

Recombinant pollen allergens from *Dactylis glomerata*: preliminary evidence that human IgE cross-reactivity between *Dac g* II and *Lol p* I/II is increased following grass pollen immunotherapy

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SUMMARY

We previously described the isolation of three identical complementary DNA (cDNA) clones, constructed from Orchard/Cocksfoot grass (*Dactylis glomerata*) anther messenger RNA (mRNA), expressing a 140,000 MW β -galactosidase fusion protein recognized by IgE antibodies in atopic sera. Partial nucleotide sequencing and inferred amino acid sequence showed > 90% homology with the group II allergen from *Lolium perenne* (*Lol* II) indicating they encode the group II equivalent, *Dac g* II. Western blot immunoprobings of recombinant lysates with rabbit polyclonal, mouse monoclonal and human polyclonal antisera demonstrates immunological identity between recombinant *Dac g* II, *Lol p* I and *Lol p* II. Similar cross-identity is observed with pollen extracts from three other grass species: *Festuca rubra*, *Phleum pratense* and *Anthoxanthum odoratum*. Recombinant *Dac g* II was recognized by species- and group-cross-reactive human IgE antibodies in 33% (4/12) of sera randomly selected from grass-sensitive individuals and in 67% (14/21) of sera from patients receiving grass pollen immunotherapy, whilst 0/4 sera from patients receiving venom immunotherapy alone contained *Dac g* II cross-reactive IgE. Cross-reactive IgG4 antibodies were detectable in 95% of sera from grass pollen immunotherapy patients. These preliminary data suggest that conventional grass pollen allergoid desensitization immunotherapy may induce IgE responses to a cross-reactive epitope(s) co-expressed by grass pollen groups I and II (and possibly group III) allergens.

INTRODUCTION

Antibody recognition of allergen is required for both immune clearance¹ and activation of allergic sequelae.² That IgE recognition of allergen is critical in allergic provocation³ is well established, however the contribution of other antibody isotypes to immune clearance of allergen remains to be definitively established. The presence of allergen-specific IgG (particularly IgG1 and IgG4 subclass) antibodies in serum,⁴ and the immune clearance mechanisms involved, suggest that this antibody class is potentially protective against allergic provocation.^{5,6} Similarly the mechanisms involved in removal of IgA-containing immune complexes and its predominant role in mucosal immunity⁷ suggest this isotype may also be potentially protective in many allergies, especially asthma and allergic rhinitis. The development of specific immunotherapies designed to predictably adjust specific isotypes,⁸ or inhibit antibody–allergen interaction⁹

requires precise knowledge of allergenic epitopes recognized by each antibody class (and subclass). Furthermore, the contribution of individual species unique and cross-reactive allergenic epitopes, which may significantly affect the efficacy of any immunotherapy, requires determination.

The apparent complexity of allergenic proteins, and antibody specificities within human and rodent immune responses, has reduced availability of sufficiently purified protein from which sequence and structural information can be gained.¹⁰ The definitive identification of antigenic determinants is complicated by the demonstration that biochemically/immunologically separated, apparently homogeneous, native allergens may be contaminated by allergens of similar sequence, structural characteristics, and/or immunoreactivity. Nevertheless, putative antibody isotype-specific and T-cell recognized allergenic epitopes¹¹ have been identified based on amino acid sequence determination of purified native allergen extracts. The application of recombinant DNA technologies to the isolation of homogeneous allergen preparations provides several insights into allergen sequence diversity, and species similarities,^{12,13} and their relative importance *in vivo* during allergy.¹⁴ Thus, full-

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length cDNA clones encoding allergens from several plant species have been generated and isolated.¹²⁻¹⁴ *In vitro* expression of the cDNA clones also allows investigation of *in vivo* relevant allergen unique and cross-reactive epitopes.¹²

Pollen allergens from Orchard/Cocksfoot grass (*Dactylis glomerata*) have been demonstrated to be significant in asthma and allergic rhinitis.¹⁵ Close resemblance between major allergens in various grass species from group I and group V (including *Dac g I* and *Dac g V*) has been demonstrated in terms of expression of cross-reactive epitopes¹⁶⁻²² and N-terminal amino acid sequence.³⁵ We previously reported the construction of a cDNA library in λ gt11 and the detection of three identical clones bearing a 700 base pair (bp) insert encoding an IgE reactive *D. glomerata* pollen allergen.¹² The 24,000 MW recombinant allergens were recognized by six out of eight pollen-sensitive sera and a polyclonal rabbit antiserum against *Lol p I*²³ suggesting the recombinant allergen could be a group I member. Here we report partial nucleotide sequencing and immunoreactivity with rabbit polyclonal, murine monoclonal and human IgE and IgG4 antibodies which indicate these proteins represent the group II allergen, *Dac g II* (designated *Dac g II*_(Rec)). Furthermore, the data suggest that both human serum IgE and IgG4 antibody recognition of *Dac g II*_(Rec) is increased during immunotherapy.

METHODS AND MATERIALS

Pollen

Grass pollens were obtained from Diephuis Laboratory (Groningen, The Netherlands). Crude aqueous extracts were obtained from pollen from *Lolium perenne*, *D. glomerata*, *Festuca rubra*, *Phleum pratense* and *Anthoxanthum odoratum* as previously described.^{23,24} Isolation of *Lol p I* from extracts has also been previously described.²³ Purified *Lol p II* was obtained as reference reagent (A612-901-185) from National Institute of Health (Bethesda, MD).

Antisera

Rabbit polyclonal anti-*Lol p I* and *Dac g V* antisera were prepared by immunization with purified *Lol p I* and *Dac g V* respectively, as previously described.²³ The anti-*Lol p I* antiserum was highly reactive with *Lol p II* as demonstrated by SDS-PAGE/autoradiography analysis of [¹²⁵I] *Lol p II* binding to antiserum after precipitation with Sepharose-coupled protein A (not shown). Rabbit polyclonal antiserum [designated anti-*Lol p*_(LMW)] recognizing the low molecular weight (10,000–12,000 MW), acidic allergen fraction of *Lol p* was produced similarly by immunization with a partially purified pollen extract. Purification of this fraction was performed with a gel-filtration step (Sephacryl HR S-100: Pharmacia/LKB, Uppsala, Sweden), followed by three subsequent ion-exchange chromatographic steps (FAST-Q, FAST-S and FAST-Q, Pharmacia/LKB). This immunogen was shown to contain three allergens: *Lol p II*, an 11,000 MW polypeptide identified as a degradation product of *Lol p V*, and a so far unidentified 17,000 MW allergen.²⁵ All three components were recognized by the anti-*Lol p*_(LMW) antiserum which also weakly cross-reacted with *Lol p I*: only at high concentrations (1:10) could binding of [¹²⁵I] *Lol p I* to protein A Sepharose-precipitated antibodies be demonstrated (not shown). Monoclonal antibodies (mAb) 4B1²³ and

*Dac g II*_(Rec) nucleotide sequence (1-108):

gaggcccccgc tgaccttcac ggtggagaag ggttcgcacg agaagaacct ggcgctgctg
atcaagtaca acaaggaggc cgactccatg gcggaggtgg agctcaag

Protein sequence alignments:

	1	20
<i>Dac g II</i> _(Rec)	E A P V T F T V E K G S D E K N L <u>A L</u> S I K Y N K E	
<i>Lol p II</i>	A A P V E F T V E K G S D E K N L <u>A L</u> S I K Y N K E	
<i>Lol p III</i>	- T K V D L T V E K G S D A K T L <u>V L</u> N I K Y T R P	
<i>Lol p I</i> *	D T K P T F H V E K G S N P N Y L <u>A I</u> L V K Y V A G	
	27	36
<i>Dac g II</i> _(Rec)	- G D S M A E V E L K	
<i>Lol p II</i>	- G D S M A E V E L K	
<i>Lol p III</i>	- G D T L A E V E L R	
<i>Lol p I</i>	D G D - V V A V D I K	

* (starting at residue 144)

Figure 1. Nucleotide (5') and inferred amino acid sequence (N-terminal) of *Dac g II*_(Rec) compared with *Lol p I*, II and III allergens aligned for maximal homology. Residues in bold represent identity whereas conservative changes are underlined. Sequences for *Lol p I*, II and III were taken from refs 11, 13, 27.

IB9²⁴ recognize species-cross-reactive epitopes expressed on group V grass pollen allergens of a broad and narrow range of species (respectively). Monoclonal antibody 24-64 directed to group I grass pollen allergens was kindly provided by Dr R. E. Esch (Chapel Hill, NC).²⁰ Secondary antisera used in RAST and immunoblotting were ¹²⁵I-labelled polyclonal sheep anti-human IgE, polyclonal sheep anti-rabbit IgG, polyclonal goat anti-mouse IgG, and monoclonal murine anti-human IgG4 (CLB, Amsterdam, The Netherlands). Polyclonal antisera were affinity purified, using human IgE, total rabbit immunoglobulin and mouse IgGκ immunosorbents respectively, prior to use.

Human sera

Human serum samples from grass pollen-sensitive patients were selected from sera submitted for allergy serology (CLB) on the basis of high anti-grass pollen IgE titres (>10% binding in RAST for grass pollen). Serum samples from individual receiving grass pollen allergoid or wasp (*Vespula*) venom desensitization immunotherapy were obtained from general practitioners throughout The Netherlands. However no further clinical data regarding the type, source or amount of the allergoid extracts used, or the stage during immunotherapy at which samples were taken, are presently available.

Recombinant fusion proteins

The *D. glomerata* pollen allergen fusion proteins used in this study have been previously described.¹² Essentially, lysates were prepared according to Gupta *et al.*²⁶ from *Escherichia coli* Y1089 infected by recombinant or non-recombinant λ gt11 clones and induced by addition of 10 mM isopropyl β -D thiogalactoside (IPTG) prior to incubation at 37° for 1 hr.

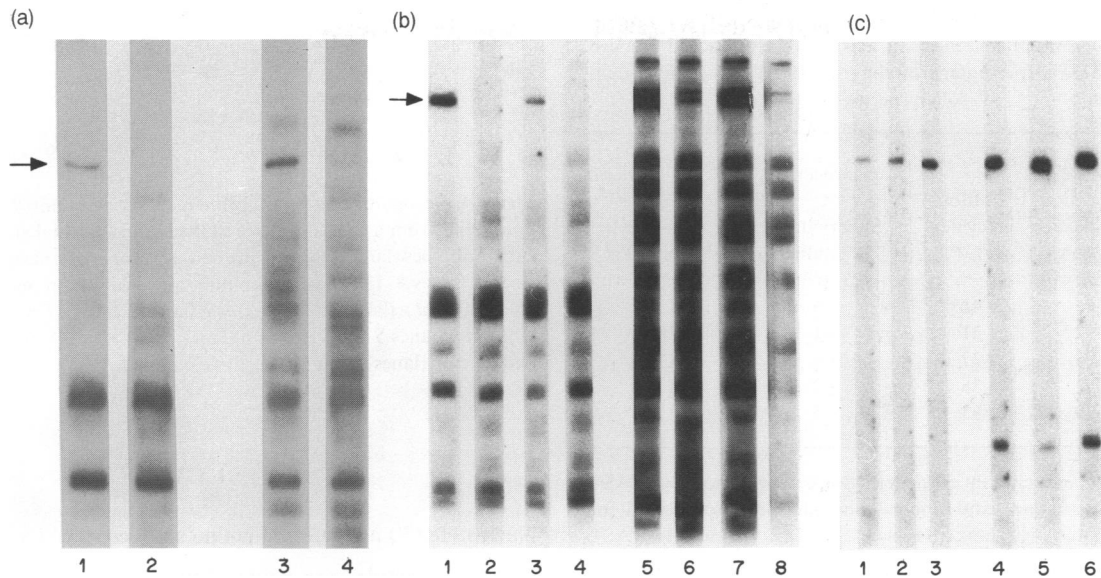


Figure 2. (a) Immunoprobings of Western blots from SDS-PAGE separated recombinant (lanes 1 and 3), and non-recombinant (lanes 2 and 4) lysates with rabbit polyclonal anti-*Lol p I* (lanes 1 and 2), and anti-*Lol p_(LMW)* (lanes 3 and 4). (b) Inhibition of rabbit polyclonal anti-*Lol p I* (lanes 1-4) and anti-*Lol p_(LMW)* (lanes 5-8) binding to the fusion protein (arrowed) in Western blots. Lanes 1 and 5 = no inhibitor; lanes 2 and 6 = *Dac g* total extract; lanes 3 and 7 = purified *Lol p I*; lanes 4 and 8 = purified *Lol p II* as inhibitors. (c) Fusion protein reactivity with rabbit polyclonal anti-*Lol p I* (lanes 1-3) and anti-*Lol p_(LMW)* (lanes 4-6) antibodies affinity-purified using Sepharose coupled to total *Lol p* extract (lanes 1 and 4), *Lol p I* (lanes 2 and 5) and *Lol p II* (lanes 3 and 6).

Nucleotide sequencing

DNA insert sequences from recombinant clones 7·8, 8·1 and 8·3 were amplified *in vitro* using the polymerase chain reaction as previously described¹² and nucleotide sequence directly determined using a Sequenase™ kit (United States Biochemicals, Cambridge, U.K.) employing a modified protocol which included the addition of 1 µg *E. coli* single-stranded binding protein (United States Biochemicals, Cambridge, U.K.) to each sequencing reaction.

Immuno-affinity purification of human and rabbit antibodies

Five milligrams (dry weight) of pollen extracts (*Dac g*, *Lol p*, *Fes r*, *Phl p*, *Ant o*), 1·2 mg (determined spectrophotometrically) of *Lol p I* and 1 mg of *Lol p II* were individually coupled to 1 g CNBr-activated Sepharose 4B (Pharmacia/LKB). Glycine-inactivated Sepharose was used as a negative control. One hundred milligrams of the prepared Sepharose was incubated with antisera for 2 hr at room temperature with end-over-end rotation in a total volume of 10 ml [phosphate-buffered saline (PBS)/10 mM EDTA/0·1% Tween 20/0·05% NaN₃, pH 7·4]. The volume of antisera used varied according to titres: 2 ml (using *Lol p I* Sepharose) or 1 ml (using total pollen Sepharose) serum for human IgE, 250 µl for human IgG4 and 25 µl for rabbit IgG. Incubations were stopped by centrifugation, supernatants were removed for separate analysis and the Sepharose washed five times with PBS. Bound immunoglobulin was eluted by 3 × 1 min incubations with 2 ml 0·1 M glycine/HCl/0·3% bovine serum albumin (BSA) pH 2·5. The eluates were pooled and neutralized (pH 7·0) with 1 M Tris (325 µl).

SDS-PAGE/Western blotting

Recombinant and non-recombinant lysates (500 µl/gel), or crude aqueous extract of *Dac g* pollen (500 µl of 2 mg/ml) were

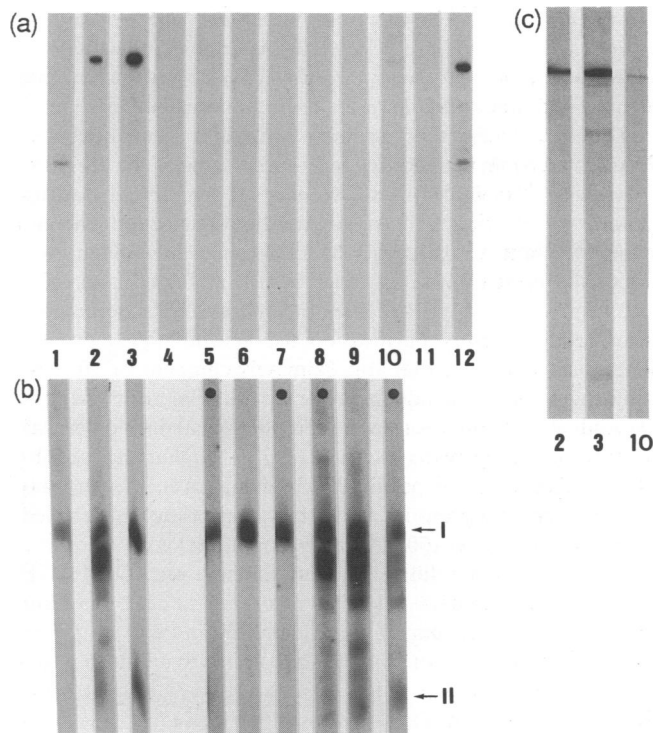


Figure 3. (a) Recognition of fusion protein on Western blots by IgE in serum from 12 grass pollen sensitive (untreated) individuals, showing recognition of the 140,000 MW band in lanes 2, 3, 10 and 12. (b) Binding of antibodies in the same sera (except numbers 4, 11 and 12) to *Dac g* total extracts. Positions of *Dac g I* and *II* proteins are indicated. (c) Fusion protein reactivity with IgE antibodies affinity purified from sera 2, 3 and 10 using Sepharose-coupled *Lol p I*. Lanes subject to 72 hr autoradiography (●) compared to unmarked lanes obtained using 18 hr exposure.

Table 1. RAST analysis of cross-reactivity of human IgE between *Dac g*, *Lol p*, *Fes r*, *Phl p* and *Ant o* pollen extracts. The starting serum showed similar (28.1–31.0%) percentage binding in RAST for all five grass pollen species

Affinity matrix	Depletion efficiency (%) [*]	Elution efficiency (%) [*]	Cross-reactivity in RAST (<i>Dac g</i>)	
			Supernatant % binding	Eluate % binding
<i>Dac g</i>	95	42	1.6	13.0
<i>Lol p</i>	92	31	5.1	10.2
<i>Fes r</i>	93	37	8.9	11.6
<i>Phl p</i>	92	35	9.0	12.5
<i>Ant o</i>	90	35	9.5	12.2

^{*} Depletion and elution efficiencies were determined by RAST analysis of supernatants and eluates using the Sepharoses used for affinity purification, whereas cross-reactivity was determined in RAST using Sepharose coupled to *Dac g* total pollen extract.

heated for 5 min (90°) in an equal volume of sample disruption buffer containing 20 mM dithiothreitol prior to electrophoresis under reducing conditions in a 3% stacking and a 7% resolving gel. Electrophoretic transfer to nitrocellulose (1 mA/cm² for 1 hr) was performed semi-dry on a Novablot™ electrophoretic transfer apparatus (Pharmacia/LKB). After blocking with PBS/1.8% (w/v) BSA/0.1% (v/v) Tween 20, the blots were cut into strips of 4–6 mm × 12.5 cm prior to immunoprobings.

Differing volumes of primary antibodies were incubated overnight at room temperature in a total volume of 5 ml [in PBS/0.3% (w/v) BSA/0.1% (v/v) Tween 20]. Thus, 250 µl starting serum was added per strip for IgE and IgG4 detection whereas one-third of antibodies affinity-purified using *Lol p* I Sepharose was used (purified from 2 ml and 250 µl serum for IgE and IgG4 detection respectively). Volumes of eluates and supernatants derived from affinity purification using total pollen protein Sepharoses were used that approximately compensated for the dilution of 600 µl starting human serum. Similar adjustment of the volume of affinity-purified rabbit polyclonal antibodies was used to correlate with the use of 25 µl starting serum. Immunodetection was performed following overnight incubation at room temperature with the appropriate ¹²⁵I-labelled secondary antibodies (50,000 c.p.m./strip).

Inhibition of binding of rabbit antisera to SDS-PAGE separated recombinant allergen was performed by a 2-hr preincubation of serum (5 µl) and inhibitor (500 µg *Dac g* extract, 100 µg *Lol p* I or 50 µg *Lol p* II) prior to addition to the nitrocellulose strip.

RAST

RAST was performed as previously described²³ using Sepharose-coupled allergens using the same amounts of crude pollen extracts (*Dac g*, *Lol p*, *Fes r*, *Phl p* and *Ant o*) in the same amounts used for antibody immunoaffinity purification (above). Per test, 1.25 mg Sepharose and 50 µl human serum were used, whereas supernatants and eluates obtained by affinity purification were used in volumes corrected for dilution such that they were equivalent to 50 µl of starting serum.

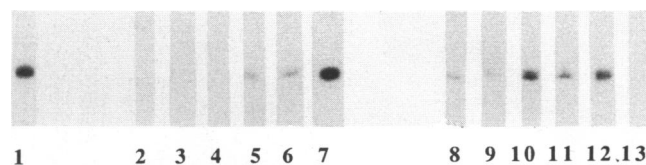


Figure 4. Recognition of fusion protein on Western blots by IgE serum antibodies from a grass-sensitive individual using total starting serum IgE antibodies (lane 1), or IgE antibodies in supernatants (lanes 2–7) or eluates (lanes 8–13) following immunopurification on Sepharose coupled with *Lol p* (lanes 2 and 8), *Dac g* (lanes 3 and 9), *Fes r* (lanes 4 and 10), *Phl p* (lanes 5 and 11), *Ant o* (lanes 6 and 12) grass pollen extracts, and glycine (lanes 7 and 13) negative controls.

RESULTS

Nucleotide (5') and inferred amino acid sequence (N-terminal)

Partial nucleotide sequencing of the 5' end of the three cDNA clones¹² was determined and the consensus sequence of the first 108 bp is shown in Fig. 1 together with the inferred amino acid sequence. Aligning published amino acid sequences for *Lol p* I, II and III^{11,13,27} for maximum homology demonstrates that the recombinant *Dac g* allergen is >90% homologous with *Lol p* II. There is also considerable homology with *Lol p* I and III, as previously described for *Lol p* II.

Reactivity with rabbit polyclonal and murine monoclonal antibodies

Immunoprobings of recombinant and non-recombinant lysates following Western blotting reveals that the 140,000 MW fusion protein is recognized by cross-reactive antibodies in rabbit polyclonal anti-*Lol p* I and *Lol p* (LMW) antisera (Fig. 2a). The fusion protein was unreactive with anti-group I mAb 24-64, anti-group V mAb 4B1 and 1B9, and rabbit polyclonal anti-*Dac g* V antibodies (not shown).

Binding of anti-*Lol p* I antiserum to the recombinant *Dac g* fusion protein could be totally inhibited by prior incubation with purified *Dac g* total pollen extract and purified *Lol p* II, and partially inhibited with equivalent concentrations of purified *Lol p* I (Fig. 2b, tracks 1–4). Binding of anti-*Lol p* (LMW) antiserum to the fusion protein was almost completely inhibited by co-incubation with purified *Lol p* II and partially inhibited by *Dac g* total pollen extract whilst purified *Lol p* I had little inhibitory effect (Fig. 2b, tracks 5–8). Both antisera detected additional molecular weight species present in the recombinant lysates. Figure 2c shows the results obtained using antibodies purified from both antisera using *Lol p* total extract (tracks 1 and 4), purified *Lol p* I (tracks 2 and 5) and *Lol p* II (tracks 3 and 6) coupled to Sepharose. In all cases the 140,000 MW fusion protein was recognized (to varying degrees) whilst the additional lower molecular weight bands observed using total antiserum were absent; the exception being affinity-purified IgG from anti-*Lol p* (LMW) antiserum which consistently recognized an additional low molecular weight band of approximately 50,000 MW (see tracks 4–6), presumed to be a cleavage product derived from the fusion protein.

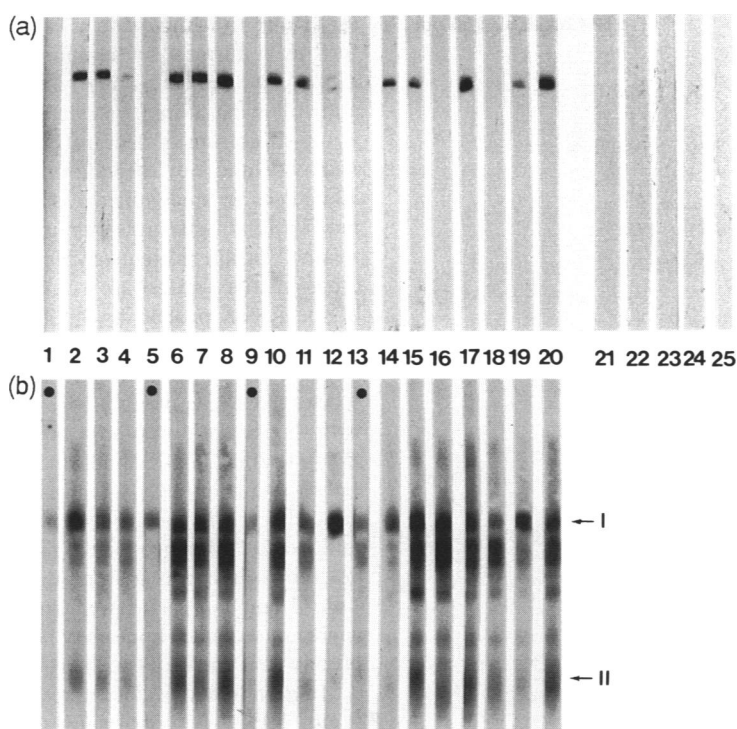


Figure 5. Recognition of the fusion protein (a) and total *Dac g* extract (b) in Western blots by human IgE in the sera from grass-sensitive individuals receiving grass pollen hyposensitization immunotherapy (lanes 1–20) and control sera of patients receiving wasp venom immunotherapy (lanes 21–25). Patient 22 received both grass pollen and wasp venom immunotherapy. Lanes subject to 72 hr autoradiography (●) compared to unmarked lanes obtained using 18 hr exposure. Positions of *Dac g* I and *Dac g* II proteins are also indicated.

Reactivity of fusion protein with human serum IgE antibodies

Western blot immunoprobings of fusion protein reactivity with antibodies in individual grass-sensitive patients demonstrated that 4/12 (33%) contained reactive IgE antibodies, one of which (track 10) gave a very low signal (Fig. 3a). Nine of these sera, including three of the four sera with *Dac g* II_(Rec)-reactive IgE antibodies, were also tested for IgE recognition of Western blots of total *Dac g* extract (Fig. 3b). All nine sera recognized a band at the *Dac g* I level while only five sera (including the three with fusion protein-reactive IgE antibodies) contained IgE antibodies binding in the group II molecular weight range. Anti-*Lol p* I IgE antibodies affinity purified from the fusion protein-reactive sera retained cross-reactivity with the fusion protein (Fig. 3c).

The extent to which *Dac g* II_(Rec) expressed cross-reactivity epitopes on allergens from other grass species was further investigated using IgE affinity purified from one of the sera using Sepharose coupled to total pollen extracts from five grass species. As can be seen from Table 1, RAST analysis revealed the efficiency of depletion was >90% for all extracts whereas between 31 and 42% of the original IgE anti-pollen activity was retained after elution. This discrepancy can be attributed to losses in recovery of the IgE antibodies during elution making this a qualitative rather than quantitative analysis. However, IgE in all eluates bound *Dac g* total extract in RAST and *Dac g* II_(Rec) in immunoblotting (Fig. 4). Both assays demonstrated supernatant IgE binding increased in the following order: *Dac g*, *Lol p*, *Fes r*, *Phl p* and *Ant o*.

IgE reactivity with *Dac g* II_(Rec) fusion protein was demonstrable in 14 out of 21 (67%) sera from grass pollen-sensitive patients receiving hyposensitization immunotherapy (Fig. 5a). All of the sera tested (20 in total) in immunoprobings of *Dac g* total extract showed IgE reactivity with molecular weight proteins in the *Dac g* I range (Fig. 5b) and 12/14 fusion protein-reactive sera clearly showed IgE reactivity with proteins in the group II molecular weight. Of the six *Dac g* II_(Rec) fusion protein unreactive sera tested, only two clearly recognized a band at the 11,000 MW level.

Reactivity of fusion protein with human serum IgG4 antibodies

Sera from grass pollen immunotherapy patients were also tested for *Dac g* II_(Rec) fusion protein-specific IgG4 antibodies (Fig. 6a). Ninety-five per cent (20/21) contained detectable *Dac g* II_(Rec) fusion protein-specific IgG4 antibodies compared to 0/4 patients receiving wasp hyposensitization immunotherapy. As can be seen from Fig. 6b, 9/10 sera contained *Lol p* I-specific IgG4 antibodies that at least partly cross-reacted with *Dac g* II_(Rec).

DISCUSSION

Previously we described the identification of three λ gt11 cDNA clones (designated 7-8, 8-1 and 8-3) containing apparently identical 700 bp inserts encoding expression of a 24,000 MW (aglycosylated) protein expressing human IgE recognized anti-

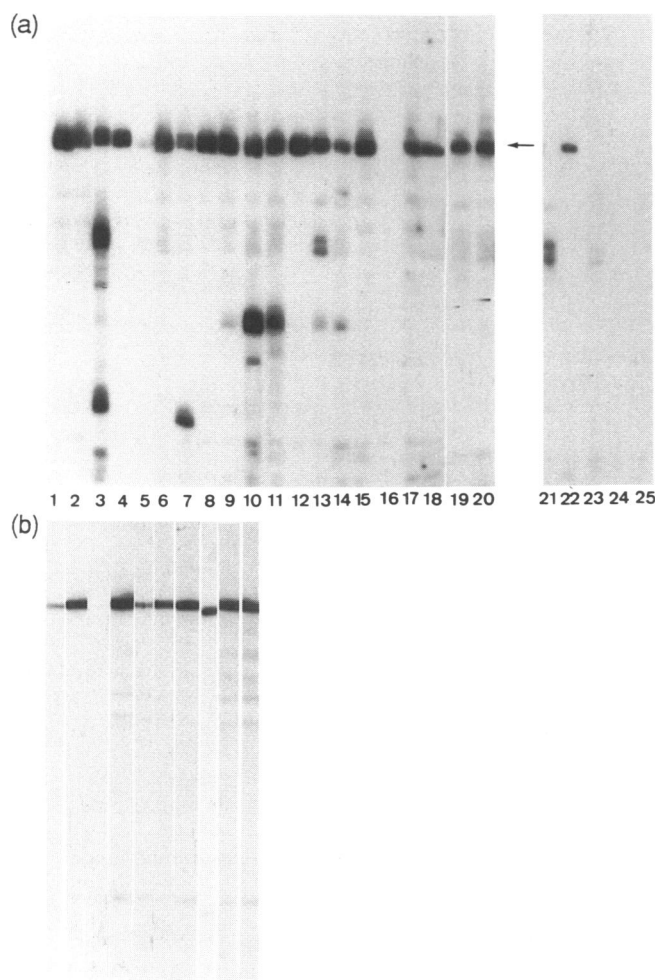


Figure 6. (a) Fusion protein (arrowed) reactivity, in Western blot immunoprobings, of IgG4 antibodies in the same immunotherapy sera as used in Fig. 5. (b) Fusion protein reactivity following affinity purification of IgG4 antibodies from sera 1–10 using Sepharose-coupled *Lol p* I.

genic determinants characteristic of a major allergen from *D. glomerata*,^{12,28–30} i.e. 27,000–33,000 MW (glycosylated).

Preliminary nucleotide sequence obtained from polymerase chain reaction amplified products and the inferred N-terminal amino acid sequence obtained within this study indicates that the inserts encode a recombinant *Dactylis* allergen homologous to the group II pollen allergen *Lol p* II (Fig. 1), i.e. *Dac g* II. Native group II grass pollen allergens are however detected as 10,000–13,000 MW (glycosylated) proteins in most assays,^{11,27} and in the case of *Lol p* II composed of 97 amino acids.²⁷ Whether the 24,000 MW protein expressed from these inserts reflects a full-length *Dac g* II transcript encoding a precursor protein that is subsequently cleaved *in vivo* or *in vitro* to yield the classical 10,000–13,000 MW group II allergen, or the addition of non-allergen encoding sequence C-terminally, remains to be determined. In either case, immunoreactivity studies using rabbit and human polyclonal antisera provides little evidence of antigenic modification between native and recombinant *Dac g* II.

Group II membership of the recombinant *Dac g* allergen is further confirmed by immunoreactivity (and inhibition) of rabbit polyclonal anti-*Lol p* I and anti-*Lol p*_(LMW) antisera before and following affinity purification. Reactivity of the anti-*Lol p*_(LMW) antiserum is demonstrated to be due to anti-*Lol p* II reactivity and not recognition of a subproduct of group V allergen or an unidentified 17,000 MW allergen.²⁵ Thus, affinity purification of this antiserum using Sepharose-coupled *Lol p* II retained fusion protein reactivity (Fig. 2c) whilst two species cross-reactive anti-group V mAb did not bind. The recognition of the fusion protein by IgG antibodies in the anti-*Lol p* I antiserum before (Fig. 2a) and after affinity purification using Sepharose-coupled *Lol p* I or *Lol p* II (Fig. 2c), but not by the species cross-reactive anti-*Lol p* I mAb 26-64,²⁰ is also consistent with the recognition of cross-reactive sites shared by *Lol p* II, *Dac g* II and *Lol p* I. Similarly the complete inhibition of anti-*Lol p* I and anti-*Lol p*_(LMW) antisera recognition of the recombinant allergen by purified *Lol p* II, and partial inhibition by purified *Lol p* I suggests recognition of cross-reactive *Dac g* II determinants, consistent with the sequence homologies between *Lol p* I and *Lol p* II. It remains to be determined if the high degree of sequence homology between *Lol p* III and the fusion protein also correlates with cross-reactive epitope expression.

Immunoreactivity of the recombinant allergen with human serum IgE also supports the recombinant *Dactylis* allergen designation as *Dac g* II_(Rec). The frequency of specific IgE against the fusion protein in a group of grass-sensitive patients is far too low (33%) compared to the usually reported frequency of recognition of group I allergen (80–90%).¹⁷ Whilst all 29 grass-sensitive sera (treated and untreated) recognized *Dac g* I in crude *Dac g* pollen extracts, only 17 were reactive with the fusion protein. Of these, 15 clearly, and two weakly, showed reactivity in the group II molecular weight range, and none of the seven sera unreactive with the fusion protein were reactive with native *Dac g* allergens at this level. Human IgE reactivity is also demonstrated to be largely due to the recognition of species cross-reactive group I and group II epitopes with cross-reactivities observed using sera directly or IgE antibodies immuno-affinity purified using Sepharose coupled with native *Lol p*, *Dac g*, *Fes r*, *Ant o* or *Phl p*. This cross-reactivity can also be seen to decrease with phylogenetic affinity to *Dac g*: in decreasing order *Lol p*, *Fes r*, *Phl p* and *Ant o*.³¹ Insufficient data are currently available to enable sequence correlation with this pattern of reactivity.

Cross-reactivity of human IgG4 antibodies in 20/21 (95%) sera from immunotherapy patients with *Dac g* II_(Rec) and *Lol p* I is also demonstrated. That these sera also showed a higher degree of IgE recognition of *Dac g* II_(Rec) (66% compared with 33% in the untreated group), but a generally lower titre of grass pollen reactive IgE antibodies (R. Van Ree, unpublished data), suggests induction of IgE by hyposensitization immunotherapy (Fig. 5a). The data also suggest a qualitative increase in IgG4 in these sera, generally exceeding the apparent increase in IgE recognition which may sufficiently compensate *in vivo* to allow clinical improvement. Measurement of IgE versus IgG4 responses to *Dac g* II_(Rec) may therefore have utility in monitoring immunotherapy efficacy. However, in the absence of data regarding the clinical benefits obtained, form and timing of the immunotherapy administered prior to sampling, this is at present difficult to prove. Further longitudinal patient samples,

for which clinical data are available, require analysis to enable quantification of the amount, and clinical significance, of immunotherapy-elicited IgE and IgG4 responses to groups I, II and possibly III pollen allergen cross-reactive epitopes. Such essential further studies are currently underway.

Nevertheless, the preliminary data presented suggest that current grass pollen allergoids may increase potentially deleterious IgE responses to groups I/II/III unique and/or cross-reactive epitopes; envisaged to arise from the presentation of the altered conformations of chemically denatured, degraded and/or polymerized native allergen comprising allergoid preparations.^{32,33} The molecular basis of such changes in conformation remain to be established, as do the contributions of the species sequence homologies between groups I, II and III grass pollen previously suggested to represent B-cell and T-cell epitopes.^{11,27,34} Further investigation of the immunoreactivity of defined human antibody probes with *Dac g* II_(Rec) (and engineered/mutated gene constructs), will facilitate unravelling of the molecular basis of IgE/IgG recognition of groups I and II grass pollen allergens *in vivo*. The demonstration of species conservation of such epitopes suggest that these data may contribute to the generation of specific and effective allergen polypeptide-based immunotherapeutic agents, of utility to the majority of grass-sensitive individuals.

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