

ORIGINAL ARTICLE

Molecular variability of group 1 and 5 grass pollen allergens between Pooideae species: implications for immunotherapy

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Summary

Background Differences between major allergens from distinct grass species remain to be investigated, both in terms of structure and antigenicity.

Methods Group 1 and 5 allergens purified from five common Pooideae species were analysed by mass spectrometry (MS). Major histocompatibility complex (MHC) class II-restricted T cell epitopes were identified using predictive algorithms and human leucocyte antigen (HLA)-binding assays. CD4⁺ T cell reactivity and IgE binding were assessed based on the induction of CD154 expression in peripheral blood mononuclear cells and using competitive ELISA assays, respectively.

Results MS analysis of group 5 pollen allergens reveals considerable intra- and inter-species variability in amino acid sequence, with 30–50 predominant isoforms found for each species. Differences in the amino acid sequence as well as *N*- and *O*-glycosylation contribute to the variability of group 1 allergens, yielding 5–10 main isoforms, depending on the species. Out of 14 MHC class II-restricted T cell epitopes identified within group 1, only one is conserved among the five grass species. Significant differences in binding affinities for HLA-DR molecules result in variable CD4⁺ T cell recognition of group 1 and 5 allergens purified from the various species. Up to 38% and 85% of patients exhibit seric IgE responses to species-restricted (or semi-restricted) epitopes associated with group 1 or 5 allergens, respectively.

Conclusion Major pollen allergens from distinct grass species bear both shared and species-restricted T and B cell immune epitopes. When compared with single extracts, a five grass pollen extract is thus more suitable for specific immunotherapy, as it contains a broader repertoire of the IgE epitopes to which patients are sensitized.

Keywords grass pollen, immunotherapy, major allergens, Pooideae

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Introduction

Grass pollen allergy is a frequent type I respiratory hypersensitivity, involving broadly distributed grasses belonging to the Pooideae subfamily [1, 2]. The latter represent a coherent phylogenetic group within the Poaceae family, encompassing most common allergenic grasses [3]. As a consequence, extracts made from Pooideae pollens exhibit significant IgE cross-reactivity, thus reflecting the well-known homology between allergens from Pooideae [4–11]. Up to 13 families of allergens have been described within Pooideae, out of which group 1 and 5 allergens appear to be the most clinically relevant, since 95 and 65–85% of patients, respectively, are sensitized to

them [1, 9, 12]. IgEs against those two major allergens routinely represent 80% or more of specific IgEs in patients' sera [12].

Both group 1 and 5 allergens are well conserved within Pooideae, with an amino acid sequence homology in the range of 90 and 55–80%, respectively [9, 12]. Nonetheless, some molecular differences have been reported for each of these two allergens when originating from distinct grass species, which impact antibody and T cell recognition [13–15]. These observations provide evidence that specific allergen isoforms are present in pollens from individual grass species, thus contributing unique epitopes to patient sensitization. Because all patients are polyexposed and polysensitized to multiple pollens, understanding in detail

the structural and antigenic variability of major pollen allergens obtained from various grass species is critical for designing appropriate specific immunotherapies [2].

In this context, the present study addresses the following two questions: (i) to which extent are group 1 or 5 major allergens molecularly diverse within and between distinct grass species? (ii) are such molecular differences recognized by the patients' immune system? Herein, we document a substantial molecular variability for both group 1 and 5 allergens purified from five common grass species. We further establish that such molecular differences translate into species-specific epitopes on such allergens, recognized by B or T cells from most allergic patients.

Methods

Allergen extracts and purification

Allergens were purified from three batches of pollen for each grass species, purchased from either Allergon (Angelholm, Sweden) or Pharmallerga (Lisov, Czech Republic). Pollens from *Anthoxanthum odoratum* (Sweet vernal), *Dactylis glomerata* (Orchard or Cocksfoot), *Lolium perenne* (Perennial rye), *Phleum pratense* (Timothy) and *Poa pratensis* (Kentucky blue grass) or a mixture (w/w) of the five pollens (five grass mix) were incubated for 24 h with a 50 mM ammonium bicarbonate solution (1 g pollen/20 mL solution) at $5 \pm 3^\circ\text{C}$ under magnetic stirring. After centrifugation, the supernatants were stored at -20°C until use. Group 1 and 5 allergen concentrations routinely obtained in such extracts are reported elsewhere [2].

Pollen extracts were applied on a phenyl-sepharose hydrophobic interaction column (GE Healthcare, Orsay, France), using an elution gradient based on 0.02M sodium phosphate, and 5% isopropanol, pH 7.6. This material was applied on a Superdex S-75 superfine column (GE Healthcare), and fractions containing group 1 or 5 allergens were pooled and concentrated by ultrafiltration, using an Amicon PM-10 membrane (Millipore, Saint-Quentin-en-Yvelines, France). Protein samples obtained after purification were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), followed by silver staining and Western blot.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blot

Purified allergens (3 µg) diluted in sample buffer (62 mM Tris, 1% SDS, 0.01% bromophenol blue) was loaded onto 12.5% Excel-gels (GE Healthcare). Electrophoresis was carried out under reducing conditions for 1 h at 80 V in parallel with molecular weight markers, and proteins were detected using a silver staining kit (GE Healthcare). For

Western blot, proteins were transferred onto a nitrocellulose membrane (Whatman, Versailles, France). After a blocking step, membranes were cut into strips and incubated overnight with anti group 1 or 5 (0.5 µg/mL) Tim 130B and Stal 15A murine monoclonal antibodies (Stallergenes, Antony, France), respectively. These antibodies were obtained after immunizing with either purified Dac g 1 or a *D. glomerata* extract, respectively, selecting hybridomas producing antibodies reactive with purified allergens from the five grass species. After washing, membranes were incubated with peroxidase-labelled sheep anti mouse IgG diluted 1 : 1000, before a revealing step using West pico substrate (Pierce, Rockford, USA). Luminescence signals were detected using a Chemismart 2000 camera and analysed with the Bio-1D software (Vilber Lourmat, Marne la vallée, France).

Proteolytic digestions

Ten microlitre of purified group 1 allergen ($\sim 0.5\text{--}1\text{ }\mu\text{g}/\mu\text{L}$) was mixed with 10 µL of a 0.2% (w/v) RapiGest SF (Waters, Versailles, France) solution in 50 mM ammonium bicarbonate. Disulphide bond reduction was performed after adding 10 µL of a 10 mM DTT solution in water and incubating the resulting mixture for 1 h at 60°C . The solution was allowed to cool at room temperature, before adding 10 µL of a 24 mM iodoacetamide solution in water, and incubating at room temperature for 45 min in the dark. After addition of 50 mM ammonium bicarbonate (15 µL), digestion with either trypsin (5 µL of a 100 ng/µL aqueous solution) or Asp-N (5 µL of a 40 ng/µL aqueous solution) was performed overnight at 37°C . Protein digests were acidified with 5 µL of 1 M HCl, further incubated at 37°C for 45 min and centrifuged at 13 000 g for 5 min to remove insoluble material.

Mass spectrometry analyses

For intact protein analysis by LC-ESIMS, whole-protein analysis was performed using an Ultimate 3000 LC system (Dionex, Sunnyvale, CA, USA) coupled with a QTOF-I (Waters, Milford, MA, USA) mass spectrometer. Chromatographic separations were performed on a Zorbax 300SB C18 HPLC column ($2.1 \times 150\text{ mm}$, 5 µm, 300 Å, Interchim, Montluçon, France) as described previously [16]. Mobile phases consisted of 0.1% formic acid (A) and ACN containing 0.1% formic acid (B). Proteins were eluted from the column at 300 µL/min using a 5–60% phase B gradient over 25 min. Ionization on the QTOF-I was performed in the positive mode using a capillary voltage of 2900 V, with the cone voltage set at 30 V. Mass calibration was performed externally on the multiple charged ions of myoglobin. Data were acquired by the TOF analyzer from m/z 700 to 2000 with a cycle time of 2 s.

Protein mass spectra were deconvoluted using the MaxEnt maximum entropy software (Waters).

For peptide analysis by LC-ESIMS/MS, the experimental conditions for liquid chromatography were as above, excepted that the gradient lasted 35 min. The HPLC eluent was directly connected to the ESI probe of an LCQ-Duo ion trap mass spectrometer (Thermo, San Jose, CA, USA). Source conditions were as follows: capillary temperature, 275 °C; sheath gas flow, 80 arbitrary units; auxiliary gas flow, 20 arbitrary units; and ESI spray voltage, 4.5 kV. The target was fixed at 1×10^7 ions and the automatic gain control was turned on. The LCQ was operated in a data-dependent mode, in which the instrument was set up to automatically acquire a full scan over the 300–2000 m/z range, followed by ZoomScan (high-resolution mass spectrum) and MS/MS spectra (with a normalized collision energy of 35% and an isolation width of 2.0 Th) for the two most intense ions. An additional experiment was performed using the in-source voltage set to 60 V, in order to generate carbohydrate-derived product ions and specifically identify glycopeptides.

In parallel, enzymatic digests were also analysed by matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOFMS). Samples were first desalted using C18 pipette tips (ZipTip, Millipore, Saint-Quentin en Yvelines, France) and further analysed on a Voyager-DE STR instrument (Applied Biosystems, Les Ulis, France) equipped with a pulsed nitrogen laser (337 nm). The instrument was operated in the delayed extraction reflector mode with a 20 kV acceleration voltage and an extraction delay of 140 ns. The optimized laser power was ~ 1500 arbitrary units. Data were accumulated over the m/z ratio window of 500–4000 Th. All samples were prepared in duplicate and spotted. Spectra comprising 500 averaged laser shots were acquired for each sample. Mass spectra were internally calibrated with trypsin autolysis peaks at m/z 842.51 and 2211.10.

Modelization and bioinformatics

Models of secondary and tertiary structures of group 1 allergens were produced based on the published Phl p 1 X-ray structure (PDB ID code 1N10) [17] using PyMOL software (DeLano Scientific, San Carlos, CA, USA). Potential Phl p 1.0101 epitopes restricted to human leucocyte antigen (HLA)-DRB1 molecules were selected by bioinformatic analyses performed using Syfpeithi (<http://www.syfpeithi.de>) and Propred (<http://www.imtech.res.in/raghava/propred>) algorithms on the basis of a score superior to 20 for at least one DRB1 haplotype. Published amino acid sequences of group 1 allergens from Timothy (isoforms Phl p 1.0101 and 1.0102), Orchard (isoforms Dac g 1.0101 and 1.0102), Perennial Rye (Lol p 1.0101 and 1.0103) and Kentucky blue grass (isoform Poa p 1.0101)

were aligned using the CLC Combined Workbench Software (CLC Bio, Aarhus, Denmark).

Human leucocyte antigen-DR-peptide binding assays

Peptides containing putative anchor motifs for common HLA-DRB1 molecules were synthesized by NeoSystem (Strasbourg, France) with a purity grade $> 75\%$ confirmed by HPLC. Binding assays were conducted using HLA-DR molecules purified from human EBV-transformed B cell lines, as described elsewhere [18]. Biotinylated reporter peptides were incubated in the presence of serial dilutions of group 1 allergen peptides tested together with appropriate purified major histocompatibility complex (MHC) class II molecules. Specifically, the flu haemagglutinin HA_{306–318} peptide was used as a reporter peptide for binding to both the HLA-DRB1*0101 and the HLA-DRB1*0401 molecules. HLA-A3_{152–166} and *Mycobacterium tuberculosis* MT_{2–16} were used as reporter peptides for binding to either the HLA-DRB1*1501 or the HLA-DRB1*0301 molecule [18]. Bound biotinylated peptides were detected after adding a streptavidin-alkaline phosphatase conjugate (Amersham, Little Chalfont, UK) and 4-methyl umbelliferyl phosphate (Sigma, St Quentin-Fallavier, France) as a substrate. Emitted fluorescence was measured at 450 nm following excitation at 365 nm on a Gemini Spectramax Fluorimeter (Molecular Devices, St Gregoire, France). Peptide concentrations inhibiting 50% binding of biotinylated reporter peptides (IC₅₀) were obtained and data were expressed as ratios between IC₅₀ values obtained for the test vs. the reference peptide [18].

T cell responses to group 1 and 5 grass pollen allergens

Citrated peripheral blood was obtained after informed consent from four grass pollen-allergic patients. The study was approved by the Comité Consultatif pour la Protection des Patients dans la Recherche Biomédicale (CCPPRB, Hôpital Bécélère, no. 05-44). Freshly isolated peripheral blood mononuclear cells (PBMCs) ($2.5 \times 10^6/\text{mL}$) were stimulated in 96-well plates with each of group 1 or 5 natural purified allergens ($10 \mu\text{g}/\text{mL}$) in 0.2 mL complete RPMI 1640 medium. After 5 days at 37 °C, 10 U/mL IL-2 (Roche, Meylan, France) was added to cell cultures every 3 days. Allergen-specific T cells were identified based on CD154 (CD40 ligand) expression [19] after stimulation with grass pollen allergens. On day 13, anti CD40 blocking monoclonal antibodies (Beckman Coulter, Villepinte, France) were added in each well for 16 h to prevent subsequent internalization of CD154. PBMCs were then harvested and incubated with PE-labelled anti-CD154, FITC-labelled anti-CD4, ECD-labelled anti-CD14, PC7-labelled anti-CD3 (all from Beckman Coulter) antibodies and ViaProbe reagent (BD, San Diego, CA, USA) for 20 min in the dark at 4 °C. After washing, samples were

analysed using an FC500 flow cytometer (Beckman Coulter), after exclusion of dead cells and CD14⁺ monocytes.

Immunoglobulin E-binding experiments

Twenty seven sera from grass pollen-allergic patients (IgE class 2–5) living either in the United States ($n = 10$) or in France ($n = 17$) were obtained from Plasmalab, Everett, WA, or Etablissements Français du Sang, Rungis, France, respectively, to perform IgE-binding experiments. Allergen cross-reactivity was assessed by a competitive ELISA assay [20]. To this end, purified group 1 or 5 allergens obtained from the five grass species (1 µg protein/well) were coated in PBS overnight at +4 °C onto a 96-well plastic microtitre plate. Individual sera obtained from grass pollen-allergic patients were diluted at 1 : 20 in EIA buffer (0.1 M potassium phosphate pH 7.4, 0.4 M sodium chloride, 0.78 mM EDTA, 0.01% sodium azide, 0.1% bovine serum albumin) and incubated for 1 h at room temperature with 100 µL of an excess concentration of either the five individual pollen extracts, the five grass mix extract or with buffer alone as a control, and then added for 2 h to group 1 or 5 purified allergens coated on a 96-well plate. Maximum extract concentrations allowing complete dissolution were used for inhibition. The latter corresponded to 0.3–2.5 µg/mL group 1 and 29–98 µg/mL group 5 concentration, with the exception of Ant o 5, found only at a 2 µg/mL concentration. After washing, bound IgE antibodies were revealed for 1 h at room temperature with a 1 : 3000 dilution of a goat IgG anti-human IgE antibody labelled with horseradish peroxidase, using tetramethylbenzidine as a substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The reaction was stopped with 1 M phosphoric acid, and plates were read at 450 nm on a Multiskan ascent spectrophotometer (Labsystems, Saint Herblain, France).

Results

Molecular characterization of purified group 1 and 5 allergens

To assess intra- and inter-species molecular variability, group 1 and 5 allergens were purified from three batches of pollens obtained from each of five common grass species as described in methods. Results from only a single representative set of allergens are shown below for each analysis, because no differences were observed between batches. After purification, all allergens were at least 95% pure, as confirmed by silver staining (Fig. 1a). Such allergens were also resolved by SDS-PAGE and detected with either anti-group 1 or 5 monoclonal antibodies. As shown in Fig. 1b, all group 1 allergens resolve as a single band with similar molecular masses, with the exception of Phl p 1, which appears as a doublet. In contrast, group 5

allergens purified from pollens obtained from the various grass species resolve as two major bands exhibiting dramatically different molecular masses. Such differences were not due to protein degradation during purification, because similar mass patterns were found in allergens present in raw extracts (not shown).

These purified allergens were further compared by MS. Figure 2 summarizes the characterization of intact group 1 allergens by LC-ESIMS. Taking as an example Lol p 1 purified from perennial rye (*L. perenne*) pollen, the protein was eluted after 16.6 min of RP-HPLC (Fig. 2a) as a single peak. The corresponding deconvoluted mass spectrum (Fig. 2b) revealed multiple protein isoforms with two prominent ones at 27135 and 27241 Da. The latter differ from calculated masses at 26109 and 26186 Da for published amino acid sequences (access numbers Q9SC98 and P14946, respectively). These mass increments likely represent an additional M0XF3 *N*-glycan motif on Asn 9 (i.e. +1008 Da), as described previously in grass pollens [21], along with the presence of two hydroxyproline residues in positions 5 and 8 (i.e. +32 Da) [22]. This hypothesis was further corroborated by 162 Da mass increments observed between isoforms in the deconvoluted spectrum (Fig. 2b), likely corresponding to hexose addition, in agreement with *N*-glycan structures reported previously in various grass pollens [20]. Lastly, additional ~20–60 Da increments likely originate from primary sequence variation, as suggested by LC-MS/MS analysis of corresponding tryptic peptides (Table 1 and data not shown).

The RP-HPLC analysis of purified Phl p 1 (Fig. 2c) shows an even more heterogeneous product when compared with Lol p 1, with two distinct chromatographic peaks. Figure 2d shows the deconvoluted mass spectrum from the peak eluted at 20 min, also documenting variability in primary structure and *N*-glycosylation, with mass shifts of ~132, 146 and 162 Da, potentially corresponding to pentose (e.g. xylose, arabinose), deoxyhexose (e.g. fucose) and hexose (e.g. mannose, galactose) addition, respectively. Altogether, > 10 isoforms are observed for Phl p 1. All observed masses are above the theoretical average masses reported for Phl p 1 at 26 151 and 25 925 Da (access numbers P43213 and Q40967, respectively), again likely as a consequence of additional *N*-glycans and hydroxyproline residues. Similarly, purified Dac g 1, Ant o 1 and Poa p 1 also show heterogeneous profiles (Fig. 2e–j), all demonstrating intraspecies variability in terms of primary sequence and glycosylation, leading to the presence of at least 5–10 main isoforms depending on the grass species. Our detailed mass spectrometric investigation of the molecular variability documented for group 1 grass pollen allergens is reported elsewhere [23].

Importantly, this variability translates into molecular differences between species themselves. Table 1 displays the alignment of group 1 amino acid sequences obtained

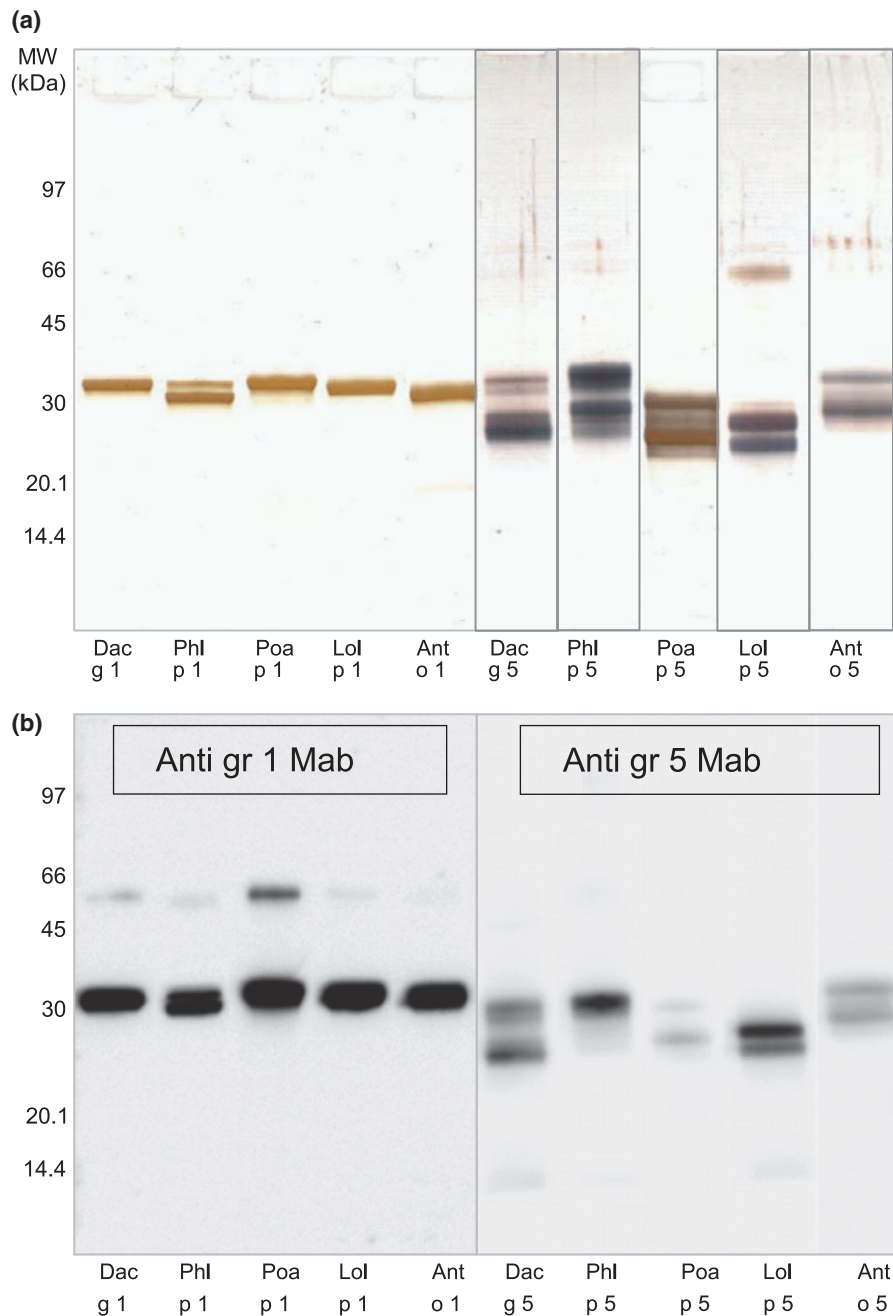


Fig. 1. Analysis of group 1 and 5 allergens purified from pollens of individual grass species. Allergens were purified from either *Anthoxanthum odoratum*, *Dactylis glomerata*, *Lolium perenne*, *Phleum pratense* or *Poa pratensis*. (a) Purified proteins (0.15 µg/lane) were submitted to SDS-PAGE and silver staining. (b) Purified proteins (3 µg/lane) were submitted to SDS-PAGE and Western blotting. Blots were revealed with anti-group 1 (Tim 130B) or anti-group 5 (Stal 15A) murine monoclonal antibodies. Bands in the 60–70 kDa range correspond to dimers.

from our MS/MS analysis for each of the five grass species, with most of the protein sequences confirmed in those experiments. Amino acids highlighted in red differ between the grass species tested. Also, for a given species, numerous sequence variants are found (with the most frequent amino acid shown in green). Altogether, the present results demonstrate that despite being the most conserved allergen between Pooideae species, group 1

exhibits a high degree of structural heterogeneity, linked with primary sequence variations (Table 1), as well as post-translational modifications such as *N*-glycosylation, *O*-glycosylation and proline hydroxylation. Importantly, modelization of the varying amino acids based on the known Phl p 1 three-dimensional (3D) structure reveals that most of them are surface exposed (Fig. 3), thus likely to influence both IgE and IgG responses.

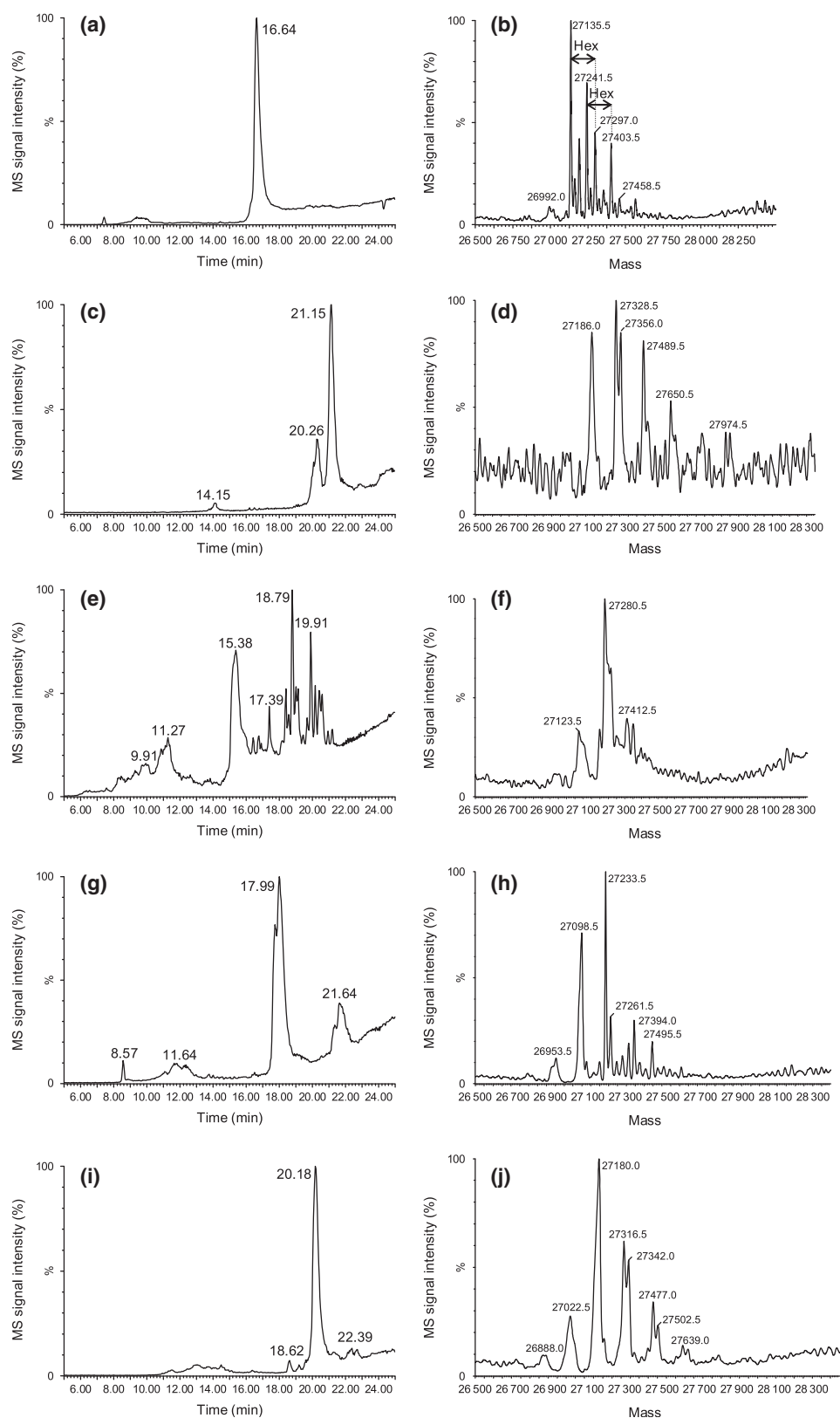


Fig. 2. LC-ESI-MS analysis of group 1 allergens purified from individual grass species. RP-HPLC chromatograms (left panels, with peaks labelled with retention times in minutes) and corresponding deconvoluted mass spectra (right panels, with peaks labelled with masses in Da) obtained with group 1 allergens from *Lolium perenne* (a and b), *Phleum pratense* (c and d), *Poa pratensis* (e and f), *Anthoxanthum odoratum* (g and h) and *Dactylis glomerata* (i and j). Mass increments associated with hexose (Hex) addition are shown with double arrows in (b).

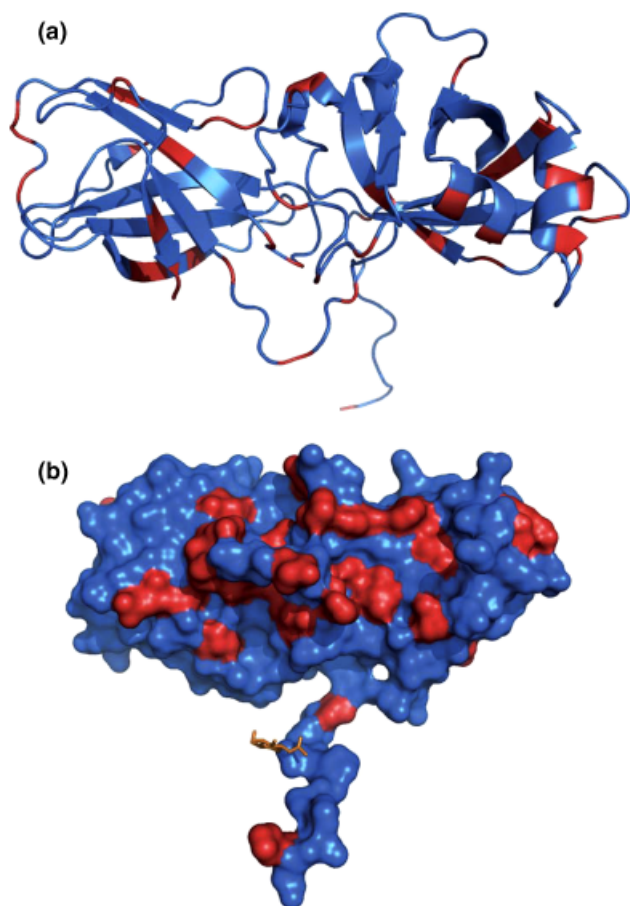


Fig. 3. Tridimensional modelization of varying amino acids within group 1 allergens. The tridimensional structure shown as a ribbon (a) or a space filling model (b) is based on the known Phl p 1 X-Ray structure (PDB code 1N10). Both intra- and inter-species variable amino acids are shown in red. The N-linked N-acetylglucosamine is shown in orange.

81 and >4807 for Phl p 1_{59–73} and Dac g 1 (or Lol p 1)_{59–73}, respectively. In contrast, Dac g 1 (or Lol p 1)_{59–73} display a much higher affinity for HLA-DRB1*0301, when compared with Phl p 1_{59–73}, with IC₅₀ ratios of 15 and >357, respectively, due to the presence of an aspartate residue acting as a major anchor amino acid for this HLA-DR molecule. Similarly, differences in affinity for HLA-DR molecules between species-specific peptides were also noticed for group 1_{100–114} and group 1_{121–135} epitopes, whereas the binding characteristics of group 1_{23–27} were not significantly impacted by amino acid substitutions (Table 3). These results unambiguously establish that primary sequence acid variations dramatically influence group 1 presentation and thus recognition by CD4⁺ T cells.

To further assess whether grass pollen-allergic patients exhibit distinct patterns of T cell responses to group 1 and 5 allergens depending on the grass species, we monitored the expression of the activation marker CD154 (CD40L) on CD4⁺ T cells [19] following incubation of PBMCs from

eight grass pollen-allergic patients with the 10 purified allergens. As shown in Fig. 6, patterns of T cell recognition of group 1 or 5 allergens differed significantly, depending on both the patient and the grass pollen species, and this heterogeneity in T cell responses was observed for the two major allergens. Not a single patient had CD4⁺ T cells recognizing all group 1 or all group 5 allergens in a comparable manner (Fig. 6).

Analysis of immunoglobulin E reactivity with purified group 1 and 5 allergens

To further analyse whether the patients' immune system can discriminate molecular differences between species-specific group 1 and 5 allergens, we performed competition experiments (as described in "Methods") to investigate IgE binding to the 10 purified allergens coated on a 96-well plate. These studies were conducted with 27 sera from European and North American patients selected for their high-IgE reactivity. Excess concentrations of inhibiting extracts (i.e. maximum concentrations allowing complete dissolution) were used in those experiments, as confirmed by complete (i.e. ≥90%) inhibition by a given extract (e.g. *P. pratense*) of IgE binding to corresponding purified allergens (i.e. Phl p 1 or Phl p 5). For both group 1 (Fig. 7a) and 5 (Fig. 7b), two patterns of IgE binding were observed. Up to 62% and 15% patients (upper panels, profile 1) had IgEs to group 1 and 5, respectively, which could be inhibited completely (i.e. ≥90% inhibition) by either one of the individual extracts or the five grass mix. We conclude that such patients have only IgEs cross-reactive to all group 1 or 5 allergens (i.e. directed to shared epitopes) in their sera. In contrast, 38% and 85% patients had IgEs against group 1 or 5 allergens, respectively, which were not totally blocked (i.e. with <90% inhibition) by at least one or most individual extracts (Fig. 7, lower panels, profile 2). The proportion of patients with the latter pattern is higher with respect to IgE responses to group 5 (85%) when compared with group 1 (38%), in agreement with the relative levels of molecular variability demonstrated for those molecules. Interestingly, inhibition with an extract made from the five grass pollen mix was always complete. We conclude that patients with profile 2 exhibit IgE responses to epitopes restricted (or semi-restricted) to selected grass species, and that all IgE epitopes to which such patients are sensitized are present in the five grass mix. Although not shown, no differences in the overall patterns of IgE recognition were found between European and North American patients.

Discussion

A majority of grass pollen-allergic patients are sensitized to both group 1 and 5 allergens, thus termed major allergens [1, 9, 12]. Group 1 allergens are highly

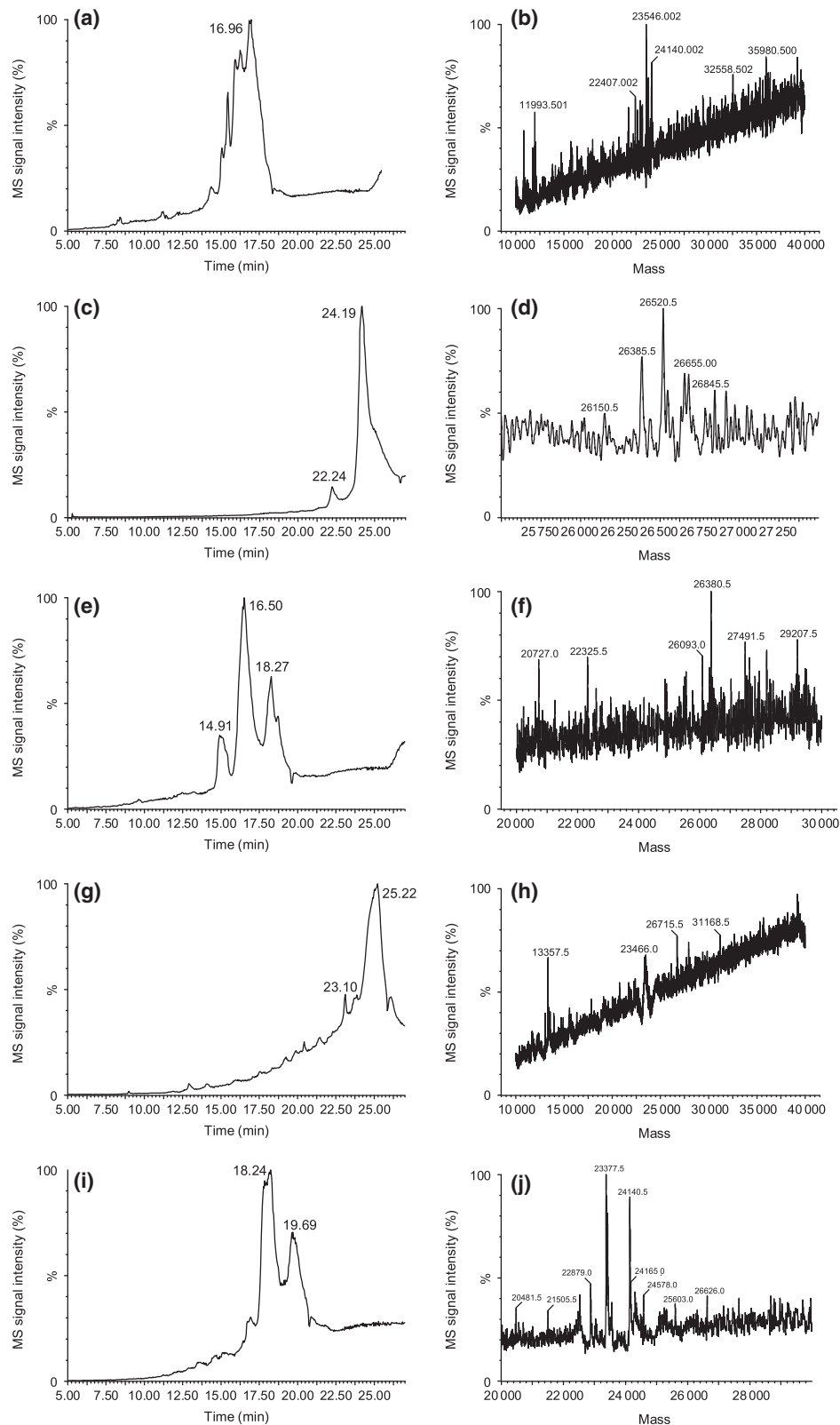


Fig. 4. LC-ESI-MS analyses of group 5 allergens purified from individual grass species. RP-HPLC chromatograms (left panels, with peaks labelled with retention times in minutes) and corresponding deconvoluted mass spectra (right panels, with peaks labelled with masses in Da) obtained with group 5 allergens from *Lolium perenne* (a and b), *Phleum pratense* (c and d), *Poa pratensis* (e and f) *Anthoxanthum odoratum* (g and h) and *Dactylis glomerata* (i and j).

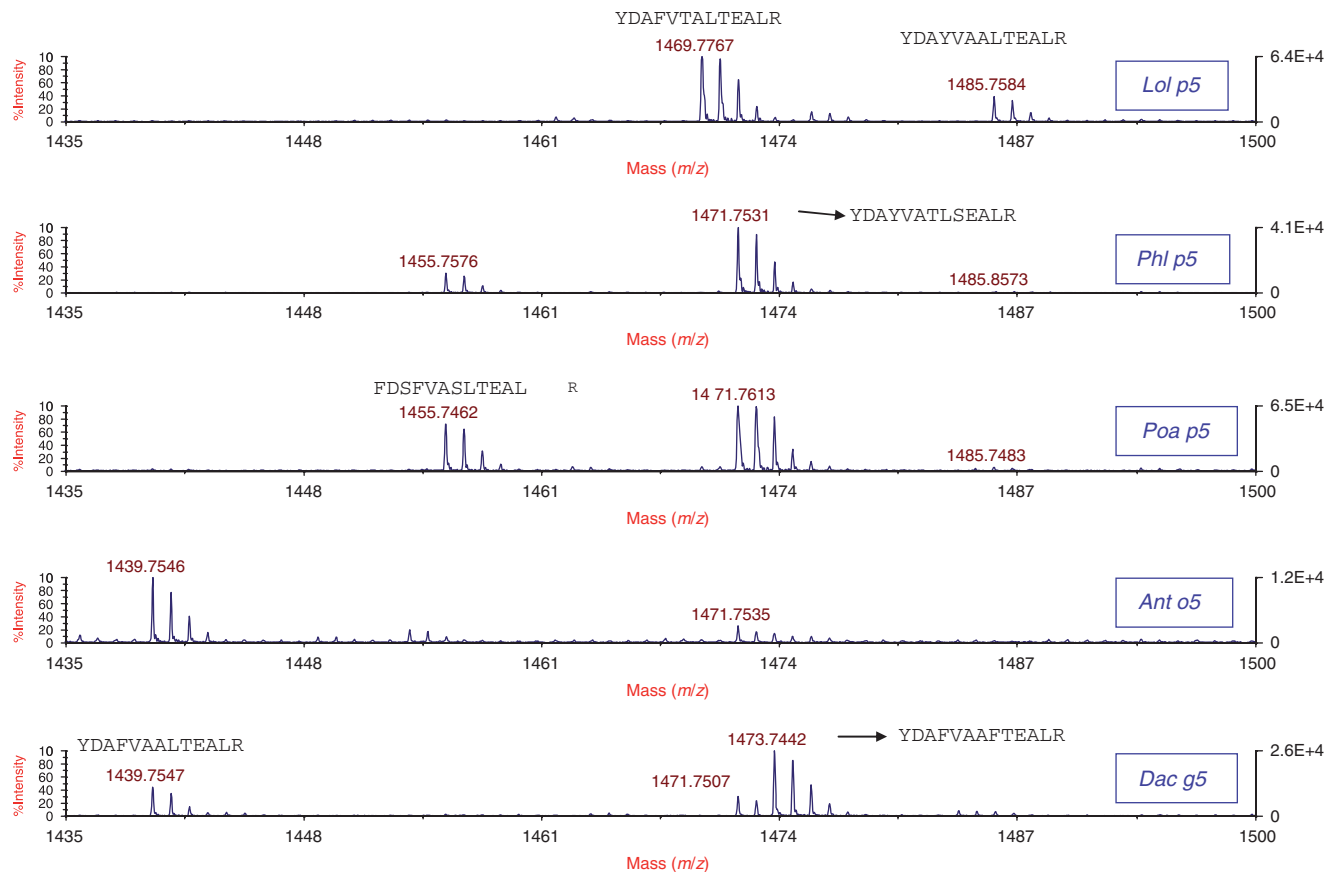


Fig. 5. Primary amino acid sequence variations within group 5 allergens from distinct grass species. MALDI-TOF mass spectra illustrating the considerable amino acid sequence heterogeneity of group 5 allergens. Species-specific peptide sequences (and corresponding masses) were deduced from LC-MS/MS experiments.

homologous among Pooideae, with an amino acid sequence homology in the range of 90%. As such, group 1 is the most conserved allergen, whereas group 5 only displays 55–80% sequence homology among species, due to deletion or insertion of stretches of amino acids depending on the grass species [9, 12]. Understanding the extent of molecular heterogeneity for those major allergens, both within and between grass species is critical, given that patients are concomitantly exposed to pollens from multiple Pooideae species [3]. Specifically, a key question is whether major allergens from distinct grass species contribute specific immune epitopes leading to patient polysensitization, as it has been suggested by previous reports [13–15].

In the present study, we assessed intra- and inter-species variability for both major group 1 and 5 allergens, using powerful analytical methods to characterize molecules purified from three distinct batches of each pollen. Within a given grass species, molecular variability was confirmed beyond expectation for group 5 allergen, and was also unambiguously demonstrated for group 1 allergens, with the presence of at least 30–50 and 5–10 prominent isoforms, respectively, within each individual

grass species. LC-MS analyses established the presence of numerous primary sequence variants for both group 1 and 5 allergens, and *N*- and *O*-glycoforms further contribute to the heterogeneity of group 1. Our observation is reminiscent of other studies demonstrating the presence of multiple allergen isoforms (e.g. for Der p 2, Bet v 1 or Ole e 1) in common allergen sources such as mites or tree pollens [26–29]. Our present results also establish that group 1 and 5 isoforms exhibit inter-species differences. In addition, as reported elsewhere, Pooideae species contain varying amounts of group 1 and 5 allergens in their pollens [2]. Although not shown, we also observed significant qualitative and quantitative differences between grass species regarding other allergens (our unpublished data) in agreement with previous reports focusing on group 2, 4 or 13 molecules [30–33].

Such an impressive molecular variability raises the possibility that allergens from various grass species bear distinct immune epitopes eliciting specific immune responses. Modelization of the 3D structure of group 1 allergens based on our experimental data confirms the presence of intra and inter-species variable amino acids at the molecular surface, which likely impact IgE and IgG

Table 2. Variability of MHC-class II restricted T cell epitopes within group 1 allergen

	Candidate T cell epitopes (identified within Phl p 1.0101)	Amino acid sequence variability	Corresponding grass species
1–15	IPKVPPGNITATYG	IAKVPPGNITAAYG	Lol p 1
		IAKVPPGNITAAYG	Poa p 1
23–37	STWYGKPTAAGPKDN	STWYGKPTGAGPKDN	Phl p 1 0102, Dac g 1, Lol p 1, Poa p 1, Ant o 1
43–57	YKDVDKPPFSGMTGC	YKDVDKAPFNGMTGC	Dac g 1, Ant o 1
		YKNVDKAPFNGMTGC	Lol p 1
		YKDVDKAPFSGMTGC	Poa p 1
59–73	NTPIFKSGRGCGSCF	NTPIFKDGRGCGSCF	Dac g 1, Lol p 1
70–84	GSCFEIKCTKPEACS	GSCFEIKCTKPESCS	Dac g 1, Lol p 1, Poa p 1
100–114	IAAYHFDLSGIAFGS	IAPYHFDLSGHAFGA	Phl p 1 0102
		IAPYHFDLSGHAFGS	Dac g 1, Lol p 1
		IAAYHFDLSGKAFGA	Poa p 1
104–118	HFDLSGIAFGSMAKK	HFDLSGHAFGAMAKK	Phl p 1 0102
		HFDLSGHAFGSMAKK	Dac g 1, Lol p 1
		HFDLSGKAFGAMAKK	Poa p 1
121–135	EQKLRSAGEVEIQFR	EQKLRSAGELELQFR	Phl p 1 0102, Dac g 1
		EQNVRSAAGELELQFR	Lol p 1
		EQKLRSAGELELKFR	Poa p 1
131–145	EIQFRRVKCKYPEGT	ELQFRRVKCKYPEGT	Phl p 1 0102, Dac g 1
		ELQFRRVKCKYPDDT	Lol p 1
		ELKFRRVKCEYPEGT	Poa p 1
156–170	NPNYLALLVKFVAGD	NPNYLALLVKYVNGD	Phl p 1 0102
		NPNYLALLVKYVDGD	Dac g 1, Ant o 1
		NPNYLAILVKYVDGD	Lol p 1
		NPNYLALLVKYVTGD	Poa p 1
160–174	LALLVKFVAGDGDVV	LALLVKYVNGDGDVV	Phl p 1 0102
		LALLVKYVDGDGDVV	Dac g 1, Ant o 1
		LAILVKYVDGDGDVV	Lol p 1
		LALLVKYVTGDGDVV	Poa p 1
171–185	GDVVAVDIKEKGKDK	Common to the 5 species	
194–208	GAIWRIDTPEVLKGP	GAIWRIDTPDKLTGP	Phl p 1 0102
		GAIWRVDTPDKLTGP	Dac g 1, Ant o 1
		GAVWRIDTPDKLTGP	Lol p 1
		GSIWRVDTPDKLTGP	Poa p 1
210–224	TVRYTTEGGTKGEAK	TVRYTTEGGTKTEAE	Phl p 1 0102
		TVRYTTEGGTKSEVE	Dac g 1
		TVRYTTEGGTKSEFE	Lol p 1
		TVRYTTEGGTKVEAE	Ant o 1
		TVRYTTEGGTKGEAE	Poa p 1

T cell epitope restricted to HLA-DRB1 molecules were identified from species-specific group 1 amino acid sequences identified by MS (cf. Table 1), using the T-epitope and proper algorithms. T cell epitopes described in the literature are also included.

responses during natural exposure or immunotherapy. Furthermore, amino acid sequence variations in class II-restricted T cell epitopes within group 1 result in significant differences in binding to common MHC class II haplotypes. As a consequence, dramatic differences are easily detected for all allergic patients tested regarding CD4⁺ T cell reactivity to group 1 or 5 allergens, depending on the grass species considered. These results are in agreement with other studies reporting isoform-specific T cell responses directed to group 1 [15] and 5 [14, 34, 35] grass pollen allergens. Also, ELISA competition experiments demonstrate that both European and North

American patients are polysensitized to species-restricted or semi-restricted epitopes within grass pollen allergens. This observation corroborates previous findings that each of group 1 and 5 allergens contains both cross-reactive as well as isoform-specific IgE epitopes [7–9, 11, 36, 37]. Whereas, all patients exhibit IgEs directed to shared epitopes, we estimate that at least 85% of them also have IgE reactivity to restricted or semi-restricted epitopes, in particular from group 5 allergens. Importantly, whereas no single extract can totally inhibit IgE binding for all the sera tested, a five grass mix always blocks IgE reactivity completely.

Table 3. *In vitro* HLA DRB1 peptide-binding assays

T cell epitope (amino acid position)	Peptide sequence	Corresponding grass species	HLA-DRB1 alleles (IC50 ratios)			
			0101	0301	0401	1501
23–37	STWYGKPTAAGPKDN	Phl p 1.0101	> 4300	> 357	533	> 276
	STWYGKPTGAGPKDN	Phl p 1.0102, Dac g 1, Lol p 1, Poa p 1, Ant o 1	> 4807	> 357	> 868	> 693
59–73	NTPIFKSGRGCGSCF	Phl p 1.0101	81	> 357	> 1 760	> 276
	NTPIFKDGRGCGSCF	Dac g 1, Lol p 1	> 4807	15	> 868	> 693
100–114	IAAYHFDLSGIAFGS	Phl p 1.0101	73	> 357	4	> 276
	IAPYHFDLSGHAFGS	Dac g 1, Lol p 1	133	125	0.4	637
121–135	EQKLRSAGEVEIQFR	Phl p 1.0101	4703	> 357	> 1 760	239
	EQNVRSAGELELQFR	Lol p 1	2415	> 357	225	84

Peptide-binding capacities were evaluated for common DRB1 molecules in a competitive ELISA assay as described in Methods. Results are expressed as a ratio between IC50 (peptide concentration inhibiting 50% binding of a biotinylated reporter peptide) values obtained with tested peptides versus reporter peptides.

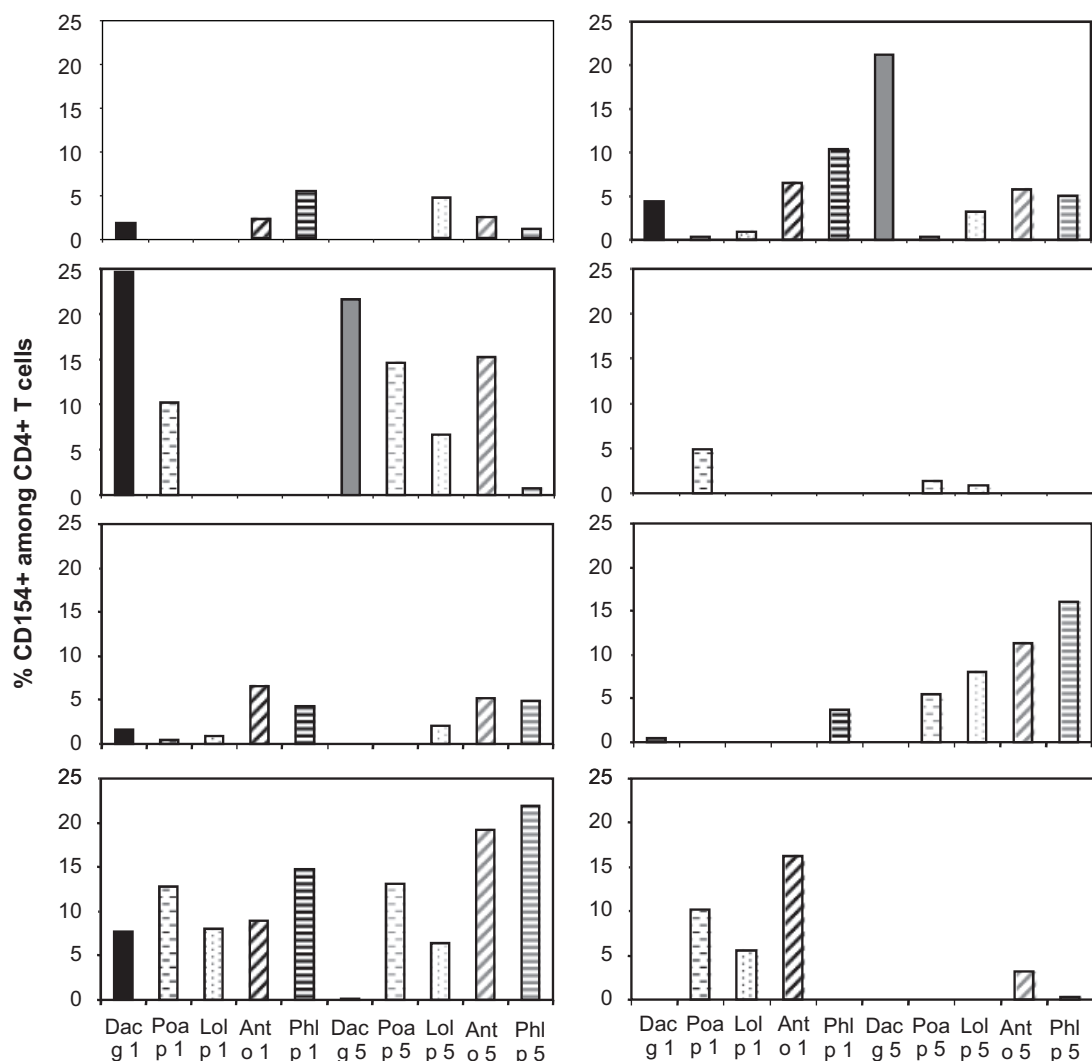


Fig. 6. T cell responses from grass pollen-allergic patients against purified group 1 and 5 allergens. PBMCs from four grass pollen-allergic patients were stimulated with each of group 1 and 5 natural purified allergens (10 µg/mL) for 14 days. Allergen-specific T cells were identified by cytofluorometry based on CD154 (CD40 ligand) expression. Background percentages of CD154-expressing cells among CD4⁺ cells obtained with unstimulated cells were subtracted from those obtained with cells stimulated with purified allergens.

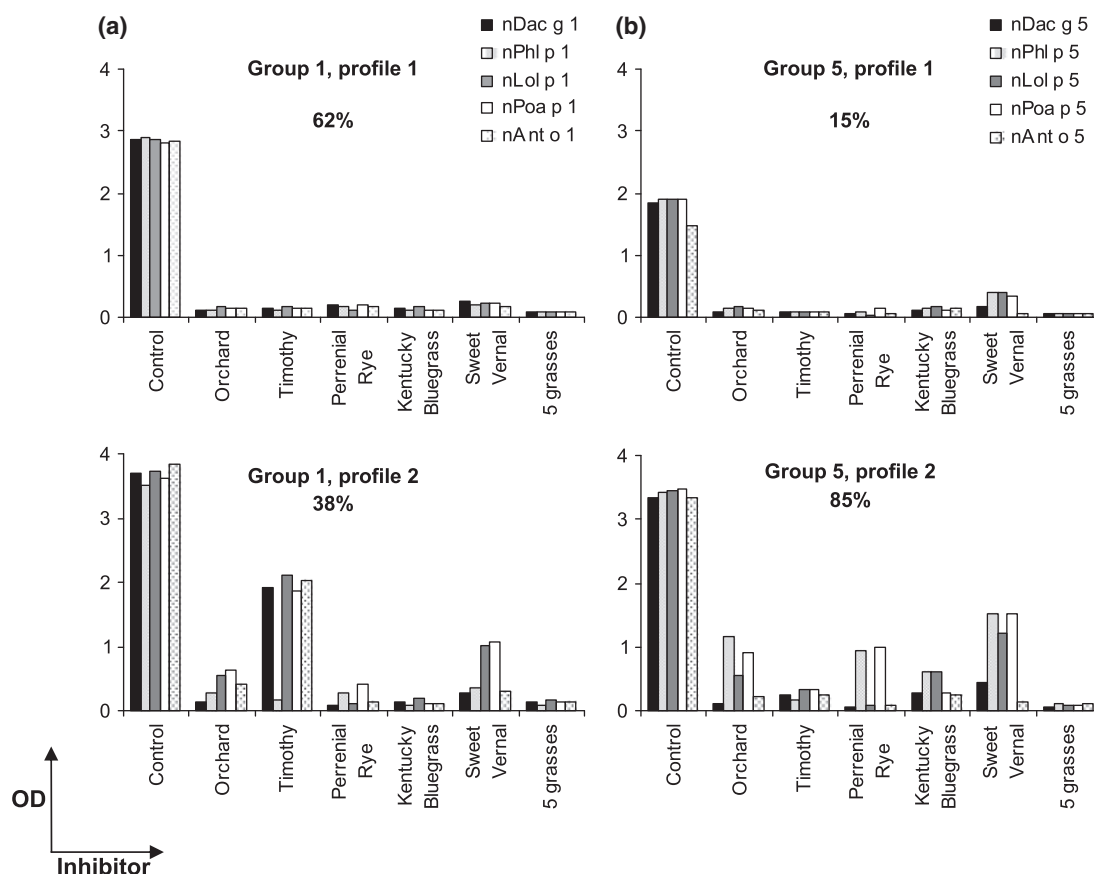


Fig. 7. IgE binding to purified group 1 or 5 allergens: competition experiments with individual or five grass extracts. In those experiments, complete inhibition was defined as $\geq 90\%$ inhibition of IgE binding to purified allergens, when compared with control samples without a competing extract. Percentages of sera displaying the various patterns illustrated above are indicated within each quadrant (out of $n = 27$). Individual sera diluted at 1 : 20 were incubated with either the five individual pollen extracts or the five-grass mixture or with buffer alone, and further added to group 1 or 5 purified allergens coated onto a 96-well plate. The absorbance (optical density, OD) corresponding to IgE antibodies bound in the absence of inhibitors was compared with the ones obtained with individual pollen extracts as an inhibitor. Percentages of sera exhibiting a complete (profile 1) or an incomplete (profile 2) inhibition of IgE binding by individual extracts (cut-off $> 10\%$ of negative control) are shown in each quadrant. The lower inhibition of IgE binding to purified group 5 allergens observed with *A. odoratum* (upper panel) is due to the low amounts of Ant o 5 in this extract. Patients with profile 1 have IgEs cross-reactive to all group 1 or group 5 allergens (i.e. directed to shared epitopes). Patients with profile 2 exhibit IgE responses to epitopes restricted, or semi-restricted to selected grass species.

Those differences in terms of molecular structures and antigenicity properties between allergens derived from distinct grass species have major implications in vaccine design. Because individual grass pollen extracts contribute shared but also species-restricted T or B cell epitopes, a five-grass pollen mix extract is more appropriate than any single grass pollen extract to treat patients exposed concomitantly to multiple allergens from various Pooideae, as it contains a broader spectrum of immune epitopes. As such, it better recapitulates natural exposure and sensitization conditions. Noteworthy, the clinical efficacy of immunotherapy protocols relying on a single (Timothy) grass pollen extract has nonetheless been documented in humans [11, 38, 39]. Although the relative efficacy of a single vs. a mixed pollen vaccine has not been assessed in a comparative trial, we infer from the present study that the latter would likely be more

efficacious than the former. Lastly, there is a growing interest in the development of allergy vaccines based on recombinant allergens [40]. The existence of numerous allergen isoforms for each allergen makes it difficult, albeit crucial, to select the proper isoform for vaccination purposes, based on abundance in natural sources and clinical relevance.

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