

ORIGINAL ARTICLE

Phleum pratense alone is sufficient for allergen-specific immunotherapy against allergy to Pooideae grass pollens

C. Hejl*, P. A. Wurtzen*, J. Kleine-Tebbe†, N. Johansen*, L. Broge* and H. Ipsen*

*ALK-Abelló A/S, Hørsholm, Denmark and †Allergy and Asthma Center Westend, Berlin, Germany

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Summary

Background Specific immunotherapy is the only causal treatment of allergy available today. Traditionally, therapeutic products based on either a single grass species or a mix of such extracts are used for grass pollen immunotherapy. Investigations comparing the immunological response to these allergen preparations are needed to ensure optimal treatment. The objective of this study was to investigate patterns of T and B cell cross-reactivity to Pooideae single-species extracts and to extract mixes.

Methods IgG4 induced by immunotherapy with *Phleum pratense* extract was investigated for cross-reactivity using nine single-species extracts and four mixes. For the mixes, studies of IgE cross-reactivity were also performed. T cell cross-reactivity was investigated in lines specific to nPhl p 1 or nPhl p 5 allergens, and the amounts of group 1 and 5 allergens in the extracts were quantified by a single radial immunodiffusion.

Results The levels of treatment-induced IgG4 detected by all the extracts displayed a clear correlation to that detected by the *P. pratense* pollen extract. The IgE studies confirmed the cross-reactivity of *P. pratense*-specific B cells towards the allergens contained in the mixes, and the T cell studies demonstrated cross-reactivity towards group 1 and 5 major allergens in extracts of six temperate grass species.

Conclusion Extensive T and B cell cross-reactivity was observed towards the allergens of the Pooideae grasses, and the degree of B cell cross-reactivity was independent of the number of species included in the extract mixes. This implies that treatment with pollen extract of just one Pooideae species will affect the allergic responses caused by any of the temperate grasses in this subfamily.

Keywords cross-reactivity, grass, IgE, IgG4, immunotherapy, mix, *Phleum pratense*, Pooideae, T cell

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Correspondence:

Henrik Ipsen, Bøge Allé 6–8, DK 2970 Hørsholm, Denmark.

E-mail: hi@dk.alk-abello.com

Introduction

Allergen-specific immunotherapy is widely used and is recognized by the WHO as the only causal treatment for Type 1 allergy available today [1]. The choice of allergen source for immunotherapeutic products is straightforward if the allergen source is well defined and contains only a few major allergens. However, closely related allergen sources often induce similar symptoms. This phenomenon results from cross-reactivity, defined as binding to the same receptors [i.e. T cell receptors (TCR) or antibody (Ig)] by structurally related allergens from different sources. Specific immunotherapy may be performed with allergen preparations composed of mixes of extracts from several

related species or preparations based on a single extract from an individual species. The use of single-species products simplifies standardization procedures, reducing the risk of side-effects and avoiding dilution of the individual allergens [2]. However, species distribution and prevalence are geographically non-uniform, leading to heterogeneity in exposure profiles, and immunotherapy with combined extracts (mixes) might ensure that patients with different sensitization patterns benefit equally from the treatment. This option poses the question of how many species to include in such combined products – and which species to select. Investigations of the immunological responses towards the various species in each allergen family are needed in order to find allergen extracts

leading to optimal effect of treatment in patients with diverse sensitization patterns.

The grasses constitute more than 10 000 individual species, and the choice of an optimal treatment is not immediately obvious. Investigations of B and T cell cross-reactivity for a series of Pooideae grasses were therefore initiated and are reported in this work and a recently submitted manuscript (N. Johansen *et al.*, personal communication).

The present report includes the investigation of the T cell reactivity to grass allergen extracts of nPhl p 1- and nPhl p 5-specific T cell lines from grass pollen-sensitized subjects. Also included are measurements of the binding of IgG4 from post-immunotherapy sera to the allergens of pollen extracts from individual Pooideae grasses, in order to investigate the cross-reactivity of antibodies induced by a clinically effective treatment with *Phleum pratense* pollen extract.

Furthermore, binding and inhibition studies of IgE cross-reactivity are reported, searching for possible differences in the cross-reactivity patterns for pollen extracts of individual grass species vs. four different mixes. The above-mentioned IgG4 experiments were also performed for the mixes. The constituents of the mixes were chosen to mimic the contents of commercially available mixed products. Finally, the content of group 1 and 5 allergens in nine grass allergen extracts was determined by single radial immunodiffusion (SRID) to determine the absolute content and the ratio between the two major allergens in the individual extracts. In all experiments, *P. pratense* was chosen as the reference due to the documented clinical efficacy of immunotherapy with this species (Table 1).

Methods

Study populations and samples

B cell cross-reactivity. Samples drawn before immunotherapy were used for the IgE determinations, and

samples drawn after immunotherapy were used for the IgG4 determinations. Two groups of serum samples were used:

Group A: Serum samples from 29 subjects participating in a clinical trial with 28 days of sublingual, tablet-based specific immunotherapy based on *P. pratense* pollen extract. The trial included children from Germany aged 5–12 years with grass pollen-induced rhinoconjunctivitis (with/without mild to moderate asthma) [3].

Group B: Serum samples from an in-house serum bank, drawn from 20 grass pollen-sensitized subjects in Denmark or Germany, with 1–3 years of treatment with subcutaneous specific immunotherapy based on *P. pratense* pollen extract. All sera displayed *in vitro* reactivity to *P. pratense* (IgE $\geq$ 3.5 kU/L).

T cell stimulation. Blood samples were obtained from a total of 20 grass pollen-sensitized subjects (*P. pratense* IgE $\geq$ 0.7 kU/L, no history of grass pollen immunotherapy), shown to release histamine in response to purified nPhl p 1 and/or nPhl p 5 (> 10 nm histamine at 5 ng/mL major allergen), as described previously [4].

Statistics

IgE and IgG4 responses were analysed by Spearman's rank correlation test. The Spearman rank correlation coefficients are presented with two-sided 95% confidence intervals. All stochastic probabilities < 0.05 were considered significant.

Methods and materials

Pollen extracts were obtained from ALK-Abelló, Hørsholm, Denmark, manufactured as described previously [5]. *P. pratense* extract was taken from a production batch for the ALK grass allergen tablet, Grazax[®]. Batches of the different extracts used for the antibody measurements were selected at random.

Table 1. Symptom and medication reduction documented for registered immunotherapy products based on the single species *Phleum pratense*

	Number of subjects	Nationality of the subjects (number of subjects)	Reduction symptom score (%)	Reduction medication score (%)
<i>Tablet-based immunotherapy</i>				
Grazax [®] (<i>P. pratense</i>) [10]	277	DE (60), BE (12), DK (43), SE (64), AU (17), NO (14), UK (20), CA (47)	21	29
Grazax [®] (<i>P. pratense</i>) [36]	114	DK (96), SE (18)	37	41
Grazax [®] (<i>P. pratense</i>) [9]	634	AU (25), DK (122), DE (152), IT (70), NL (40), ES (46), SE (63), UK (116)	30	38
<i>Subcutaneous immunotherapy</i>				
Alutard ^{®*} (<i>P. pratense</i>) [11]	347	UK (347)	29	32

AU, Austria; BE, Belgium; CA, Canada; DE, Germany; DK, Denmark; ES, Spain; IT, Italy; NL, the Netherlands; NO, Norway; SE, Sweden; UK, United Kingdom.

*ALK-Abelló, Hørsholm, Denmark.

All reagents were used without further modification.

IgE studies. The four mixes investigated consisted of pollen extracts from 5 to 10 grass species, all from the Pooideae subfamily. The pollen extracts of the individual constituent species were mixed in equal quantities on a milligram basis. The species contained in each of the four combined grass pollen extracts are listed below:

Mix 1: *Arrhenatherum elatius* (*Avena elatior*), *Dactylis glomerata*, *Festuca pratensis*, *Lolium perenne*, *P. pratense*, *Poa pratensis* and *Secale cereale*.

Mix 2: *Anthoxanthum odoratum*, *D. glomerata*, *L. perenne*, *P. pratense* and *P. pratensis*.

Mix 3: *D. glomerata*, *F. pratensis*, *Holcus lanatus*, *L. perenne*, *P. pratense* and *P. pratensis*.

Mix 4: *Agrostis stolonifera*, *A. odoratum*, *A. elatius* (*A. elatior*), *D. glomerata*, *F. pratensis*, *H. lanatus*, *L. perenne*, *P. pratense*, *P. pratensis* and *S. cereale*.

Specific IgE was determined with the ADVIA Centaur immunoassay system (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA) as described previously by Petersen et al. [6].

In the inhibition studies, specific IgE was determined in individual sera before and after addition of non-labelled *P. pratense* extract (2 mg, 5000-fold excess) to the biotinylated allergen extract reagent under investigation. The 5000-fold excess of *P. pratense* in the inhibition experiments was primarily used to ensure capture of high- and low-affinity grass allergen-specific IgE antibodies. Also, the presence of non-IgE antibodies, e.g. IgG4 in sera from SIT patients, may reduce the relative inhibitor surplus to an unknown extent.

IgG4 studies. Single-species extracts: *P. pratense*, *P. pratensis*, *F. pratensis*, *D. glomerata*, *A. elatius*, *L. perenne*, *H. lanatus*, *A. stolonifera*, *A. odoratum* and *S. cereale*.

Combined extracts: Identical to those used for the IgE measurements.

Specific IgG4 was determined using the ADVIA Centaur immunoassay system (Siemens Medical Solutions Diagnostics). The assay was similar to that used for determination of specific IgE, with a few modifications: Anti-human IgE was replaced with murine monoclonal anti-human IgG4, and to counteract the presence of a large excess of non-specific IgG4, the serum samples were diluted 1 : 30 before analysis. For each extract (individual grass species and combinations of extracts), a standard curve ranging from a dilution factor of 12.5 to 10 000 was constructed from a standard serum pool based on nine serum samples drawn from four patients during specific immunotherapy treatment. The amount in each serum sample of IgG4 specific to a particular extract was then determined relative to the standard curve for that extract.

Establishment of nPhl p 1- and nPhl p 5-specific T cell lines. Freshly isolated peripheral blood mononuclear cells (PBMCs) were CD8-depleted by Macs magnetic depletion (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. CD8-depleted PBMC (2×10^6 /mL) were stimulated with purified Phl p 1 or Phl p 5 (1 µg/mL) for 14 days. Recombinant IL-2 (Chiron, Emeryville, CA, USA) was added on days 5, 6 and 7. After 14 days, T cells were isolated, washed and restimulated with autologous PBMCs (10^6 /well, irradiated 2500 Rad), nPhl p 1 or nPhl p 5 (1 µg/mL) and phytohaemagglutinin P (PHA-P) (0.5 µg/mL, Difco, Detroit, MI, USA). Recombinant IL-2 was added on days 3, 4 and 5. The lines were kept in culture with restimulation every 10 days with allogeneic Epstein-Barr virus (EBV) B cells (10^5 /well, irradiated 3500 Rad), allogeneic PBMCs (10^6 /well, irradiated 2500 Rad) and 0.5 µg/mL PHA-P and addition of recombinant IL-2 at days 3–5. The cell line cultures were terminated after a period of 8 weeks.

T cell stimulation assay. The capability of six different single-species grass pollen extracts to stimulate allergen-specific T cells was analysed in a standard 72-h T cell stimulation assay as described previously [7]. Ten and 1 µg/mL of each allergen extract were used for T cell stimulation. Purified nPhl p 1 or nPhl p 5 was used as a positive control, and cells cultured in medium alone were used to determine background proliferation. For all extracts, except one, the lipopolysaccharide (LPS) content was <50 EU/mg, yielding general final LPS concentrations in the tests of <0.5 or 0.05 EU/mL for the 10 and 1 µg/mL stimulations, respectively. *A. odoratum*: LPS <400 EU/mg. All assays were repeated twice as three or four replica cultures, and the results are expressed as the stimulation index (SI = c.p.m. stimulated cultures / c.p.m. medium control). Assays with SI < 3 in response to the positive control were discarded.

Quantification of Group 1 and 5 allergens. The amounts of grass group 1 and 5 allergens in 10 different single-species grass pollen extracts were determined by SRID [8]. The pollen extracts from the individual grass species were applied in different concentrations selected to be within the range of the amounts of either nPhl p 1 or nPhl p 5 used to obtain a standard curve (determined from pilot studies). The amount of group 1 or 5 allergens was determined from four SRID precipitates for each species, with the exception of *P. pratense*, for which eight applications were used. The SRIDs were performed with polyclonal, monospecific rabbit antibodies raised towards nPhl p 1 or nPhl p 5, using purified preparations of the allergens nPhl p 1 or nPhl p 5 to construct the four-point standard curves.

The quantification may exhibit a bias if groups 1 and/or 5 are precipitated differently. Therefore, the amounts are reported as nPhl p 1- or nPhl p 5-equivalent amounts.

However, the precipitates exhibit a high degree of morphological similarity, indicating a high degree of homology within groups 1 and 5 for the species tested.

Results

Direct IgE cross-reactivity

The IgE response in sera from grass pollen-sensitized subjects to four different combined extracts (mixes) of Pooideae grass pollen was correlated to the response to *P. pratense* pollen extract (Fig. 1).

As expected from identical studies concerning the individual constituents of the mixes (results submitted for publication, N. Johansen et al., personal communication), the data points cluster around the line of identity, with comparable Spearman rank correlation factors. The close correlation implies substantial cross-reactivity of the IgE detected by *P. pratense* extract towards the allergens of the combined extracts. The observed cross-reactivity was not related to the number of species included in the mix.

IgE inhibition studies

The IgE response in sera from grass pollen-sensitized subjects to four different combined extracts of Pooideae grass pollen was determined before and after addition of non-labelled *P. pratense* extract, to test the proportion of IgE potentially binding to *P. pratense* allergens (Fig. 2).

For all four mixed extracts, *P. pratense* extract completely inhibited the binding of specific IgE to the allergens in the extracts to a level below the limit of quantification for all serum samples, as expected from the identical experiments concerning the individual constituents of the combined extracts (results submitted for publication, N. Johansen et al., personal communication).

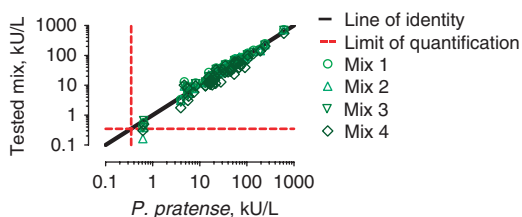


Fig. 1. Specific IgE response to pollen extract of *Phleum pratense* (x-axis) and specific IgE response to four combined grass pollen extracts from the Pooideae subfamily. The threshold for a positive result is 0.35 kU/L. The diagonal represents the line of identity. Spearman rank correlation factors [95% confidence interval]: Mix 1, 0.98 [0.97–0.99]; Mix 2, 0.98 [0.97–0.99]; Mix 3, 0.98 [0.97–0.99]; and Mix 4, 0.96 [0.92–0.98].

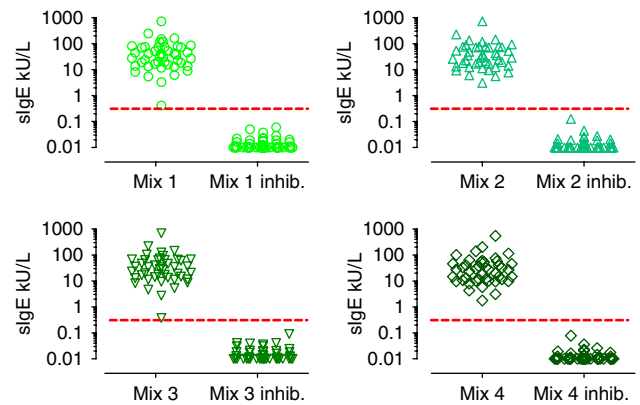


Fig. 2. Specific serum IgE concentrations from grass pollen-sensitized subjects measured before and after addition of non-labelled *Phleum pratense* extract to four combined pollen extracts of grasses from the Pooideae subfamily.

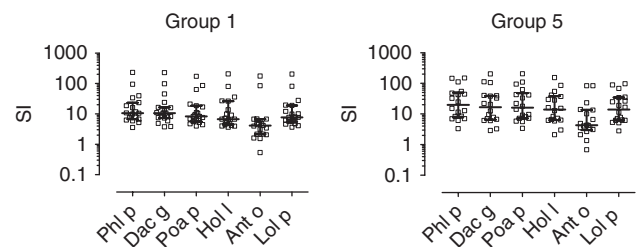


Fig. 3. Stimulation of T cell lines specific to nPhl p 1 ($n = 19$, left panel) and B) nPhl p 5 ($n = 18$, right panel) by 10 μ g of *P. pratense*, *D. glomerata*, *P. pratensis*, *H. lanatus*, *A. odoratum* and *L. perenne*. Results are given as stimulation indices (SI), to normalize all assays with respect to variations in background values. Medians and interquartile ranges are indicated. Assays with $SI < 3$ in response to the positive control were discarded. Endotoxin/lipopolysaccharide (LPS) concentrations were < 0.5 EU/mL in all assays apart from *Anthoxanthum odoratum* (LPS < 4 EU/mL). Almost identical responses were also observed with extract concentrations at 1 μ g/mL where the endotoxin levels were reduced by a factor 10. All experiments were conducted in three or four replica cultures performed twice, with comparable results.

Stimulation of T cell lines

T cell lines specific to nPhl p 1 or nPhl p 5 allergens were stimulated with six single-species grass pollen extracts to investigate whether T cells specific to allergens from one species are activated by allergens from related Pooideae grass species. The results obtained from stimulation with 1 and 10 μ g/mL allergen extract were similar; the latter are shown in Fig. 3. Stimulation of Bet v 1-specific T cell lines with these grass extracts in various concentrations did not result in any increase or reduction in proliferation, which clearly excludes any mitogenic or inhibitory effects of the extracts investigated (data not shown).

Strong T cell proliferation of both nPhl p 1- and nPhl p 5-specific T cells was observed upon stimulation with the pollen extract of five out of six Pooideae grasses. Even the extract of pollen from *A. odoratum*, the grass species most

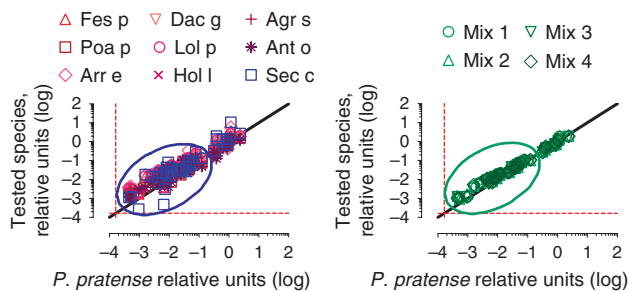


Fig. 4. *Phleum pratense*-specific IgG4 plotted vs. specific IgG4 towards (A) *P. pratense*, *F. pratensis*, *D. glomerata*, *A. elatius*, *L. perenne*, *H. lanatus*, *A. stolonifera*, *A. odoratum* and *S. cereale* (B) Mix 1, Mix 2, Mix 3 and Mix 4. Serum samples were from patients treated with *P. pratense* pollen extract, either subcutaneously for at least 1 year or sublingually for at least 4 weeks. The group receiving sublingual treatment is marked in both panels. A point on the line of identity indicates complete cross-reactivity of the *P. pratense*-specific IgG4 antibodies towards the allergens of the tested extract. Spearman rank correlation coefficients [95% confidence interval]: *P. pratensis*, 0.94 [0.90–0.97]; *F. pratensis*, 0.97 [0.94–0.98]; *D. glomerata*, 0.98 [0.91–0.98]; *A. elatius*, 0.94 [0.89–0.97]; *L. perenne*, 0.96 [0.92–0.98]; *H. lanatus*, 0.93 [0.87–0.96]; *A. stolonifera*, 0.94 [0.88–0.97]; *A. odoratum*, 0.92 [0.85–0.96]; *S. cereale*, 0.92 [0.86–0.96]; Mix 1, 0.99 [0.98–0.99]; Mix 2, 0.99 [0.98–0.99]; Mix 3, 0.99 [0.98–0.99]; and Mix 4, 0.99 [0.98–1.00].

distantly related to *P. pratense*, clearly induced significant T cell activation (SI > 2) in 24 out of 29 T cell lines tested, although the mean response was significantly reduced compared with all the other grasses tested ($P < 0.05$).

Cross-reactivity of treatment-induced IgG4

Allergen-specific IgG4 in serum samples from patients treated subcutaneously or sublingually with *P. pratense* pollen extract was detected and quantified by nine different pollen extracts of individual grass species and the four mixes. The results were correlated to the IgG4 response to *P. pratense* pollen extract (Fig. 4).

In general, the data points cluster around the line of identity for the individual pollen extracts of the Pooideae grass species and for combinations of these, implying cross-reactivity of the treatment-induced IgG4 antibodies as well. There appear to be no significant differences between the correlation for the single-species extracts compared with the mixes.

Quantification of allergen content

The amounts of grass group 1 and 5 allergens in the extracts of the 10 species were quantified by SRID [8]. Purified nPhl p 1 and nPhl p 5 were used to construct linear standard curves, and the concentration of group 1 and 5 allergens was determined from the standard curves and expressed as nPhl p 1 or nPhl p 5 equivalent amounts (Fig. 5).

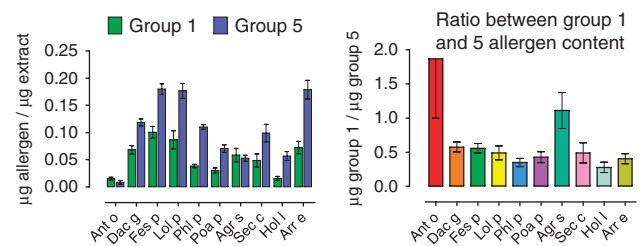


Fig. 5. Left panel: Amount (mass) of grass group 1 and 5 allergens in pollen extracts from 10 different Pooideae grasses. The individual amounts are expressed as μg major allergen per μg dry weight of extract. Right panel: Ratio between the amount of group 1 and 5 allergen (μg group 1/ μg group 5). The mass comparison is valid because the molecular weights of the allergens are comparable (approximately 27 kDa) – in fact, the mass ratio is equivalent to the molar ratio between the two allergens. The error bars represent the 95% confidence intervals of 4–8 (*P. pratense* only) independent amount estimates in one batch of each of the grass extracts. The confidence limits of the ratio were estimated assuming a covariance of zero.

The contents of group 1 and 5 allergens were observed to vary between species but the ratio between the two allergens was close to constant, with *A. odoratum* and *A. stolonifera* as exceptions.

Discussion

Traditionally, grass pollen-allergic subjects have been treated with extracts of pollen from either a single grass species or with a combination of grass pollen extracts from different species. The clinical efficacy of using pollen extract of *P. pratense* alone has been thoroughly documented, for subcutaneous as well as sublingual treatment [9–15]. This is illustrated in Table 1. Allergen exposure was diverse and was not restricted to *P. pratense* for the subjects enrolled in these studies, indicating that treatment with vaccines based on extract from one species will influence allergic symptoms caused by related species.

However, the use of mixes vs. single-species products in grass pollen immunotherapy is still debated [16], and in order to investigate any possible differences in response to the two types of treatment, the IgE response to four combined allergen extracts (mixes) was determined in sera from grass pollen-sensitized subjects. The combined extracts contained pollen extract from 5 to 10 grass species, all from the Pooideae subfamily, and the combinations were chosen to mimic four commercial mix products.

Identical experiments were performed previously with the same serum samples and with samples from other regions such as South Africa and the United States, using single-species extracts of the individual components of the mixes. These results have been reported in a recently submitted manuscript (N. Johansen et al., personal communication) and indicated extensive IgE cross-reactivity

towards the Pooideae allergens (> 19 000 data pairs), with *P. pratense* as the reference.

The patterns observed in the studies with the combined extracts are identical to those observed with the single-species constituents, both for the direct binding (Fig. 1) and for the inhibition experiments (Fig. 2). The findings are in line with indications from previous smaller studies [17–22], and the clinical consequences for the patient are the occurrence of allergic symptoms in the presence of pollen from any of the Pooideae grasses.

Both T and B cell epitopes recognized by the individual patient have to be included in the allergen preparations used for therapy, and clinically effective immunotherapy has been shown to modulate allergen-specific T cell responses observed as a down-regulation of the allergen-specific response through induction of regulatory T cells and/or a shift from a T helper type 2 (Th2) to a Th1 response [23–27]. To determine whether the T cell response of grass pollen-sensitized subjects is different for allergens from different Pooideae grasses, T cell cross-reactivity was investigated by stimulation of T cell lines specific to group 1 or 5 allergens with six different grass pollen extracts. This resulted in almost identical levels of T cell proliferation for five out of the six single-species extracts tested (Fig. 3). Significantly lower average values were seen for *A. odoratum*, and this may reflect the low content of the two major allergens for this species (Fig. 5). Altogether, true cross-reactivity towards pollen extracts of six Pooideae grasses is demonstrated at the T cell level in the current investigation, confirming the results from previous investigations with a focus on grass group 1 or 5 allergens [28–31]. In addition, the cross-reactivity was further substantiated in these previous studies, which investigated T cell clones and identified common T cell epitopes in grass group 1 or 5 allergens from related grass species [28–31].

Furthermore, induction of allergen-specific non-IgE antibodies has been a consistent finding in numerous trials during the last 30–40 years [27, 32, 33]. The non-IgE antibodies have been shown to compete with IgE with respect to allergen binding, thus inhibiting IgE-mediated mechanisms involved in both immediate and late-phase allergic responses [23, 25, 31]. Therefore, specific IgG4 was determined in patient sera after immunotherapy with pollen extract from *P. pratense*. As can be seen from Fig. 4, these treatment-induced IgG4 antibodies responded equally to pollen extracts from eight other Pooideae grasses (*P. pratense*, *F. pratensis*, *D. glomerata*, *A. elatius*, *L. perenne*, *H. lanatus*, *A. stolonifera* and *A. odoratum*), indicating cross-reactivity of these antibodies as well. As expected, identical results were obtained for combined pollen extracts of these species (Fig. 4). These results are in full accordance with previous observations made by van Ree et al. [34] where IgG induced by immunotherapy with a combined extract (grass mix) was

shown to display extensive cross-reactivity towards the grass group 5 major allergens.

One single batch of *P. pratense* extract was used in the IgE and IgG4 investigations, yielding a high degree of correlation to various randomly selected batches of extract from other grass species. High levels of IgG4 specific to the *P. pratense* batch used for analysis were observed, even though patients were treated with products based on a different batch of *P. pratense* extract. This makes it highly unlikely that the data presented are restricted to the single batch of *P. pratense* extract used as a reference in the presently reported investigations.

The results for *S. cereale* (Fig. 4) were slightly more dispersed than for the other species tested, in agreement with the results obtained by van Ree et al. [34], who reported *L. perenne* pollen extract to be less powerful in inhibiting the binding of treatment-induced IgG to the allergens in *S. cereale* pollen extract than to the allergens of the other Pooideae species investigated in that study.

Finally, inter-species consistency within the Pooideae subfamily of the ratio of major allergen 1–5 was observed (Fig. 5), resulting in a similar distribution of these two major allergens in combined and single-species extracts. This implies equal treatment effect with respect to the major allergens regardless of the choice of species (nature and number).

In conclusion, cross-reactivity has been confirmed for both IgE and IgG4. IgE is responsible for allergic symptoms, and the shift in balance from a predominant IgE response to one based primarily on other antibody isotypes is intrinsic to the effect of immunotherapy [23, 25, 32]. The present study shows that IgG4 induced by immunotherapy with *P. pratense* will recognize allergens from the Pooideae grasses in general. The shift in antibody response is linked to shifts in the T cell response [23–25], and the present study confirms cross-reactivity at the T cell level as well. The results are in full accordance with numerous trials showing the clinical efficacy of treatment with *P. pratense* in different geographic regions [9–12, 14, 15, 35].

In accordance with the results obtained in this study, the AAAAI Joint Task Force on Practice Parameter [2] recommends treating grass pollen-allergic subjects in regions of the world dominated by temperate grasses with the pollen extract of only one species, to ensure batch-to-batch consistency and optimal efficacy and to minimize the risk of side-effects.

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non-related companies and has advised on design of immunotherapy trials. Charlotte Hejl, Peter Adler Wurtzen, Niels Johansen, Louise Broge and Henrik Ipsen are ALK employees.

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