

Characterization with monoclonal and polyclonal antibodies of a new major allergen from grass pollen in the group I molecular weight range

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*We have purified a 24/25 kd allergen from orchard grass pollen (*Dactylis glomerata*) that has an allergenic potency similar to that of the major group I allergen. We provisionally named this allergen grass 4B1 after the monoclonal antibody used for its identification and purification. This monoclonal antibody was obtained by immunizing mice with whole *Lolium perenne*-pollen extract and by screening the antibody producing hybrids for reactivity with *Dactylis glomerata*-pollen extract. Grass 4B1 is physicochemically separable from the group I allergen. Polyclonal rabbit antibodies to grass 4B1 do not react with group I allergen or vice versa. Ninety-five sera with IgE antibodies to grass pollen were tested for IgE antibodies to grass 4B1, and >90% was positive in this test. The median response to grass 4B1 was 70% of that to *Lol p I*. (*J ALLERGY CLIN IMMUNOL* 1989;83:144-51.)*

The most intensively studied major allergen of grass pollen is Rye I or *Lol p I*,^{1,2} a protein from ryegrass pollen (*Lolium perenne*). In many other grass species, a homologous protein exists,³ for example, *Dac g I* in *Dactylis glomerata*, which is immunologically very similar. For this reason, it is common to refer to this group of grass allergens as group I allergen. We produced rabbit antibodies to *Lol p I* that were found to be cross-reactive with *Dac g I*. There is no agreement about the physicochemical characteristics of *Lol p I*, notably the MW; it is referred to as a protein with an MW between 25 kd and 35 kd.⁴⁻⁸

It is agreed, however, that it is a major allergen, if it is not the most important major allergen of grass pollen. About 90% of the patients with grass-pollen allergy have IgE antibodies against it.⁹

In the present article, we describe the isolation of another major allergen in the MW region of group I that is distinct from *Lol p I*. This protein has not been

Abbreviations used

<i>Lp</i> :	<i>Lolium perenne</i>
<i>Dg</i> :	<i>Dactylis glomerata</i>
<i>Pp</i> :	<i>Phleum pratense</i>
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
McAb:	Monoclonal antibody
HPLC:	High-performance liquid chromatography
PBS:	Phosphate-buffered saline
CBB:	Coomassie brilliant blue
BSA:	Bovine serum albumin
MW:	Molecular weight

identified earlier as a separate major allergen, possibly because it has a molecular weight similar to *Lol p I*.

Throughout this article we use the allergen nomenclature recommended by the International Union of Immunological Societies.¹⁰

MATERIAL AND METHODS

Pollen extracts

Ryegrass (*Lp*) pollen and orchard grass (*Dg*) pollen were obtained from Diephuis Laboratories (Groningen, The Netherlands). The pollen was extracted with distilled water (5% wt/vol) under continuous shaking at room temperature for 2 hours. The pH was maintained at 8.5 by readjustment with 0.1 mol/L of NaOH every 15 minutes. The pollen

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Received for publication March 1, 1988.

Accepted for publication July 13, 1988.

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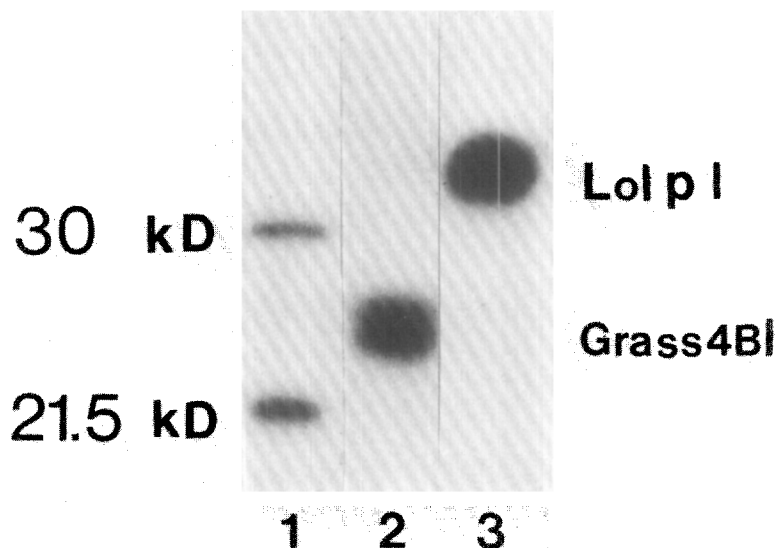


FIG. 1. Autoradiography (24 hours) of ^{125}I -labeled grass 4B1 (lane 2) and *Lol p I* (lane 3) after SDS-PAGE. In lane 1, MW markers of 30 kd (top) and 21.5 kd.

suspension was centrifuged for 15 minutes at 10,000 *g*. The supernatant was defatted with Freon TF (Brummelhuis H. G. J. Personal communication). Fifty percent (vol/vol) Freon was mixed for 2 hours with the pollen extract. The suspension was centrifuged, and the upper phase was dialyzed against distilled water and freeze-dried.

Selection of sera

The 95 sera used in the grass 4B1 and *Lol p I* RAST were obtained from Dutch patients (predominantly untreated) who demonstrated specific IgE antibodies (>2% binding) in the routine grass-pollen (*Dg* and *Pp*) RAST (see below). The 157 sera tested in the IgG antigen-binding assay were obtained from a group of Dutch patients who received immunotherapy for grass-pollen allergy.

Purification of *Lol p I* (31 kd)

For the purification of *Lol p I*, we used dialyzed and lyophilized ryegrass-pollen extract. As a first step, 350 mg in 5 ml of PBS was applied to a Sephadex G-75 column (3.5 by 90 cm) equilibrated in PBS. We analyzed the fractions by SDS-PAGE and pooled the fractions that contained the 31 kd band. Subsequently, this pool was concentrated five times by filtration over an Amicon YM10 (Amicon Corp., Danvers, Mass.) filter and dialyzed overnight against 1 mol/L of ammonium sulfate. It was then applied to a phenyl-Sepharose column (1.5 by 40 cm) equilibrated in 1 mol/L of ammonium sulfate. Under these conditions, most (glyco)proteins were bound. However, the major part of the 31 kd protein did not bind. Bound material was eluted with a linear gradient from 1 mol/L of ammonium sulfate to 50% ethylene glycol. The nonbound material was dialyzed overnight against 20 mmol/L of Tris HCl, pH 8.0, concentrated 10 times by filtration over an Amicon YM10 filter, and applied to a TSK (LKB-Produkter AB, Bromma, Sweden)-diethylaminoethyl 5 PW column (HPLC), equilibrated in

20 mmol/L of Tris HCl (pH 8.0), followed by elution with a linear NaCl gradient from 0 to 0.5 mol/L. The flow was 1 ml/min, resulting in a barometric pressure of 5. The 31 kd material eluted between 0.05 and 0.1 mol/L of NaCl.

Purification of grass 4B1 (24/25 kd)

McAbs directed against grass 4B1 (see below) were used for the purification of this allergen. For this purpose, 4 ml of ascites containing antigass 4B1 was applied to a protein A column (equilibrated in 0.1 mol/L of phosphate buffer, pH 8); bound IgG was eluted with 0.1 mol/L of citrate buffer (pH 3), followed by immediate neutralization on a Sephadex G-25 column equilibrated in PBS. The antibody-containing fractions were pooled and subsequently coupled to 1 gm of CNBr-activated Sepharose. Crude *Dg*-pollen extract, dialyzed and lyophilized (500 mg in 5 ml of PBS), was allowed to bind to this solid phase. Bound components were eluted with 0.1 mol/L of glycine HCl, pH 2.5, followed by immediate neutralization on a Sephadex G-25 column equilibrated in PBS. In this way, we collected 6.1 ml of grass 4B1 with $E_{280\text{ nm}}^{1\text{ cm}} = 0.58$.

SDS-PAGE immunoblotting

Vertical slab-gel electrophoresis was carried out on an apparatus from Bio-Rad Laboratories according to the method of Laemmli.¹¹ Gels (0.75 mm) were run at 30 mA for 4 hours. A 12.5% resolving gel and a 3% stacking gel was used, and the reducing agent was either mercaptoethanol or dithiothreitol. Staining was performed with CBB. Electrophoretic transfer to nitrocellulose was performed overnight according to the method described by Burnette.¹² Immunodetection was done with human IgE or IgG4 antibodies (150 μl and 80 μl of serum, respectively) in conjunction with the appropriate ^{125}I -labeled second antibodies (50,000 cpm per nitrocellulose strip). Both incubations (first and second antibody) were carried out overnight in 4 ml of

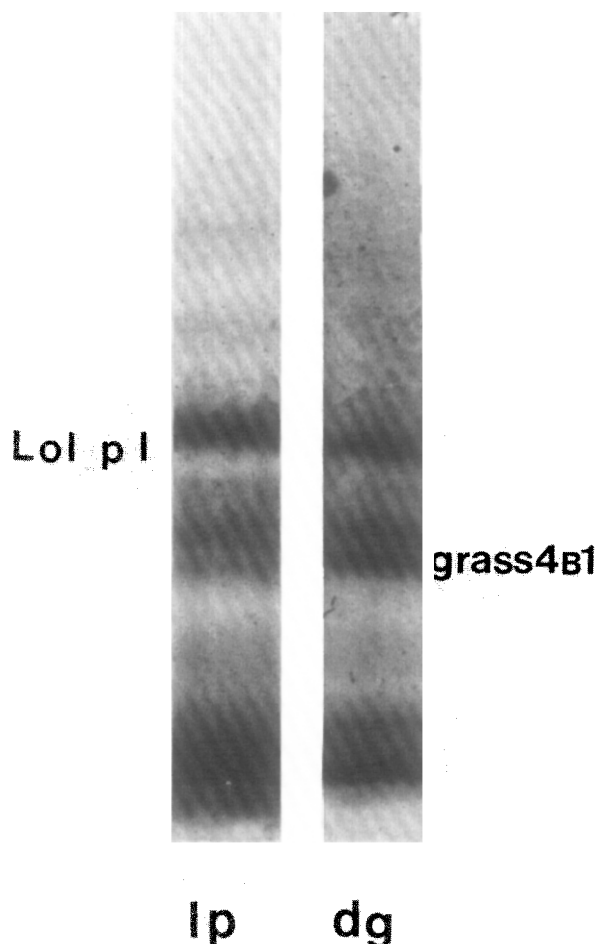


FIG. 2. SDS-PAGE (CBB staining) of *Lp* and *Dg* extract (25 μ g). The main band in the group I range (25 to 35 kD) corresponds to 31 kD. It can be observed that *Dg* contains more grass 4B1.

PBS (per strip) containing 3 mg/ml of BSA, 0.01 mol/L of ethylenediaminetetraacetic acid, 0.5 mg/ml of NaN_3 , and 0.1% Tween 20 (vol/vol). Autoradiography was performed by exposing dried gels or immunoblots to x-ray film (Kodak X-Omat S, Eastman Kodak Co., Rochester, N.Y.).

Iodination

The *Lol p I* and grass 4B1 antigen were radiolabeled by the chloramine-T method with carrier-free ^{125}I (Amersham International Ltd., Buckinghamshire, U.K.); 25 to 50 μ g of protein in 100 μ l of PBS were iodinated with 1 mCi of ^{125}I (10 μ l). The reaction was started with 20 μ g of chloramine-T (in 50 μ l of PBS) and after 1 minute, stopped by addition of 50 μ g of $\text{Na}_2\text{S}_2\text{O}_3$ (in 50 μ l of PBS), followed by addition of 100 μ l of BSA (20 mg/ml in PBS) as carrier protein. Unbound ^{125}I was separated from the labeled allergens on a Sephadex G-75 column equilibrated in PBS with BSA (3 mg/ml $^{-1}$).

Rabbit antisera

New Zealand white rabbits were immunized with 100 μ g of *Lol p I* or of grass 4B1 in Freund's complete adjuvant

on day 0, followed by three to four injections with the same amount in Freund's incomplete adjuvant at monthly intervals. Antisera with high titers were pooled.

Affinity purification of rabbit antisera

Affinity-purified antibodies to group I were obtained by incubation of rabbit antiserum to *Lol p I* with Sepharose-coupled *Dg*-pollen extract. Bound antibodies were eluted with 0.1 mol/L of glycine HCl, pH 2.5, followed by immediate neutralization on a Sephadex G-25 column equilibrated in PBS. Similarly, affinity-purified antibodies to grass 4B1 were obtained with rabbit antiserum to grass 4B1 and Sepharose-coupled *Dg*-pollen extract.

Depletion of grass-pollen extracts

Affinity-purified rabbit antibodies to group I and to grass 4B1 were coupled to Sepharose (2 mg of antibody to 100 mg of Sepharose); this adsorbent was subsequently used for depleting orchard grass-pollen extract for either *Dac g I* or grass 4B1. Depletions were carried out by incubating (end-over-end) the Sepharose with 5 ml of orchard grass-pollen extract (0.4 mg/ml in PBS) for 3 hours. Sepharose to which glycine was coupled was used as a control. Supernatants were tested for grass 4B1 and *Dac g I* content with the antigen-binding inhibition assay.

Monoclonal antibodies

Balb/c mice were immunized intraperitoneally with 50 μ g of *Lp*-pollen extract in complete Freund's adjuvant and boosted after 6 weeks with the same dose in incomplete Freund's adjuvant. An intravenous booster with the same amount of allergen was administered 4 days before the removal of the spleen. Hybridization was performed according to the method of Kohler and Milstein^{13, 14} with some modifications (Astaldi et al., 1980¹⁵). Antibody-producing hybrids were selected by a RAST procedure with Sepharose-coupled ryegrass extract and ^{125}I -labeled goat antimouse IgG. Positive wells were further tested with an indirect RAST system. Sepharose-coupled McAb (4B1) was incubated with orchard grass-pollen extract, and allergen binding was quantitated with human IgE antibodies and ^{125}I -labeled sheep anti-IgE.¹⁶ Cloning was performed by repeated limiting dilution. Antigraass 4B1 ascites was obtained from pristane-treated Balb/c mice.

Agar precipitation

Double-diffusion tests were carried out on 5 by 5 cm glass plates in 1.5% agarose (Indubiose A37; IBF, Villeneuve-la Garenne, France) in PBS. After 24 hours of incubation, the plates were washed three times for about 12 hours in PBS, dried, and stained with CBB.

RAST

RAST was performed with Sepharose-coupled allergens (*Dg/Pp*-pollen mixture or purified *Lol p I*) and ^{125}I -labeled sheep antibodies to human IgE.¹⁷ RAST results were first corrected for nonspecific binding (approximately 1%) and then expressed in classes as follows: 0, <2% binding; 1+, 2% to 5% binding; 2+, 5% to 10% binding; 3+, 10% to

TABLE I

	<i>Lol p</i> I	Grass 4B1
<i>Lol p</i> I (HPLC pool)	100	<1
Grass 4B1 (purified with monoclonal)	<1	100
Total ryegrass	8	6
Sephadex G-75 pool	39	6
Phenyl-Sepharose: nonretarded fraction	89	<1
Eluate	21	10

Units per optical density of *Lol p* I and grass 4B1 activity in an antigen-binding inhibition assay, as described in Material and Methods. Purified allergens are regarded as 100 units.

20% binding; 4+, 20% to 40% binding; 5+, >40% binding.

Allergen-binding assay

In the allergen-binding assay, protein A Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) was used as solid phase; 0.5 mg of Sepharose per test was incubated with 2.5 μ l of serum and 15,000 cpm of a ^{125} I-labeled allergen in a final volume of 0.4 ml. After overnight incubation on a shaking apparatus, the samples were washed four times, and radioactivity was counted. Results are presented as the percentage of the label added. A similar protocol was used for the inhibition assay. In this case, the sera were preincubated with 50 μ l of pollen extract or pollen fractions for 2 hours. The concentration of these pollen samples ranged from 1 mg/ml to 10 ng/ml. Next, the Sepharose and the labeled allergen were added.

RESULTS

Characterization of grass 4B1

An allergen fraction from *Dg* pollen was purified with the McAb 4B1 coupled to CNBr-activated Sepharose. Affinity chromatography of crude pollen extract resulted in the isolation of a protein fraction, grass 4B1, with high activity in the RAST. When this fraction was tested in SDS-PAGE with CBB staining and with autoradiography (after ^{125} I-labeling), two bands were found corresponding with MWs of 24 kd and 25 kd (Fig. 1). SDS-PAGE with CBB staining of *Lp* and *Dg* extract demonstrated that this doublet is present in both cases; however, *Dg* contains more grass 4B1 (as was confirmed quantitatively in an allergen-binding inhibition assay). For this reason, *Dg*-pollen extract was used for grass 4B1 purification. The most distinct protein band in *Lp* extract in the group I range has a MW of 31 kd (Fig. 2). *Dg* has its own group I equivalent with similar MWs in comparable amounts. This major allergen *Lol p* I from *Lp* (see Material and Methods) was purified and the behavior of grass 4B1 was monitored in the course of

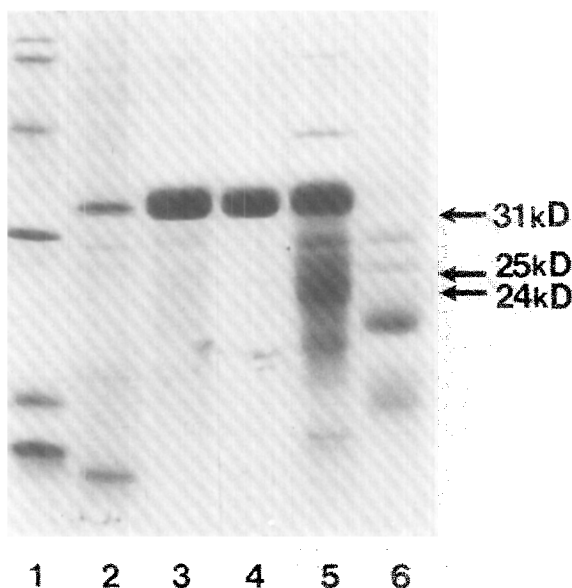


FIG. 3. SDS-PAGE (CBB staining) of the products obtained during the purification of *Lol p* I. Lane 1 contains the MW markers (from top to bottom: 92.5, 66.2, 45.0, 30.0, 21.5, and 14.4 kd). Lane 2 illustrates the starting material, *Lolium perenne* pollen extract (10 μ g). Lane 3 is the pool after Sephadex G-75 (10 μ g), lane 4, the nonretarded fraction of phenyl-Sepharose column (7.5 μ g), and lanes 5 and 6 (20 μ g and 10 μ g) illustrate two successive eluent peaks from the phenyl-Sepharose (after Amicon concentration). The separation of *Lol p* I and grass 4B1 occurs mainly during the phenyl-Sepharose step.

this purification. The purification steps on SDS-PAGE with CBB staining are illustrated in Fig. 3, and quantitative data are presented in Table I. The results of the antigen-binding inhibition assay demonstrated that *Lol p* I and grass 4B1 were separated from each other mainly on phenyl-Sepharose. The SDS-PAGE of the different samples, taken in each step of the purification, confirmed this. The HPLC step was introduced for removing some minor impurities not detectable on SDS-PAGE with CBB staining. For reference purposes, the resulting *Lol p* I was compared to a *Lol p* I preparation, kindly provided by Dr. D. Marsh (Baltimore, Md.). In a double-immunodiffusion assay with antiserum precipitation, lines completely merged, pointing to immunologic identity.

Human antibodies to grass 4B1 and *Lol p* I

Ninety-five sera that were positive in a grass-pollen RAST (with *Dg*/*Pp*-pollen mixture coupled to the Sepharose) were tested in an indirect RAST with Sepharose-coupled McAb 4B1 as the capturing antibody. The adsorbent was incubated with an excess of *Lp* extract for 2 hours and washed. As a control Sepharose was used with an unrelated McAb (Anti-*Der p* II, directed against a house dust-mite allergen);

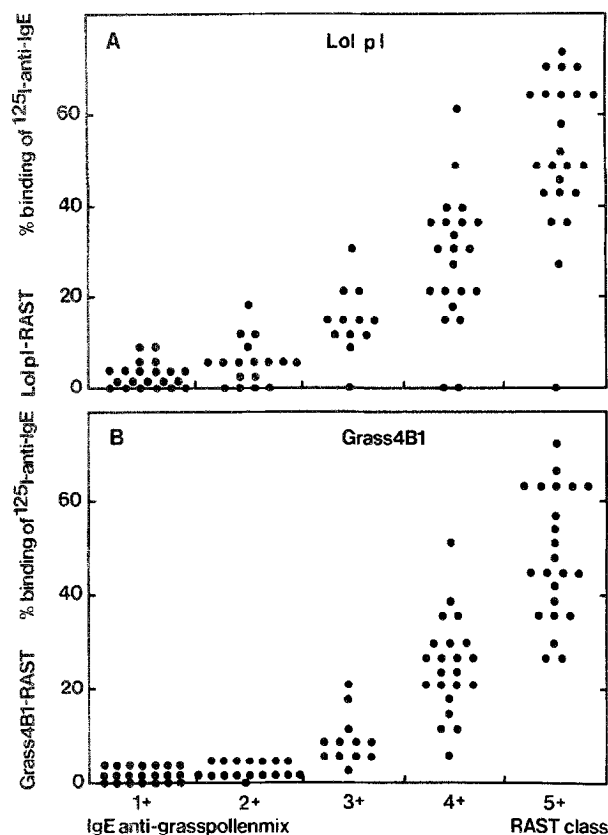


FIG. 4. A, The binding of *Lol p I* by specific IgE antibodies of grass pollen-sensitive patients. On the x-axis, the different RAST classes are presented: 1+, 2% to 5% binding; 2+, 5% to 10% binding; 3+, 10% to 20% binding; 4+, 20% to 40% binding; 5+, >40% binding. B, The same analysis as in A but now for grass 4B1.

>90% of the patients were found to have specific IgE antibodies to grass 4B1, as detected by this test (Fig. 4, B). In order to exclude nonspecific binding, 10 sera were tested with total IgE >2000 IU/ml that were negative in the grass-pollen RAST. Binding was <1.5%. Another control test was performed by use of McAb 4B1 Sepharose without pollen extract. The reason for not using a normal grass 4B1 RAST was the lack of material. The same sera were tested in a *Lol p I* RAST, which demonstrated that in this case also >90% was positive (Fig. 4, A). To determine an estimate of the amount of antibodies to grass 4B1 relative to the amount to *Lol p I*, the IgE-antibody titers to *Lol p I* and to grass 4B1 for 44 sera of the above-mentioned group of patients were compared that had binding percentages between 5% and 50% in the *Lol p I* RAST. For each serum the antibody titer was calculated to grass 4B1 as a percentage of the antibody titer to *Lol p I*. The median of that calculation was 70% (25 percentile, 50%; 75 percentile, 90%); that is, the median response to grass 4B1 was 70% of the response to *Lol p I*.

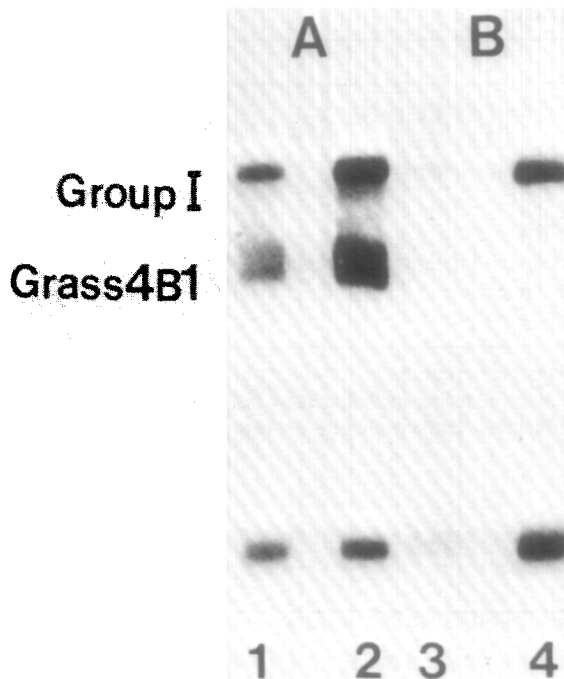


FIG. 5. Binding pattern of serum from two patients to a dactylis blot. Autoradiography was performed for 48 hours. Lanes 1 and 3 are labeled with a ^{125}I McAb against IgG4, and lanes 2 and 4 with ^{125}I -labeled sheep antibodies against IgE. In the group I region, sera from both patients react with the 31 kd band. Patient A has antibodies against the 24/25-kd doublet, also. This is a very common pattern among the patients we tested. Patient B misses those antibodies.

In sera from patients receiving immunotherapy with grass-pollen extract, IgG antibodies (IgG1 as well as IgG4) to both allergens can usually be demonstrated (besides the IgE antibodies), e.g., on Dg blots that were incubated with patient sera and subsequently with ^{125}I -labeled anti-IgG4 (or anti-IgE). A minority of those sera lacks antibodies to one of these two allergens (Fig. 5). Grass 4B1 and *Lol p I* were radiolabeled and used in the allergen-binding assay. About 10% of the sera tested in this assay ($n = 157$) had IgG antibodies against only one of the two allergens (Fig. 6).

Mouse monoclonal and rabbit polyclonal antibodies to grass 4B1 and group I

For further comparison we analyzed the binding of the two antigens to polyclonal and monoclonal antibodies. Goat antimouse IgG, coupled to Sepharose, was used as the precipitating reagent for the analysis of the binding to the McAbs. The grass 4B1 allergen bound strongly (80% binding) to the 4B1 mouse McAb (in a 1/1000 antiserum dilution), whereas *Lol p I* binding was <2%. With the antigroup I McAb

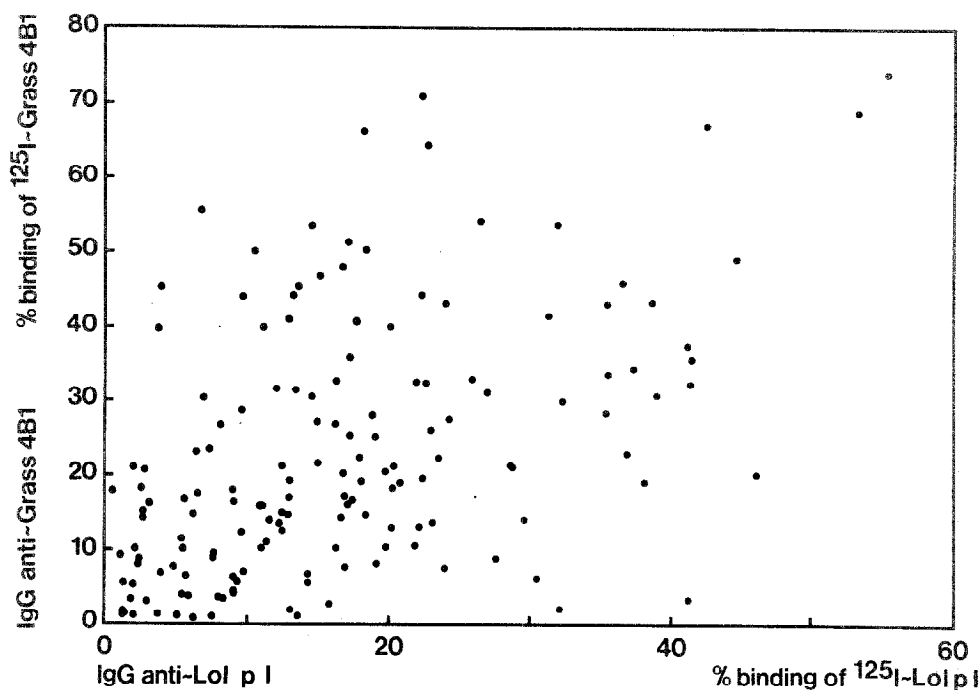


FIG. 6. Correlation between titers of IgG antibodies directed against the two major allergens, *Lol p I* and grass 4B1. Sera from 157 grass pollen-sensitive patients were tested in an allergen-binding assay with purified ^{125}I -labeled allergen in combination with Sepharose-coupled protein A.

(kindly provided by Dr. R. Esch, Chapel Hill, N.C.) in a 1/1000 dilution, 20% binding of *Lol p I* was obtained, whereas <2% binding of the grass 4B1 antigen was found. Protein A Sepharose was the precipitating reagent in the binding assay with the polyclonal antibodies, rabbit antigroup I and rabbit antigrass 4B1. The latter only binds to the 4B1 protein (75% binding in a 1/500 antiserum dilution). Binding of *Lol p I* was not detectable. With the rabbit antibodies against group I, 75% binding of *Lol p I* was obtained with a 1/100 dilution of the antiserum, whereas <2% of grass 4B1 was bound.

When the allergen preparations were tested in a double-immunodiffusion assay, crossing precipitation lines were found (Fig. 7). Here, a mixture of rabbit antigrass 4B1 and goat antigroup I (kindly provided by Dr. D. Marsh) was used instead of the rabbit antibodies.

In the antigen-binding inhibition assay with ^{125}I -labeled grass 4B1, *Lol p I* caused <5% inhibition at 5 $\mu\text{g}/\text{ml}$, whereas grass 4B1 still elicited an inhibition of 10% at 5 ng/ml. With grass 4B1, 50% inhibition could be achieved at a concentration of 15 ng/ml. *Dg* pollen extract was depleted for either group I allergen or grass 4B1, with affinity-purified rabbit antibodies to both allergens. The results in Table II demonstrate the specificity of the adsorption. In the case of

Grass4B1



FIG. 7. Agar immunoprecipitation test. The center well contained a mixture of 35 μl of undiluted rabbit antigrass 4B1 and 35 μl of undiluted goat anti-*Lol p I*; the top well, 50 μl of grass 4B1 (40 $\mu\text{g}/\text{ml}$), and the well at the right, 50 μl of *Lol p I* (50 $\mu\text{g}/\text{ml}$). No evidence of antigenic similarity is obtained.

Dac g I, <2% is left after depletion; with grass 4B1, <0.2%.

DISCUSSION

In agreement with previous studies, we found that approximately 90% of the grass pollen-sensitive patients had specific IgE antibodies against group I allergen; >90% of the sera with IgE antibodies against grass pollen had specific antibodies against grass 4B1

TABLE II

	<i>Dac g I</i>	Grass 4B1
Glycine control	100	100
<i>Dac g I</i> depletion	<2	109
Grass 4B1 depletion	107	<0.2

Units per optical density of *Dac g I* and Grass 4B1 activity in an antigen binding inhibition assay as described in Material and Methods. The glycine control in the depletion experiment is regarded as 100 units of both *Dac g I* and grass 4B1 activity.

(Fig. 4). Therefore, this allergen qualifies as a major allergen. The response to grass 4B1, as detected in the RAST, was about 70% relative to that to *Lol p I*. Patients with low levels of IgE antibodies (RAST classes 1+ and 2+) to a grass-pollen mix (*Dg* and *Pp*) have less IgE antibodies to grass 4B1 than to *Lol p I*. Immunoblots for detecting specific IgE antibodies confirm that most grass pollen-sensitive patients have antibodies against both allergens. In the few sera in which IgE antibodies against only one of the two proteins are detectable, it more often concerns *Lol p I*. Although it is important to remember that during SDS-PAGE the proteins are denatured and therefore possibly less effectively detected, these immunoblots support the conclusion that group I is a more potent allergen. However, the response to grass 4B1 is still so pronounced that it is remarkable that it appears to have escaped attracting attention. Part of the explanation undoubtedly is the similarity in the MW, especially when it is noted that various investigations mention MWs for *Lol p I* <31 kd. Another potentially contributing factor might be a cross-reactivity at the level of human antibodies between group I and grass 4B1 (still in study), although no such cross-reactivity with rabbit and goat antibodies was demonstrable.

It is very unlikely that grass 4B1 is a degradation product of group I, since in this case rabbit antibodies to grass 4B1 would be expected to be at least partially cross-reactive with group I. In the double-diffusion experiment, the pattern of the precipitation lines was of the nonidentity type; the lines were clearly crossing. Depletion of *Dg* extract with rabbit antibodies against group I or against grass 4B1 resulted in specific depletion for *Dac g I* and grass 4B1, respectively. In an antigen-binding assay performed with both rabbit antibodies, cross-reactivity was <0.3%. These experiments demonstrate that the two proteins are distinct.

The monoclonal antibody to grass 4B1 was raised by immunization with whole *Lolium perenne*-pollen extract. Screening of the hybrids was done with *Dactylis glomerata*-pollen extract. Therefore only cross-

reacting antibodies were expected. This resulted in 30 positive clones, five with antigrass 4B1 specificity. Surprisingly, no clones were found with antibodies to group I. With the selected McAb 4B1, cross-reactive material was found in *Dactylis glomerata*, *Lolium perenne*, *Phleum pratense*, *Secale cereale*, and *Anthoxanthum odoratum* in decreasing order of reactivity.

On SDS-PAGE and immunoblot patterns, in studies published by different authors,^{3, 4, 18, 19, 20} grass 4B1 bands can usually be detected. However, until now in the 25 to 35 kd range, the group I allergen is the only identified major allergen in grass pollen. On the basis of published MWs, identity of grass 4B1 with other major allergens would appear to be excluded. However, it is probable that the MW of group V allergen (Ag B,²¹ Ag 25,²² or *Phl p V*²³) has to be revised. Originally, the MW was listed as 15 kd for group V allergen from *Phleum pratense*. According to recent data from Dr. F. Matthiesen and Dr. H. Lowenstein, the MW of *Phl p V* is likely to be approximately 25 kd. Furthermore, in crossed immunoelectrophoresis, antibodies to grass 4B1 were found to precipitate group V components in both *Pp* and *Dg* in the same way as rabbit anti-*Phl p V*.

Data presented in this article make it clear that a considerable fraction of the allergenic activity of grass-pollen extract in the 25 to 35 kd range is not due to group I but to another allergen, provisionally named grass 4B1.

We thank W. A. van Leeuwen for carrying out a major part of the experiments, Dr. D. G. Marsh for his *Lol p I* preparation and goat antibodies, Dr. R. E. Esch for his monoclonal antibodies, Dr. T.A. Out and Dr. C. Aaij for critically reviewing the typescript, and Mrs. J. Gerritsen for typing the manuscript.

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