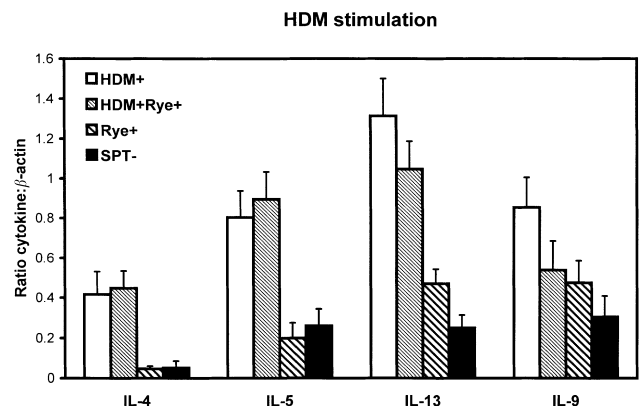


Figure 3. IL-4, IL-5, IL-13, and IL-9 responses to HDM allergen stimulation in PBMCs from groups of 6-year-old children sensitized to HDM allergen, ryegrass allergen, or both, and nonatopic controls (SPT-). Cytokine-specific mRNA relative to mRNA specific for housekeeping gene β -actin was determined by semiquantitative RT-PCR. Data shown are group means with SEM bars. Kruskal-Wallis tests showed that statistically significant difference among all four groups of children for IL-4 ($P=0.0002$), IL-5 ($P<0.0001$), IL-13 ($P<0.0001$), and IL-9 ($P=0.027$). When each atopic group was compared to nonatopic group by Mann-Whitney *U*-test, following *P* values were obtained: HDM + group IL-4 (**0.0006**), IL-5 (**0.0018**), IL-13 (**<0.0001**), IL-9 (**0.0063**); HDM + Rye + group IL-4 (**0.0022**), IL-5 (**0.0033**), IL-13 (**0.0001**), IL-9 (0.15); Rye + group IL-4 (0.08), IL-5 (0.98), IL-13 (0.075) IL-9 (0.13). Boldface denotes statistically significant values.

not after stimulation with an allergen to which they are not sensitized. Thus, the finding that the levels of several Th2 cytokines were lower in the SPT- group than the SPT+ group after polyclonal stimulation does not indicate that the Th2 cytokine response is a generalized

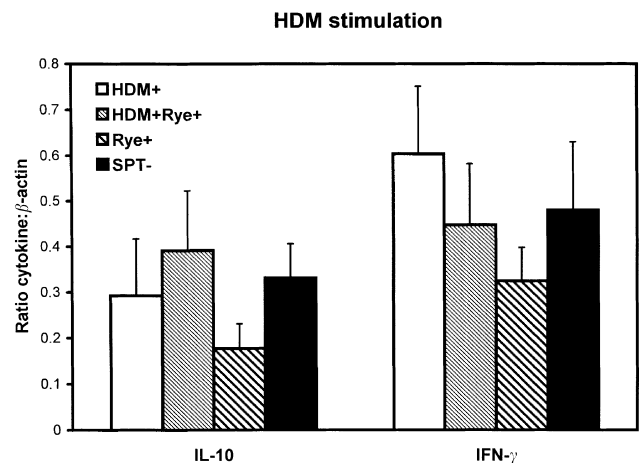


Figure 4. IL-10 and IFN- γ responses to HDM allergen stimulation in PBMCs from groups of 6-year-old children sensitized to HDM allergen, ryegrass allergen or both, and nonatopic controls (SPT-). Cytokine-specific mRNA relative to mRNA specific for housekeeping gene β -actin was determined by semiquantitative RT-PCR. Data shown are group means with SEM bars. Kruskal-Wallis tests showed no statistically significant differences among all four groups of children for IL-10 ($P=0.37$) or IFN- γ ($P=0.67$). When each atopic group was compared to nonatopic group by Mann-Whitney *U*-test, no significant *P* values were obtained.

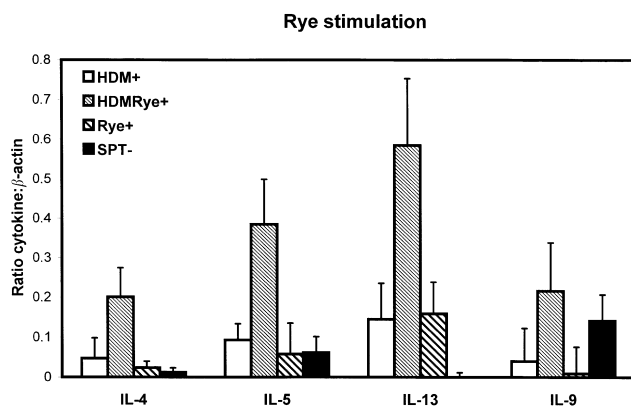


Figure 5. IL-4, IL-5, IL-13 and IL-9 responses to ryegrass allergen stimulation in PBMCs from groups of 6-year-old children sensitized to HDM allergen, ryegrass allergen, or both, and nonatopic controls (SPT –). Cytokine-specific mRNA relative to mRNA specific for housekeeping gene β -actin was determined by semiquantitative RT-PCR. Data shown are group means with SEM bars. Kruskal–Wallis tests showed no statistically significant differences among all four groups of children for IL-4 ($P=0.16$), IL-5 ($P=0.12$), IL-13 ($P=0.053$), and IL-9 ($P=0.71$). When each atopic group was compared to nonatopic group by Mann–Whitney U -test, following P values were obtained: HDM + group IL-4 (0.80), IL-5 (0.76), IL-13 (**0.028**), IL-9 (0.60); HDM + Rye + group IL-4 (0.088), IL-5 (**0.029**), IL-13 (**0.002**), IL-9 (0.83); Rye + group IL-4 (0.91), IL-5 (0.79), IL-13 (0.53), IL-9 (0.50). Boldface denotes statistically significant values.

response; rather, it indicates that this response is confined to specific allergens. However, the IFN- γ levels were unaltered when both polyclonal and allergen-specific stimulation of the SPT + and the SPT– groups were compared, a finding that has been reported previously by our group as well as by others (3, 6). Taken together, our results indicate an increased frequency of allergen-specific Th2 cells, but not Th1 cells, in the circulation, but only if the children are sensitized to that allergen, showing that the cytokine response after *in vitro* stimulation of PBMCs with allergen reflects the effector phase of allergic sensitization *in vivo*.

We were initially surprised that ryegrass stimulation in the group sensitized only to ryegrass did not evoke increased Th2 cytokine expression. We could exclude that this was due to the use of defective allergen for stimulation since cells from the group sensitized to both HDM and ryegrass expressed increased levels of Th2 cytokines after culture in the ryegrass allergen. We think that the lack of increased Th2 cytokine expression in the PBMCs from the ryegrass-positive groups was caused by a lack of exposure of the children to the allergen at the time of blood sampling. Indeed, only a few samples were taken around the time of potential ryegrass exposure in both ryegrass-positive groups. When mucosal surfaces do not encounter seasonal environmental allergens, the allergen-specific B and T cells are probably not activated and thus not induced to circulate in the blood, and the specific Th2 cytokine-producing cells are therefore not present in the circulation. An analogous

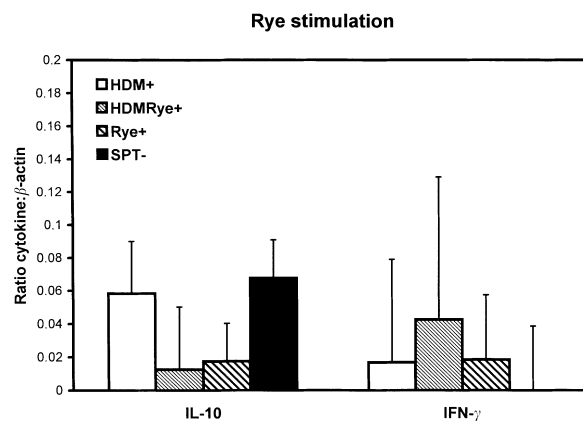


Figure 6. IL-10 and IFN- γ responses to ryegrass allergen stimulation in PBMCs from groups of 6-year-old children sensitized to HDM allergen, ryegrass allergen, or both, and nonatopic controls (SPT –). Cytokine-specific mRNA relative to mRNA specific for housekeeping gene β -actin was determined by semiquantitative RT-PCR. Data shown are group means with SEM bars. Kruskal–Wallis tests showed no statistically significant differences among all four groups of children for IL-10 ($P=0.17$) or IFN- γ ($P=0.77$). When each atopic group was compared to nonatopic group by Mann–Whitney U -test, no significant P values were obtained.

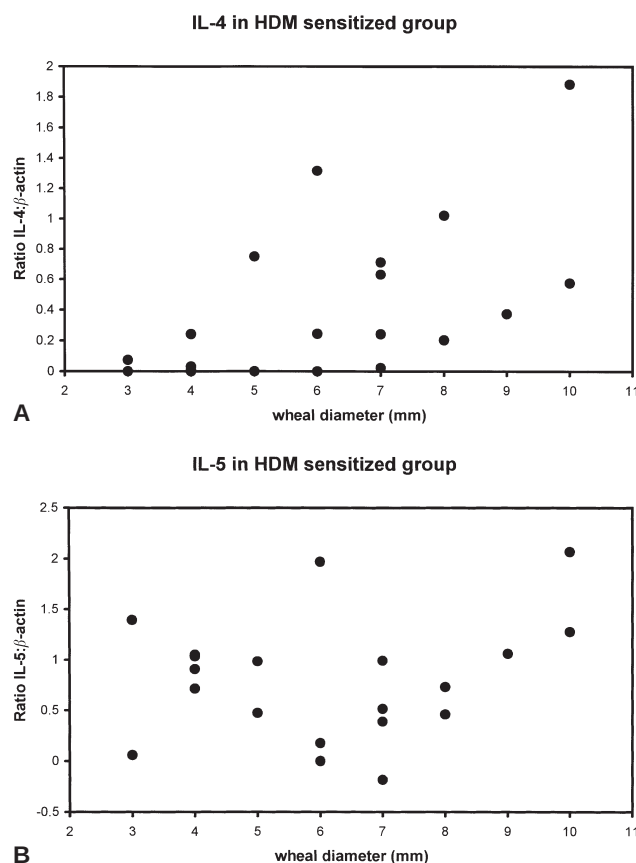


Figure 7. A) HDM antigen-stimulated IL-4 responses in HDM-sensitized children correlated with SPT wheal diameter (Spearman's rank correlation, $r=0.60$, $P=0.009$). B) Expression of IL-5 (Spearman's rank correlation, $r=0.118$, $P=0.61$), IL-13, IL-10, and IFN- γ (not shown) did not correlate with wheal diameter.

situation is mucosal vaccination of the respiratory tract when specific antibody-producing cells are found only in the peripheral blood of human subjects a few weeks after antigenic stimulation (17). Indeed, two previous studies of adults with pollen allergy show that production of IL-4 and IL-13 is undetectable or very low in the circulating cells when these are sampled out of season, whereas the same individuals have a clear increase in both Th2 cytokine production and IgE production during the season (18, 19). In contrast, a previous study showed that circulating Th2 cells are depleted from the circulation and recruited to the lung in asthmatic adult subjects challenged *in vivo* with allergen (20). It cannot be excluded, however, that the lack of Th2 cells was an effect of hyporesponsiveness to allergen *in vitro* due to the massive allergen challenge *in vivo* rather than true depletion of the allergen-specific cells from the circulation.

Although the children monosensitized only to ryegrass had similar SPT reactivity and wheal diameter to the other two sensitized groups, the specific IgE levels were considerably lower in the group reacting to ryegrass only, with some subjects being RAST negative. One explanation for the SPT reactivity in the latter subgroup is that allergen-specific IgG antibodies induced degranulation of mast cells upon the binding of allergen. Another possibility is that the amount of specific IgE in the blood of these subjects was under detection limits in the RAST but that sufficient IgE still coated the mast cells in the skin to elicit an acute-phase response (21). Thus, we speculate that when exposure to the seasonal allergen ceases, the allergen-specific IgE levels in serum gradually decrease as the allergen-specific T cells are no longer activated and stop circulating. It is interesting that we found increased Th2 expression after *in vitro* ryegrass stimulation in the individuals who were also sensitized to the perennial allergen HDM. Perhaps the HDM allergen stimulating the respiratory mucosa continuously induces a Th2-type microenvironment in regional lymph nodes, which might in turn cause "bystander" activation of T cells specific for other allergens encountered at mucosal surfaces such as ryegrass, and induce them to migrate into the circulation. This bystander activation could also explain why

the ryegrass-specific IgE levels were lower in the group sensitized only to ryegrass than in the group sensitized to both HDM and ryegrass.

Although it was not the primary purpose of this study, we studied the association between cytokine levels and wheal diameter to ensure that our results agreed with other studies. We showed that the expression of IL-4 correlates with the wheal diameter in the SPT, as well as with the level of specific IgE, but we did not obtain any significant correlation between the expression of IL-5 or IL-13 and the markers of atopy. Since IL-4 is the main switch factor for IgE, while IL-13 has an ancillary role, these results are not entirely surprising. A recent study also showed that IL-4 correlates with specific IgE levels to a much higher degree than IL-13 (22). In a previous paper, we also described that the magnitude of the IL-4 response is more strongly associated with wheal diameter than the magnitude of IL-5 and IL-13 (3). Our positive correlation between the expression of IL-5 and IL-13, but not between these cytokines and IL-4, also agrees with previous studies measuring the protein levels of the cytokines (1, 2).

In summary, the main findings of this study are, firstly, that the expression of Th2 cytokines in atopic children is increased after stimulation only with the allergen the subject is sensitized to, indicating that atopic status per se does not affect the type of T-cell response. Secondly, we conclude that when the mucosal surfaces of an atopic individual are not exposed to a seasonal allergen, *in vitro* responsiveness of PBMC to the allergen is not found, suggesting that the allergen-specific cells do not circulate. However, in polysensitized subjects responsive also to perennial allergens, continuous exposure to the perennial allergens may induce the reactivation of initially quiescent seasonal allergen-reactive T cells via bystander stimulation.

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