

Conversion of grass pollen allergen-specific human IgE into a protective IgG₁ antibody

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More than 100 million individuals exhibit IgE-mediated allergic reactions against Phl p 2, a major allergen from timothy grass pollen. We isolated cDNA coding for three Phl p 2-specific human IgE antibodies from a combinatorial library, which was constructed from lymphocytes of a grass pollen-allergic patient. Recombinant Phl p 2-specific IgE antibody fragments (Fab) recognized a fragment comprising the 64 N-terminal amino acids of Phl p 2 and cross-reacted with group 2 allergens from seven grass species. cDNA coding for the variable regions of one of the IgE Fab were cloned into a plasmid vector expressing the constant region of human IgG₁ to obtain a complete, recombinant Phl p 2-specific human IgG₁. This antibody blocked the binding of grass pollen-allergic patients IgE ($n=26$; mean inhibition: 58%) to Phl p 2 and caused a 100-fold reduction of Phl p 2-induced basophil histamine release. The recombinant human Phl p 2-specific IgG₁ may be used for environmental allergen detection, for standardization of diagnostic as well as therapeutic grass pollen allergen preparations and for passive therapy of grass pollen allergy.

Key words: Allergy / IgE / Allergen / Combinatorial cloning / Recombinant antibody

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1 Introduction

Almost 500 million individuals suffer from Type I allergy, a genetically determined hypersensitivity disease, which is based on the formation of IgE antibodies against *per se* harmless antigens (*i.e.* allergens) [1]. IgE represents the least abundant class of immunoglobulins but it can induce severe inflammatory reactions via receptor-mediated activation of immune cells [2–4]. IgE has thus a central pathogenetic role in Type I allergy, but almost no information is available regarding the primary structure of human allergen-specific IgE antibodies, which is due to their extremely low concentration in serum.

The only curative therapy of Type I allergy, allergen-specific immunotherapy, has been introduced in 1911 [5]. It is based on the administration of allergens in the form of vaccines and clinical efficacy has been demonstrated in numerous clinical studies [6, 7]. One of the first immunological mechanisms revealed to be operative in

specific immunotherapy was the induction of allergen-specific blocking antibodies that antagonize the pathogenetic effects of allergen-specific IgE. More than 30 years before the discovery of IgE antibodies, Cooke et al. [8] reported cure of a hay fever patient by transfer of blood from a patient who had been successfully treated by specific immunotherapy and, thus, suggested that the induction of antibodies may be important for successful immunotherapy. A few years later, Loveless [9] demonstrated that the protective effects observed by Cooke were due to blocking antibodies that specifically suppressed cutaneous hypersensitivity. Now it is established that immunotherapy-induced antibodies belong mainly to the IgG₄ and IgG₁ subclasses [10, 11]. However, not all therapy-induced allergen-specific IgG antibodies exhibit protective activity. Detailed studies performed with defined recombinant allergen molecules and epitopes thereof have shown that allergen-specific IgG may be directed to epitopes different from those recognized by IgE [12]. Antibodies of the latter type cannot compete with IgE and hence have neither beneficial nor harmful effects. However, certain allergen-specific IgG antibodies have been demonstrated to augment IgE binding to allergens, most likely by inducing changes in the allergen conformation, and thus can even augment anaphylactic reactions [13].

[1 22903]

Abbreviations: TBST: Tris-buffered saline + 0.05% Tween
cDNA: Complementary DNA

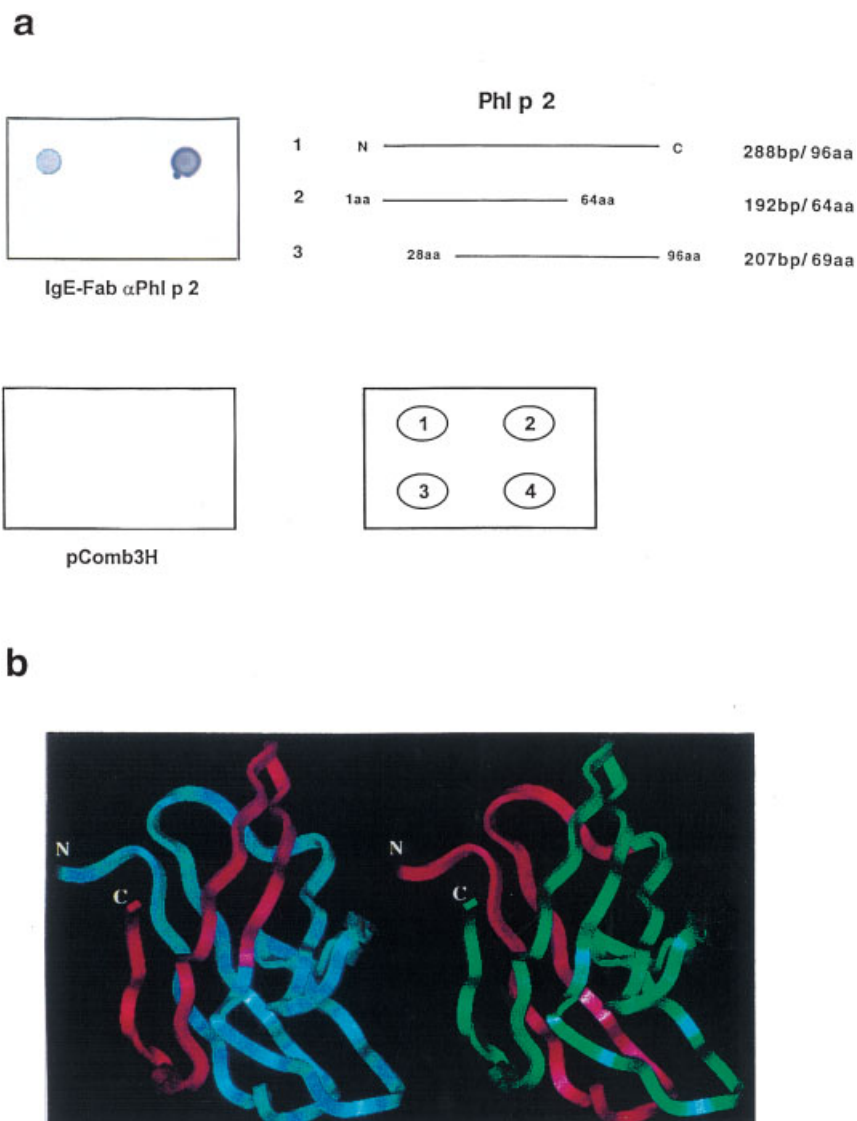


Fig. 1. Mapping of the binding site of the Phl p 2-specific human IgE Fab on the Phl p 2 allergen. (a) An overview of the rPhl p 2 fragments comprising the N-terminal 64 aa and the C-terminal 69 aa and the binding of the clone 94-derived IgE Fab to nitrocellulose filters containing complete rPhl p 2 (1), the N-terminal (2), C-terminal (3) Phl p 2-derived fragments and β -galactosidase alone (4). For control purposes, filter-bound clones were exposed to bacterial extracts from *E. coli* transformed with empty plasmid (pComb3H). (b) A ribbon representation of the three-dimensional structure of the Phl p 2 allergen in which the N-terminal (blue) and C-terminal (green) fragments have been indicated.

To obtain specific competitors for the interaction between patients IgE and the corresponding allergens, we isolated human IgE antibodies specific for the major timothy grass pollen allergen Phl p 2 [14, 15] using the combinatorial library technology [16]. Subsequently these IgE antibodies were converted by genetic engineering into protective IgG₁.

2 Results

2.1 The three Phl p 2-specific IgE Fab consist of closely related heavy chain fragments which have recombined with different light chains: recognition of an N-terminal Phl p 2 fragment

The analysis of the cDNA coding for the three heavy chain variable regions showed that they differed only in few nucleotides [27 out of 342 bp (8%) for clone 94

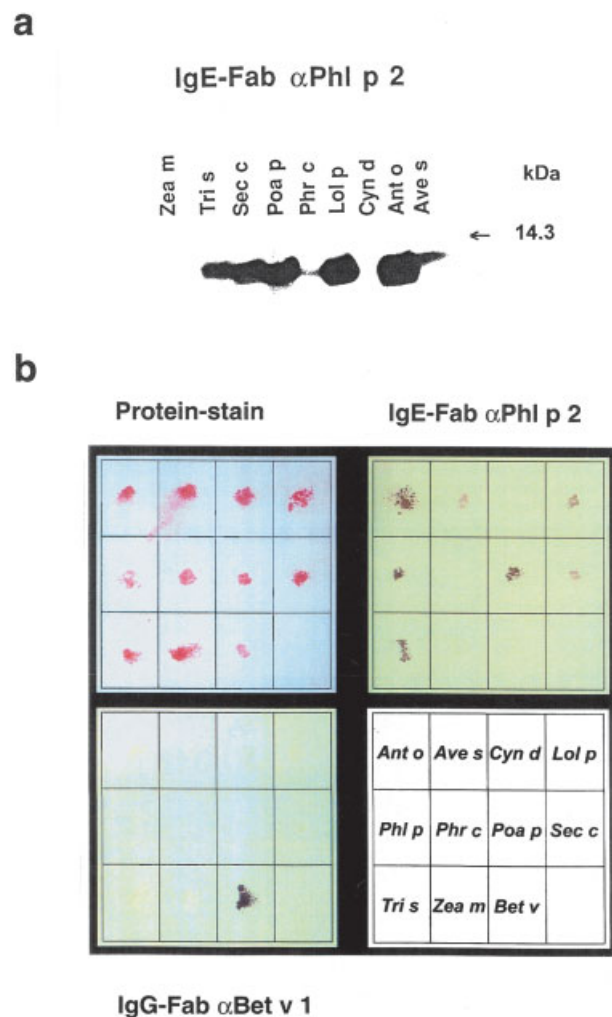


Fig. 2. (a) rPhl p 2-specific IgE Fab cross-react with natural group 2 allergens from different grass and corn species (Ant o: sweet vernal grass; Ave s: oat; Cyn d: Bermuda grass; Lol p: rye grass; Phr c: common reed; Poa p: Kentucky Bluegrass; Sec c: rye; Tri s: wheat; Zea m: maize; Phl p: timothy grass). The position of the 14.3-kDa marker is indicated with an arrow on the right margin. (b) Detection of pollen grains containing group 2 allergens by particle blotting. Pollen grains from ten different monocots (Ant o: sweet vernal grass; Ave s: oat; Cyn d: Bermuda grass; Lol p: rye grass; Phl p: timothy grass; Phr c: common reed; Poa p: Kentucky Bluegrass; Sec c: rye; Tri s: wheat; Zea m: maize) and birch (Bet v) were dotted to nitrocellulose. Released proteins were stained with Ponceau S. Group 2 allergens were detected with Phl p 2-specific IgE Fab, the major birch pollen allergen, Bet v 1, with Bet v 1-specific Fab.

versus clone 60; 18 out of 342 bp (5%) for clone 94 versus clone 100; 9 out of 342 bp (3%) for clone 60 versus clone 100; accession nos. AJ458382; AJ458383; AJ458384]. The three light chains (accession nos. AJ458379, AJ458380, AJ458381) belonged to the kappa

Table 1. Inhibition of grass pollen-allergic patients' IgE binding to Phl p 2 by Phl p 2-specific rIgG₁, rIgE Fab or rabbit antibodies^{a)}

Patient	Inhibition of IgE binding		
	IgG ₁ anti-Phl p 2	IgE-Fab anti-Phl p 2	Rabbit anti-Phl p 2
1	70	22	36
2	70	35	37
3	57	22	57
4	68	25	22
5	57	24	10
6	69	33	35
7	62	26	39
8	72	15	25
9	39	14	53
10	48	22	31
11	29	5	42
12	47	19	12
13	57	22	31
14	61	10	49
15	52	14	1
16	67	34	34
17	84	62	63
18	36	15	37
19	75	27	27
20	35	20	4
21	71	25	22
22	72	24	27
23	65	25	24
24	56	28	39
25	83	46	30
26	70	33	30
% mean inhibition	58	25	31

a) The percentages inhibition of IgE binding to Phl p 2 obtained after preincubation with IgG₁ anti-Phl p 2, IgE Fab anti-Phl p 2 and rabbit anti-Phl p 2 are displayed for sera from 26 grass pollen-allergic patients. Mean percentage inhibitions for the 26 sera are displayed in the bottom line.

family and showed a much greater sequence variation than was observed among the heavy chains.

The complete recombinant Phl p 2 (rPhl p 2) allergen [96 amino acids (aa)], a N-terminal rPhl p 2 fragment

comprising the first 64 aa portion (1–64) and a 69 aa long C-terminal rPhl p 2 fragment (28–96 aa) were expressed as β -galactosidase fusion proteins and exposed to the Fab to characterize the binding site for the rPhl p 2-specific IgE Fab (Fig. 1a). Fig. 1b shows a ribbon representation of the complete Phl p 2 allergen, which is similar to an immunoglobulin domain and consists mainly of β -sheet structure [15]. The N-terminal and C-terminal fragment have been indicated in the model (Fig. 1b). As exemplified for clone 94, all three Phl p 2-specific IgE Fab recognized the complete rPhl p 2 [1] and the N-terminal rPhl p 2 fragment [2] but did not bind to the C-terminal fragment [3] nor to λ gt11 phage control proteins [4] (Fig. 1a).

2.2 Phl p 2-specific IgE Fab cross-react with natural group 2 allergens from seven grass species and identify group 2 allergen-containing pollens by particle blotting

The IgE Fab reacted strongly with group 2 allergens in sweet vernal grass, rye grass, Kentucky Bluegrass, rye and wheat (Fig. 2a). Only weak reactivity was observed with oat and common reed. No reactivity was seen with pollen extracts from monocots reportedly lacking or containing low levels of group 2 allergens (e.g. Bermuda grass, maize) [17].

We further demonstrate that Phl p 2-specific Fab can be used for the immunological identification of pollen grains containing group 2 allergens by particle blotting (Fig. 2b). Phl p 2-specific Fab identified exclusively grass pollen grains containing group 2 allergens (sweet vernal grass, rye grass, Kentucky Bluegrass, rye, wheat and oat) (Fig. 2b). A recombinant mouse IgG Fab with specificity for the major birch pollen allergen, Bet v 1, reacted only with birch pollen confirming the specificity of the assay (Fig. 2b).

2.3 Clone 94-derived Phl p 2-specific IgG₁ antibodies inhibit the binding of grass pollen-allergic patients IgE antibodies to Phl p 2

Table 1 displays the percentage inhibition of IgE binding obtained for sera from 26 grass pollen-allergic patients with Phl p 2-specific recombinant IgG₁, IgE Fab and polyclonal rabbit antibodies. Clone 94-derived rPhl p 2-specific IgG₁ inhibited IgE binding to Phl p 2 stronger (mean % inhibition: 58%) than polyclonal Phl p 2-specific rabbit antibodies (mean % inhibition: 31%) or clone 94-derived IgE Fab (mean % inhibition: 25%). Moreover, Phl p 2-specific IgG₁ caused a significant reduction of IgE binding for all 26 sera tested. A reduction of IgE binding of >70% was observed for 9 sera

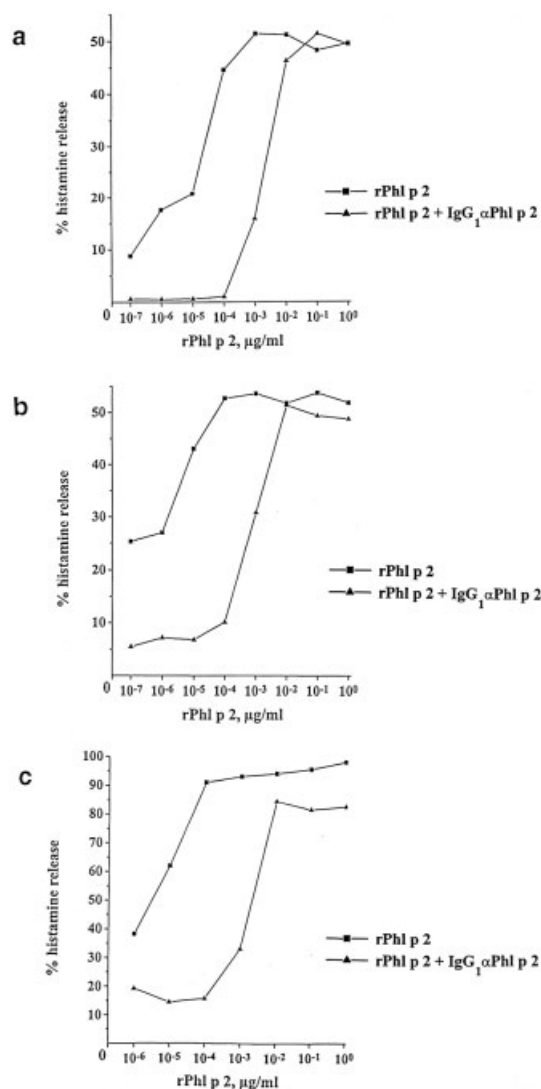


Fig. 3. Inhibition of histamine release by preincubation of rPhl p 2 with rPhl p 2-specific IgG₁ antibodies. Granulocytes from three grass pollen-allergic patients (a–c) were incubated with various concentrations of rPhl p 2 (10⁻⁷–10⁰ µg/ml) which were preincubated with (triangles) or without (squares) rPhl p 2-specific IgG₁ antibodies (x-axis). Histamine release is expressed as percentage of total histamine on the y-axis.

(35%), a >50% reduction of IgE binding was obtained for 11 sera (42%) and the inhibition of IgE binding for the remaining 6 sera (23%) ranged from 29–48% (Table 1).

2.4 The rPhl p 2-specific IgG₁ antibody reduces rPhl p 2-induced histamine release from basophils of grass pollen-allergic patients

Fig. 3 shows that the presence of rPhl p 2-specific IgG₁ in µg/ml concentrations completely suppressed Phl p 2-

induced basophil histamine release up to allergen concentrations up to 10^{-4} $\mu\text{g/ml}$. Without addition of rPhl p 2-specific IgG₁, maximal histamine release was observed at concentrations 10^{-4} $\mu\text{g/ml}$ – 10^{-3} $\mu\text{g/ml}$ of Phl p 2 whereas addition of the blocking antibody shifted the maximal histamine release to allergen concentrations between 10^{-2} $\mu\text{g/ml}$ – 10^{-1} $\mu\text{g/ml}$. Control experiments performed with anti-IgE antibodies showed that the Phl p 2-specific IgG₁ did not affect anti-IgE-induced basophil histamine release (data not shown).

3 Discussion

We report the molecular and immunological characterization of human IgE antibodies with specificity for one of the most important environmental allergens, the major timothy grass pollen allergen, Phl p 2, and convert by genetic engineering allergen-specific IgE into a protective IgG₁ antibody. The IgE Fab were isolated from an IgE combinatorial library, which was constructed from lymphocytes of a grass pollen-allergic patient via panning to the purified Phl p 2 allergen.

The rPhl p 2-specific IgE Fab obtained from the combinatorial library cross-reacted with group 2 allergens from a variety of grass species and thus closely mimicked the reactivity profile of the polyclonal IgE response of grass pollen-allergic patients. rPhl p 2-specific IgE Fab may therefore be used for the quality control of natural grass pollen extracts which are currently used for *in vitro* and *in vivo* diagnosis as well as for specific immunotherapy of grass pollen allergy to determine the presence and concentration of group 2 allergens. The latter procedure will allow to standardize diagnostic and therapeutic grass pollen extracts regarding their allergen contents. Our finding that the Phl p 2-specific IgE Fab could be used to identify grass pollen grains releasing group 2 allergens by particle blotting indicates that they will be also useful tools for the detection and, perhaps, quantification of group 2 allergens in environmental air samples. The measurement of the loads of group 2 allergens in our environment may then form a basis for preventive measures against grass pollen allergy.

The perhaps most important and unexpected finding was that a complete rPhl p 2-specific human IgG₁ antibody obtained by grafting of the IgE Fab variable regions onto human IgG₁ strongly inhibited the binding of allergic patients IgE to the Phl p 2 allergen. This result is unexpected because it has been demonstrated that even small allergens or allergen fragments ranging from 10–30 kDa contain simultaneously the binding sites for several different IgE antibodies and thus can elicit strong effector cell activation [18]. The consistent and strong

inhibition of IgE binding to Phl p 2 obtained with the rPhl p 2-specific IgG₁ antibody may be due to the fact that this antibody reacted with a major IgE epitope-containing domain (*i.e.* the N-terminal portion of Phl p 2) [15]. Support for the assumption that the Phl p 2-specific IgG₁ may be useful for passive therapy of grass pollen allergy comes from basophil histamine release experiments. Phl p 2-specific IgG₁ led to a 100-fold suppression of Phl p 2-induced histamine release from basophils of grass pollen-allergic patients. At physiological allergen concentrations (10^{-4} $\mu\text{g/ml}$) to be expected in the target organs of atopy [19] a complete suppression of histamine release could be achieved with $\mu\text{g/ml}$ concentrations of Phl p 2-specific IgG₁. We therefore consider to further engineer the rPhl p 2-specific IgG₁ antibodies to allow targeting to epithelial cells or antigen-presenting cells on mucosal surfaces. Such manipulation will prevent the washing out of the therapeutic antibody and allow to build up a stable defense line against intruding allergens and, perhaps, to mistarget the allergens into the proteolytic compartment of antigen-presenting cells without activation of effector cells. The latter approach may not only inactivate the allergen by proteolysis but may be also useful for the induction of a protective allergen-specific mucosal immune response. The rPhl p 2-specific human IgG₁ therefore represents a candidate molecule for the therapy of grass pollen allergy and will represent a paradigmatic tool to explore *in vivo* the usefulness of concepts for therapy of allergy which are based on allergen-specific blocking antibodies.

4 Materials and methods

4.1 Biological materials, patients' sera, antibodies

Pollen from sweet vernal grass, oat, Bermuda grass, rye grass, common reed, Kentucky Bluegrass, rye, wheat and maize were purchased from Allergon (Välinge, Sweden). Sera were collected from grass pollen-allergic patients who were characterized by case history, skin prick testing, radio-immunosorbent allergy test, and by testing with recombinant grass pollen allergens as described [17]. A rabbit anti-rPhl p 2 antiserum was obtained by immunization of a rabbit with purified rPhl p 2 using complete Freund's adjuvant (CFA) (Charles River, Kisslegg, Germany).

4.2 Sequence analysis of the cDNA coding for IgE Fab and light chains; expression of rPhl p 2-specific IgE Fab in *E. coli*

Three phage clones expressing antibody fragments with specificity for Phl p 2 were isolated from an IgE combinatorial library by panning to rPhl p 2 as described [16]. All three clones were checked for the production of Phl p 2-specific

Fab by ELISA and for the correct insertion of cDNA coding for the heavy chain fragments and the light chains by restriction analysis before sequencing [16]. rPhl p 2-specific IgE Fab were prepared as described [18].

4.3 Mapping of the binding sites of the human IgE Fab; identification of group 2 allergens by immunoblotting and particle blotting

λ gt11 phage clones expressing β -galactosidase-fused complete Phl p 2 and Phl p 2 fragments were generated as described [15]. Filter replicas containing rPhl p 2 fragments [18] were washed twice for 5 min and once for 30 min with Tris-buffered saline + 0.05% Tween (TBST) containing 0.5% w/v BSA at room temperature and then probed overnight with *E. coli* extracts containing anti-Phl p 2 Fab and, for control purposes, with extracts from *E. coli* transformed with the empty pComb3H plasmid at 4°C. After washing, bound Fab were detected with an alkaline phosphatase-coupled goat anti-human Fab antiserum (Pierce, Rockland, IL) diluted 1:5,000 in TBST/0.5% w/v BSA.

Group 2 allergens were detected in nitrocellulose-blotted grass pollen extracts with IgE Fab as described for the domain mapping. Pollen grains from sweet vernal grass, oat, Bermuda grass, rye grass, timothy grass, common reed, Kentucky Bluegrass, rye, wheat, maize and birch (control) were applied to a nitrocellulose membrane in defined order using toothpicks. Membranes were placed on water-soaked Whatman paper for 30 min to allow the release of allergens. Released proteins were detected with Ponceau S (Boehringer, Mannheim, Germany). Group 2 allergens were detected with Phl p 2-specific IgE Fab as described for the immunoblotting. For the identification of pollen grains releasing the major birch pollen allergen, Bet v 1, a recombinant mouse anti-Bet v 1 Fab [20] was used which was detected with an alkaline phosphatase-conjugated goat anti-mouse Fab antiserum (Pierce).

4.4 Construction and expression of a complete rPhl p 2-specific IgG₁ antibody

Complete recombinant IgG₁ antibodies containing the Fab-derived variable regions of clone 94 were obtained by expression in COS-7 cells as described [18]. cDNA coding for the heavy and light chain variable regions of the IgE Fab were amplified from the IgE Fab-expressing pComb3H plasmid using the V_H (5' V_H primer: 5'-CGGAATTCGTCATTCGAGGTGCAGCTGCTCGAG-3'; 3' V_H: 5'-CGGAATTCGACGTACGACTCACCTGAGGAGACGGTGACCAG-3') and V_K (5' V_K primer: 5'-CGGAATTCGTCATTCGACATCCAGATGACTCAGTCTCCATCCTCC-3'; 3' V_K primer: 5'-CGGAATTCACGTACGTTCTACTCACGTTTGATCTCCACCTT-3') primer pairs, respectively. The primers contained BsmI (underlined) and BsiWI (italics) restriction sites to allow subcloning of the V_H products into plasmid pLNOH2 and the V_K product into

plasmid pLNOK [21]. Plasmids pLNOH2 and pLNOK expressing the variable regions of the Phl p 2-specific IgE were co-transfected into COS-7 cells using DEAE-dextran to obtain complete Phl p 2-specific IgG₁ antibodies as described [18].

4.5 IgE competition experiments

Hundred ng purified rPhl p 2 per well was coated to ELISA plates (Nunc-Immuno, Maxisorp, Greiner, Kremsmünster, Austria) in 0.1 M sodium bicarbonate pH 9.6 at 4°C overnight. Plates were washed twice with TBST containing 0.5% w/v BSA and blocked with TBST containing 3% w/v BSA at 37°C for 3 h. After blocking, the following preincubations were performed in duplicates: 1.) incubation with 1:1 in TBST diluted COS-7 cell culture supernatants containing approximately 5 μ g/ml rPhl p 2-specific IgG₁ antibodies, or, for control purposes, IgG₁ antibodies with specificity for an immunologically unrelated grass pollen allergen, Phl p 5 [18]; 2.) addition of 1:1 in TBST-diluted bacterial extract containing Phl p 2-specific rIgE Fab (approximately 5 μ g/ml) or, for control purposes, bacterial extract without rIgE Fab and 3.) incubation with a 1:100 in TBST diluted rabbit anti-rPhl p 2 antiserum or, for control purposes, with the rabbit's preimmune serum. After washing five times with TBST, plates were incubated in duplicates with sera from Phl p 2 allergic patients diluted 1:3 in TBST/0.5% w/v BSA and, for control purposes, with serum from a non-allergic individual overnight at 4°C. Bound serum IgE was detected with an alkaline phosphatase-conjugated mouse monoclonal anti-human IgE antibody as described [13]. To avoid plate-to-plate variability experiments were performed for each patient's serum on the same plate. Results represented means of duplicate determinations with variations of less than 10%. The percentage inhibition of IgE binding was calculated as follows: Percentage inhibition = $100 - (\text{OD}_{\text{competitor}} \times 100 / \text{OD}_{\text{control}})$.

4.6 Basophil histamine release assay

Granulocytes were isolated by dextran sedimentation from Phl p 2 allergic patients and exposed to different concentrations of the purified rPhl p 2 allergen (10^{-7} – 10^0 μ g/ml). rPhl p 2 dilutions were preincubated for 5 min at room temperature with COS-7 supernatants containing rPhl p 2-specific IgG₁ antibodies (5 μ g/ml), or, for control purposes, with equal volumes of histamine release buffer (HRB: 25 mmol/l Tris pH 7.6, 5 mmol/l KCl, 130 mmol/l NaCl, 0.33 mg/ml human serum albumin). In addition, granulocytes were exposed to different concentrations of a monoclonal anti-human IgE antibody (E-124-2-8/D ϵ 2 Immuno-tech, Marseille, France) which were also preincubated with rPhl p 2-containing COS-7 supernatants or HRB to exclude nonspecific inhibition of basophil histamine release by the rPhl p 2-containing COS-7 supernatants. Histamine released in the cell-free supernatants was determined by

radioimmunoassay (Immunotech) and is expressed as percentage of total histamine after cell lysis [22].

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