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Hyposensitization Therapy with Whole Pollen Extract or Purified Allergens Monitored by Immunoblotting

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Abstract. Patients allergic to grass pollen were hyposensitized with two major allergenic components or whole extract of timothy grass pollen. Specific IgE, IgG1, and IgG4 formed during immunotherapy were analyzed by immunoblotting. Similar antibody-binding patterns were observed in both patient groups. Inhibition experiments using allergenic components isolated by preparative sodium dodecyl sulphate-polyacrylamide gel electrophoresis indicated that all antigenic components of timothy grass pollen detected in immunoblot dispose of private and cross-reactive determinants for binding of human IgE. The worse clinical outcome of immunotherapy after hyposensitization with two cross-reactive components did, therefore, not correlate with a specific antibody formation pattern.

Introduction

Pollen of grasses of the botanical family *Graminae* have been known since long to represent a major source for type I reactions [1]. Grass pollen contain distinct groups of antigenic molecules. The same antigenic groups are present in pollen of different species, but with varying distribution [2-4]. These antigen groups cause cross-reactivity which is more pronounced between grass species closely related within the botanical family [5-7]. Beyond other species, timothy grass (*Phleum pratense*) of the subfamily *Festucoideae* represents one of the most common grasses in temperate climate. It was described to possess unique antigenic determinants [8]. Important allergens of timothy grass pollen, Ag 19 and Ag 25, were defined by means of crossed radioimmuno-electrophoresis [9], and their relative molecular mass was determined to be 15 kD in gel filtration chromatography [10]. In the new allergen nomenclature they are designated as *Phl p* VI (Ag 19) and *Phl p* V (Ag 25) [11]. Partial identity of Ag 19 and Ag 25 has been claimed [9, 10] and their presence in other related grasses documented [12]. Recently, it has been shown that *Phl p* V represents an independent allergenic group with molecular weights of 29-31 kD [13].

In a double-blind immunotherapy (IT) study two major allergens (*Phl p* VI and *Phl p* V) or a 20-component pollen extract of timothy grass have been used for hyposensitizing two groups of patients [14]. The patients immunized with two allergens only showed the worse clinical outcome of therapy. The aim of the present study was to find an antibody-binding profile in immunoblot which would correspond with the clinical outcome of IT. It became evident that antibody responses induced during IT were never confined to single antigens. Furthermore, similar antibody-binding patterns were observed in both patient groups.

We investigated, therefore, the apparent cross-reactivities between different antigens of timothy pollen detected in immunoblot. Purification of single timothy grass pollen components was a prerequisite. Many different technical approaches have been made to isolate grass pollen allergens [5, 7, 10, 15-17]. We isolated allergenic polypeptides of timothy grass pollen extract by means of preparative sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [18, 19]. These polypeptides were used in inhibition experiments. Based on the results of the inhibitions, it became obvious that allergens of timothy grass detected in immunoblot dispose of private and cross-reactive determinants for IgE binding. Im-

munization with two major allergens led to induction of IgE also directed against cross-reactive determinants. As a consequence, the worse clinical outcome of IT after hyposensitization with two components only did not correlate with a specific antibody formation pattern.

Materials and Methods

Chemicals and General Reagents

Chemicals such as Tris, NaCl, HCl, glycine, acetic acid, methanol, ethanol, 2-propanol, bromophenol blue, ethylenediamine-tetraacetic acid (EDTA), ϵ -aminocaproic acid (EACA), Tween 20, 2-mercaptoethanol, and N,N,N,N-tetramethylethylenediamine were obtained from Merck (Darmstadt, FRG); acrylamide was purchased from Serva Feinbiochemicals (Heidelberg/New York); ammoniumperoxodisulphate was obtained from Bio-Rad Laboratories (Richmond, Calif., USA); SDS and glycerol were from BDH Chemicals (Poole, England), and bisacrylamide and Coomassie brilliant blue R 250 were from Sigma (St. Louis, Mo., USA).

Serum Samples

Sera from 40 grass pollen allergic patients included in an IT study [14] were re-examined in the present study. Serum samples obtained before start, 12 weeks after start, and at the end of therapy (week 116) were subjected to immunoblotting. The sera have been stored at -20°C until use. The allergogram (see Results) was constructed from samples drawn before start of IT. Five sera of week 12 of IT were pooled at equal parts (serum pool) and were used as screening medium in immunoblotting analysis of isolated timothy grass pollen allergens. Inhibition experiments were performed using individual sera drawn at weeks 0, 12, or 116 of IT or serum pool, respectively. As control was used a pool of sera negative in radioallergosorbent tests (RAST) towards common allergens [20].

Blood Samples

Two patients with established grass pollen allergy, 1 of them (patient B) also reacting against cat dander, were selected for histamine release test according to their IgE-binding patterns in immunoblot. As controls we used blood of 1 healthy donor without history of atopic disease and negative reaction to grass pollen extract in histamine release test.

Extract

Extract of timothy grass pollen (*P. pratense*) was a kind gift from ALK (Allergologisk Laboratorium Koebenhavn, Hoersholm, Denmark) and was prepared as follows: pollen were obtained from Allergon (Uppsala, Sweden), extracted at 5°C for 20 h in a carbonate buffer solution (10% w/v in 0.125 M NH_4HCO_3 and 0.015 M NaN_3), centrifuged (23,000 g for 1 h at 0°C), and then the extract was dialyzed and lyophilized.

SDS-PAGE

SDS-PAGE was performed in a vertical slab gel apparatus (SE 600; Hoefer Scientific Instruments) according to the method of Laemmli [21]. 16% separation gels ($180 \times 150 \times 1.5$) and a 5% stacking gels were used in a discontinuous buffer system (0.025 M Tris,

0.2 M glycine, 0.1% SDS; w/v). Samples for gels subjected to preparative SDS-PAGE and immunoblotting were prepared by boiling in Laemmli sample buffer devoid of mercaptoethanol. 2.4 mg timothy grass pollen extract was loaded onto one gel subjected to immunoblotting (100 $\mu\text{g}/0.5$ cm gel), 10 mg/gel in preparative electrophoresis. Samples from preparative SDS-PAGE were prepared as follows: 60 μl of eluent (estimated timothy grass pollen content 10 ng/ μl) was boiled for 5 min at 95°C in fourfold Laemmli sample buffer, with or without mercaptoethanol. Molecular weights were determined using prestained protein standards (14.3–200 kD) from Amersham International.

Preparative SDS-PAGE

This has been performed essentially according to the method of Mendel-Hartvig [22], with slight modifications; in short: dimensions of $180 \times 160 \times 4.5$ mm were used for the separations gels. 10 mg extract was loaded per gel. Two gels were run at a time at 40 mA overnight. Staining was done in 45% (v/v) 2-propanol, 10% (v/v) acetic acid (96%), and 0.125% (w/v) Coomassie brilliant blue R 250, for 1 h at room temperature. Destaining was done in 10% 2-propanol and 7% acetic acid overnight at room temperature. Thirteen of the stained bands and one gel strip above the stained parts of the gel (200 kD; blank) were cut out on a light screen and stored at 4°C in a humid chamber until elution. Corresponding gel strips of ten separation runs were equilibrated for 30 min in running buffer containing 0.5% (w/v) bromophenol blue and were applied onto a 30% supporting gel. Stacking gel (4.5%) was added in two layers. Running buffer was used double concentrated in contrast to normal SDS-PAGE. Poles were reversed, and electrophoresis was run at 150 mA constant current. Proteins were eluted into a layer of stacking gel buffer (Tris-HCl, pH 6.8) containing 30% (v/v) glycerol, until stacking gels were destained completely. Volumes of 300 ml were collected. 5 mM EACA and 20 mM EDTA were added to prevent enzymatic degradation of the probes during storage periods [23]. Equal volumes of eluents were subjected to analysis using SDS-PAGE/immunoblotting. 250 ml of each sample was dialyzed (dialysis membranes from Struers, Denmark; cutoff 5 kD) and then lyophilized.

Electroblotting

Electroblotting was performed in a semidry electroblotter (JKA Biotech, Denmark) according to the manufacturer's instructions. Briefly, the proteins were transferred to nitrocellulose (pore size 0.2 μm ; Schleicher & Schüll, Dassel, FRG) in a discontinuous buffer system [anodic buffer 1: 0.3 M Tris, 20% (v/v) methanol, pH 10.4; anodic buffer 2: 25 mM Tris, 20% (v/v) methanol, pH 10.4; cathodic buffer: 25 mM Tris, 40 mM EACA, 20% (v/v) methanol, pH 9.4] for 1.5 h at 200 mA.

Immunoblotting

Blot strips (11.5×0.5 cm) and blot sheets (16.0×18 cm) were blocked in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) and 0.5% Tween 20 containing 3% powdered milk for 30 min. All incubation and washing steps were performed at room temperature on a rocking platform. Washing was done with TBS and 0.5% Tween 20 for 30 min.

Detection of Specific IgE

For IgE detection, individual sera were diluted 1:10 in TBS/Tween/powdered milk (1:10 or 1:150 for inhibition experiments);

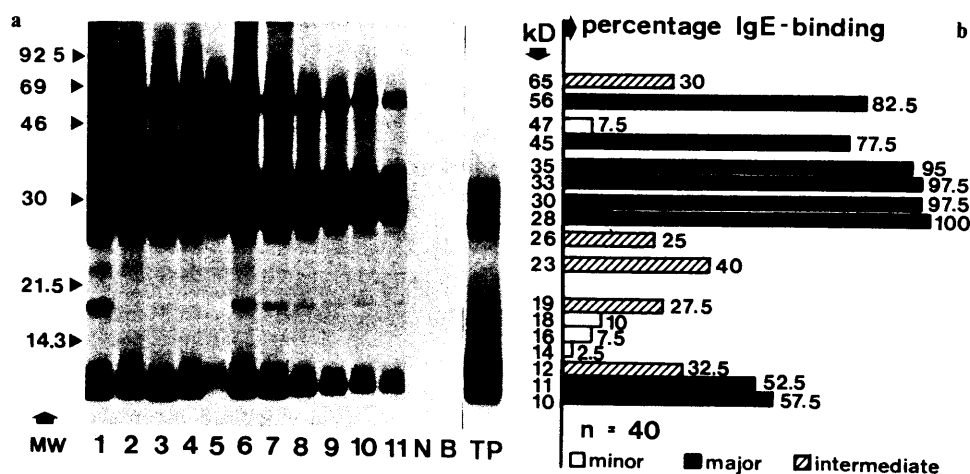


Fig. 1. **a** IgE-binding patterns to timothy grass pollen extract of five individual sera and serum pool in immunoblot and protein pattern of timothy pollen extract in silver-stained SDS-PAGE. Lanes 1-5: individual sera (diluted 1:10); lanes 6-11: serum pool (consisting of equal volumes of sera 1-5) at dilutions of 1:10, 1:20, 1:40, 1:60, 1:80, and 1:100, respectively; lane TP: protein pattern of timothy pollen extract (10 µg) in silver-stained SDS-PAGE; lane N: normal human serum pool; lane B: buffer control. MW = Molecular weight standards in kilodaltons. **b** Percentage IgE binding to timothy grass pollen allergens in immunoblotting, testing sera of 40 patients allergic to grass pollen before hyposensitization therapy. Allergens were classified as minor (2.5-10% of sera tested show IgE binding), intermediate (10-50% IgE binding), or major allergens (50-100% IgE binding), kD = Relative molecular mass of timothy grass pollen allergens in immunoblot.

the serum pool was diluted 1:20 (1:100 for inhibition experiments). Negative control serum pool was used at a 1:10 dilution. For inhibition experiments 1 ml diluted serum or serum pool was preincubated with either 1 mg of isolated polypeptides or 1 mg of whole timothy grass pollen extract (overnight at 4°C). Then blot strips were incubated with the samples for 24 h. After washing, bound IgE was detected with ^{125} I-labelled rabbit antihuman IgE (Phadebas RAST 100, Pharmacia, Uppsala, Sweden; 100,000 cpm/ml TBS/Tween/powdered milk) overnight.

Detection of Specific IgG1 and IgG4

For detection of IgG1 and IgG4, sera were diluted 1:100 and 1:50, respectively, in blocking solution and applied to strips overnight. Bound IgG1 and IgG4 were detected by monoclonal antibodies (Seward Laboratory, London, England). Mouse antihuman IgG1 (BAM 15) was diluted 1:2,000 and mouse anti-human IgG4 (BAM 11) 1:1,000 in TBS/Tween/powdered milk and applied to blot strips for 2 h. After washing, bound mouse IgG was detected by ^{125} I-labelled rabbit antimouse immunoglobulin, γ -chain specific, purified as described previously [24]. Labelled antibody was added to strips for 1 h (100,000 cpm/ml blocking solution).

Strips/sheets from IgE or IgG detection procedures then were washed again in TBS/Tween for 30 min, dried, and exposed to Hyperfilm MP (Amersham) in Neto exposure cassettes (Nielsen & Olsen, Denmark) containing intensifying screens at -80°C.

Histamine Release Test

This was performed as described earlier [25]. Briefly for histamine release experiments Pipes-AMC was used as buffer: 10 mM piperazine-N,N-(bisethanol sulphonic acid), 140 mM sodium acetate, 5 mM potassium acetate, 0.6 mM CaCl_2 , 1 mg/ml glucose, 0.3

mg/ml human serum albumin, and 15 IU/ml heparin (Leo, Denmark; pH 7.4). 10-ml blood samples were collected in plastic tubes containing 0.5 ml of 0.2 M EDTA, (pH 7.2). 1.3 ml of the samples was washed three times for 6 min at room temperature at 250 g with 9 ml of Pipes-AMC. The samples were resuspended in this buffer to a final volume of 1.3 ml. 50 µl Pipes-AMC was added per well to glass microfibre prepared microtitre plates (US patent No. 4,550,085). Then 50 µl of prepared blood samples was incubated with a 20-µl dilution of lyophilized polypeptides (10, 11, 12, 14, 16, 19, 23, 26, 28, 30, 35 and 45 kD) or crude extract of timothy grass pollen (2 mg/ml in Pipes-AMC; dilution series of 10^0 , 10^{-1} , 10^{-2} and 10^{-3} were tested) and added to the microtitre plates. After incubation, released histamine was bound to the microfibres. After washing with deionized water, histamine was released from the fibres by addition of 20 µl of HClO_4 /OPT mixture; 2 mg *o*-phthaldialdehyde, (Fluka) dissolved in 2 ml methanol and 8 ml of 0.59% HClO_4 . After 10 min at room temperature, 65 µl of 0.05 N NaOH was added and the coupling reaction stopped after 10 min by addition of 65 µl of 0.59% HClO_4 . Positive reactivity in comparison to a histamine standard curve was determined in a Farrand spectrofluorometer provided with microflow cell and autosampler.

Results

Allergens of Timothy Grass Pollen in Immunoblot

The patients had been hyposensitized with extract of timothy grass pollen (n = 20) or two major allergens only (n = 20) [14]. We intended to study the anti-

body responses of the two patient groups by immunoblotting. First we investigated which and how many potential allergenic molecules were detected by this technique. Testing sera of the 40 patients before hyposensitization treatment revealed that 17 components of timothy grass pollen extract were capable of binding IgE. Typical IgE-binding patterns of five sera and serum pool are shown in figure 1a. Allergenic molecules were observed with molecular weights of 10, 11, 12, 14, 16, 18, 19, 23, 26, 28, 30, 33, 35, 45, 47, 56, and 65 kD. These allergenic compounds were recognized by IgE of 2.5% (1 of 40) to 100% (40 of 40 patients; fig. 1b). Up to 10% of the sera investigated showed IgE binding to the 14-, 16-, 18-, and 47-kD proteins. They are, therefore, classified as minor allergens. 25–40% of the sera contained IgE directed against the 12-, 19-, 23-, 26- and 65-kD proteins, which accordingly are classified as intermediate allergens. The 10-, 11-, 28-, 30-, 33-, 35-, 45- and 56-kD allergens were predominantly recognized by IgE. IgE binding occurred in 52.5% of the patients (21 of 40) to the 10-kD allergen and in up to 95–100% to the 28-, 30-, 33- and 35-kD proteins. They represent major allergens of timothy grass pollen in immunoblot. No IgE binding could be detected testing the normal human serum pool and in reagent control strips. Knowing the IgE-binding profile of timothy grass pollen extract, we started to test the patient sera for IgE-, IgG1-, and IgG4-binding patterns during hyposensitization treatment.

Specific IgE, IgG1, and IgG4 in IT

We have recently shown that hyposensitization with timothy grass pollen extract results in the formation of specific IgE, IgG1, and IgG4 [26]. Hyposensitization with the two major allergens *Phl p* V and *Phl p* VI was expected to lead to formation of antibodies directed against two components only, i.e., against 15-kD [10] or 27-kD allergens [13], respectively. Sera of patients treated with two components or whole extract of timothy grass pollen were tested by immunoblotting. For each group antibody-binding profiles were determined by adding up binding profiles of the individual patients. This was done for allergens recognized by IgE, IgG1, and IgG4. The changes of antibody-binding profiles were monitored during immunotherapy (fig. 2).

Before start of the therapy, IgE-binding patterns varied individually. By now all allergenic compounds were recognized by IgE. There were no differences be-

tween the two patient groups. Twelve weeks after the outset of therapy, IgE was enhanced against most possible allergenic components in both patient groups. Until the end of therapy, IgE-binding profiles in both patient groups did no more vary.

Before starting the therapy, allergens of timothy grass pollen were weakly recognized by IgG1. In week 12 after the start of therapy, the number of bands recognized by IgG1 increased. IgG1 against allergens of 10, 26, 28, 29, 30, 33, 35, 45, and 56 kD was detected in both patient groups. IgG1 was not induced against the 12-, 14-, 16-, 18-, 23-, and 47-kD allergens in the group immunized with two major allergens only. In week 12, the binding patterns of patients hyposensitized with whole extract or two components of timothy grass pollen extract only were very similar. At the end of therapy, still the same allergens were recognized by IgG1, as in week 12, by both groups. By now, in sera from patients treated with two allergens only, IgG1 was also directed against minor allergens of 12, 14, and 16 kD.

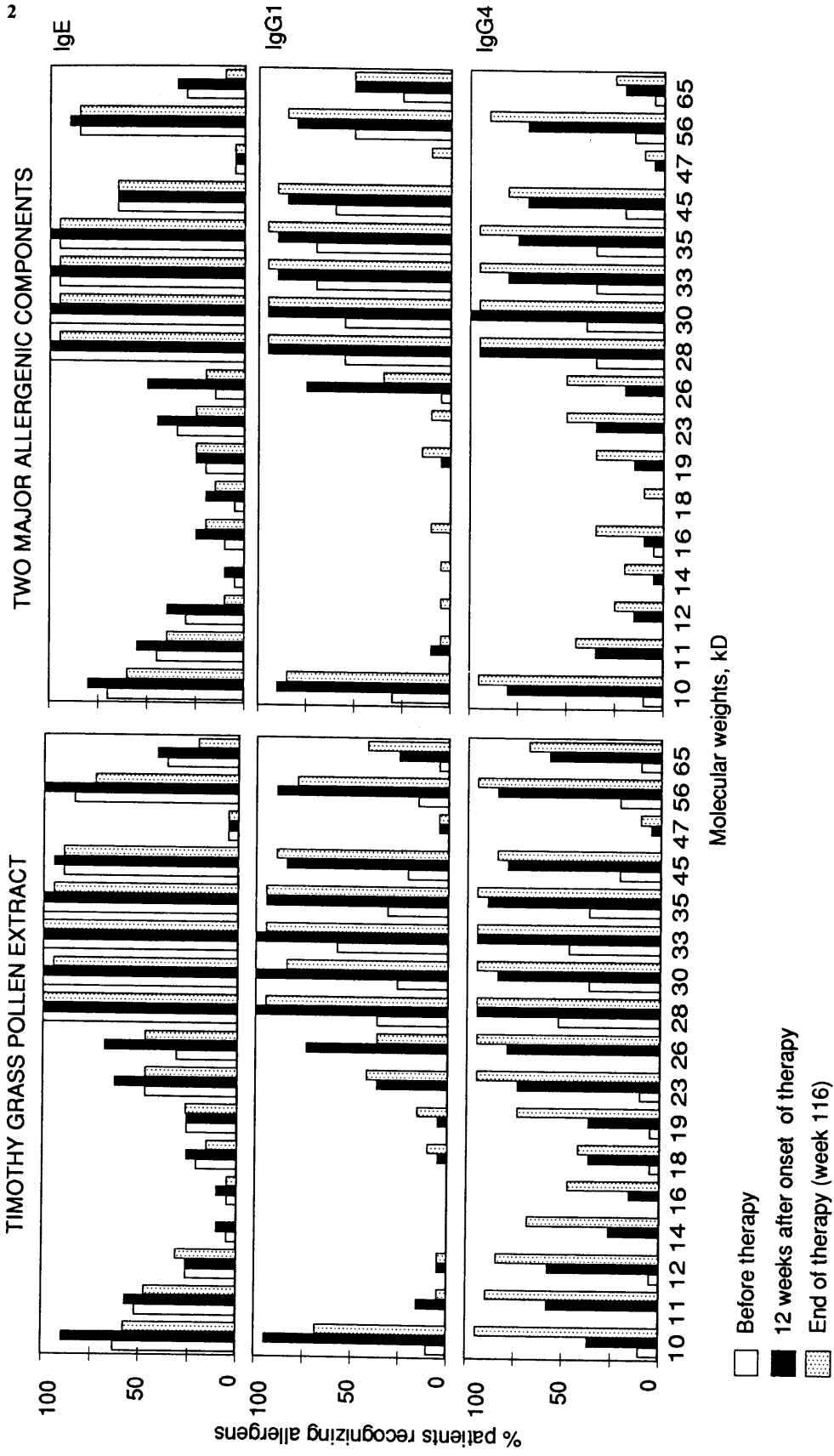
In both patient groups IgG4 binding was enhanced after initiation of therapy (week 12). IgG4 was not induced against the 18-kD allergen and was less induced against allergens of 11, 12, 14, 19, 23, 26, and 56 kD in the group immunized with the two components only. In the same patients, IgG4 against the 10-kD allergen was more often enhanced than in the patients hyposensitized with whole extract. At the end of IT, 2 years later, again all allergenic compounds were recognized by IgG4 in both patient groups.

Comparing IgE-, IgG1-, and IgG4-binding profiles of the two patient groups, none of the components was preferentially recognized by the patients immunized with the two major allergenic components only. Also in this patient group, all allergens were detected by IgE, IgG1, and/or IgG4. Furthermore, it became clear that induction of IgE, IgG1, and IgG4 was never confined to single allergen bands. We investigated the apparent cross-reactivities by preparative SDS-PAGE.

Analysis of Fractions from Preparative SDS-PAGE

Allergen samples from preparative SDS-PAGE were tested in immunoblot (fig. 3). IgE binding to reduced and unreduced samples was compared directly. Dense IgE binding was detected against fractions of 10, 11, 28, 30, 33, and 35 kD, whereas only weak IgE binding was directed against fractions with molecular weights of 12, 14, 16, 19, 23, 26, and 45 kD. The 19-kD

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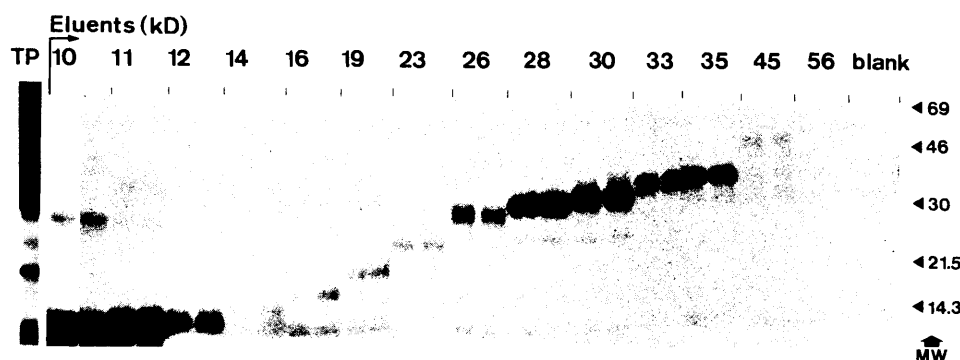


Fig. 3. IgE binding to polypeptides isolated by preparative SDS-PAGE and to crude extract of timothy grass pollen, testing with a serum pool. Bound IgE was detected with ^{125}I -labelled rabbit antihuman IgE. Eluents (10–56 kD) and blank fraction were subjected unreduced (left-hand lanes) or reduced (right-hand lanes) to SDS-PAGE. TP = Timothy pollen extract; WM = molecular weight standards in kilodaltons.

allergen was better recognized by IgE in crude extract (lane TP) than after SDS-PAGE preparation. The 10-kD preparation contained also a 26-kD band; preparations of 28 and 30 kD contained 23-kD IgE-binding bands which persisted in reduced condition. The 12-, 14-, 16-, 19-, and 45-kD preparations showed also weak IgE binding in the 30-kD area which disappeared in the 12-, 14-, 16- and 19-kD preparations after treatment with mercaptoethanol. Simultaneously, IgE binding directed against the isolated polypeptides increased. Additional bands in the 45-kD preparation persisted after treatment with mercaptoethanol. The 56-kD allergen could not be detected; the 26- and 33-kD allergens contained the neighbouring bands. No IgE binding was observed in the blank preparation. Knowing the composition of the isolated allergen preparations, we tested their activity triggering specifically histamine release from patients' leucocytes.

Histamine Release Test

For examining cross-reactivities, it was crucial to control if IgE would still recognize the isolated polypeptides specifically. We compared, therefore, the reactivity patterns in histamine release tests of 2 pa-

tients (A and B), with the IgE-binding patterns in immunoblot of the same patients' sera (fig. 4, lanes A and B).

Patient A. Histamine was released probing with timothy grass pollen allergens with a molecular weight of 11, 28, 30, and 35 kD, in accordance with the IgE-binding pattern in immunoblotting, and in addition with the 10-kD fraction. No histamine release was obtained probing the 12-, 14-, 19-, 23-, and 26-kD fractions which were not recognized by IgE in immunoblot.

Patient B. Histamine release was provoked by allergen fractions with a molecular weight of 11, 19, 26, 28, 30, and 35 kD, in accordance with immunoblotting results, and in addition by the 10-kD fraction. No histamine release was observed testing the 12-, 14-, 16-, and 23-kD fractions. They were not recognized by patient B's IgE in immunoblot.

IgE-binding patterns of patients A and B in immunoblot paralleled their individual reactivity pattern in histamine release test; e.g., the 19-kD preparation was capable to cross-link cell-bound IgE in patient B, but not in patient A. Exclusively, the 10-kD preparation was capable to release histamine without being recognized in immunoblotting. IgE binding to the 28-, 30-, 33-, and 35-kD allergens was more intense in patient B, and, consecutively, lower concentrations of timothy grass pollen fractions sufficiently cross-bridging patient B's IgE as compared with patient A. However, testing crude extract of timothy grass pollen in histamine, a tenfold less amount than of the isolated polypeptides was sufficient for triggering histamine release from the same patients' leuco-

Fig. 2. Sera of 20 patients hyposensitized with timothy grass pollen extract and sera of 20 patients hyposensitized with two major allergens only were tested in immunoblot. Percentage of sera (y axis) showing IgE, IgG1 or IgG4 binding to allergenic compounds of timothy grass pollen extract (x axis). Values before (open columns), 12 weeks after (black columns), and at the end of IT (shaded columns) are shown.

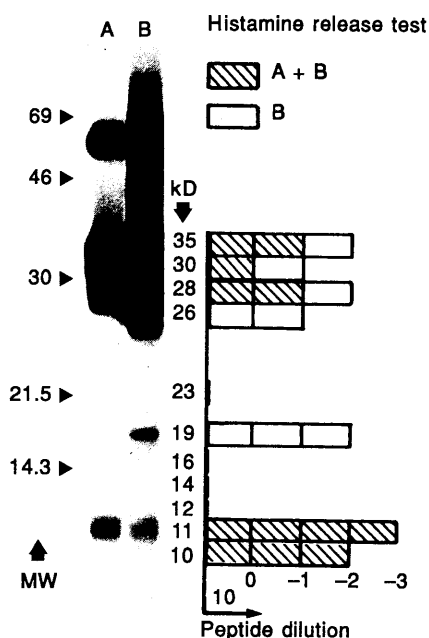


Fig. 4. IgE binding patterns of 2 patients (A and B) in immunoblot in comparison to the reactivity of the same patients' leucocytes in histamine release test probing allergenic compounds isolated from extract of timothy grass pollen. Left panel: IgE binding patterns of patients A and B to crude timothy grass pollen extract in immunoblot. MW = Molecular weight standards in kilodaltons. Right panel: relative reactivity of patients A and B in histamine release, testing different dilutions of isolated timothy grass pollen polypeptides. X axis: dilution of timothy grass pollen allergens ($10^0 = 2 \text{ mg/ml}$); y axis: allergen fractions tested (kD).

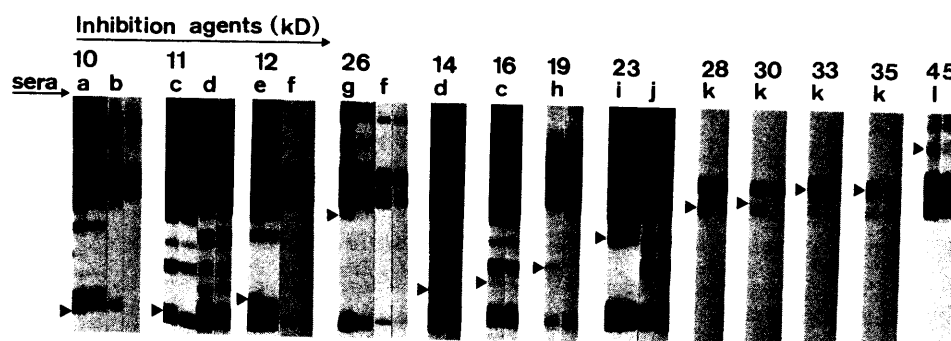


Fig. 5. IgE binding of individual sera inhibited by polypeptides isolated from timothy grass pollen extract. IgE binding is shown before (left) and after addition of the isolated allergens (right). 1 mg lyophilized timothy pollen allergen per millilitre diluted serum was used (sera a, c, d, e, and g-l). In case of the 10-, 12-, and 26-kD components, in addition inhibitions using 2 mg are shown (sera b and f). Sera were diluted 1:10 (10, 11, 12, 14, 16, 19, 26, 45 kD) or 1:150 (28, 30, 33, 35 kD). Arrows indicate molecular weights of the polypeptides used to inhibit IgE binding.

cytes. Neither the isolated polypeptides nor crude extract of timothy grass pollen released histamine from leucocytes of a nonallergic volunteer.

Combining histamine release test and immunoblot revealed that on the one hand the polypeptides had lost some of their allergenic potency; on the other hand, we could show their remaining specificity which made them useful tools for studying cross-reactivity patterns in crude extract of timothy grass pollen.

Inhibition Experiments in Immunoblot Using Individual Sera

Thirteen allergen fractions of timothy grass pollen have been used in immunoblotting experiments (fig. 5). Original IgE-binding patterns of sera (lanes a-l) are shown in direct comparison to IgE binding of the same sera after inhibition. They were diluted 1:10 for incubation with the 10-, 11-, 12-, 14-, 16-, 18-, 19-, 23-, and 45-kD fractions. For allergens exhibiting a high IgE-binding capability (26, 28, 30, and 35 kD),

the sera were diluted 1:150. This was done in order to produce an inhibition by the limited amount of purified allergen. The effects on the IgE-binding patterns using 1 mg of the isolated polypeptides per millilitre serum are shown, for the 10-, 12-, and 26-kD allergens also using 2 mg. All polypeptides reduced markedly IgE binding directed against their counterparts in blotted whole extract.

In addition to component-specific inhibitions, also IgE binding to allergens other than themselves was affected: the 11-kD fraction reduced also binding of IgE to the 10-kD allergen (shown in serum d). The 10- and 26-kD allergens were capable of inhibiting IgE binding to each other, as shown in sera b and f. Two milligrams of the 26-kD polypeptide reduced in addition IgE binding to the 28- and 30-kD allergens (serum f). Also the 10- and 12-kD polypeptides reduced IgE binding to additional allergens (to the 28-, 30-, 33-, 35-, 45-, 47-, 56-kD allergens in serum b and to the 10-, 28-, 30-, and 56-kD allergens in serum f). The 14-kD polypeptide reduced IgE binding to the 16- and 18-kD allergens (serum d), and the 16-kD polypeptide inhibited IgE binding to the 18- and 19-kD allergens (serum c). IgE binding to the 14-, 16-, 18-, 19-kD group was not reduced by the 23-kD polypeptide (serum j). The 28- and 30-kD fractions inhibited binding of IgE to each other and reduced in addition IgE binding to the 33- and 35-kD allergens (serum k). The 35-kD fraction inhibited binding of IgE to the 33- and 35-kD allergens (serum k). The 33-kD fraction (containing the 30-kD allergen) reacted with the 28-, 30-, 33-, and 35-kD allergens. The 45-kD fraction (containing the 28-, 30-, 33- and 35-kD allergens) did not react with the 56- and 65-kD bands (serum l).

These experiments showed that besides component-specific inhibition also IgE binding to related allergens was affected. The extract of timothy grass pollen seemed to contain three groups of allergens: the fractions with molecular weights of 10, 11, 12, 26, and 56 kD, the 14-, 16-, 18-, 19-kD family, and the fractions with a molecular weight of 23, 28, 30, 33, 35, and 45 kD. However, these results were obtained testing sera individually.

Inhibition Experiments in Immunoblot Using a Serum Pool

In order to produce a more representative pattern, we used a pool of sera of allergic patients, diluted 1:100. This diluted serum pool was incubated with 1 mg each of the 10-, 11-, 12-, 16-, 19-, 26-, 28-, 30-,

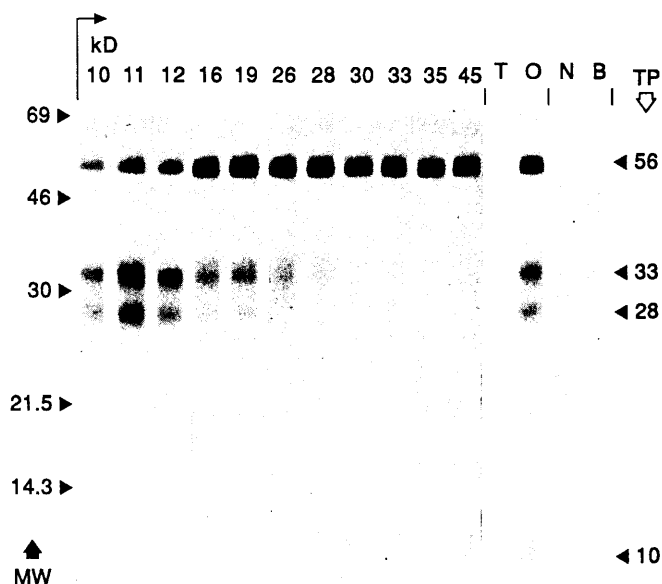


Fig. 6. IgE binding pattern of a serum pool to crude timothy grass pollen extract before and after incubation with different polypeptides isolated from the same extract. Serum pool was diluted 1:100; 1 mg of each polypeptide was used. Effects on IgE binding in serum pool by incubation with the 10-, 11-, 12-, 16-, 19-, 26-, 28-, 30-, 33-, 35-, and 45-kD allergens are shown. T = 100 μ g timothy grass pollen extract inhibited IgE binding in serum pool; O = Original IgE binding in serum pool before inhibition; N = Normal human serum pool; B = buffer control; TP = molecular weights of four important timothy grass pollen allergens in kilodaltons; MW = molecular weight standards in kilodaltons.

33-, 35-, or 45-kD timothy grass pollen polypeptides (fig. 6). All fractions were capable to inhibit IgE binding to the 10-kD allergen. The 10-, 11-, and 12-kD polypeptides reduced also IgE binding to the 56-kD allergen. Testing the 16-kD and 19-kD polypeptides revealed that binding of IgE to the 28-, 30-, 33-, and 35-kD allergens was reduced. On the contrary, IgE binding to the 56-kD allergen was not affected. The 26-, 28-, 30-, 33-, 35-, and 45-kD allergens reduced IgE binding to the 28-, 30-, 33-, and 35-kD allergens. 100 μ g of whole timothy grass pollen extract per millilitre serum dilution neutralized IgE binding completely. No IgE binding was observed in normal human serum pool (N) and reagent control strip (B).

Testing with a serum pool containing IgE with a broad variety of specificities confirmed that allergens of timothy grass pollen also dispose of cross-reactive determinants for binding of IgE.

Discussion

In the present study, we compared the antibody-binding profiles of patients hyposensitized with whole extract of timothy grass pollen and of patients immunized with only two major allergenic components [14] in immunoblotting experiments. During IT, IgE and IgG4 were formed against all allergens. Also IgG1 increased, but did not bind to some minor allergens, in accordance with previous findings [26]. The most essential changes of antibody-binding patterns were observed in the samples of week 12 after initiation of IT. Until the end of therapy, 2 years later, the binding patterns did no more vary markedly. However, due to possible interference of two pollen seasons with antibody-binding profiles seen at the end of IT, we decided to comment mainly upon variations of profiles occurring already 12 weeks after outset of IT.

We had expected to find antibody-binding profiles typical for each patient group. But, surprisingly, the group immunized with two components only showed enhancement of IgE, IgG1, and IgG4-binding against the same components as the patients immunized with whole allergen extract. Cross-reactivities could be due to this phenomenon.

Antigenic groups of grass have been defined, e.g., by the use of monoclonal antibodies or amino acid sequence analysis [13, 18, 27, 28]. We decided to use sera of allergic patients in our experiments to examine cross-reactivities. The whole antigen surface has been considered to be potentially immunogenic [reviewed in ref. 29], thus being recognized by antibodies with many different specificities. This repertoire might be multiplied by the dimension of antibody diversity and flexibility: one antibody has been suggested to potentially react with a variety of epitopes [30]. Using sera of allergic patients, we took advantage of the repertoire of IgE, simultaneously favouring determinants relevant *in vivo* [31].

Prerequisite for investigating cross-reactivities were single allergenic compounds from timothy grass pollen extract which were isolated according to molecular weights by preparative SDS-PAGE. In all preparations we observed IgE binding to the actually prepared band, but also IgE binding to additional components. We observed, e.g., 23-kD bands in the 28- and 30-kD preparations. A relationship of components with comparable molecular weights has been described before: using a monoclonal antibody di-

rected against group 1 allergens of ryegrass for affinity chromatography, a 34-kD protein together with a 23-kD protein was eluted [6]. Our 10-kD preparation contained also a 26-kD IgE-binding band in immunoblot. This was similar to properties of Kentucky bluegrass allergens. A low molecular weight antigen containing cysteine in its amino acid composition was capable to reassemble to a 31-kD allergen [32]. The observed additional components in our preparations might, therefore, (1) represent polymeric isoallergens which are first separated from each other in a second-step SDS-PAGE; (2) trailing effects might be responsible for the presence of additional allergenic molecules in some of the preparations. However, when separating the 14-, 16-, and 19-kD preparations in reduced condition, additional allergenic molecules disappeared in favour of the binding intensity on the actually prepared bands. Oligomeric proteins consisting of several polypeptide chains can dissociate in SDS-PAGE into their subunits. The subunits could also reassemble to longer polypeptide chains based on rearrangements of disulphide bonds. This fact may indicate that the 28- to 33-kD major allergens partly consist of the subunits of 14, 16, and 19 kD. Their relationship has been confirmed by inhibition experiments using a pool of sera.

The 10-kD allergen was capable of triggering histamine release. This was in discrepancy to immunoblotting results: the same patients' sera did not show IgE binding to the 10-kD allergen. Epitopes have been characterized to be of topographic nature [33, 34], with the antigen interaction surface consisting of several loops of the peptide fold. The 10-kD polypeptide in blotted extract as well as the 10-kD polypeptide tested in histamine release had undergone boiling in the presence of SDS. Thereby, they both did certainly loose conformational determinants responsible for antibody binding. Exclusively the 10-kD polypeptide examined in histamine release test was then allowed to reconstitute its tertiary fold under physiological buffer conditions.

The 56-kD molecule may correspond to a high molecular weight basic allergen from ryegrass [35]. IgE binding to the 56-kD allergen of timothy extract occurred in 82.5% of 40 patients. Thereby, it was shown to represent an allergen of major importance in extracts of timothy grass pollen. However, preparation of the 56-kD allergen did not succeed. This might be due to labile conformational determinants [35].

By inhibition experiments testing sera individu-

ally, timothy grass pollen allergens were allocated to three allergenic groups. Probing with a pool of sera, these groups could be no more distinguished. These data suggest that binding specificities of IgE might overlap the different grass groups. Besides private determinants for binding of IgE, timothy grass pollen allergens dispose of cross-reactive determinants. Furthermore, results of inhibition experiments explained the similar antibody-binding profiles of the two patient groups during hyposensitization. *Phl p V* and *Phl p VI* which represent major allergens of timothy grass, induced an IgE, IgG1, and IgG4 response quite similar to immunization with whole allergen extract. However, patients immunized with two major allergens only showed the worse clinical outcome of IT [14]. This was not correlated with a specific antibody formation pattern.

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