Immunization with Purified Natural and Recombinant Allergens Induces Mouse IgG1 Antibodies That Recognize Similar Epitopes as Human IgE and Inhibit the Human IgE-Allergen Interaction and Allergen-Induced Basophil Degranulation

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Susanne Vrtala,* Tanja Ball,* Susanne Spitzauer,† Budhi Pandjaitan,‡ Cenk Suphioglu,§ Bruce Knox,§ Wolfgang R. Sperr,‡ Peter Valent,‡ Dietrich Kraft,* and Rudolf Valenta2*

Molecular characterization of allergens by recombinant DNA technology has made rapid progress in the recent few years. In the present study we immunized mice with aluminum hydroxide-adsorbed purified recombinant major timothy grass pollen allergens (rPhl p 1, rPhl p 2, rPhl p 5), dog albumin, a major animal dander allergen, and proteins with low (β-lactoglobulin) or no (ribulose diphosphate carboxylase) allergenic potential in humans. Allergens that bind high levels of IgE in humans (Phl p 1, Phl p 5, dog albumin) induced high IgE and IgG1 levels in mice, whereas proteins with little or no allergenic activity in humans failed to induce significant IgE and IgG1 levels in mice. Continuous immunization for a period of 27 wk resulted in the production of mouse IgG1 Abs that recognized recombinant allergen fragments/epitopes defined by IgE Abs of allergic patients. As a consequence, allergen-specific mouse Abs strongly inhibited human IgE binding to the allergens and suppressed the allergen-induced histamine release from human basophils. In summary, our data indicate that 1) the allergenic potency of a protein may be related to its overall specific mouse Abs strongly inhibited human IgE binding to the allergens and suppressed the allergen-induced histamine release from human basophils. 

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A

Most 20% of the population suffers from Type I allergy (1). The symptoms of Type I allergy (allergic rhinitis, conjunctivitis, asthma, and anaphylaxis) result from the cross-link of effector cell-bound IgE Abs by per se innocuous Ags (allergens). While the role of IgE Abs in the pathogenesis of Type I allergy is well established, much less is known concerning the effects of allergen-specific IgG Abs. In 1935, Cooke and colleagues had already described successful therapy of an allergic patient by transfer of blood from a hyporesensitized patient (2), and it could be later shown that the transferable protective factor represented a thermostable “blocking Ab” that suppressed skin responses to injected allergens (3–5). In humans, blocking Abs were found to belong essentially to the IgG class (6–9), and it could be demonstrated that successful immunotherapy is paralleled by an increase of IgG, in particular IgG4 Abs (10). A later study monitoring pa-

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Materials and Methods

Characterization of allergic patients and sera

Allergic patients were characterized by positive case history, skin prick testing, and radioallergosorbent test (RAST)\(^1\) analysis of the sera as described (19, 20). In addition, sera were tested for reactivity with natural allergen extracts by IgE immunoblotting and for IgE reactivity to purified recombinant timothy grass pollen allergens and dog albumin as described (20, 18).

**Allergen extracts, purified recombinant timothy grass pollen allergens, dog albumin, and control proteins**

Pollen from timothy grass (*Phleum pratense*), Kentucky bluegrass (*Poa pratensis*), rye grass (*Lolium perenne*), and rye (*Secale cereale*) were purchased from Allergon AB (Välinge, Sweden). Aqueous protein extracts were prepared (21), checked by SDS-PAGE (22) and Coomassie Blue staining (23), and stored lyophilized at −20°C until use. Purified dog albumin, β-lactoglobulin, and ribulose diphosphate carboxylase were purchased from Sigma (St. Louis, MO). Recombinant timothy grass pollen allergens (Php p 1, Php p 2, and Php p 5) were expressed as nonfusion proteins in *E. coli* BL21 (DE3) and purified as described (24). Before immunization, recombinant allergens were tested for their capacity to bind human IgE and to induce specific histamine release from allergic patients’ basophils.

**Plasmids, phage, and *E. coli* strains**

The cDNAs coding for the major timothy grass pollen allergens Php p 1, Php p 2, and Php p 5 were inserted into plasmid pMW 172 (25), a derivative of pRK 172 (26). Plasmids were transformed into *E. coli* BL21 (DE3), derived from *E. coli* strain B (27) (Novagen, Madison, WI). EcoRI-cut, dephosphorylated agt11 DNA was purchased from Pharmacia (Uppsala, Sweden). *E. coli* strain Y1090 (hsdR m<b>+</b> <i>hfr</i>) was obtained from Amersham (Amersham, Buckinghamshire, U.K.). *E. coli* XL-1 Blue recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac (F' proAB lacZAM15 Tn10 (Tet)) was purchased from Stratagene (La Jolla, CA), and plasmid pUC18 from Boehringer (Mannheim, Germany).

**Immunization of mice and measurement of specific IgE and IgG Ab levels**

Eight-week-old BALB/c mice were obtained from Charles River (Kislegg, Germany). Animals were maintained in the animal care unit of the Institute of General and Experimental Pathological Research of the University of Vienna according to the local guidelines for animal care. Groups of five mice each were immunized for 27 wk with 5 µg of each purified protein (recombinant timothy grass pollen allergens Php p 1, Php p 2, and Php p 5; dog albumin, ribulose-diphosphate carboxylase; and β-lactoglobulin) adsorbed to 200 µl of AluGel-S (Serva, Heidelberg, Germany) s.c. in the neck as described (28, 29). Mice were immunized and bled at approximately 3-wk intervals. Sera were stored at −20°C until analysis. IgE and IgG1 responses were measured by ELISA as described (28, 29).

**SDS-PAGE and immunoblotting**

IgE and IgG1 detection of nitrocellulose-blotted allergens and allergen extracts was performed by immunoblotting. Proteins were separated by SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany) by electroblotting (30). Nitrocellulose filters were blocked in buffer A (50 mM sodium phosphate, pH 7.5, 0.5% w/v BSA, 0.5% v/v Tween-20, 0.05% NaN<sub>3</sub>) two times for 5 min and once for 30 min and incubated for 1:20 (IgE) or 1:1000 (IgG1) in buffer A-diluted mouse sera at 4°C overnight. Nitrocellulose filters were then washed three times in buffer A and exposed to 1:1000 in buffer A-diluted monoclonal rat anti-mouse IgE or anti-IgG1 Abs (Pharmingen, San Diego, CA) at 4°C overnight. After washing as above, bound secondary Abs were detected with a 125<sup>I</sup>-labeled sheep anti-rat IgG antiserum (Amersham) and visualized by autoradiography.

**IgE epitope mapping of recombinant Php p 1, Lol p 5, and dog albumin by gene fragmentation**

**Recombinant IgE epitopes of Php p 1**

A random fragment expression cDNA library was constructed using the cDNA coding for the major timothy grass pollen allergen, Php p 1, as a source (31). Phage clones coding for IgE epitopes were isolated by immunoscreening with serum IgE from grass pollen-allergic individuals. IgE-binding phage clones were characterized by sequence analysis of the inserted cDNA fragments (Ref. 31; and T. Ball and R. Valeta, unpublished observations).

**Recombinant IgE epitopes of Lol p 5**

Phage clones containing IgE-binding portions of the major rye grass pollen isoallergens, Lol p 5a and Lol p 5b were isolated and shared IgE epitopes and sequence homology with the major timothy grass pollen allergen, Phl p 5, were isolated by IgE immunoscreening of a random fragment expression cDNA library prepared from the Lol p 5a and 5b cDNAs as described (32). The IgE-binding phage clones were purified to homogeneity by rescreening with serum IgE from a grass pollen-allergic individual. cDNAs coding for Lol p 5 IgE epitopes were amplified by PCR using agt11 forward (5′-CGG GAT CCC GGT TTC CAT ATG GGG ATT GGT GCT G 3′) and reversed (5′-CGC GGA TCC GTG TCA TAT GGA ATT GGT GCT G 3′) primers and phage DNA as a template. Both primers contained BamHI restriction sites (underlined) that allowed subcloning of the PCR products into plasmid pUC18 (33). Plasmids were transformed into *E. coli* XL-1 Blue using the calcium chloride method, and plasmid DNA was isolated using Qiagen tips (Qiagen, Hilden, Germany). The sequence of the subcloned fragments was determined by DNA sequence analysis according to the method of Sanger et al. (34) using the 111 primers described above. [35S]dCTP, and a T7 polymerase sequencing kit (Pharmacia).

**Recombinant IgE epitopes of dog albumin**

IgE binding fragments comprising different regions of dog albumin were obtained by IgE immunoscreening of a dog liver expression cDNA library constructed in phage agt11 (Ref. 17; and B. Pandajatan and R. Valeta, unpublished observation). Representative clone fragments from the N terminus, C terminus, and a middle region of dog albumin as well as a clone expressing complete dog albumin were used in this study.

**Comparison of human IgE and mouse IgG1 binding to recombinant allergen fragments**

Eight-week-old BALB/c mice were obtained from Charles River (Kislegg, Germany). Animals were maintained in the animal care unit of the Institute of General and Experimental Pathological Research of the University of Vienna according to the local guidelines for animal care. Groups of five mice each were immunized for 27 wk with 5 µg of each purified protein (recombinant timothy grass pollen allergens Php p 1, Php p 2, and Php p 5; dog albumin, ribulose-diphosphate carboxylase; and β-lactoglobulin) adsorbed to 200 µl of AluGel-S (Serva, Heidelberg, Germany) s.c. in the neck as described (28, 29). Mice were immunized and bled at approximately 3-wk intervals. Sera were stored at −20°C until analysis. IgE and IgG1 responses were measured by ELISA as described (28, 29).

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**IgE epitope mapping of recombinant Php p 1, Lol p 5, and dog albumin by gene fragmentation**

Recombinant IgE epitopes of Php p 1, Lol p 5, and dog albumin by gene fragmentation

1 Abbreviations used in this paper: RAST, radioallergosorbent test; β-gal, β-galactosidase.
Granulocytes were then incubated with recombinant allergens dissolved in histamine release buffer (20 mM PIPES, pH 7.4, 110 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 g/L glucose, 0.3 mg/ml human serum albumin) at different concentrations (0.1, 0.01, and 0.001 mg/ml) that had been preincubated with an equal volume of mouse immune serum, or, for control purposes, with mouse preimmune serum or buffer alone, for 1 h at room temperature. Histamine released into the cell-free supernatant was determined by radioimmunoassay (Immunotech, Marseille, France) and is expressed as percentage of total histamine release determined after cell lysis. All measurements represent means of triplicate determinations.

Results

Kinetics, levels, and cross-reactivity of mouse IgE and IgG1

Allergen-specific IgE and IgG1 responses were studied after immunization of mice with purified allergens (recombinant timothy grass pollen allergens rPhl p 1, rPhl p 2, rPhl p 5, dog albumin, D-ribulose 1,5-diphosphate carboxylase, and β-lactoglobulin, respectively. IgE (A) and IgG1 (B) levels of sera collected at different times (x-axis) were determined by ELISA. The mean levels of the allergen-specific IgE and IgG1 levels in each group are displayed on the y-axis as ODs.

FIGURE 1. Time course and intensity of mouse IgE and IgG1 responses. Groups of five mice each were immunized with rPhl p 1, rPhl p 2, rPhl p 5, dog albumin, D-ribulose 1,5-diphosphate carboxylase, and β-lactoglobulin, respectively. IgE (A) and IgG1 (B) levels of sera collected at different times (x-axis) were determined by ELISA. The mean levels of the allergen-specific IgE and IgG1 levels in each group are displayed on the y-axis as ODs.

FIGURE 2. Cross-reactivity of mouse IgE and IgG1 Abs with homologous allergens. The preimmune sera (lane 1) and immune sera (lane 2) of a representative rPhl p 5 and dog albumin-immunized mouse were tested for IgE and IgG1 reactivity with blotted natural allergens by immunoblotting. Serum from a rPhl p 5-immunized mouse showed IgE and IgG1 reactivity to natural group 5 allergens at approximately 30 kDa in timothy grass, rye grass, and rye pollen extracts (A). The dog albumin-immunized mouse serum displays IgE and IgG1 cross-reactivity with cat, horse, pig, and rabbit albumin at approximately 69 kDa (B).

As exemplified for a Phl p 5- and dog albumin-immunized mouse (Fig. 2, A and B), IgE and IgG1 Abs cross-reacted with homologous allergens from different sources. The deduced amino acid sequences of group 5 allergens from various grass species share an average sequence identity of 75% (16). Accordingly, rPhl p 5-induced mouse IgE and IgG1 Abs cross-reacted with natural group 5 allergens present in timothy grass, rye grass, and rye pollen (Fig. 2A). An average sequence identity of 80% exists among albumins from various animals (17). As a consequence, dog albumin-induced IgE and IgG1 cross-reacted with cat, horse, pig, and rabbit albumin (Fig. 2B).
Mapping of mouse IgG1 epitopes using recombinant allergen fragments: mouse IgG1 Abs recognize similar epitopes as human IgE Abs

We have expressed IgE binding fragments of dog albumin, and the major grass pollen allergens, Phl p 1 and Lol p 5, as β-gal fusion proteins using phage λgt11 and E. coli Y1090. Fragments of the allergen-encoding cDNAs were inserted into phage λgt11 and clones that bound IgE Abs from allergic patients were selected. IgE-binding regions in dog albumin were found at the N terminus, C terminus, and in the middle of the protein (Fig. 3A). IgE epitopes of Phl p 1 were found at the N and C termini as well as in the center of the protein (Fig. 4A). Lol p 5a contains a major IgE binding site in the center, close to the C terminus (Fig. 5A, upper part). Major IgE binding areas of Lol p 5b were found in the center of the molecule, one represented by clones 123 and 81 at the more N-terminal part and one represented by clone 21 at the C-terminal portion (Fig. 5A, lower part). The testing of the recombinant allergen fragments with serum IgE from allergic patients showed that certain immunodominant portions (e.g., the C-terminus of dog albumin, the central portion of Phl p 1, and the two central portions of Lol p 5 represented by clones 81 and 117) were recognized by all sera. While no significant IgG1, IgG2, and IgG3 reactivity to...
nitrocellulose-immobilized recombinant allergen fragments could be detected in sera from nonatopic and allergic individuals, sera from allergic patients who had received immunotherapy contained IgG4 Abs that strongly reacted with IgE epitopes (Ref. 36, and R. Valenta and T. Ball, unpublished observations). The immunodominant IgE epitopes were also detected by IgG1 Abs of all mouse sera. Serum IgE from nonallergic individuals and mouse preimmune sera failed to react with the epitope clones. Phage clones with control inserts or without inserts were not bound by any serum tested.

Allergen-specific mouse Abs block binding of human IgE to purified allergens and allergen extracts

To investigate whether mouse IgG1 Abs are able to inhibit the binding of human IgE Abs to allergens, competition experiments were performed. In the first set of experiments, nitrocellulose-blotted purified allergens were preincubated with sera from immunized mice, and for control purposes, with preimmune sera. As exemplified in Figure 6, IgE binding of six allergic patients (panels A–F) to purified allergens (panels: rPhl p 1, rPhl p 5, dog albumin) was strongly inhibited by preincubation of the respective allergen with the mouse immune sera (lane 2) but not after preincubation with the preimmune sera (lane 1). Table I displays the percentage of inhibition of IgE binding to recombinant Phl p 1 and Phl p 5 determined for sera from several grass pollen-allergic patients by gamma counting. Phl p 5-specific mouse sera inhibited human IgE binding to recombinant Phl p 5 between 45 and 89%.

FIGURE 5. Localization of IgE-binding fragments on Lol p 5a (276 amino acids) and Lol p 5b (314 amino acids). The localization and length of rIgE binding fragments are displayed (A). Nitrocellulose-dotted rLol p 5a fragments (11, 14, 26, 47, 50, 57, 68, 117), rLol p 5b fragments (21, 81, 120, 123), two β-gal-fused control proteins (29, 87), and β-gal alone (0) were exposed to serum IgE from grass pollen-allergic patients (A–C), a nonallergic individual (N), serum IgG1 from Phl p 5-immunized mice (a–c), and mouse preimmune serum (n) (B).

FIGURE 6. Mouse Ig inhibits human IgE binding to purified allergens. Nitrocellulose-blotted rPhl p 1, rPhl p 5, and dog albumin were preincubated with preimmune sera (lane 1) and sera from immunized mice (lane 2). Strips were then exposed to sera from six allergic patients (panels A–F) and bound IgE was detected with 125I-labeled anti-human IgE Abs and visualized by autoradiography.
and Phl p 1-specific mouse sera reduced human IgE binding to recombinant Phl p 1 between 15% and 77%. Serum Ig from rPhl p 5-immunized mice neither cross-reacted with rPhl p 1 nor inhibited human IgE binding to Phl p 1. Likewise, rPhl p 1-specific mouse Abs failed to react with Phl p 5 and to inhibit human IgE binding to Phl p 5 (data not shown).

Sera from mice that were immunized with single purified recombinant grass pollen allergens also significantly blocked IgE binding to natural grass pollen extracts. Serum Ig from a Phl p 5-immunized mouse but not the preimmune Ig blocked IgE binding of two sera from grass pollen-allergic patients (C and D) to natural pollen extracts from Kentucky blue grass, rye grass, and rye (Fig. 7). The quantification of the inhibition of IgE binding of an additional 13 sera from grass pollen-allergic patients to natural grass pollen extracts is summarized in Table II. Sera from rPhl p 5-immunized mice inhibited IgE binding to natural grass pollen extracts up to 66% and sera from rPhl p 1-immunized mice suppressed IgE binding up to 76%. Although IgE binding of several sera to certain extracts was not inhibited (Table II, dashes) a significant inhibition of IgE binding was observed for most of the sera tested.

Preincubation of rPhl p 5 with mouse immune sera inhibits the allergen-induced histamine release from human basophils

To evaluate whether blocking mouse Igs are also able to inhibit allergen-induced effector reactions, histamine release experiments were performed with basophils from a grass pollen-allergic individual. As exemplified in Figure 8, preincubation of the major timothy grass pollen allergen Phl p 5 with serum from a Phl p 5-immunized mouse, but not with buffer alone or preimmune serum, strongly suppressed the allergen-induced histamine release in a dose-dependent manner.

### Discussion

In the present study, we investigated whether it is possible to use single purified natural or recombinant allergens to induce blocking mouse Abs that interfere with the interaction of allergic patients’ IgE and allergens. Mice were immunized with aluminum hydroxide-adsorbed purified recombinant major timothy grass pollen allergens, rPhl p 1 (14), rPhl p 2 (15), rPhl p 5 (16), dog albumin (a relevant pet allergen (17, 18)), and two proteins with low (β-lactoglobulin) or no (D-ribulose-1,5-diphosphate carboxylase) allergenic activity in humans. Results obtained indicated that there is a trend supporting the contention that allergens that cause greater IgE responses in humans (Phl p 1, Phl p 5, dog albumin) (14, 16–18, 24) induce greater IgE and IgG1 responses in mice. Abs that bind lower levels of IgE (Phl p 2) (24) and represent rare (β-lactoglobulin) or no (D-ribulose 1, 5-diphosphate carboxylase) targets for human IgE Abs induced low or no detectable Ab responses in mice. In a previous study performed with the recombinant birch pollen allergens rBet v 1 and rBet v 2 in mice (29) and rhesus monkeys (37), comparable results were obtained for the two birch pollen allergens. Bet v 1 (38), the major birch pollen allergen, binds high levels of IgE in humans (39, 40) and induced higher IgE levels both in mice and rhesus monkeys, compared with Bet v 2 (41), a less frequently detected allergen (40). Taken together, these data and the present results suggest immunogenicity as a general feature of a prominent allergen.

In this study, we noted pronounced cross-reactivity of timothy grass pollen-specific and dog albumin-specific IgE and IgG1 Abs with homologous allergens from other sources. Also, this finding was not limited to the allergen panel investigated in this study as we had previously noted that IgE and IgG Abs induced in mice and rhesus monkeys with recombinant birch pollen allergens, Bet v 1 and Bet v 2, cross-reacted with immunologically related allergens present in various pollens and plant-derived food (29, 37). On the basis of the presence of cross-reactive IgE Abs, sensitized rhesus monkeys displayed skin reactivity with a series of allergen sources containing Bet v 1 and Bet v 2-related proteins (37). The present and previously obtained data would indicate that many of the B cell epitopes present on structurally related tree pollen, grass pollen, plant food, and animal hair/dander allergens are similar. The concept that relevant allergens bear a limited number of prominent B cell epitopes gained further support by our demonstration that mouse IgG1 Abs recognized recombinant allergen fragments/epitopes defined by IgE Abs of allergic patients. These data strongly suggest that major allergens contain a few prominent B cell epitopes that may represent conserved immunodominant structures. In fact, the cDNA and deduced amino acid sequences of
the relevant IgE epitopes of the major timothy grass pollen allergen, Phl p 1 and dog albumin, are highly homologous to those found in related allergens (31, 17). More formal proof for the assumption that B cell epitopes of major allergens are conserved among their homologues will certainly come from the combined structural (x-ray, nuclear magnetic resonance) and epitope analysis of relevant allergens, as has been recently reported for the major birch pollen allergen Bet v 1 (42) and birch profilin (43). The analysis of the three-dimensional structures of both allergens indicated that the relevant B cell epitopes mapped to surface-exposed areas that are highly conserved across species and overlap with the binding sites of natural ligands.

The fact that IgG1 Abs of immunized mice strongly recognized epitopes defined by human IgE Abs that are present on homologous allergens of various origin (e.g., group 1 or 5 allergens of different grass species) explains why preincubation of purified allergens as well as of natural allergen extracts with mouse immune sera lead to a strong reduction of human IgE binding. Support for the biologic relevance of the in vitro IgE competition comes from our finding that the relevant B cell epitopes mapped to surface-exposed areas that are highly conserved across species and overlap with the binding sites of natural ligands.

FIGURE 8. Preincubation of Phl p 5 with serum from a Phl p 5-immunized mouse inhibits the allergen-induced histamine release from basophils of a grass-pollen-allergic patient. Different concentrations of rPhl p 5 (0.001 μg/ml, 0.01 μg/ml, 0.1 μg/ml; x-axis) were preincubated with buffer alone (b), mouse preimmune serum (ps), or serum from the Phl p 5-immunized mouse (s). Histamine release is displayed on the y-axis as a percentage of the total histamine release measured after cell lysis.

<table>
<thead>
<tr>
<th>% Inhibition of IgE binding</th>
<th>Preincubation with Phl p 1-Specific Mouse Ig</th>
<th>Preincubation with Phl p 5-Specific Mouse Ig</th>
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<tr>
<td>Patients’ serum</td>
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<td>Rye grass</td>
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<tr>
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"The percentage inhibition of human IgE binding to the allergens after preincubation with mouse sera is shown. Dashes indicate no inhibition. Capital letters indicate the patients’ sera and correspond to Figures 6 and 7.

References

