

Dissection of the grass allergen-specific immune response in patients with allergies and control subjects: T-cell proliferation in patients does not correlate with specific serum IgE and skin reactivity

P. A. Würtzen, MSc,^a R. J. J. van Neerven, PhD,^a J. Arned, MD,^b H. Ipsen, MSc,^a and S. H. Sparholt, PhD^a *Hørsholm and Copenhagen, Denmark*

Background: Pollinosis, caused by grasses of the Poaceae family, is a problem worldwide. The relative importance of grass groups 1 and 5 major allergens is well established. However, not much is known about the recognition of these allergens by T cells and whether this T-cell reactivity correlates with skin reactivity and serum IgE levels.

Objectives: The aim of this study was to characterize the cross-reactive, grass allergen-specific T-cell responses from patients allergic to grass and nonatopic individuals and to investigate whether these responses correlate with grass-specific IgE and skin reactivity.

Methods: Skin prick test wheal areas and grass-specific serum IgE levels were determined in all patients ($n = 21$) and nonallergic control donors ($n = 20$). Peripheral blood mononuclear cells were isolated and stimulated with grass allergen extracts (*Phleum pratense*, *Poa pratensis*, *Lolium perenne*) and immunoaffinity-purified group 5 allergens, and the production of type 1 and type 2 cytokines was determined in the patient group.

Results: Donors allergic to grass showed increased T-cell-proliferative responses to grass allergens compared with nonatopic control subjects. We find it interesting that the magnitude of the patients' T-cell responses could not be correlated with the individual skin prick test areas and specific serum IgE levels, and several patients with allergies to grass had group 5-specific T-cell responses in the absence of group 5-specific IgE. The absence of a correlation between T-cell proliferation and IgE levels or skin prick test results may in part be explained by the finding that patients predominantly produced IL-5 in response to Phl p 5, the major allergen, and predominantly IFN- γ in response to Phl p extract. In general, all donors responded equally well to all three grasses. Additional experiments with Phl p 5-specific T-cell lines indicated that the equal proliferation of peripheral blood mononuclear cells to all three species is the direct result of cross-reactivity.

Conclusions: Grass allergen-specific T-cell responses are highly cross-reactive, and patients with allergies exhibit higher responses than nonallergic donors, suggesting that T cells are involved in the allergic reaction to grass group 5

allergens. However, group 5-specific T-cell responses are also found in donors without group 5-specific IgE, and the patients' grass-specific T-cell responses and cytokine production do not correlate to skin reactivity or to concentrations of grass-specific IgE. (*J Allergy Clin Immunol* 1998;101:241-9.)

Key words: T cells, Poacea grasses, grass group 5 allergens, cross-reactivity, skin prick test, IgE

Grass pollen derived from grasses of the Poaceae family are a major source of airborne allergens, giving rise to seasonal allergies with clinical manifestations such as rhinitis and asthma.¹ The IgE reactivity of patients who are allergic to grass pollen allergens is well documented,² and group 1 and 5 allergens have been shown to be the most important, clinically relevant allergens. Both group 1 and group 5 allergens are proteins with molecular weights in the range of 30 kD, showing a wide isoelectric point (pI) variation in each of the Poaceae grass species.²⁻⁴ Interspecies sequence homology of grass group 5 and group 1 allergens⁴⁻⁶ suggests that members of these allergen groups induce immune responses that cross-react with the homologous allergens from other grasses. Several studies have reported this cross-reactive recognition of grass allergens for IgE reactivity^{2,4} as well as for T-cell reactivity.⁷⁻⁹ As a result of this cross-reactivity, patients with allergies respond to different grasses with different geographic distributions, which complicates allergen avoidance. Therefore, more detailed studies on the cross-reactivity between different grass species are important and may eventually lead to the use of a single grass species for diagnosis and specific treatment of allergy.

It is well established that allergen-specific T_{H2}-like cells play an important role in the induction of IgE synthesis (IL4) and eosinophilia (IL5).¹⁰⁻¹³ Furthermore, a higher frequency of infiltrating T cells producing T_{H1} cytokines (e.g., IFN- γ)¹⁴⁻¹⁶ and an increase in allergen-specific IgG¹⁷ are found after successful immunotherapy. This stresses the importance of T cells in the allergic immune responsiveness. Furthermore, enhanced allergen-specific T-cell proliferation in atopic patients as compared with nonatopic control subjects indicates that T cells are involved in the allergic reaction. Increased proliferative responses have been documented by sev-

From ^aALK ABELLÓ Group, Hørsholm, and ^bLunge og Allergiklinikken, Copenhagen.

Received for publication April 8, 1997; revised Sept. 8, 1997; accepted for publication Sept. 19, 1997.

Reprint requests: P. A. Würtzen, ALK-Abelló group, Bøge Allé 10-12, 2970 Hørsholm, Denmark.

Copyright © 1998 by Mosby, Inc.

0091-6749/98 \$5.00 + 0 1/1/86350

Abbreviations used

AB-serum:	AB-negative human serum
PBMC:	Peripheral blood mononuclear cell
PBS:	Phosphate-buffered saline
PpV ₈ :	Monoclonal antibody to Phl p 5
SI:	Stimulation index
SPT:	Skin prick test
TT:	Tetanus toxoid

eral investigations,¹⁸⁻²⁴ whereas this could not be demonstrated in other studies.²⁵⁻²⁷

Skin prick test (SPT) results and allergen-specific IgE measurements are widely used for the diagnosis of type 1 allergies, and even though there are some discrepancies,²⁸ the concordance between a positive skin test reaction and the detection of specific IgE is high.²⁹⁻³² Not much is known about a possible correlation between allergen-specific T-cell proliferation, cytokine production, allergen-specific IgE, and SPTs. Such information may be of importance for diagnosis and specific treatment of grass pollen allergies.

In this study we have addressed this question by studying the T-cell response of patients with allergies and control donors without allergies to grass pollen extracts and purified group 5 major allergens. The responses of patients and control donors were compared, and the level of T-cell responsiveness of the patients with allergies was compared with IgE reactivity and SPT results to investigate a possible correlation, whereas the cytokine production of the responding T cells of atopic patients was determined to relate T-cell reactivity to the atopic phenotype. In addition, the T-cell responses to three different grass species were studied to gain more insight into the cross-reactive nature of immune responses to grasses.

METHODS

Characterization of blood donors

Blood samples from 21 patients with allergies and 20 nonatopic persons were collected during the Danish grass pollen season. All patients had a clinical history of allergy to grass pollen and had high serum levels of *Phleum pratense*-specific IgE (minimum MagicLite class 2, ALK A/S).²⁹ None of the patients had received specific immunotherapy. All patients had a positive SPT result (≥ 5 mm²)²⁹ when tested with doses of 10 HEP *Phleum pratense* pollen extract (SoluPrick SQ, ALK A/S), and the wheal reaction area was evaluated relative to a positive histamine control (10 µg/ml). No patients showed skin reactions when tested with SoluPrick dilution (ALK A/S) as a negative control.

None of the control donors had a clinical history of allergic reactions, and none had *Phleum pratense*-specific serum IgE (MagicLite, ALK A/S) or a positive SPT reaction to Phl p. All subjects provided written informed consent, and the protocol was approved by the Danish ethic committee.

Allergens

Pollen extracts (Phl p, Poa p, and Lol p). Pollens from *Phleum pratense*, *Poa pratensis*, and *Lolium perenne* were obtained from

Greer Laboratories and extracted as described elsewhere.² Working solutions (1 mg lyophilized material/ml RPMI 1640 medium (Gipco) were sterile filtered and stored at -20° C until used. The extracts contained all important allergens as evaluated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblotting.

Affinity purification of grass group 5 allergens. Group 5 allergens (Phl p 5, Poa p 5, and Lol p 5) were affinity purified on a HiTrap affinity column (Pharmacia, Uppsala, Sweden) according to the manufacturer's description by use of Phl p 5-specific mouse monoclonal antibody PpV₈.⁵ Briefly, 10 mg of grass pollen extract, 10 mg/ml in PBS, 0.5 mol/L NaCl (pH 7.2) was added to the column (10 ml), and the column was washed 10 times with 10 ml of phosphate-buffered saline (PBS) and 0.10 mol/L glycine (pH 2.7). Group 5 allergen was eluted by adding 10 times 10 ml of PBS, 0.10 mol/L glycine, 0.15 mol/L NaCl (pH 11) and collected in buffer for neutralization. The fractions containing group 5 allergen identified as a homogeneous band pattern in sodium dodecyl sulfate-polyacrylamide gel silver staining³³ were pooled and lyophilized. The group 5 allergens were dissolved in RPMI 1640 medium, sterile filtered, and stored at -20° C until used.

The concentrations of group 5 allergens were determined by evaluation in rocket immunoelectrophoresis³⁴ with PpV₈ in the gel.

PBMC stimulation assay

PBMCs were isolated from freshly drawn heparinized blood by gradient centrifugation on lymphoprep (Nycomed), washed twice with RPMI (Gibco), and resuspended in RPMI medium supplemented with 5% vol/vol AB-serum (AH Diagnostics), 0.29 (µg/ml l-glutamine (Gibco) 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Sigma, St. Louis, Mo.) (referred to as AB-medium). The PBMCs were stimulated with serial dilutions of grass allergen extracts (100 to 6.25 µg/ml of Phl p, Poa p, and Lol p) and purified group 5 allergens (60 to 1.6 µg/ml) in 96-well, round-bottomed microtiter plates with 2×10^5 cells/well in 200 µl AB-medium/well. Optimal conditions for the proliferation assays were determined by pilot studies with allergen concentrations from 250 to 1 µg/ml and harvest of the cells at day 4, 5, 6, 7, or 8 with PBMCs from donors with allergies and nonatopic donors. PBMCs in cultures without Ag were used as negative controls; PBMCs stimulated with 5 µg/ml tetanus toxoid (TT) (Serum Institutttet) were used as positive controls. The cells were cultured for 6 days in a humidified atmosphere at 37° C, 5% CO₂, followed by an 18-hour pulse in the presence of 0.5 µCi of ³H-thymidine (NEN DuPont) per well. The cells were harvested onto nitrocellulose filters and thymidine incorporation was determined by liquid scintillation counting. Maximal stimulation index (SI) values were calculated as the ratio of cpm at the optimal antigen concentration in the stimulated cultures relative to the cpm in the unstimulated cultures. All results represent the mean values of triplicate cultures.

SDS-PAGE and electroblotting

SDS-PAGE and electroblotting were performed essentially as described previously³⁵ with the use of 400 µg crude grass allergen extract in a 10 cm broad well in sodium dodecylsulfate-polyacrylamide gel. After blotting, the nitrocellulose sheets were cut into 0.5 cm lanes with approximately 20 µg grass allergen extract per lane to probe with human serum samples followed by detection with ¹²⁵I-labeled anti-IgE (Pharmacia) by autoradiography at -80° C for 10 days.

Cytokine production

PBMCs were thawed and stimulated in AB-medium with 50 $\mu\text{g/ml}$ Phl p, 30 $\mu\text{g/ml}$ Phl p 5, or medium control in 1 ml cultures with 2 to 5×10^6 cells/ml. The cells were cultured in humidified atmosphere at 37°C , 5% CO_2 ; at day 6, supernatants were collected. Cells were resuspended in 500 μl AB-medium and transferred to 96-well, round-bottomed microtiter plates with 50 μl culture/well and pulsed for 18 hours with 0.5 μCi of ^3H -thymidine per well to determine proliferation as described for the PBMC assay.

Cytokine concentrations in supernatants were determined by ELISA for IL-5 (Immunotech, Marseille, France) and for IL-4 and IFN- γ (Mabtech, Nacka, Sweden) according to the manufacturer's description.

Establishment of Phl p 5-specific T-cell lines

T-cell lines specific to Phl p 5 were established from PBMCs of two patients allergic to grass. Isolated PBMCs ($2 \times 10^6/\text{ml}$) were stimulated in 1 ml bulk cultures in 24-well plates (Nunc) with Phl p 5 (20 $\mu\text{g/ml}$) for 14 days with 10 IU, 5 IU, and 5 IU r-IL2 (Chiron) added per well at days 5, 6, and 7, respectively. After 14 days, T cells were isolated, washed, and restimulated in culture with autologous EBV B-cells ($10^5/\text{well}$, irradiated 3,500 Rad), allogeneic PBMCs ($10^6/\text{well}$, irradiated 2,500 Rad), 10 $\mu\text{g/ml}$ PHA-C (Boehringer Mannheim, Germany), and Phl p 5 (20 $\mu\text{g/ml}$), and r-IL2 was added at days 5, 6, and 7. The lines were kept in culture with restimulation every 10 days and addition of r-IL2 at days 3 to 5. The cell line cultures were terminated after a period of 8 weeks.

T-cell stimulation assay

On day 10 after restimulation, T cells ($2 \times 10^4/\text{well}$) were cultured with autologous PBMCs ($10^5/\text{well}$, irradiated 2,500 Rad) in 200 μl AB-medium with or without antigen in 96-well, round-bottomed microtiter plates (Nunc). Cells were cultured for 72 hours in a humidified atmosphere at 37°C , 5% CO_2 followed by an 18-hour pulse with 0.5 μCi ^3H -thymidine/well to determine proliferation as described for the PBMC assay. Results are expressed as mean cpm values of triplicate cultures.

Statistical analysis

The Mann Whitney U test was used to compare groups of data with nonnormal distribution. Spearman rank order correlation was used to detect correlation between different data sets. All stochastic probabilities less than or equal to 0.05 were considered significant. Analyses were performed with Statistica (Stat Soft, Tulsa, Okla.).

RESULTS

SPTs and allergen-specific IgE

All patients had a positive skin reaction to *Phleum pratense* and grass allergen-specific IgE in serum, but no direct correlation between the patients' area of SPT wheal reactions and concentration of grass-specific IgE in serum was found (Fig. 1). Total serum IgE levels varied from 5 to 952.2 IU/ml and *Phleum pratense*-specific IgE from 10 to 522 SU/ml. SPT areas varied from 14 to 55 mm^2 for the histamine control and from 6 to 60 mm^2 for *Phleum pratense*. The negative control (PBS) was less than 1 mm^2 in all cases.

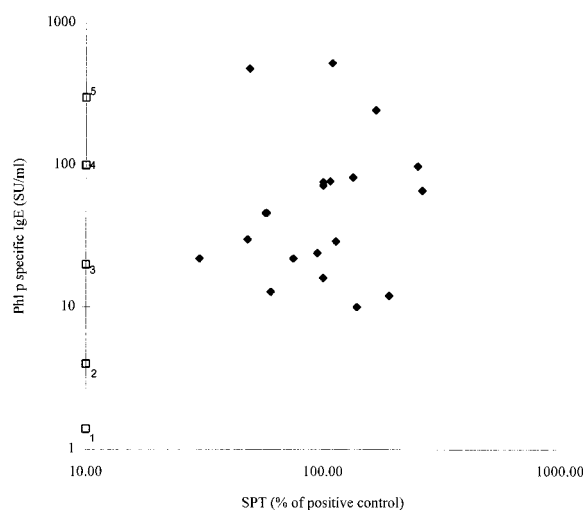


FIG. 1. SPT versus specific IgE. SPT results are expressed as Phl p wheal area relative to histamine control wheal area. Phl p-specific IgE concentrations were determined in MagicLite and are expressed in standard units.²⁹ Lower limits for MagicLite allergy classes 1 through 5 are indicated by squares at the y-axis. Spearman rank order correlation: $r = 0.15$, $p = 0.58$.

Proliferative T-cell responses to grass pollen allergens

To detect differences in the T-cell responses of patients allergic to grass compared with nonatopic control subjects, the proliferation of T cells in PBMC cultures stimulated with various concentrations of grass allergens was investigated. Both patients and control subjects exhibited pronounced proliferative T-cell responses to the allergen extracts with maximal stimulation in the range of 5 to 50 μg allergen/ml (data not shown). Similar dose-response curves were also found in the two groups when PBMCs were stimulated with purified group 5 allergens, with maximal responses in the range of 5 to 30 μg allergen/ml (data not shown). T cells from the majority of the donors from both groups were equally activated by all the grass allergen extracts, but some donors only recognized one or two of the purified group 5 allergens. The fact that all donors reacted to grass allergens was not the result of mitogenic activity of the preparations, because no mitogenic effect was found when a Bet v 1-specific T-cell line³⁶ was cultured in the presence of different concentrations of Phl p 5 or Phl p (results not shown). To ensure that the results are not influenced by the variation in background counts, they are expressed as maximal SI (Fig. 2). It should be noted, however, that background values in the patients with allergies were generally higher than in control subjects (see legend of Fig. 2), resulting in lower stimulation indices in respect to total counts. The mean responses expressed as SI to grass allergens (Fig. 2, A and B) were in all cases higher in the group with allergies than the responses seen in the group without allergies. The mean responses to the purified allergens Phl p 5 and Poa p 5 were significantly higher in the atopic group (Fig. 2, B).

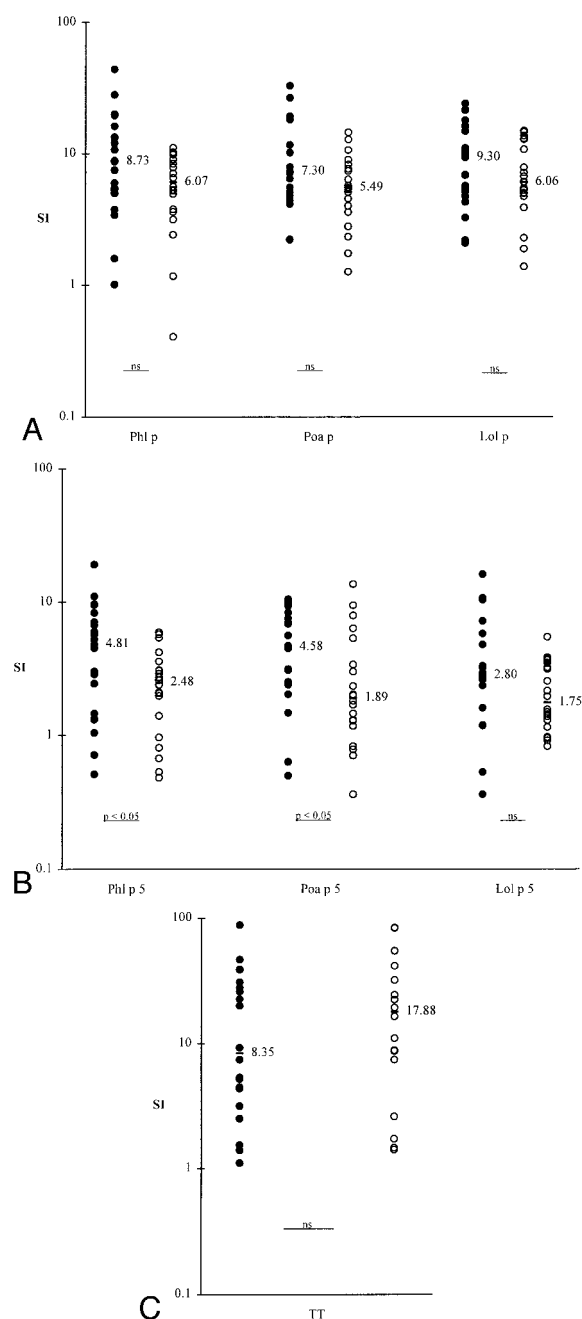


FIG. 2. Specific T-cell responses to grass allergen extracts (**A**), grass group 5 allergens (**B**), and tetanus toxoid (**C**) in PBMC cultures from patients with allergies to grass (closed circles) and nonatopic control subjects (open circles). The maximal stimulations with crude extracts were 1000 to 40,000 cpm in PBMCs from donors with allergies, whereas PBMCs from individuals without allergies in general show maximal responses between 100 and 5000 cpm. Maximal responses to purified group 5 allergens were in general between 1000 and 5000 cpm in the atopic group and 100 and 2000 cpm in the group without allergies. The mean PBMC responses to TT (in cpm) were similar in the two groups. The mean background value was higher in the group with allergies (920 cpm compared with 450 cpm), but the difference was not significant. Results are expressed as maximal SI. Median values are indicated for each group. Median values in bold are significantly higher than the median values of the control group ($p \leq 0.05$).

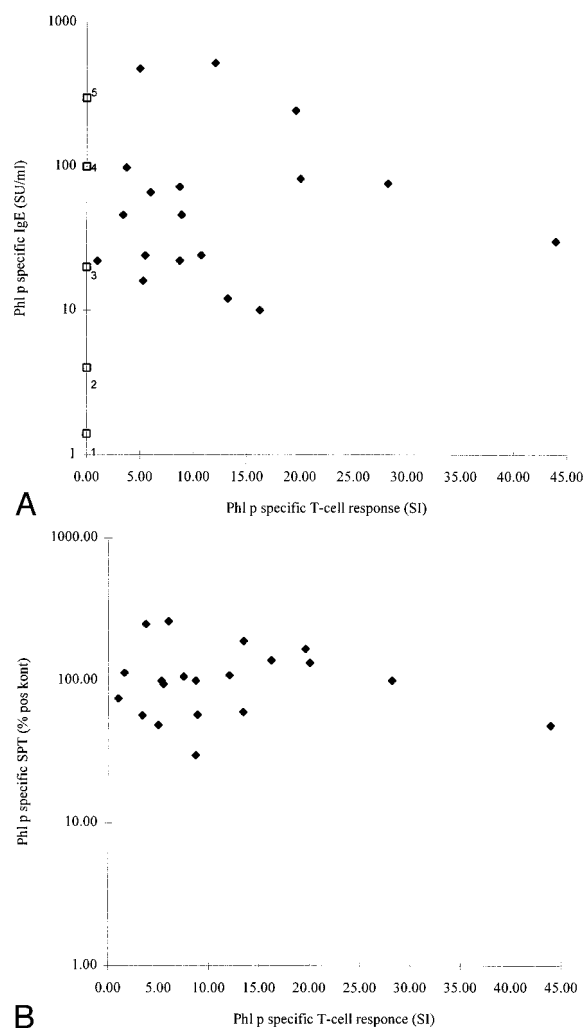


FIG. 3. **A**, T-cell response versus specific IgE concentrations, Phl p-specific T-cell responses in PBMC cultures expressed as maximum SI and Phl p-specific IgE concentrations were determined in MagicLite and are expressed in standard units.²⁹ Lower limits for MagicLite allergy classes 1 through 5 are indicated by squares at the y-axis. Spearman rank order correlation: $r = 0.06$, $p = 0.80$. **B**, T-cell response versus SPT response. Phl p-specific T-cell responses in PBMC cultures expressed as max. SI and SPT results are expressed as Phl p wheal area relative to histamine control wheal area. Spearman rank order correlation: $r = 0.07$, $p = 0.78$.

In contrast, the response to TT as a control was higher in the nonatopic group, but this difference was not significant. Interestingly, when the results were expressed as Δ cpm, all allergen-specific responses were found to be significantly higher in the atopic group ($p < 0.01$), and only the difference in responses to Lol p 5 failed to reach significance ($p < 0.06$). Responses to the control antigen TT were identical in both groups ($p = 0.92$) when expressed as Δ cpm (not shown).

Each donor group showed comparable mean responses to all three grass allergen extracts as well as to the three purified group 5 allergens (Fig. 2, **A** and **B**). The high level of *Phleum pratense*-specific T-cell prolifer-

TABLE I. T-cell and IgE reactivity of 13 patients to grass allergen extracts and group 5 allergens

Patient	Allergen					
	Phl p	Poa p	Lol p	Phl p 5	Poa p 5	Lol p
6	19.57	19.25	14.77	11.04	9.35	10.68
7	12.04	7.30	9.30	5.98	4.47	5.79
8	10.73	10.18	16.22	7.07	6.83	2.95
9	8.89	7.12	6.86	6.70	6.99	2.74
10	3.41	4.39	3.23	4.51	3.07	2.35
12	20.01	18.12	21.31	19.13	9.81	4.78
13	8.73	10.18	21.54	9.64	7.52	2.61
14	43.91	32.89	17.90	8.30	4.70	3.19
15	28.17	26.59	24.05	4.81	10.47	10.37
18	5.99	5.52	4.72	2.86	3.13	3.80
19	8.70	11.63	9.78	2.44	2.50	3.24
20	13.22	19.21	16.01	5.24	5.60	2.69
21	5.49	5.07	4.26	9.53	8.32	16.15

T-cell responses are expressed as maximum. SI found in PBMC cultures stimulated with various concentrations of each allergen and IgE reactivity was detected by SDS-PAGE immunoblotting. Numbers indicate T-cell responses combined with IgE reactivity; numbers in bold indicate T-cell responses without simultaneous IgE reactivity.

eration did not correlate with specific IgE concentrations (Fig. 3, *A*) or SPT data (Fig. 3, *B*) of the individual patients. Similar results were obtained when Phl p 5-specific T-cell proliferation was correlated with SPT and IgE concentrations (not shown).

Grass group 5 allergen-specific IgE

Because allergen-specific T-cells induce the production of specific IgE antibodies, one expects a correlation between T-cell reactivity and IgE levels. Therefore the sera of 13 patients with high T-cell responses to grass allergens were selected to investigate the patients' IgE reactivity by SDS-PAGE immunoblotting. All patients (except patient 10) had detectable IgE reactivity to one or more allergens in all three grasses. IgE reactivity to group 5 allergens were seen in most patients' sera, but the sera of some patients only contained detectable IgE specific to the group 5 allergen in one or two of the grasses. Interestingly, 5 of 13 patients who had good proliferative responses to group 5 allergens had no detectable group 5-specific IgE (Table I). These results were confirmed by a fluid-phase assay (data not shown).

Cytokine profiles of the allergen-specific T-cell response

To determine whether the cytokine production of the patients was consistent with the atopic phenotype, supernatants from Phl p 5- and Phl p-stimulated PBMCs from 15 patients were assayed for production of IL-4, IL-5, and IFN- γ . Unexpectedly, all patients produced significant amounts of IFN- γ in response to Phl p, whereas IL-5 was only produced by eight of 15 patients. In contrast, in cultures stimulated with Phl p 5, 13 of 15 patients had significant IL-5 production, whereas low levels of IFN- γ were measured in only seven of 15 (Fig. 4). IL-4 could not be measured in any of the supernatants. The levels of IL-5 or IFN- γ production did not

correlate to SPT reactions or specific IgE concentrations.

Cross-reactivity of Phl p 5-specific T-cell lines

To distinguish between actual cross-reactivity of individual T cells and separate T-cell populations' equal responses to different group 5 allergens, the responses of T-cell lines were investigated. T-cell lines raised against Phl p 5 exhibited extensive cross-reactivity with specific responses to all crude grass extracts and all immunoaffinity purified grass group 5 allergens (Fig. 5, *A* and *B*). T-cell reactivity of pt AGC and pt TL in the PBMC cultures show a similar reactivity pattern (not shown). The specificity of the cell lines did not change after several restimulations.

DISCUSSION

This study showed that patients allergic to grass have a slightly increased T-cell proliferative response to grass allergens compared with control subjects without allergies and that this difference reaches significance when the responses to single grass group 5 allergens are compared, even though grass allergen-specific T-cell responses are present in both groups. The enhanced T-cell proliferation of the atopic patients could neither be correlated to skin reactivity nor to specific serum IgE measurements, and some patients even had T-cell proliferative responses to group 5 allergens in the absence of specific IgE. Unexpectedly, the cytokine production of patients allergic to grass in response to Phl p 5 was dominated by the T_{H2} cytokine IL-5, whereas the cytokine productions in response to Phl p was dominated by the T_{H1} cytokine IFN- γ . In addition, the present data suggest that a major part of the simultaneous reactivity of peripheral T cells to related grasses is the direct result of cross-reactivity.

CD4+ T cells have been shown to play an important

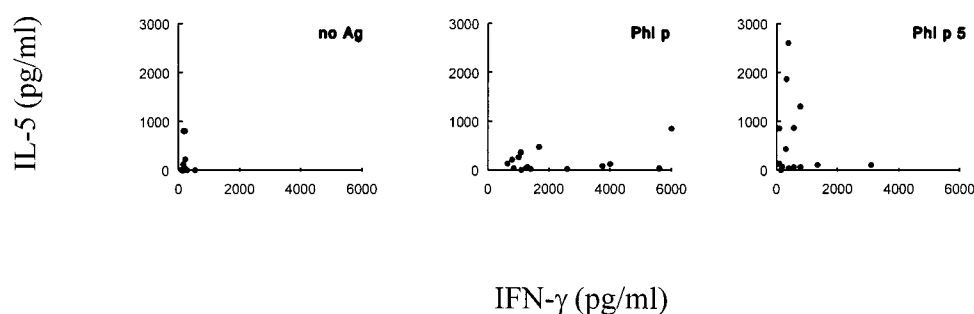


FIG. 4. IL-5 (*y*-axis) and IFN- γ (*x*-axis) production of PBMCs from 15 patients with allergies to grass in cultures with no allergen (*left*), 50 μ g/ml Phl p (*middle*), or 30 μ g/ml Phl p 5 (*right*). Supernatants were collected at day 6 after stimulation, and the concentrations of IL-5 and IFN- γ were determined by ELISA. Ag, Antigen.

role in the pathophysiology of allergic disease,¹⁰⁻¹³ and a shift in their cytokine profile has been suggested as a possible mechanism for successful specific immunotherapy.¹⁴⁻¹⁶ The involvement of T cells in the regulation of allergic disease would suggest that there is a direct correlation between allergen-specific T-cell activation and allergen-specific IgE-mediated reactions.

T-cell reactivity to the three grass extracts tested could be demonstrated in the patient group as well as in the control group. However, responses in the patient group were higher than responses in the control group. When grass group 5-specific T-cell reactivity was studied, significantly higher responses to Phl p 5 and Poa p 5 were found in the atopic group and low mean responses to all three group 5 allergens were found in the nonatopic group (Fig. 2, *A* and *B*). The lack of a significantly higher mean response to Lol p 5 in the atopic group may be caused by the relatively low SI from a few allergic individuals (Fig. 2), indicating that the difference might reach significance in larger populations. The elevated allergen-specific T-cell responses found in the atopic group indicate that T cells from patients with allergies are involved in the mechanisms of the allergic reaction and therefore may be the actual target for specific therapy.

Several studies have demonstrated that atopic patients display higher T-cell-proliferative responses to allergenic extracts than control subjects without allergies.¹⁸⁻²⁴ Some other studies, however, have not been able to confirm this.²⁵⁻²⁷ Similarly, the data presented here show that proliferative T-cell responses of T cells from donors with allergies to grass pollen extracts were higher (but not significantly higher) than responses from donors without allergies. A possible explanation for this is that allergenic extracts consist of complex mixtures of proteins, possibly masking a significantly different response to single major allergens. This is consistent with the finding of other groups that a more pronounced difference between the T-cell reactivity to purified major allergens compared with allergen extracts exists.^{37, 38} Because allergen-specific IgE can facilitate the presentation of allergens through Fc ϵ RI or RII, resulting in a higher T-cell-proliferative response,³⁹⁻⁴¹ studies per-

formed in autologous serum might be more relevant to the *in vivo* situation. However, IgE-facilitated allergen presentation is dependent on high levels of specific IgE and may only be relevant in atopic dermatitis.⁴⁰

Both significant and nonsignificant differences in mean SI are further underscored by the fact that the mean response to TT was (nonsignificantly) higher in the nonatopic group, and, in addition, that the higher background values in the atopic group have lowered the SI values in this group. In addition, when data were expressed as Δ cpm, significant differences were found for both allergen extracts and purified allergens, but the significance of the differences in the responses to the allergen extracts were lost when the variability of the baseline was included in the evaluation of the data (i.e., when data were expressed as SI). Responses to Phl p 5 and Poa p 5, however, remained significantly higher in the atopic group. The enhanced T-cell responses to grass allergens in the patient group could not be explained by different dose-response curves (data not shown) but rather indicate an increased frequency of allergen-specific T cells,²² implying that not only the cytokines these allergen-specific T cells produce are affected in allergic disease but that their frequency also is enhanced in the peripheral blood of patients with allergies.

A positive SPT result and the presence of allergen-specific serum IgE are normally concordant.²⁹⁻³² The inclusion criteria for patients of the present investigation was positive SPT reaction (≥ 5 mm²) to Phl p and specific IgE concentrations above 4 SU/ml ($\geq$ class 2). However, no clear-cut correlation between the level of SPT and the IgE concentrations of individual patients could be found (Fig. 1). When SPT areas and specific IgE determination are compared, a correlation was found when SPT is expressed as end point titration.^{30, 42} However, the direct correlation of SPT with IgE measurements is complicated by many different factors involved in the determination of specific IgE concentrations and skin reactivity, such as IgE affinity, total IgE levels, the presence of blocking IgG, and varying mast cell releaseability in the skin.⁴²⁻⁴⁴ The fact that enhanced grass allergen-specific T-cell responses are found in the peripheral blood of subjects with allergies suggests that

the responding T cells are inducing the production of the grass-specific IgE by B cells and subsequent skin reactivity. Therefore a direct correlation between the level of T-cell reactivity and allergen-specific serum IgE and SPT areas could be expected. A correlation between IgE and T-cell proliferative responses has been suggested,^{37, 45} whereas other studies indicate that allergen extracts also contain proteins that induce T-cell proliferation in the absence of IgE.⁴⁶⁻⁴⁸ However, in this study neither specific serum IgE levels nor SPT areas could be correlated to the level of Phl p or Phl p 5-specific T-cell responses of the individual patients. In addition to the parameters influencing SPT and IgE determination, T-cell reactivity to proteins other than major allergens in the grass extracts may complicate this kind of correlation.

Interestingly, the fact that some patients had Phl p 5-specific T-cell responses in the absence of Phl p 5-specific IgE (Fig. 5) might indicate that these T cells are not able to induce IgE production. In addition, the group 5-specific T-cell proliferation found in several nonatopic individuals (Fig. 2, B) indicates that a nonallergic T-cell response to allergens is present in nonatopic donors as well as in some patients with allergies to grass without IgE specific to the allergen in question. This is in concordance with findings by Spiegelberg et al.⁷ and Schenk et al.,⁸ who showed that both allergen-specific T_{H1} and T_{H2} clones can be established from the peripheral blood of patients with allergies to grass. However, the specificity of the T cells and the IgE specificity of the patients was not compared in these studies.

The production of IL-5 (typical T_{H2} cytokine) and IFN- γ (typical T_{H1} cytokine) was used to determine whether the cytokine profile of the responding T-cell population reflects the allergic phenotype of the patients. The production of IL-5 by PBMCs has recently been reported to be found only in atopic patients and to be correlated to the severity of the disease, indicating that it is an excellent marker for T_{H2} activation in PBMC cultures.⁴⁹ The lack of detectable IL-4 production in the allergen-stimulated PBMC cultures found here may be the result of consumption in the culture, and it has previously been shown that detectable IL-4 production may require several allergen-specific T-cell stimulations.⁵⁰

The differential production of IL-5 (to Phl p 5) and IFN- γ (to Phl p extract) is puzzling. It may in part be explained by the fact that the extract contains proteins that activate T_{H1}-like T cells that possibly are important in skewing the T-cell response after specific immunotherapy. The production of IFN- γ by the activated T_{H1} cells may directly inhibit the proliferation and production of IL-5 by the Phl p 5-specific T cells, resulting in the IFN- γ -dominated response to Phl p. This is supported by a recent study demonstrating that IL-5 production by PBMCs in response to allergens is completely inhibited by the addition of IFN- γ .⁵¹ However, neither IFN- γ responses nor IL-5 responses correlated with the presence of specific IgE or SPT values. These results

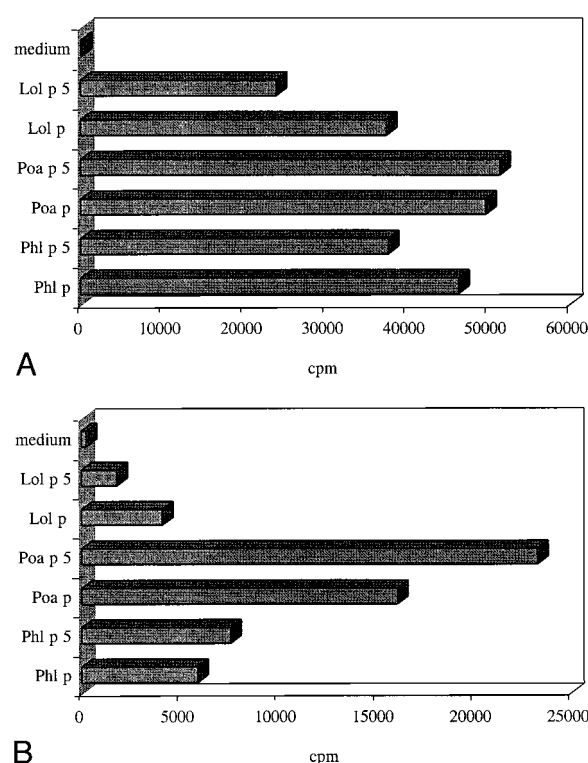


FIG. 5. Grass-specific T-cell reactivity of Phl p 5-specific T-cell lines established from the peripheral blood of two patients. Proliferation is expressed as mean cpm of triplicate cultures, and results represent two or more identical experiments. Similar reactivity patterns were obtained when PBMCs from the two patients were stimulated with the same allergens.

show that even the production of a typical T_{H2} cytokine as IL-5 does not correlate to specific IgE levels or SPT results, as was also found for T-cell proliferation.

All patients and donors without allergies tested exhibited equally strong T-cell responses to all three related grass allergens. The extensive cross-reactivity of T-cell lines generated by stimulation with Phl p 5 (Fig. 5, B) shows that Phl p 5-specific T cells do react with group 5 allergens from other grass species, and it indicates that the simultaneous response of PBMCs to multiple grasses is in part the direct result of cross-reactivity. The cross-reactivity was strongest between Phl p 5 and Poa p 5. Even though these data are obtained from experiments with a limited number of T-cell lines, the results are in accordance with earlier findings by Müller et al.⁹ and directly reflect the sequence homology between the different grass allergens.⁶ Furthermore, cross-reactivity of T-cell lines and clones specific to other allergens have been reported,^{7, 8, 36} confirming that individual T cells recognize homologous allergens from related allergen sources.

The identified T-cell cross-reactivity pattern is similar to the cross-reactivity pattern described on the IgE level,⁴ and it may therefore be expected that these Phl p 5-specific T cells will provide help for the production of Phl p 5-specific as well as Poa p 5- and Lol p 5-specific

IgE by B lymphocytes. Subsequently, allergen extracts from different Poaceae grasses may be expected to function equally well in diagnosis and treatment of grass allergy. However, the T-cell and IgE reactivity patterns of individual patients stress the importance of species-specific diagnosis if purified single allergens are considered for treatment.

In conclusion, the results of this investigation show that the cross-reactive grass group 5 allergen-specific T-cell response of patients with allergies to grass is higher than the response of nonatopic control subjects. The lack of correlation between the skin reactivity and T-cell reactivity or IgE concentrations is most likely to reflect the complexity of the skin reactivity, whereas the fact that the specific T-cell responses did not correlate to the patients' serum IgE indicates that at least some of the responding T cells may be involved in immune reactions other than the support of IgE production, which was supported by the differential production of the T_{H2} cytokine IL-5 (Phl p 5) and the T_{H1} cytokine IFN- γ (Phl p) by the responding T-cell populations.

The authors would like to thank Dr. J. S. van der Zee for critically reviewing the manuscript.

REFERENCES

- Løwenstein H, Østerballe O. Standardized grass pollen extracts. *Clin Rev Allergy* 1986;4:405-533.
- Matthiesen F, Løwenstein H. Group V allergens in grass pollen. II. investigation of group V allergens in pollen from 10 grasses. *Clin Exp Allergy* 1991;21:309-20.
- Petersen A, Schramm G, Becker W, Schlaak M. Comparison of four grass pollen species concerning their allergens of grass group V by 2D immunoblotting and microsequencing. *Biol Chem Hoppe-Seyler* 1993;374:855-61.
- Smith PM, Ong EK, Knox RB, Singh MB. Immunological relationship among group I and group V allergens from grass pollen. *Mol Immunol* 1994;31:491-8.
- Klysner S, Welinder KG, Løwenstein H, Matthiesen F. Group V allergens in grass pollens. IV. similarities in amino acid compositions and NH_2 -terminal sequences of the group V allergens from *Lolium perenne*, *Poa pratensis*, and *Dactylis glomerata*. *Clin Exp Allergy* 1992;22:491-7.
- Vrtala S, Sperr WR, Remitzer I, van Ree R, Laffer S, Müller W, et al. cDNA cloning of a major allergen from timothy grass (*Phleum pratense*) pollen: characterization of the recombinant Phl p V allergen. *J Immunol* 1993;151:4773-81.
- Spiegelberg HL, Beck L, Stevenson RD, Ishioka GY. Recognition of T cell epitopes and lymphokine secretion by rye grass allergen *Lolium perenne* I-specific human T cell clones. *J Immunol* 1994;152:4706-11.
- Schenk S, Breiteneder H, Susani M, Najafian N, Laffer S, Duchene M, et al. T-cell epitopes of Phl p 1, major pollen allergen of timothy grass (*Phleum pratense*): evidence for cross-reacting and non-cross-reacting T-cell epitopes within grass group I allergens. *J Allergy Clin Immunol* 1995;96:986-96.
- Müller W, Karamfilov T, Fahlbusch B, Vogelsang H, Jäger L. Analysis of human T cell clones reactive with group V grass pollen allergens. *Int Arch Allergy Immunol* 1994;105:391-6.
- Kapsenberg ML, van der Pouw-Kraan T, Stickema FE, Schootmeyer A, Bos JD. Direct and indirect nickel-specific stimulation of T lymphocytes from patients with allergic contact dermatitis to nickel. *Eur J Immunol* 1988;18:977-82.
- Parronchi P, Macchia D, Piccinni M, Biswas P, Simonelli C, Maggi E, et al. Allergen and bacterial antigen-specific T-cell clones established from atopic donor show a different profile of cytokine production. *Proc Natl Acad Sci U S A* 1991;88:4538.
- Secrist H, Chelen CJ, Wen Y, Marshall JD, Umetsu DT. Allergen immunotherapy decreases interleukin-4 production in CD4+ T cells from allergic individuals. *J Exp Med* 1993;178:2123.
- Wierenga EA, Snoek M, de Groot C, Chrétien I, Bos JD, Jansen HM, et al. Evidence for compartmentalization of functional subsets of CD4+ T lymphocytes in atopic patients. *J Immunol* 1990;144:4651-6.
- Varney VA, Hamid QA, Gaga M, Ying S, Jacobsen M, Frew AJ, et al. Influence of grass pollen immunotherapy on cellular infiltration and cytokine mRNA expression during allergen-induced late-phase cutaneous responses. *J Clin Invest* 1993;92:644-51.
- Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Müller UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN- γ secretion in specific allergen stimulated T cell cultures. *J Immunol* 1995;154:4187-94.
- Durham SR, Ying S, Varney VA, Jacobsen M, Sudderick RM, Macay IS, et al. Grass pollen immunotherapy inhibits allergen-induced infiltration of CD4+ T lymphocytes and eosinophils in the nasal mucosa and increases the number of cells expressing messenger RNA for interferon- γ . *J Allergy Clin Immunol* 1996;97:1356-65.
- Creticos PS, Norman PS. Immunotherapy in inhalant allergy. In: Lessof MH, Lee TH, Kemeny DM, editors. *Allergy: an international textbook*. New York: John Wiley & Sons, Ltd; 1987. p. 617-630.
- Hiratani M, Muto K, Oshida Y, Ito S, Kasei M. Lymphocyte responsiveness to *Dermatophagoides farinae* extract in mite sensitive patients: effect of immunotherapy on cellular proliferative response and specific immunoglobulin E antibody (RAST score). *J Allergy Clin Immunol* 1981;68:205-11.
- Walker CA, Fitzharris P, Longbottom JL, Newman Tayler AJ. Lymphocyte sensitization to *Aspergillus fumigatus* in allergic bronchopulmonary aspergillosis. *Clin Exp Immunol* 1989;76:34-40.
- Nagaya H, Lee SK, Reddy PH, Pascual H, Jerome D, Sadai J, et al. Lymphocyte response to grass pollen allergens: a correlation with radioallergosorbent test and effect of immunotherapy. *Ann Allergy* 1996;39:246-52.
- Gatien JG, Merler E, Colten HR. Allergy to ragweed antigen E: effect of specific immunotherapy on the reactivity of human T lymphocytes in vitro. *Clin Immunol Immunopathol* 1975;4:32-7.
- Burastero SE, Fenoglio D, Crimi E, Brusasco V, Rossi GA. Frequency of allergen-specific T lymphocytes in blood and bronchial response to allergen in asthma. *J Allergy Clin Immunol* 1993;91:1075-81.
- Eisenbrey AB, Agarwal MK, Offord KP, Adolphson CR, Yunginger JW, Gleich GJ. Seasonal variation of in vitro lymphocyte proliferative response to ragweed antigen E. *J Allergy Clin Immunol* 1985;75:84-90.
- Rocklin RE, Pence H, Kaplan H, Evans R. In vitro transformation and elaboration of lymphocyte mediators. *J Clin Invest* 1974;53:735-44.
- Ownby DR, Buckley RH. Lymphocyte responses to ragweed allergens from different sources. *J Allergy Clin Immunol* 1979;63:65-6.
- Di Felica G, Mari A, Mucci N, Afferni C, Bruno G, Pini C. T cell responses to a *Parietaria judaica* pollen extract: comparison between *Parietaria*-sensitive patients, other atopics, and healthy controls. *Allergy* 1989;44:322-9.
- Buckley RH, Seymour F, Sanal SO, Ownby DR, Becker WG. Lymphocyte responses to purified ragweed allergens in vitro. I. proliferative responses in normal, newborn, agammaglobulinemic, and atopic subjects. *J Allergy Clin Immunol* 1977;59:70-8.
- van der Zee JS, de Groot H, Van Sweiten HM, Jansen HM, Aalberse RC. Discrepancies between the skin test and IgE antibody assays: study of histamine release, complement activation in vitro, and occurrence of allergen-specific IgE. *J Allergy Clin Immunol* 1982;82:270-81.
- Eiken P, Mosbech H, Jacobsen L, Larsen P, Scharling B, Svendsen UG, et al. Comparison between two different assays for measurements of allergen-specific IgE and skin prick test in the diagnosis of insect venom allergy. *Allergy* 1992;47:495-7.
- Businco L, Borsetto-Ménghi AM, Lucarelli S, Frediani T, Salvati L,

- Businco E. Intradermal skin tests with *Dermatogoides pteronyssinus* in asthmatic children: correlation with specific IgE and bronchial provocation tests. *Clin Allergy* 1979;9:459-63.
31. Moxnes A, Dale S, Andrew E, Halvorsen R. A new purified *Dermatogoides farinae* allergen preparation. *Allergy* 1984;39:339-49.
32. Stenius B, Wide L, Seymour WM, Holford-Strevens V, Pepys J. Clinical significance of specific IgE to common allergens. *Clin Allergy* 1971;1:37-55.
33. Heukeshoven J, Dernick R. Silverstaining. *Electrophoresis* 1985;6:103.
34. Axelsen NH, Bock E. Electroimmunoassay (rocket immunoelectrophoresis). *Scand J Immunol* 1984;17:103-7.
35. Würtzen PA, Nelson HS, Løwenstein H, Ipsen H. Characterization of chenopodiales (*Amaranthus retroflexus*, *Chenopodium Album*, *Kochia scoparia*, *Salsola pestifer*) pollen allergens. *Allergy* 1995;50:489-97.
36. Sparholt SH, Larsen JN, Ipsen H, Schou C, van Neerven RJJ. Crossreactivity and T cell epitope specificity of Bet v 1-specific T cells suggests the involvement of multiple isoallergens in sensitization to birch pollen. *Clin Exp Allergy* 1997;27:932-41.
37. O'Brien RM, Thomas WR, Wootton AM. T cell responses to the purified major allergens from the house dust mite *Dermatophagoides pteronyssinus*. *J Allergy Clin Immunol* 1992;89:1021-31.
38. Rawle FC, Mitchell EB, Platts-Mills TAE. T cell responses to the major allergen from the house dust mite *Dermatophagoides pteronyssinus*, antigen P1: comparison of patients with asthma, atopic dermatitis, and perennial rhinitis. *J Immunol* 1984;133:195-201.
39. van der Heijden FL, van Neerven RJJ, Kapsenberg ML. Relationship between facilitated antigen presentation and the presence of allergen-specific IgE in serum of atopic patients. *Clin Exp Immunol* 1995;99:289-93.
40. van der Heijden FL, van Neerven RJJ, van Katwijk M, Bos JD, Kapsenberg ML. Serum-IgE-facilitated allergen presentation in atopic disease. *J Immunol* 1993;150:3643-50.
41. Maurer D, Ebner C, Reininger B, Fribiger E, Kraft D, Kinet J, et al. The high affinity IgE receptor (FcεRI) mediates IgE-dependent allergen presentation. *J Immunol* 1995;154:6285-90.
42. Witteman AM, Stapel SO, Perdok GJ, Sjamsoedin DHS, Jansen HM, Aalberse RC, et al. The relationship between RAST and skin test results in patients with asthma or rhinitis: a quantitative study with purified major allergens. *J Allergy Clin Immunol* 1996;97:16-25.
43. Aalberse RC, Reerink-Brongers EE, Vermeulen E. RAST-inhibiting factors in human serum. *Int Arch Allergy Immunol* 1973;45:46-9.
44. Pequet C, Murrieta M, Michelen V, Leynadier F, Dry J, Allary M, et al. Blocking activity of mite-specific IgG antibodies studied by skin tests. *Allergy* 1989;44:427-31.
45. O'Hehir RE, Young DB, Kay AB, Lamb JR. Cloned human T lymphocytes reactive with *Dermatophagoides farinae* (house dust mite): a comparison of T- and B-cell antigen recognition. *Immunology* 1987;62:635-40.
46. Kimura JY, Ohta N, Ishii A, Nagano T, Usui M. Functional characterization of lymphocyte response to fractionated house dust mite antigens (*Dermatophagoides pteronyssinus*) in atopic and non-atopic individuals. *Immunology* 1990;70:385-90.
47. Sallusto F, Quintieri F, Pugliese O, Reale G, Pini C, Di Felica G. T-cell and antibody response to parietaria judica allergenic fractions in atopic and nonatopic individuals. *Allergy* 1993;48:37-44.
48. Rawle FC, Burr ML, Platts-Mills TAE. Long-term falls in antibodies to dust mite and pollen allergens in patients with asthma or hay fever. *Clin Allergy* 1983;13:409-17.
49. Till S, Dickason R, Huston D, Humbert M, Robinson D, Larché M, et al. IL-5 secretion by allergen-stimulated CD4⁺ T cells in primary culture: relationship to expression of allergic disease. *J Allergy Clin Immunol* 1997;99:563-9.
50. Marshall JD, Wen Y, Umetsu DT. In vitro synthesis of IL4 by human CD4⁺ T cells requires repeated antigenic stimulation. *Cell Immunol* 1993;152:18-34.
51. Till S, Durham SR, Dickason R, Huston D, Bungre J, Walker CA, et al. IL-13 production by allergen-stimulated T cells is increased in allergic disease and associated with IL-5 but not IFN-γ expression. *Immunology* 1997;91:53-7.