

Phleum pratense-specific T cells of allergic rhinitis patients display a broader recognition pattern than *Phleum pratense*-specific serum immunoglobulin E

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Summary

Background The role of allergen-specific CD4⁺ T lymphocytes in the pathophysiology of atopic disease is well established. Previous studies on allergen-specific T-cell responses have focused on the recognition of single major allergens to identify T-cell epitopes.

Objective However, it is not clear whether immune responses to allergen extracts are exclusively targeted at major allergens or whether additional proteins are recognized.

Methods Here we describe the *Phleum pratense*-specific immunoglobulin E (IgE) and T-cell responses of six allergic rhinitis patients. Reactivity was measured to size-separated fractions of a *P. pratense* extract as well as to the purified major allergens Phl p 1, Phl p 2/3 and Phl p 5.

Results The specificity of the patients' serum IgE, measured in a fluid phase assay, was restricted to one or two of the major allergens. Even though the majority of the patients had IgE antibodies reactive with a single major allergen, one patient reacted with both Phl p 5 and with Phl p 2/3. Analysis of the T-cell repertoire with *P. pratense*-specific T-cell lines (TCLs) and CD4⁺ T-cell clones (TCCs) revealed that at least six different proteins were recognized, including the three major allergens, most notably Phl p 5. Simultaneous production of IL-5 and interferon (IFN) γ was detected in supernatants of the TCLs stimulated with *P. pratense* extract and the major allergens.

Conclusion These results indicate that allergic rhinitis patients have a large pool of circulating allergen-specific CD4⁺ T cells that recognize many different proteins in an allergenic extract, whereas only a small number of these proteins are recognized by serum IgE.

Keywords: allergen-specific T lymphocytes, cytokines, immunoglobulin E, *Phleum pratense*

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Introduction

Grass pollen allergens from grasses belonging to the Poaceae family are a major cause of seasonal allergies, giving rise to clinical manifestations such as rhinitis, rhinoconjunctivitis and asthma. Atopic allergies are characterized by elevated levels of allergen-specific serum immunoglobulin (Ig) E and tissue eosinophilia, two parameters

that are responsible for the immediate and late phase allergic responses, respectively.

It is well established that both IgE production and eosinophilia are dependent on the presence of allergen-specific CD4⁺ T lymphocytes with a bias towards the production of TH2 cytokines [1–3]. These TH2 cells produce interleukin (IL)-4 that induces B lymphocytes to switch to the production of IgE and IL-5 that plays an important role in the differentiation and maturation of eosinophilic granulocytes. Allergen-specific TH2 cells

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have been identified in the peripheral blood as well as in disease sites of allergic patients [4–8] and have been shown to play a direct role in the mucus production in affected airways [9], further demonstrating the clinical relevance of these cells.

Because these TH2-like T cells induce the production of IgE in B cells, most studies characterizing T-cell responses to allergens have focused on major allergens (reviewed in [10]), i.e. allergens to which more than 50% of all patients have IgE. The cloning and sequencing of cDNAs encoding many of these major allergens has prompted the study of T-cell epitopes on major allergens. Recombinant major allergens are currently considered as a potential alternative to allergenic extracts for allergy vaccination [11,12]. T-cell responses to many of these major allergens have therefore been studied in detail and multiple T-cell epitopes have been identified [10].

T-cell reactivity of grass-allergic rhinitis patients with a grass major allergen in the absence of specific serum IgE was recently reported, suggesting that allergen-specific IgE reactivity and T-cell reactivity may not have a complete overlap [13]. Allergenic extracts are complex mixtures of multiple relevant allergens, including major allergens. Characterization of immune responses to the proteins in these extracts is pivotal for our understanding of the disease and for the design of novel allergy vaccination protocols, such as peptide therapy [14,15] or the use of recombinant allergens [11,12].

Allergenic extracts of *Phleum pratense* pollen contain several important allergens, namely Phl p 1 (26–33 kDa), Phl p 2/3 (both at 11 kDa, proteins cannot be separated from each other), Phl p 4 (50–60 kDa), Phl p 5 (a double band at 31–33 kDa, but also a clear breakdown product at 15 kDa) and Phl p 6 (11 kDa). Of these allergens, Phl p 1 and Phl p 5 are the most clinically relevant major allergens [16]. Approximately 80% of grass-allergic patients have IgE to both of these allergens. Group 2/3 allergens bind IgE in the sera of approximately 50% of the patients, whereas group 4 and group 6 allergens are minor allergens.

Grass allergens of the Poaceae family have a high degree of sequence homology between the different species i.e. *Lolium perennis*, *Phleum pratense*, *Poa pratensis* and *Dactylis glomerata* [17–19]. In addition to tree pollen allergens [20–22], house dust mite [23,24] and ragweed allergens [25] and multiple isoforms of grass group 1 and 5 allergens have been shown to exist in nature [18,26–28]. As a result of these sequence homologies and isoallergenic variation, specific IgE antibodies as well as specific T cells display a high level of cross-reactivity, that is highly clinically relevant.

However, T-cell responses to *P. pratense* have only been addressed in studies describing responses to the major allergens Phl p 1 and Phl p 5 [13,29,30]. In the current

study we tried to determine whether the T- and B-cell responses of *P. pratense*-allergic individuals were restricted to the major allergens or if other proteins present in *P. pratense* allergen extract were also the target for immune recognition.

Materials and methods

Patients and IgE reactivity

All six patients (a–f) are clinically allergic to grass pollen and did not receive specific immunotherapy. Type I allergy was further demonstrated by positive skin prick test to *P. pratense* (Soluprick SQ, ALK, Hørsholm, Denmark) and by the presence of grass- (Phl p) pollen specific IgE (Magic Lite SQ specific IgE, ALK). Blood samples were drawn during the pollen season [13]. The study was approved by the local ethics committee.

Grass pollen extract

Pollens from *P. pratense* (Phl p) were obtained from Allergon A/B (Engelholm, Sweden). The pollens were extracted 1:10 (w/v) in 0.125 NH₄HCO₃, pH 8.3 at 4 °C for 24 h and particulate matter was removed by centrifugation (15 000 g). The supernatant was filtered through Whatman filter paper, dialysed against 0.005 mol/L NH₄HCO₃, pH 8.3 overnight and lyophilized.

Size exclusion chromatography (SEC)

The crude Phl p extract (200 mg) were subjected to SEC on a Superdex 75 16/60 column (Pharmacia, Uppsala, Sweden) equilibrated with PBS pH 7.2. The column eluate was monitored by UV (λ = 280 and 225 nm), SDS-PAGE (silver stained, Fig. 1) and fused rocket immunoelectrophoresis (FRIE) to identify known allergen groups. Fractions containing proteins were pooled and diluted to the same OD₂₈₀ value (0.2) for use in IgE binding and T-cell assays.

Purification of Phl p 1, Phl p 5 and Phl p 2/3

The major allergens of Phl p were isolated from the crude pollen extract by a combination of hydrophobic interaction chromatography and SEC. Phl p extract (200 mg) was dissolved in 0.1 mol/L Na acetate pH 5.0, diluted 1:1 (v/v) with 2.0 mol/L (NH₄)₂SO₄ and applied (10 milligrams extract per millilitre column material) on a Octyl-Sepharose column (Pharmacia) equilibrated with 0.05 mol/L Na acetate pH 5.0. The application volume and one column volume (V_t) of 0.05 mol/L Na acetate pH 5.0 were pooled (V₁) and the column was further eluted with two V_t

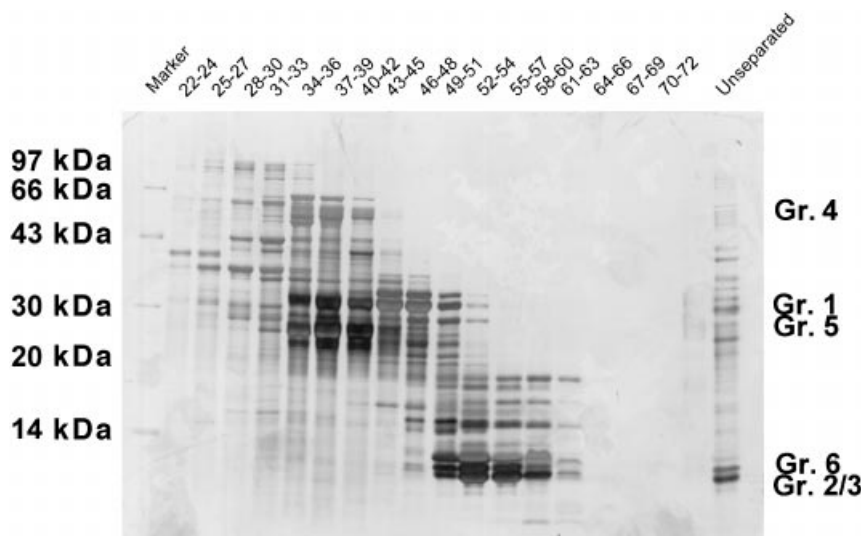


Fig. 1. Silver-stained SDS-PAGE of size-separated *P. pratense* allergen extract. *P. pratense* allergen extract was fractionated by size exclusion chromatography on a Superdex S75 column in PBS. Fractions containing protein were pooled and visualized on a reduced SDS-PAGE gel (20 μ L/lane undiluted). The mobility of known allergen groups are indicated on the right of the gel.

0.05 mol/L phosphate buffer pH 7.2 (V2), two Vt 0.05 mol/L NaHCO₃ pH 8.3 (V3) and finally two Vt 0.05 mol/L NaHCO₃ pH 8.3, 50% (v/v) ethylene glycol (V4). The V4 pool containing only Phl p 5 was dialysed against water and lyophilized. The V1 fraction containing primarily Phl p 1 and Phl p 2/3 was subjected to SEC on a Superdex S75 column equilibrated with 0.05 mol/L NH₄HCO₃ pH 8.3 and the fractions containing either Phl p 1 or Phl p 2/3 were pooled and lyophilized. The allergens were identified by silver-stained SDS-PAGE and immunoblots of the eluted fractions. The purity of the isolated allergens was certified by silver-stained SDS-PAGE of the lyophilized preparations.

IgE inhibition assay

The inhibition of the binding of biotinylated crude Phl p pollen extract to IgE by purified Phl p allergens and pooled SEC fraction was measured in a fluid phase assay. In short, murine monoclonal anti IgE, covalently coupled to paramagnetic particles (PMP, BioMag) was incubated with serum samples, washed thoroughly and further incubated with a mixture of biotinylated Phl p and inhibitor (e.g. Phl p 1, Phl p 5, Phl p 2/3 or pooled SEC fractions). A hundred fold excess (w/w) of inhibitors were used. The amount of biotinylated Phl p bound to the PMP-anti-IgE-IgE phase was estimated as the radioluminescence units (RLU) measured after incubation with streptavidin labelled with acridiniumester. All inhibition assays were performed in triplicate.

Culture conditions

T cells were cultured in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 2 mM L-glutamine (Gibco), 100 IU/mL of Penicillin-Streptomycin (Gibco)

and 5% heat-inactivated, screened human AB + serum (Harlan, UK) (later referred to as complete medium). All cultures were grown under sterile conditions at 37 °C in a humidified atmosphere of 5% CO₂.

Generation of P. pratense-reactive T-cell lines and clones

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly drawn heparinized blood by gradient centrifugation on Lymphoprep (Nycomed, Torshov, Norway). PBMCs (10⁶/mL) were stimulated with 25 μ g/mL *P. pratense* extract (Phl p) in complete medium. Phl p-reactive T-cell lines (TCLs) were established by adding IL-2 on days 5, 6 and 7 (10 U, 5 U and 5 U, respectively) (Chiron, Amsterdam, The Netherlands). This was followed by a restimulation on day 12 of T cells (2 $\times$ 10⁵/mL) with irradiated EBV (JY), allogeneic PBMCs, 10 U IL-2 and 0.5 μ g/mL PHA-P (Difco, Detroit, MI, USA) in complete medium. IL-2 was added on days 3, 4 and 5 (10 U, 5 U, 5 U, respectively). Cultures were split and expanded in complete medium every 3 days. The short-term *P. pratense*-specific T-cell lines were tested for proliferation and cytokine production after the first or second round of restimulation with feeder cells.

Phl p-reactive T-cell clones (TCC) were generated from such short-term lines by limiting dilution and plated at 0.3 or 1 cells/well in 96-well, flat-bottom tissue culture plates in the presence of irradiated EBV (JY), allogeneic PBMC, 10 U IL-2 and 0.5 μ g/mL PHA. After 7–14 days growing clones were expanded in 96-well plates and afterwards in 24-well plates. Fresh feeder cells, antigen and PHA were added every 10 days.

Proliferative responses of Phl p-specific TCLs and TCCs

T-cell lines or cloned T cells were washed twice in RPMI

1640 medium to remove excess PHA and IL-2. Antigen specific proliferation was assessed by stimulation of the T cells, $2-4 \times 10^4$ cells/well, by antigen using 3000 rad-irradiated autologous or matched PBMCs as antigen-presenting cells (10^5 cells/well). The fractionated grass extract was tested at two different concentrations in each experiment ($15 \mu\text{g/mL}$ and $1.5 \mu\text{g/mL}$). The cells were cultured for 72 h, followed by a 16-h pulse in the presence of titrated methyl thymidine ($^3\text{H-TdR}$) (NEN DuPont, Wilmington, DE, USA), $0.5 \mu\text{Ci/well}$. Proliferation was expressed as mean c.p.m. of $^3\text{H-TdR}$ incorporation of triplicate cultures.

Analysis of phenotype of T-cell clones

Phenotype analysis of the TCCs was performed using anti-CD3 (OKT3, ATCC, MD), CD4 (OKT4, Ortho), CD8 (OKT8, ATCC, MD) and anti-mouse IgG-coated Dynabeads (Dyna, Oslo, Norway), adapted from [31]. Viable T cells were obtained from TCC cultures by gradient centrifugation on lymphoprep. The cells were washed twice in RPMI-1640 medium and resuspended in complete medium at 4×10^5 cells/mL. Cells were incubated for 30 min with anti-CD3, anti-CD4 and anti-CD8 mAb and washed twice. Anti-mouse IgG-coated Dynabeads were prepared according to the manufacturer's recommendations. The TCC cells, $250 \mu\text{L/sample}$, were incubated for 10 min at room temperature with excess amounts (7×10^6 beads/sample) the anti mouse-IgG Dynabeads. Non-bound cells were magnetically separated from the Dynabead cell complex and the phenotype of the cells was established by conventional microscopy. This method was compared with regular FACS analysis with comparable results.

Production of supernatants for cytokine measurements

T cells ($2-4 \times 10^4$ /well) were activated for 24 h in the presence or absence of *P. pratense* extract ($25 \mu\text{g/mL}$) in the presence of autologous, irradiated PBMCs (10^5 cells/well) as described above. Cell-free supernatants were collected for cytokine measurements after a 24-h culture period.

IL-4, IL-5 and IFN γ ELISA assays

The presence of IL-4 and IFN γ in the TCC supernatants was measured using an IL-4, IL-5 and interferon (IFN) γ ELISA according to the manufacturer's description (Genzyme, Cambridge, MA, USA).

Results

Size exclusion chromatography

A Phl p pollen extract was subjected to size exclusion

chromatography on a Superdex 75 column and fractions were collected throughout the elution profile. Three neighbouring protein-containing fractions were pooled resulting in a total of 17 different fractions. The protein profile of these fractions are visualized in Fig. 1. The allergens were further identified by electrophoretic mobility and fused rocket immuno-electrophoresis using monospecific rabbit antibodies raised against Phl p 1, Phl p 2/3, Phl p 5 and Phl p 6 (summarized in Table 1).

IgE inhibition

The inhibitory capacity of each of the 17 pooled SEC fractions on the reaction between IgE from six individual allergic sera and biotinylated *P. pratense* pollen extract were estimated by a fluid phase inhibition assay. IgE binding of two of the patients (patients b and d) was inhibited by fractions containing Phl p 2/3 and Phl p 6, IgE binding of one patient was inhibited by fractions containing Phl p 1 (patient f), IgE binding of two patients

Table 1. Distribution of known *P. pratense* allergens in SEC fractions†

Fraction no.	Phl p 1	Phl p 2/3	Phl p 4	Phl p 5	Phl p 6
22–24	–	–	–	–	–
25–27	–	–	–	–	–
28–30	–	–	–	+	–
31–33	–	–	+	+	–
34–36	–	–	++	++	–
37–39	–	–	++	+++	–
40–42	+	–	+	+++	–
43–45	++	–	–	+	–
46–48	++	–	–	+	–
49–51	++	++	–	++	++
52–54	+	+++	–	+++	+++
55–57	–	+++	–	+++	+++
58–60	–	++	–	+++	++
61–63	–	+	–	+	+
64–66	–	–	–	–	–
67–68	–	–	–	–	–
70–72	–	–	–	–	–

†Distribution of *P. pratense* major allergens in the fractions eluted from the size exclusion column (Sephadex 75). The allergens were identified by electrophoretic mobility in silver stained SDS-PAGE, immunoblotting of the SDS-PAGE using murine mAb anti-Phl p 1 or Phl p 5 and by fused rocket immune electrophoresis (FRIE) using poly-specific rabbit anti-Phl p antibodies or with mono-specific rabbit anti-Phl p 1, Phl p 5, Phl p 6 or Phl p 2/3 in the intermediate gel. Scores represent estimated relative amounts of the major allergens

was inhibited by Phl p 5 (patients a and e) and the remaining patient (patient c) appeared to have both Phl p 5- and Phl p 2/3-specific serum IgE (Fig. 2). To exclude the presence of substantial amounts of IgE to other proteins specific to the fractions, inhibition experiments were performed with purified Phl p 1, Phl p 2/3 and Phl p 5 (Fig. 3). These experiments confirmed the IgE reactivity patterns of patients with the major allergens. Notably, patients b and d both reacted with fractions containing Phl p 2/3 and Phl p 6, and were shown to react with Phl p 2/3 (b) or with (an)other protein(s) in the fraction, most likely Phl p 6 (d).

Reactivity of Phl p-specific T-cell lines with separated Phl p fractions

P. pratense-specific T-cell lines generated from all six patients reacted with all of the SEC fractions (Fig. 4). When the TCLs were tested with purified major allergens and Phl p extract, a strikingly higher response was seen in response to the allergen extract than to any of the major allergens (Fig. 5). Reactivity with the major allergens partially reflected their IgE reactivity pattern in four of the patients (a: Phl p 5, f: Phl p 1, e: Phl p 5 and d: none of the major allergens tested). The other two patients did not have a Phl p 5/Phl p 2/3 (c or a Phl p 2/3 (b) dominant T-cell

response, as their serum IgE did. These results clearly indicate that T cells recognize additional proteins present in all TCLs.

Cytokine production by *P. pratense*-specific TCLs

The results shown above might suggest that the T cells that had the same reactivity pattern as the corresponding serum IgE were more biased to the production of TH2-type cytokines than the T cells recognizing additional proteins. To investigate this, supernatants were collected from all six T-cell lines after specific activation with *P. pratense* extract or with the purified major allergens. As is shown in Table 2, all TCLs simultaneously produced IL-5 and IFN γ in response to *P. pratense* extract, which reflects the high levels of specific proliferation to the extract. However, IL-4 was only detected in two of the lines, possibly because several *in vitro* stimulations are required before IL-4 production can be measured [32]. Production of relatively low levels of IL-5 and IFN γ were seen to each of the major allergens, reflecting a lower frequency of T cells specific for a single major allergen than an allergenic extract. This production varied between the six TCLs, dependent on the level of T-cell activation. No qualitative differences in cytokine production were observed between the extract and the major allergens in single patients.

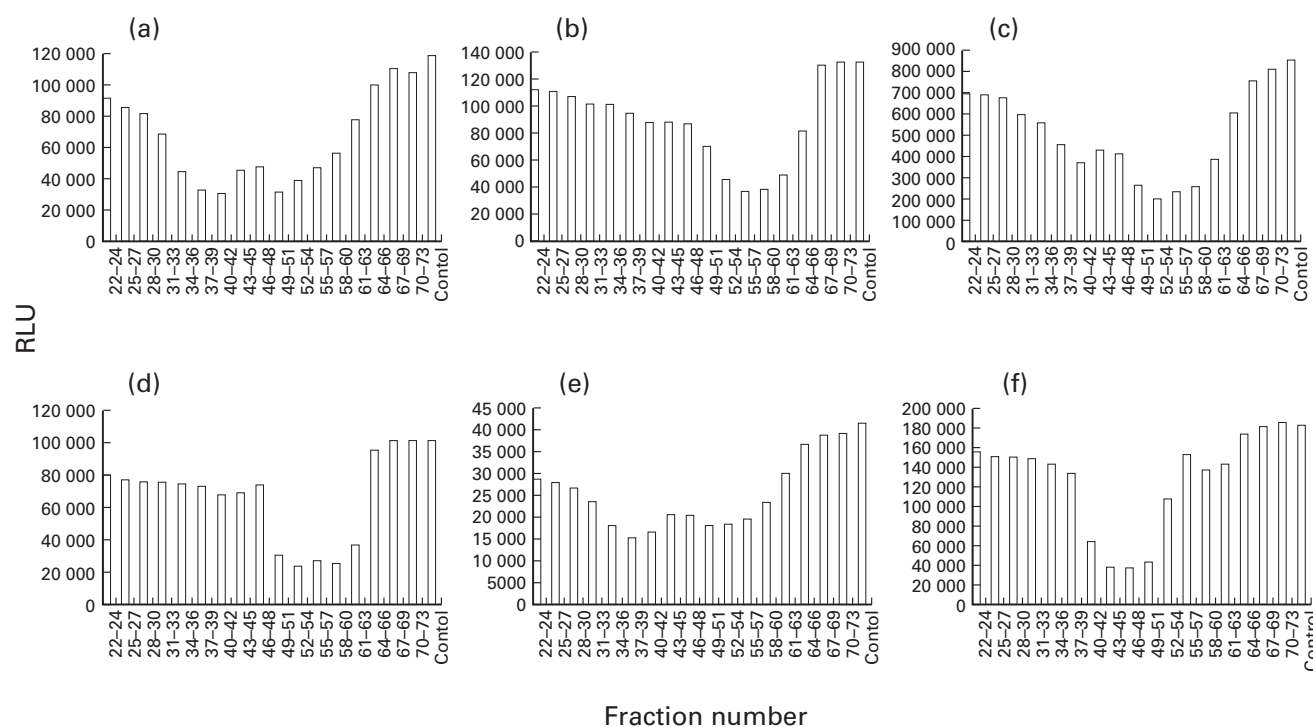


Fig. 2. Inhibition of binding of serum IgE from six allergic rhinitis patients (a–f) to *P. pratense* allergen extract by size-separated fractions of *P. pratense* allergen extract. Values shown are mean RLU of triplicate measurements. The control shown in the figure is a measurement without any inhibiting *P. pratense* proteins present. One of two experiments shown.

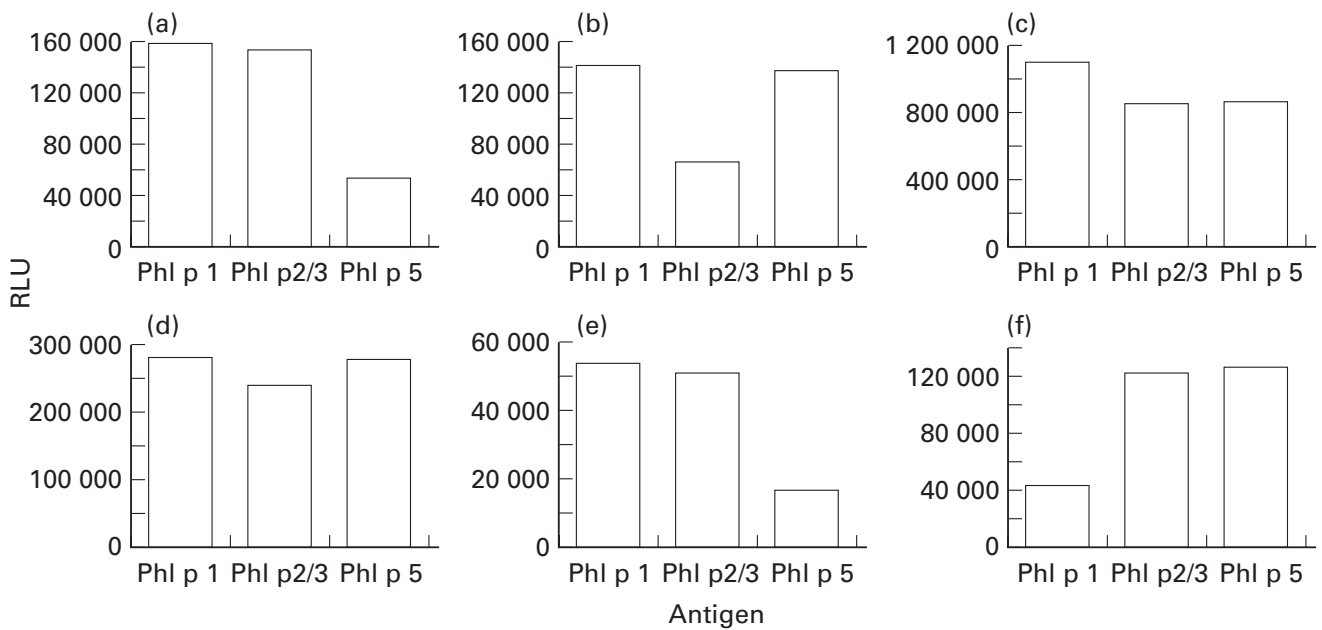


Fig. 3. Inhibition of binding of serum IgE from six allergic rhinitis patients (a-f) to *P. pratense* allergen by purified major allergens. Values shown are mean RLU of triplicate measurements.

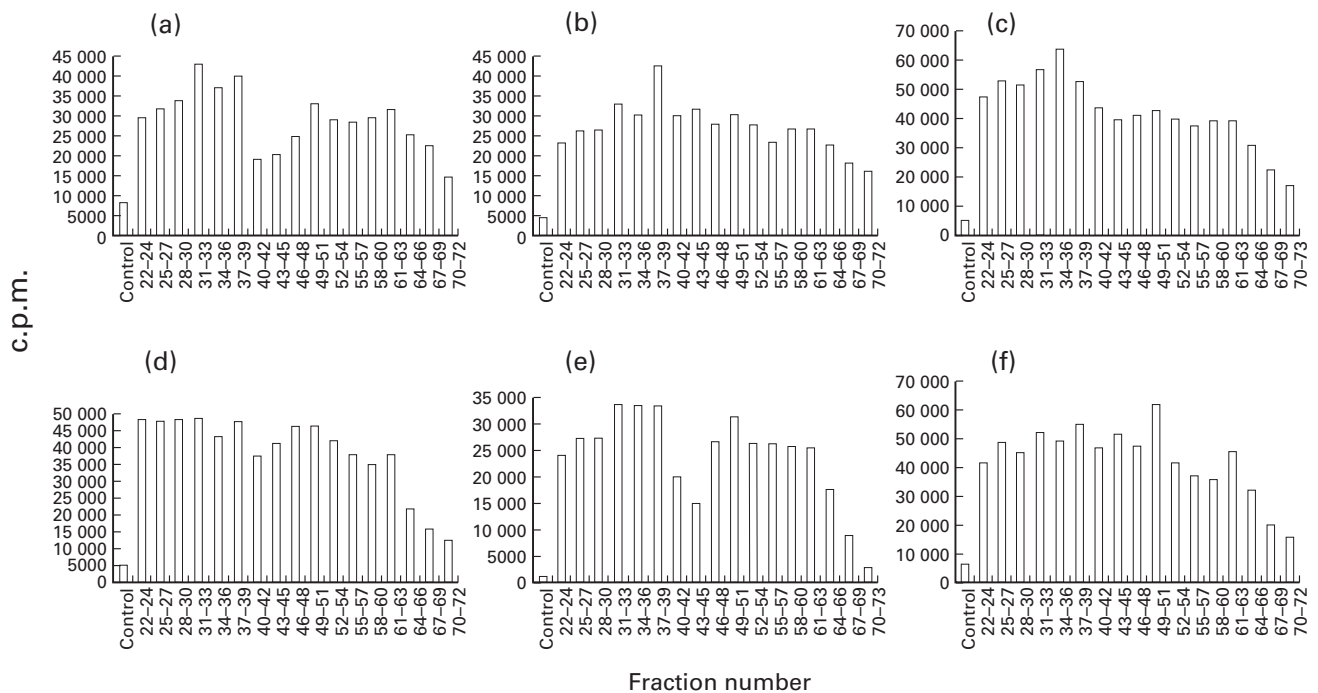


Fig. 4. Reactivity of *P. pratense*-specific T-cell lines of six allergic rhinitis patients (a-f) with size-separated fractions of *P. pratense* allergen extract. The T cells ($2-4 \times 10^4$ /well) were incubated for 48 h with autologous, irradiated PBMCs (10^5 /well) in the presence or absence (control) of pooled fractions of size-separated *P. pratense* extract. Cultures were terminated with a 16-h ^3H -TdR pulse, followed by liquid scintillation counting. Values shown are mean c.p.m. of triplicate cultures tested at a total protein concentration of $15 \mu\text{g/mL}$ for each pooled fraction. The control values shown represent the proliferation of cells without antigen. Results at other concentrations were consistent with the data presented. One out of two experiments shown.

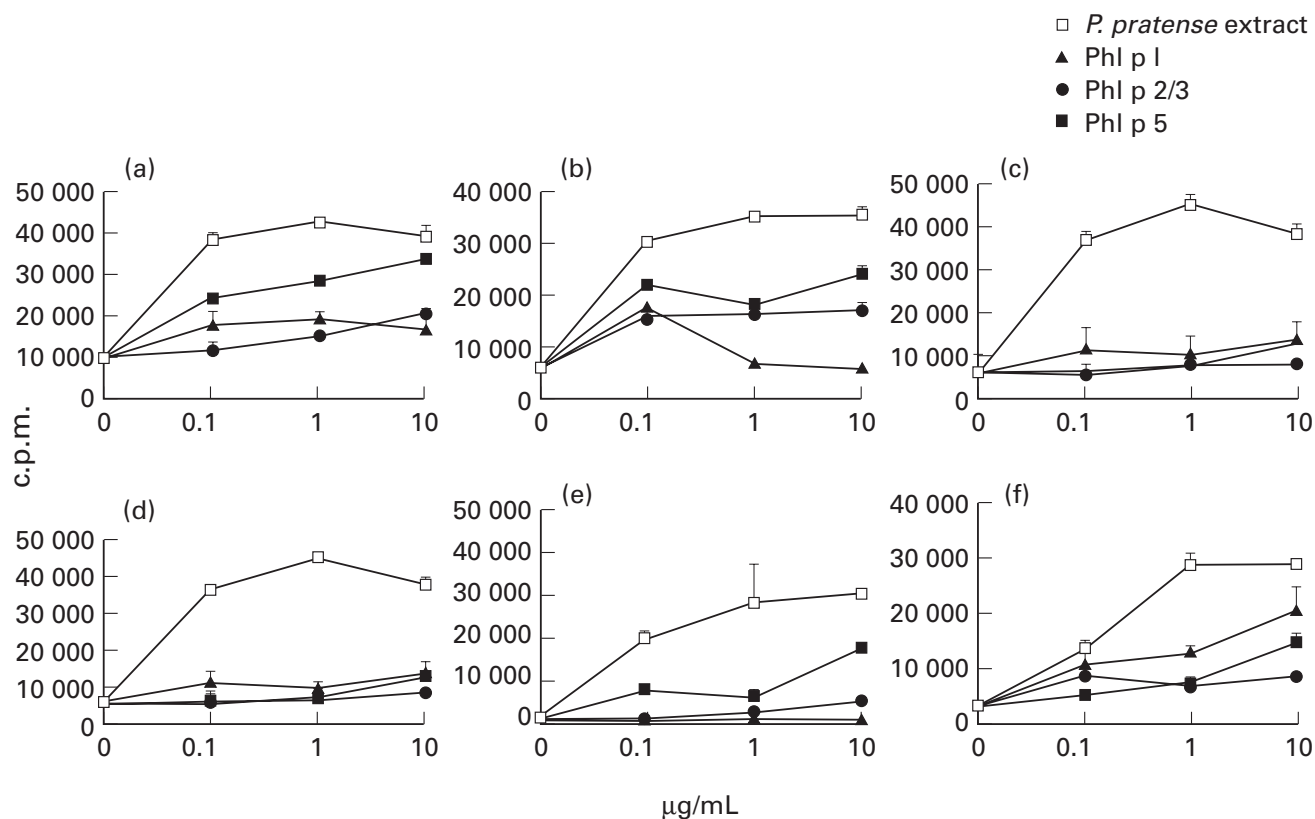


Fig. 5. Reactivity of *P. pratense*-specific T-cell lines of six allergic rhinitis patients (a–f) with *P. pratense* allergen extract and with purified major allergens. The T cells ($2\text{--}4 \times 10^4/\text{well}$) were incubated for 48 h with autologous, irradiated PBMCs ($10^5/\text{well}$) in the presence or absence of three concentrations of *P. pratense* extract, Phl p 1, Phl p 2/3 or Phl p 5. Cultures were terminated with a 16-h 3H-TdR pulse, followed by liquid scintillation counting. Values shown are mean c.p.m. of triplicate cultures. SEM are shown as error bars. One out of two experiments shown.

Reactivity of Phl p-specific TCCs with separated Phl p extract and purified major allergens

The *P. pratense*-specific T-cell lines reacted with very low concentrations of grass allergens. Therefore, one cannot completely exclude that reactivity to all of the SEC fractions was the result of reactivity to minute amounts of a single protein present in all fractions. To exclude this possibility, a panel of *P. pratense*-specific TCCs was generated from four of the patients. The patients selected for cloning had predominantly Phl p 1-specific IgE (f), Phl p 2/3-specific IgE (b), Phl p 5-specific IgE (e) or IgE that reacted with other protein(s) in the low molecular weight area (d). A total of 21 (nine of patient e, nine of patient d, two of patient f and one of patient b) *P. pratense* extract-specific TCCs was generated. All TCCs were $\text{CD}3^+$, $\text{CD}4^+$, $\text{CD}8^-$ and reacted with a limited number of SEC fractions only, with clear peak reactivities (Fig. 6). Interestingly, five of nine TCCs derived from patient e that primarily had Phl p 5-specific IgE reacted with fractions 34–42 or showed a double peak

with fractions 34–42 and 49–63, the fractions that contain Phl p 5 (34–42) or its 15-kDa breakdown product (49–63). As Phl p 5 consists of multiple isoallergenic variants, the reactivity pattern of the TCCs that react with two peaks may possibly be explained by reactivity with a subgroup of isoallergens that is more easily subjected to protein degradation.

None of three TCCs from patient f (that had Phl p 1-specific IgE) reacted with fractions containing Phl p 1 and the remaining three TCC reacted with two different proteins. Four of nine TCCs from patient d (that had IgE directed to a low molecular weight protein other than Phl p 2/3) reacted with low molecular weight fractions and none of one TCC from patient b (that had Phl p 2/3-specific IgE). The other 12 TCC (four of patient a, two of patient f, one of patient b and five of patient d) reacted with fractions that did not contain the major allergens their IgE reacted with. It is clear from Table 4 that in addition to the major allergens at least five or six other proteins are recognized by these TCCs.

Table 2. Cytokine production patterns of *P. pratense*-specific T-cell lines

T-cell line	c.p.m. ($\times 10^{-3}$)	IFN γ	IL-4	IL-5
<i>a</i>				
Control	6.3	<	<	<
<i>P. pratense</i>	34.9	2854	<	76
Phl p 1	20.2	110	<	<
Phl p 2/3	12.5	139	<	<
Phl p 5	19.8	680	<	<
<i>b</i>				
Control	8.0	<	<	<
<i>P. pratense</i>	48.1	2162	<	497
Phl p 1	8.4	<	<	<
Phl p 2/3	11.3	<	<	<
Phl p 5	29.0	726	<	227
<i>c</i>				
Control	9.4	<	<	<
<i>P. pratense</i>	37.0	3037	138	>2000
Phl p 1	13.7	79	<	81
Phl p 2/3	14.6	70	<	101
Phl p 5	15.1	132	<	121
<i>d</i>				
Control	5.3	110	<	<
<i>P. pratense</i>	45.9	1141	73	1288
Phl p 1	27.5	108	<	175
Phl p 2/3	27.1	123	<	186
Phl p 5	28.1	204	<	203
<i>e</i>				
Control	2.0	<	<	<
<i>P. pratense</i>	15.0	3900	<	1006
Phl p 1	1.1	171	<	<
Phl p 2/3	7.5	200	<	<
Phl p 5	12.0	450	<	78
<i>f</i>				
Control	5.2	107	<	<
<i>P. pratense</i>	27.4	999	<	593
Phl p 1	14.0	128	<	157
Phl p 2/3	10.5	110	<	<
Phl p 5	16.6	214	<	86

Cytokine production patterns of *P. pratense*-specific T-cell lines of six allergic rhinitis patients in response to *P. pratense* allergen extract and purified major allergens. The T cells ($2-4 \times 10^4$ /well) were incubated for 24 h with autologous, irradiated PBMCs (10^5 /well) in the presence or absence of three concentrations of *P. pratense* extract, Phl p 1, Phl p 2/3 or Phl p 5. After a 24-h culture period, supernatants were removed and the content of IL-4, IL-5 and IFN γ was measured by ELISA. <: below detection level (50 pg/mL)

When the TCCs were tested for reactivity with the purified major allergens, only eight of 20 TCCs tested were shown to react with major allergens (Table 3). Five of these TCCs reacted with Phl p 5 and three TCCs reacted with Phl p 1. The reactivity pattern of these TCCs was fully

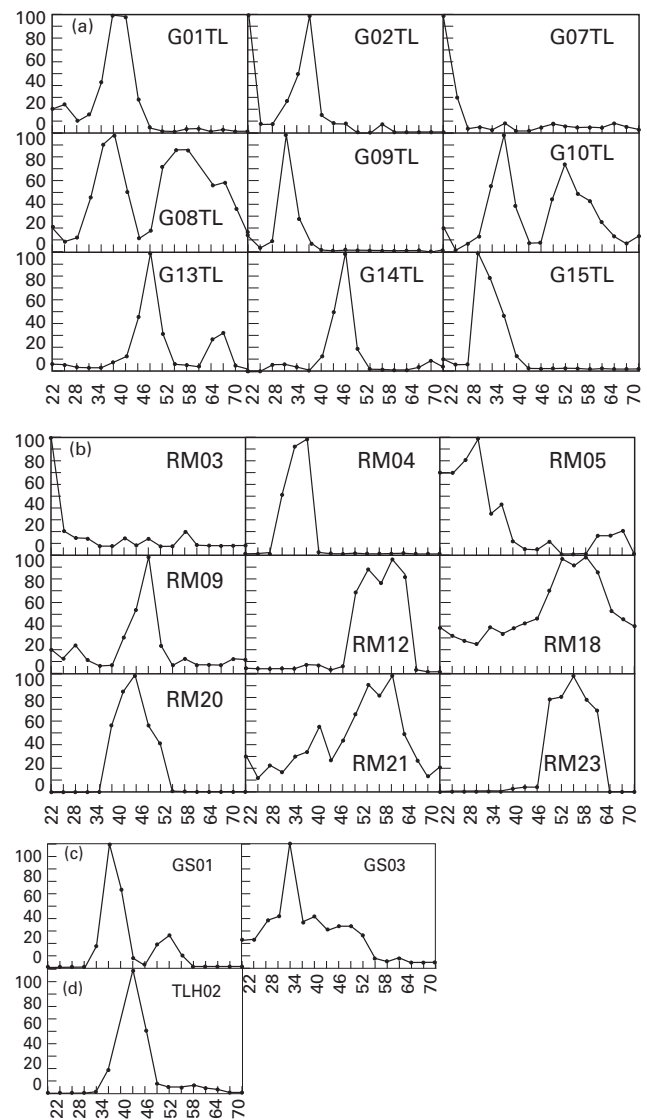


Fig. 6. Reactivity of *P. pratense*-specific T-cell clones of four allergic rhinitis patients (a) patient e, (b) patient d, (c) patient f and (d) patient with size-separated fractions of *P. pratense* allergen extract. The T cells ($2-4 \times 10^4$ /well) were incubated for 48 h with autologous, irradiated PBMCs (10^5 /well) in the presence or absence of pooled fractions of size-separated *P. pratense* extract. Cultures were terminated with a 16-h 3H-TdR pulse, followed by liquid scintillation counting. Values shown are the percentage of T-cell stimulation relative to the maximum stimulation obtained with any of the fractions. The maximum stimulation indexes obtained ranged from 12 (G02TL9–629 (RM20)). All cultures were performed as triplicate cultures.

consistent with reactivity patterns of the TCCs to the SEC fractions.

However, some TCCs reacted exclusively with major allergen-containing fractions but did not react with the corresponding purified major allergen, indicating that additional proteins are recognized. These results cannot

Table 3. Reactivity of *P. pratense*-specific TCCs with purified major allergens

TCC	Control	Phl p	Phl p 1	Phl p2/3	Phl p 5	Specificity
<i>b</i>						
TLH08	2.2	9.9	13.1	2.4	2.4	Phl p 1
<i>d</i>						
RM03	3.3	21.4	0.9	1.2	2.2	other
RM04	0.1	6.4	0.0	0.0	0.1	other
RM05	0.1	3.9	0.0	0.0	1.0	other
RM09	0.1	32.7	25.3	1.4	1.0	Phl p 1
RM12	1.2	21.7	0.1	0.7	0.6	other
RM18	0.3	17.2	0.4	0.2	1.2	other
RM20	0.1	12.5	3.5	0.0	0.0	Phl p 1
RM21	0.7	2.8	0.4	0.4	0.2	other
RM23	1.8	6.7	1.6	1.3	1.3	other
<i>e</i>						
G01TL	0.3	24.1	0.2	0.2	12.8	Phl p 5
G02TL	0.2	12.0	0.1	0.3	7.1	Phl p 5
G07TL	1.0	1.7	0.9	0.5	0.6	other
G08TL	2.9	14.3	3.5	6.4	15.1	Phl p 5
G13TL	0.3	15.1	0.4	0.6	1.2	other
G14TL	0.2	5.2	0.1	0.2	0.1	other
G15TL	0.2	18.2	0.1	0.2	0.9	other
<i>f</i>						
GS01	0.1	27.2	0.1	0.2	31.8	Phl p 5
GS03	0.4	5.9	0.7	0.6	0.5	other
GS09	0.9	17.0	1.0	0.6	18.4	Phl p 5

Reactivity of *P. pratense*-specific T-cell clones of four allergic rhinitis patients with *P. pratense* allergen extract and with purified major allergens. The T cells ($2-4 \times 10^4$ /well) were incubated for 48 h with autologous, irradiated PBMCs (10^5 /well) in the presence or absence of three concentrations of *P. pratense* extract, Phl p 1, Phl p 2/3 or Phl p 5. Cultures were terminated with a 16-h 3H-TdR pulse, followed by liquid scintillation counting. Values shown are mean c.p.m. $\times 10^{-3}$ of triplicate cultures. Values with a stimulation index >3 and a total c.p.m. of >1500 are considered positive and are shown in boldface

be explained by toxicity of the major allergen preparations, as both Phl p 1 and Phl p 2/3 are recognized in a dose-dependent fashion by the TCLs (Fig. 5).

Discussion

The present study clearly shows that the reactivity pattern of *P. pratense*-specific CD4⁺ T cells is much broader than the reactivity pattern of serum IgE of allergic rhinitis patients. In a recent study we reported that some grass-allergic rhinitis patients displayed T-cell reactivity to group 5 grass allergens in the absence of detectable group 5-specific serum IgE [13]. The present report confirms and extends this observation to additional grass allergens, indicating that T-cell reactivity to proteins in allergenic extracts in the absence of serum IgE may be a general rule rather than an exceptional finding.

The patients studied had serum IgE that was primarily directed against the major allergens Phl p 1, Phl p 2/3 and

Phl p 5. The IgE reactivity was relatively narrow, as only a few fractions and only one or two of the major allergens appeared to be able to inhibit binding of Phl p-specific serum IgE for each of the patients. In contrast T-cell reactivity was much broader with T cells recognizing not only the major allergens, but also several other proteins even including high molecular weight proteins, to which IgE reactivity has not been reported (summarized in Table 4). Only a few other studies have described T-cell reactivity towards proteins other than purified major allergens, but no comparisons with IgE from the same patients have been made [33,34].

This finding raised the question whether T cells reacting with proteins in allergenic extracts to which there is no detectable IgE is the result of a different cytokine production pattern or the mere result of a frequency that is too low to induce detectable serum IgE levels. The Phl p-specific TCLs described here all produced IFN γ and IL-5 simultaneously, but in contrast with previous observations no

Table 4. Summary of T-cell and IgE-reactivity patterns from results shown in Figs 1–6 and Tables 1–3

Patient	IgE (major)	TCL (major)	IgE (fractions) TCC (major)	TCL (fractions)	TCC (fractions)	
a	Phl p 5	Phl p 5	nd ²	34–42, 49–57 (Phl p 5)	All	ND
b	Phl p 2/3	Phl p 5, Phl p 2/3	Phl p 1	49–63 (Phl p 2/3)	All	40–48 (Phl p 1)
c	Phl p 5 + Phl p 2/3	None	ND	34–42, 49–57 (Phl p 2/3, Phl p 5)	All	ND
d	None	None	Other, Phl p 1	49–63 (not Phl p 2/3)	All	40–51 (Phl p 1) Unidentified: 49–63 (not Phl p 2/3) 22–23, 22–33, 31–39, 37–48,
e	Phl p 5	Phl p 5	Phl p 5, other	34–42, 49–57 (Phl p 5)	All	34–45 ± 49–63 (Phl p 5), Unidentified: 40–51 (not Phl p 1), 22–23, 28–33, 31–42
f	Phl p 1	Phl p 1	Phl p 5, other	40–51 (Phl p 1)*	All	34–42 (Phl p 5) Unidentified: 31–39

ND – not done. *Reactivity of TCC that did not react with any of the major allergens tested

qualitative differences were observed in responses to the major allergens. A possible explanation may be that the TCLs were generated *in vitro* with a *P. pratense* pollen allergen extract. This means that all TCLs, irrespective of their fine specificity, have been selected *in vitro* under the same circumstances, resulting in identical cytokine profiles in all TCLs.

Interestingly, evidence is accumulating from many studies that the cytokine profiles of allergen-specific T cells may vary with the allergen studied and the clinical manifestations of the allergy. Clearcut mite- and cat dander-specific TH2 cells have been isolated from the peripheral blood of allergic asthma patients or atopic dermatitis patients [35–37]. These patients have significantly higher IgE responses than do rhinitis patients. In addition, the exposure to 'typical' asthma-inducing allergens such as mite and cat is perennial, whereas exposure to 'typical' rhinitis allergens such as grass and tree pollen allergens is season related. Most studies with grass and tree pollen-specific T cells from the peripheral blood of rhinitis patients to date have demonstrated a more TH0-like response which is only slightly biased towards a higher production of TH2 cytokines than the response of control donors [38]. *In situ*, however, it appears that there is a more TH2-like response [39–42] and the production levels of IL-5, that is not detected in PBMCs of nonallergic donors, have been

linked to disease severity [43]. This may indicate that rhinitis patients have a TH2-like response in the nasal mucosa and after subcutaneous allergen challenge as a result of local end-stage differentiation or, alternatively, of specific homing of TH2 cells [44].

The recently identified differential expression of chemokine receptors and chemokine chemotaxis by TH2 vs TH1 cells may play a role in this selective homing. Most TH2 cells have been shown to preferentially express CCR8 [45] and CCR3 [44], whereas TH1 cells preferentially express CCR5 [46]. This may result in a selective influx of CCR3/CCR8-expressing TH2 cells after the production of certain chemokines in affected tissues.

This pool of tissue-residing TH2 cells may continuously be replenished by immature, circulating allergen-specific TH0-like cells that express low levels of CCR3 or CCR8 and that locally mature into TH2 cells. However, in this model there still is a large pool of circulating allergen-specific T cells that still have the ability to differentiate into TH0 or TH1, rather than TH2 cells.

Specific immunotherapy, recently termed specific allergy vaccination in the WHO report on specific allergy treatment [47], has been shown to induce a multiplicity of immunological effects, including a notable change in the production of primarily TH2 to the production of TH1 type cytokines [48–51], an increased production of IL-10 [52], a decreased

proliferative response of peripheral allergen-specific T cells [51,53], a decrease in the influx of both CD4⁺ T cells and eosinophils [48,54], a sharp increase of specific IgG4 serum antibodies [55,56], an increase in the number of IL-12⁺ cells [57] and a decreased number of circulating basophils [58]. All these changes may contribute to the improvements in symptom and medication scores and paraclinical effects such as decreased late phase reactivity. It is not clear at the present time exactly how these effects are exerted on the immune system. High antigen doses and the subcutaneous injection route, in contrast with low doses introduced on mucosal surfaces as is the case in natural exposure, may preferentially result in IFN γ -dominated responses. The results shown here may indicate that there is another factor that may have an impact on the outcome of specific allergy vaccination. The apparent lack of a complete overlap between T-cell specificity and IgE specificity may indicate that circulating allergen-specific T cells that do not induce IgE are activated when allergic patients undergo specific allergy vaccination, and that these cells may contribute to a biasing of the immune response towards a more TH1 or TH0-like response that is characterized by the production of IgG4, rather than IgE [59]. A discrepancy between the occurrence of allergen-specific IgG and IgE in allergic patients has been described previously [60,61], supporting this theory. New SIT protocols are currently focusing on the use of peptides or recombinant allergens. However, it is not clear if allergy to, for example, grass pollen allergens can be treated by a single allergen or whether the presence of additional molecules is required. A clinical study by sterballe *et al.* has shown that patients that are only sensitized to Phl p 1 and Phl p 5 can be treated equally effective with these two purified allergens, whereas patients that reacted to additional proteins were treated more effectively with the *P. pratense* extract than with the purified major allergens only [16]. The results of the present study indicate, that T-cell responses to many allergens can be detected in the peripheral blood of all allergic patients, including those patients that have specific IgE to a limited number of allergens only. This may indicate that treatment with recombinant allergens (representing single isoforms) or peptides thereof may only cover a part of the specific immune repertoire of any given patient. Additional clinical studies on the use of single major allergens vs allergenic extracts may yield further information on the feasibility of such an approach. Considering the present results, the discrepancy between T-cell specificity and IgE specificity may indicate that a large number of allergen-specific T cells do not support the induction of IgE production. These cells may become activated when allergic patients are undergoing specific allergy vaccination with allergen extracts and may be instrumental in biasing the immune response from a TH2-like profile towards the TH0 or TH1 type.

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References

- 1 Wierenga EA, Snoek M, de Groot CJ *et al.* Evidence for compartmentalization of functional subsets of CD4⁺ T lymphocytes in atopic patients. *J Immunol* 1990; 144:4651–6.
- 2 Kapsenberg ML, Wierenga EA, Bos JD, Jansen HM. Functional subsets of allergen-reactive human CD4⁺ T cells. *Immunol Today* 1991; 12:392–5.
- 3 Parronchi P, Macchia D, Piccinini M *et al.* Allergen- and bacterial antigen-specific T-cell clones established from atopic patients show a different profile of cytokine production. *Proc Natl Acad Sci USA* 1991; 88:4538–42.
- 4 Del Prete GF, De Carli M, D'Elia MM *et al.* Allergen exposure induces the activation of allergen-specific Th2 cells in the airway mucosa of patients with allergic respiratory disorders. *Eur J Immunol* 1993; 23:1445–9.
- 5 Robinson DS, Hamid Q, Ying S *et al.* Predominant Th2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992; 326:298–304.
- 6 Metz DP, Hingorani M, Calder VL, Buckley RJ, Lightman SL. T-cell cytokines in chronic allergic eye disease. *J Allergy Clin Immunol* 1997; 100:817–24.
- 7 van der Heijden FL, Wierenga EA, Bos JD, Kapsenberg ML. High frequency of IL-4-producing CD4⁺ allergen-specific T lymphocytes in atopic dermatitis lesional skin. *J Invest Dermatol* 1991; 97:389–94.
- 8 Ramb-Lindauer C, Feldmann A, Rotte M, Neumann C. Characterization of grass pollen reactive T-cell lines derived from lesional atopic skin. *Arch Dermatol Res* 1991; 283:71–6.
- 9 Cohn L, Homer RJ, Marinov A, Rankin J, Bottomly K. Induction of airway mucus production by T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J Exp Med* 1997; 186:1737–47.
- 10 van Neerven RJJ, Ebner C, Yssel H, Kapsenberg ML, Lamb JR. T lymphocyte responses to allergens: Epitope-specificity and clinical relevance. *Immunol Today* 1996; 17:526–32.
- 11 Scheiner O, Kraft D. Basic and practical aspects of recombinant allergens. *Allergy* 1995; 50:384–91.
- 12 Valenta R, Vrtala S, Spitzauer S, Kraft D. Recombinant allergens. *Allergy* 1998; 53:552–61.
- 13 Würtzen PA, van Neerven RJJ, Arnved J, Ipsen H, Sparholt SH. Dissection of the grass allergen-specific immune response of in allergic patients and controls: Elevated T cell proliferation in patients does not correlate with specific serum IgE and skin reactivity. *J Allergy Clin Immunol* 1998; 101:241–9.
- 14 Norman PS, Ohman JL, Long AA *et al.* Treatment of cat allergy with T-cell reactive peptides. *Am J Respiratory Crit Care Med* 1996; 154:1623–8.
- 15 Counsell CM, Bond JF, Ohman JLJ, Greenstein JL,

- Garman RD. Definition of the human T cell epitopes of Fel d 1, the major allergen of the domestic cat. *J Allergy Clin Immunol* 1996; 98:884–94.
- 16 sterballe O, Ipsen H, Weeke B, Lwenstein H. Specific IgE response toward allergenic molecules during perennial hypersensitization: a three year prospective double blind study. *J Allergy Clin Immunol* 1983; 71:40–6.
 - 17 Klysner S, Welinder KG, Lowenstein H, Matthiesen F. Group V allergens in grass pollens: similarities in amino acid composition and NH₂-terminal sequences of the group V allergens from *Lolium perenne*, *Poa pratensis* and *Dactylis glomerata*. *Clin Exp Allergy* 1992; 22:491–7.
 - 18 Vrtala S, Sperr WR, Reimitzer I 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.
 - 19 Smith PM, Ong EK, Knox RB, Singh MB. Immunological relationship among group 1 and group 5 allergens from grass pollen. *Mol Immunol* 1994; 31:491–8.
 - 20 Breiteneder H, Pettenburger K, Bito A et al. The gene coding for the major birch allergen, Bet v I, is highly homologous to a pea disease resistance response gene. *EMBO J* 1989; 8:1935.
 - 21 Schenk S, Hoffmann-Sommergruber K, Breiteneder H et al. Four recombinant isoforms of Cor a 1, the major allergen of hazel pollen, show different reactivity with allergen-specific T lymphocyte clones. *Eur J Biochem* 1994; 224:717.
 - 22 Ferreira F, Hirtenlehner K, Jilek A et al. Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: Potential use of hypoallergenic isoforms for immunotherapy. *J Exp Med* 1996; 183:599–609.
 - 23 Chua KY, Huang CH, Shen HD, Thomas WR. Analysis of sequence polymorphism of a major mite allergen, Der p 2. *Clin Exp Allergy* 1996; 26:829–37.
 - 24 Smith WA, Thomas WR. Sequence polymorphisms of the Der p 3 house dust mite allergen. *Clin Exp Allergy* 1996; 26:571–9.
 - 25 Rafnar T, Griffith IJ, Kuo M, Bond JF, Rogers BL, Klapper DG. Cloning of Amb a 1 (Antigen E), the major allergen family of short ragweed pollen. *J Biol Chem* 1991; 266:1229–36.
 - 26 Perez M, Ishioka GY, Walker LE, Chestnut RW. cDNA cloning and immunological characterisation of the rye grass allergen Lol p 1. *J Biol Chem* 1990; 265:16210–5.
 - 27 Bufe A, Schramm G, Keown MB, Schlaak M, Becker WM. Major allergen Phl p 5b in timothy grass is a novel pollen RNase. *FEBS Lett* 1995; 363:6.
 - 28 Laffer S, Valenta R, Vrtala S et al. Complementary cDNA cloning of the major allergen Phl p 1 from Timothy grass (*Phleum pratense*); recombinant Phl p 1 inhibits IgE binding to group 1 allergens from eight different grass species. *J Allergy Clin Immunol* 1994; 94:689–98.
 - 29 Schenk S, Breiteneder H, Susani M et al. T-cell epitopes of Phl p 1, major pollen allergen of timothy grass (*Phleum pratense*): evidence for crossreacting and non-crossreacting T-cell epitopes within grass group I allergens. *J Allergy Clin Immunol* 1995; 96:986–96.
 - 30 Muller WD, Karamfilov T, Fahlbusch B, Vogelsang H, Jager L. Analysis of human T cell clones reactive with group V grass pollen allergens. *Int Arch Allergy Immunol* 1994; 105:391–6.
 - 31 Karpati RM, Banks SM, Malissen B et al. Phenotyping characterization of murine tumor-infiltrating T lymphocytes. *J Immunol* 1991; 146:2043–51.
 - 32 Marshall JD, Wen Y, Abrams JS, Umetsu DT. In vitro synthesis of IL-4 by human CD4⁺ T cells requires repeated antigenic stimulation. *Cell Immunol* 1993; 152:18–34.
 - 33 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.
 - 34 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.
 - 35 Wierenga EA, Snoek M, Bos JD, Jansen HM, Kapsenberg ML. Comparison of diversity and function of house dust mite-specific T lymphocyte clones from atopic and non-atopic donors. *Eur J Immunol* 1990; 20:1519–26.
 - 36 van Neerven RJJ, van’t Hof W, Ringrose JH et al. T cell epitopes of house dust mite major allergen Der p II. *J Immunol* 1993; 151:2326–35.
 - 37 van Neerven RJJ, van de Pol MM, van Milligen FJ, Jansen HM, Aalberse RC, Kapsenberg ML. Characterization of cat dander-specific T lymphocytes from atopic patients. *J Immunol* 1994; 152:4203–10.
 - 38 Ebner C, Schenk S, Najafian N et al. Nonallergic individuals recognize the same T cell epitopes of Bet v 1, the major birch pollen allergen, as atopic patients. *J Immunol* 1995; 154:1932–40.
 - 39 Kay AB, Ying S, Varney V et al. Messenger RNA expression of the cytokine gene cluster, interleukin 3 (IL-3), IL-4, IL-5, and granulocyte/macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous reactions in atopic subjects. *J Exp Med* 1991; 173:775–8.
 - 40 Durham SR, Ying S, Varney VA et al. Cytokine messenger RNA expression for IL-3, IL-4, IL-5, and granulocyte/macrophage-colony-stimulating factor in the nasal mucosa after local allergen provocation: relationship to tissue eosinophilia. *J Immunol* 1992; 148:2390–4.
 - 41 Ying S, Meng Q, Barata LT, Robinson DS, Durham SR, Kay AB. Associations between IL-13 and IL-4 (mRNA and protein), vascular cell adhesion molecule-1 expression, and the infiltration of eosinophils, macrophages, and T cells in allergen-induced late-phase cutaneous reactions in atopic subjects. *J Immunol* 1997; 158:5050–7.
 - 42 Barata LT, Ying S, Meng Q et al. IL-4- and IL-5-positive T lymphocytes, eosinophils, and mast cells in allergen-induced late-phase cutaneous reactions in atopic subjects. *J Allergy Clin Immunol* 1998; 101:222–30.
 - 43 Till S, Dickason R, Huston D 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.
 - 44 Sallusto F, Mackay CR, Lanzavecchia A. Selective expression

- of the eotaxin receptor CCR3 by human Th2 cells. *Science* 1997; 277:2005–7.
- 45 Zingoni A, Soto H, Hedrick JA et al. The chemokine receptor CCR8 is preferentially expressed in Th2 but not Th1 cells. *J Immunol* 1998; 161:547–51.
- 46 Loetscher P, Uguccioni M, Bordoli L, Baggiolini M, Moser B. CCR5 is characteristic of Th1 lymphocytes. *Nature* 1998; 391:344–5.
- 47 Bousquet J, Lockey RF, Malling HJ, eds. WHO position paper allergen immunotherapy: therapeutic vaccines for allergic diseases. *Allergy* 1998; 53 (Suppl.): 1–42.
- 48 Varney VA, Hamid QA, Gaga M 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.
- 49 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; 178:2123–30.
- 50 Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Muller 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.
- 51 Ebner C, Siemann U, Bohle B et al. Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from Th2 to Th1 in T-cell clones specific for Phl p 1, a major grass pollen allergen. *Clin Exp Allergy* 1997; 27:1007–15.
- 52 Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of Interleukin 10 in specific immunotherapy. *J Clin Invest* 1998; 102:98–106.
- 53 Akdis CA, Akdis M, Blesken T et al. Epitope-specific T cell tolerance to phospholipase A2 in bee venom immunotherapy and recovery by IL-2 and IL-15 in vitro. *J Clin Invest* 1996; 98:1671–83.
- 54 Durham SR, Ying S, Varney VA 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.
- 55 Wittman AM, Stapel SO, Sjamsoedin DH, Jansen HM, Aalberse RC. Fel d 1-specific IgG antibodies induced by natural exposure have blocking activity in skin tests. *Int Arch Allergy Immunol* 1996; 109:369–75.
- 56 Golden DB, Meyers DA, Kagey-Sobotka A, Valentine MD, Lichtenstein LM. Clinical relevance of the venom-specific immunoglobulin G antibody level during immunotherapy. *J Allergy Clin Immunol* 1982; 69:489–93.
- 57 Hamid QA, Schotman E, Jacobson MR, Walker SM, Durham SR. Increases in IL-12 messenger RNA⁺ cells accompany inhibition of allergen-induced late skin responses after successful grass pollen immunotherapy. *J Allergy Clin Immunol* 1997; 99:254–60.
- 58 Jutel M, Muller UR, Fricker M, Rihs S, Pichler WJ, Dahinden C. Influence of bee venom immunotherapy on degranulation and leukotriene generation in human blood basophils. *Clin Exp Allergy* 1996; 26:1112–8.
- 59 Yamashita N, Takeno M, Kaneko S, Mizushima Y, Sakane T. Therapeutic effects of preferential induction of mite-specific T helper 0 clones. *Clin Exp Immunol* 1997; 109:332–41.
- 60 Desvaux FX, Peltre G, David B. Characterisation of grass pollen-specific IgE, IgA, IgM classes and IgG subclasses in allergic patients. *Int Arch Allergy Appl Immunol* 1989; 89:281–7.
- 61 Jarolim E, Rumpold H, Endler AT et al. IgE and IgG antibodies of patients with allergy to birch as tools to define the allergen profile of *Betula verrucosa*. *Allergy* 1989; 44:385–95.