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Diagnosis of Grass Pollen Allergy with Recombinant Timothy Grass (*Phleum pratense*) Pollen Allergens

Key Words

Grass pollen allergy
Recombinant allergens
Profilin
Timothy grass

Abstract

In order to establish a test system for grass pollen allergy based on the use of recombinant allergens we chose timothy grass (*Phleum pratense*), a widely spread grass, as a model. From a lambda gt11 cDNA expression library that we had constructed from pollen RNA of timothy grass (*P. pratense*), we had obtained with serum IgE from a grass pollen-allergic individual 60 IgE-binding clones. By differential testing with sera from different grass pollen-allergic patients, we selected three distinct clones encoding *Phl p* I (group I), *Phl p* V (group V) and profilin from timothy grass, which when used together allowed the diagnosis of grass pollen allergy in 97 out of 98 tested grass pollen-allergic patients employing a simple plaque lift technique. This recombinant test based on plaque lifts containing allergen- β -galactosidase fusion proteins was compared with IgE immunoblots using crude pollen protein extracts from timothy grass. Both methods were in good agreement with RAST scores and clinical data, and proved to be useful for the diagnosis of grass pollen allergy. Our results further indicate that a limited panel of only two recombinant grass pollen allergens, *Phl p* I and *Phl p* V, together with the plant panallergen profilin could be sufficient for the diagnosis and possibly immunotherapy of grass pollen allergy.

Introduction

Molecular characterization of atopic allergens by recombinant techniques may provide well-defined proteins for diagnosis and therapy of allergic diseases [1]. Any diagnostic or therapeutic approach that uses recombinant allergens depends on the following prerequisites: (i) natural

allergens and recombinant allergens should possess comparable antigenicity/IgE-binding capacity and functional properties (release of histamine from basophils/mast cells and capability to stimulate allergen-specific T cells), and (ii) it is essential to show that a reasonable number of relevant allergens can be defined.

In a previous study on recombinant birch pollen aller-

gens [2], we were able to show that tree pollen allergy can be diagnosed with the use of only two recombinant birch pollen allergens, *Bet v I* [3] and birch profilin, a novel plant panallergen, designated *Bet v II* [4–6]. The finding that only two recombinant allergens were sufficient for the diagnosis of tree pollen allergy encouraged us to establish such a diagnostic model for grass pollen allergy as well. The major grass pollen allergens were first characterized by proteinchemical and immunochemical methods and found to be similar in different grass species in terms of antigenicity/IgE-binding capacity [7] and primary structure (high amino acid sequence homology) [8, 9]. More recently, several grass pollen allergens were characterized by molecular cloning techniques [10–13] and found to be highly homologous between different grass species. In contrast to tree pollen allergy, grass pollen-allergic patients show IgE-binding to a large panel of different groups of grass pollen allergens. Two-dimensional electrophoresis and DNA sequence analysis revealed that even within one group of grass pollen allergens isoforms with different isoelectric points can be identified [14]. These data create a very complex and puzzling picture regarding the number and diversity of grass pollen allergens.

In order to characterize a defined and limited panel of grass pollen allergens for diagnostic and therapeutic purposes, we chose timothy grass (*Phleum pratense*) as a model. Timothy grass allergens are well characterized by protein and immunochemical techniques [9] and are widely spread in Europe. From a cDNA library that we constructed from timothy grass pollen, we could isolate and characterize the cDNAs encoding the most important allergens of timothy grass [15]. Three of these allergens, *Phl p I*, *Phl p V* and timothy grass profilin together with a fourth clone (clone 53) coding for a novel grass pollen allergen with IgE epitopes different from the above three allergens were tested for IgE-binding with 98 grass pollen-allergic patients. We found that only three recombinant timothy grass pollen allergens, *Phl p I* and *Phl p V* together with the timothy grass profilin were sufficient to diagnose grass pollen allergy in 97 out of 98 grass pollen-allergic patients.

Materials and Methods

Pollen Protein Extracts from Timothy Grass (*P. pratense*) and IgE Immunoblots

Pollen from timothy grass (*P. pratense*), determined to be 95% pure was obtained from Allergon (Engelholm, Sweden). Protein extracts were prepared as described [16] and subjected to SDS polyacrylamide gel electrophoresis according to Laemmli [17]. 1 mg pol-

len proteins from timothy grass were applied to a 20 cm wide gel and blotted onto nitrocellulose (Schleicher & Schuell, Dassel, FRG) [18]. Nitrocellulose was cut into strips, incubated with 1:10 dilutions of patients sera in 50 mM sodium phosphate buffer, pH 7.5, 0.5% Tween 20, 0.5% BSA, 0.05% sodium azide and bound IgE was detected with a ¹²⁵I-labeled rabbit anti-human IgE antibody (Pharmacia, Uppsala, Sweden) as described [16].

Preparation of Nitrocellulose Filters Containing Recombinant Timothy Grass Pollen Allergens and IgE Detection

Recombinant lambda gt11 phages capable of expressing the cDNAs encoding the major IgE-binding portion of *Phl p I*, *Phl p V* timothy grass profilin and a novel grass pollen allergen, designated c53, were isolated and characterized [15]. *Escherichia coli* Y 1090 infected with recombinant phages and phage lambda gt11 without inserted cDNA (negative control) were plated on LB plates containing 100 µg/ml ampicillin. Phages were induced to express recombinant allergens by overlay with nitrocellulose filters BA85 (Schleicher & Schuell, Dassel, FRG) that had been soaked in 10 mM isopropyl-β-thiogalactoside (IPTG; Sigma, St. Louis, Mo., USA) as described [19]. Nitrocellulose filters were then cut into sectors, and IgE-binding assays were done as described for the IgE immunoblots.

Characterization of Grass Pollen-Allergic Patients

98 grass pollen-allergic patients were characterized according to sex, age, symptoms and RAST scores to grasses. The panel of patients comprised 48% females and 52% males. The age distribution was as follows: 36% < 20 years, 37% 21–30 years, 12% 31–40 years, 9% 41–50 years, and 6% > 50 years. 100 patients suffered from rhinitis, 96 patients from conjunctivitis and 30 patients from asthma during the grass pollen season. From 98 grass pollen-allergic individuals, 14 showed a RAST score < 3 and 85 patients a RAST score > 3. All patients with RAST scores > 3 showed strong positive skin prick tests. Three patients with different allergies were included to ensure specificity of the test system. Patient number 26 suffered from asthma and had elevated total IgE levels (RIST 285 IU/ml), patient 65 suffered from Ni²⁺ contact dermatitis and patient number 101 reacted in RAST tests only with tree pollens (RAST > 4). Number 102 represents a serum pool of non-grass pollen-allergic individuals.

Results

IgE Testing of Grass Pollen-Allergic Patients with Blotted Natural Timothy Grass (*P. pratense*) Allergens

Using timothy grass pollen extract prepared as described [16] and SDS polyacrylamide gels [17], prominent bands were found in the regions of about 54, between 25 and 35, at 18 and between 14 and 10 kD (fig. 1). This extract was also blotted to nitrocellulose and used for IgE detection of allergens with sera from 98 grass pollen-allergic individuals and 3 control persons with, according to RAST and clinics, different allergic symptoms. 95% of the patients bound with their IgE to the proteins in the region of 25–35 kD corresponding to group I [20] and group V allergens [9], 75% displayed IgE reactivity with the 54-kD components known as group IV allergens [21] and about

78% gave IgE reactivity to proteins migrating in the region of 10–14 kD as described for group II and group III allergens [20, 22] as well as profilin [6]. With serum IgE from patients with no grass pollen allergy but elevated total IgE (number 26) and with a Ni²⁺ contact dermatitis (number 65), no IgE binding was found with timothy grass allergens. Patient 101 who according to RAST should represent an exclusive tree pollen-allergic patient reacted weakly with profilin from timothy grass. The normal serum control 102 and the buffer control 103 also gave no IgE reactivity on the blots. Grass pollen-allergic patients (patients No. 37, 39, 40, 42, 44, 45, 71, 74, 76, 79 and 97) with weak clinical symptoms, RAST classes <3 and weak skin prick test also showed weak IgE reactivity with blotted timothy grass pollen allergens.

IgE Testing of Grass Pollen-Allergic Patients with Nitrocellulose Filters Containing Recombinant Phl p I, Phl p V, Timothy Grass Profilin and Clone 53

The same panel of patients that had been tested with natural timothy grass pollen extracts was tested for IgE binding to recombinant Phl p I, Phl p V, timothy grass profilin as well as clone 53 that encodes a novel grass pollen allergen and lambda gt11/*E. coli* proteins (negative control). In brief, we found that three recombinant allergens Phl p I, Phl p V and timothy grass profilin are sufficient for the diagnosis of grass pollen allergy in 97 out of the 98 grass pollen-allergic patients tested. Patients 24 and 32 showed strong and exclusive reactivity with timothy grass profilin. Clone 53 represents a novel grass pollen allergen that does not share IgE epitopes with any of the other tested allergens. Although this allergen is recognized by serum IgE of 65% of the grass pollen-allergic patients, we could not identify any patient that reacted exclusively with this allergen. IgE reactivity with non-recombinant phage proteins (sector 5) could not be observed in any case. Patient 79, a patient with low amounts of IgE antibodies (RAST class 1.6) and moderate clinical symptoms (rhinitis) during the grass pollen season could not be identified with the recombinant allergens. This patient reacted only weakly with group IV allergens on the IgE immunoblot.

Comparison of the IgE-Binding Pattern of 98 Timothy Grass Pollen Allergics with Natural and Recombinant Grass Pollen Allergens and Correlation with RAST and Clinical Parameters

Comparing the intensity of IgE reactivity of the tested patients with natural and recombinant allergens, we found excellent correlation. All patients with strong IgE binding

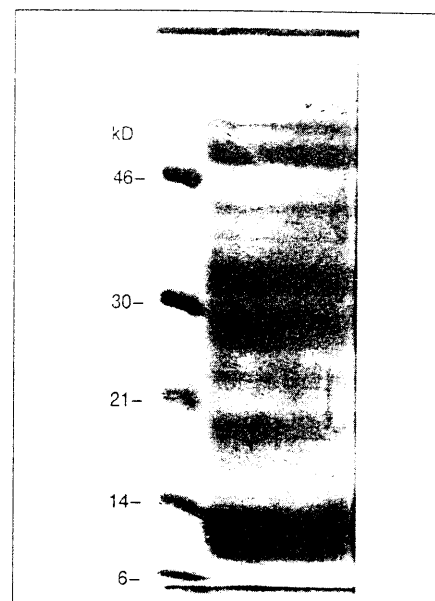
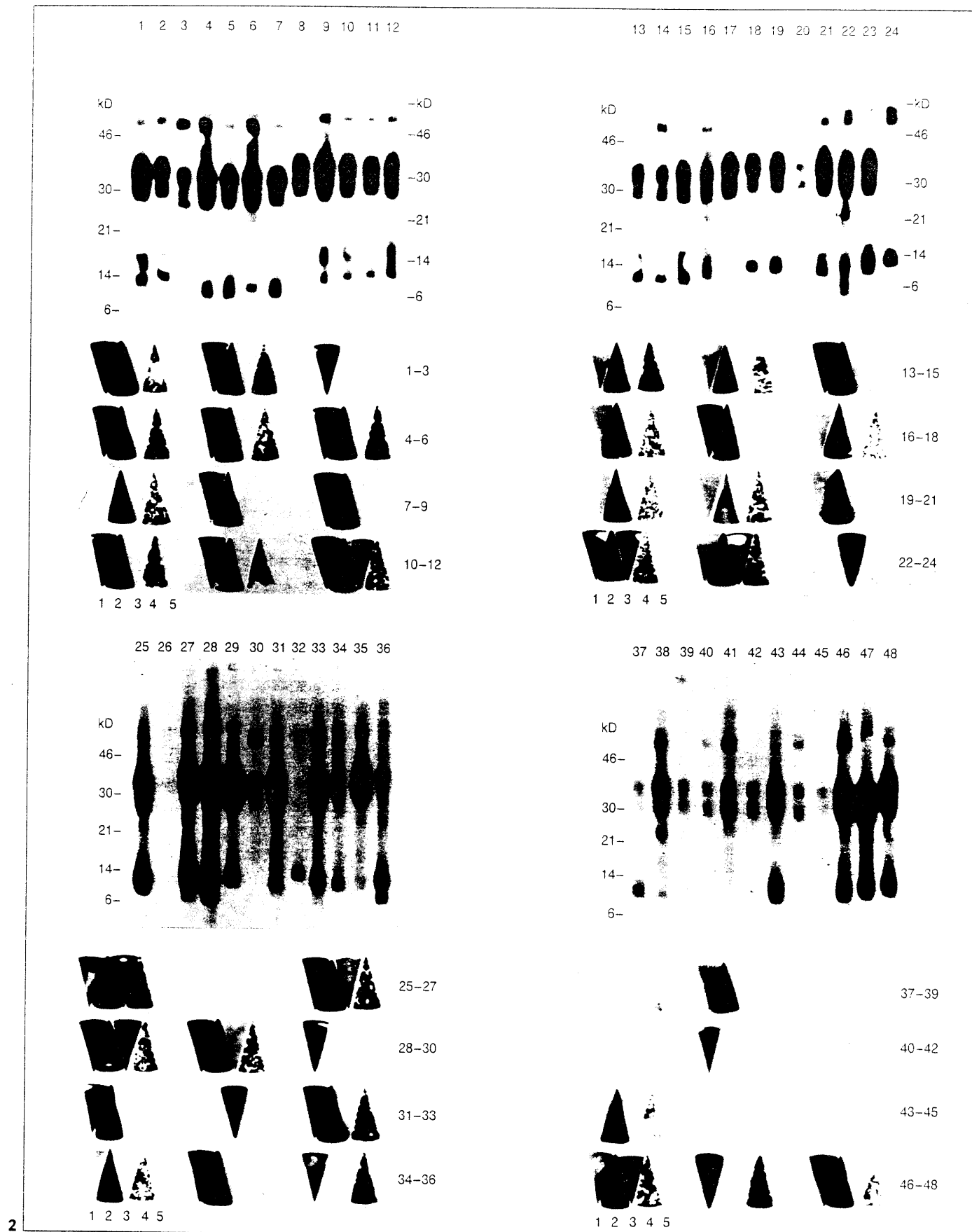


Fig. 1. Coomassie brilliant blue-stained gel containing an aqueous protein extract from pollen of timothy grass (*P. pratense*). The molecular weight marker is shown on the left side of the gel.

to natural timothy grass allergens also reacted strongly with the recombinant proteins, and these grass pollen-allergic patients showed severe clinical symptoms during the grass pollen season, positive skin prick tests and high RAST classes. Patients with less severe symptoms during the grass pollen season, with low RAST class and weak IgE binding to natural timothy grass allergens on the blot (patients 37, 39, 40, 42, 44, 45, 71, 74, 76, 97 and 101) weakly reacted with the recombinant allergens. In case of patient 101, RAST tests were not able to diagnose grass pollen allergy although weak clinical symptoms (rhinitis, conjunctivitis) were seen during the grass pollen season. Patient 79 was the only patient who reacted exclusively with group IV allergens on the IgE immunoblot and therefore could not be detected with the recombinant test system.

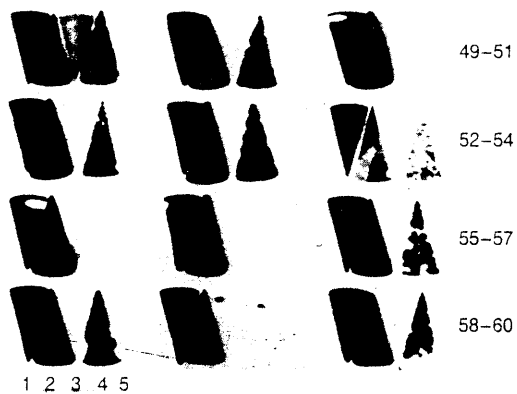
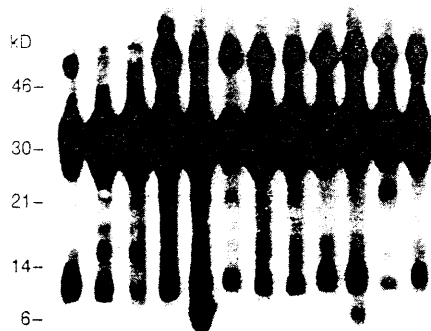
Discussion

Preparation of allergen-containing protein extracts is mostly achieved by applying aqueous extraction methods. This procedure seems to mimic the event when pollen comes into contact with the mucous membranes of the respiratory tract and liberates allergenic proteins. Most of the pollen allergens identified so far are hydrophilic proteins of low molecular weights (5–70 kD) and seem to be quite resistant against proteolytic degradation [23]. Aqueous protein extracts from timothy grass pollen (fig. 1) show

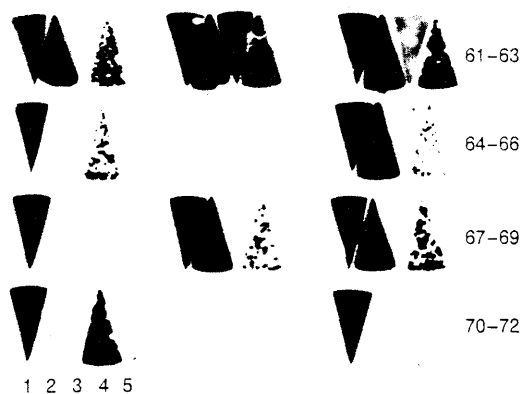


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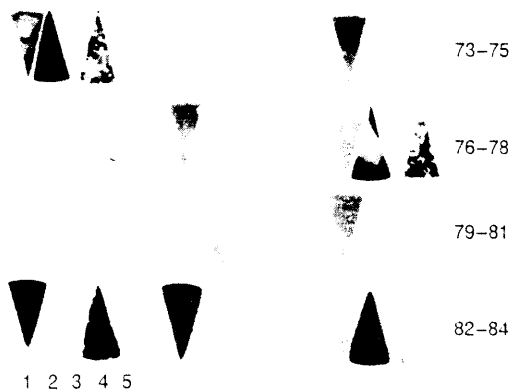
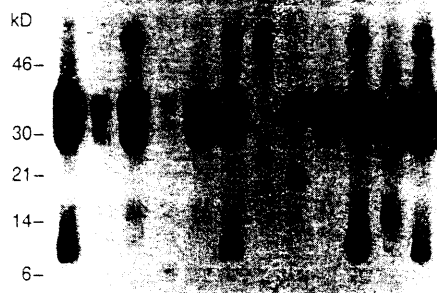
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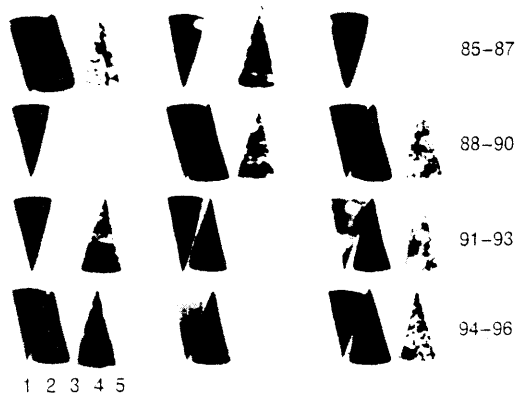
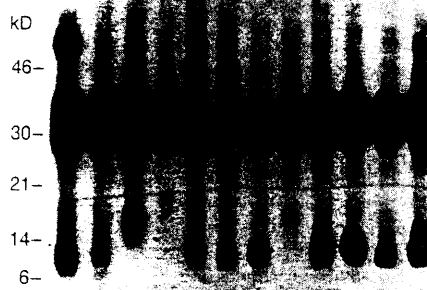
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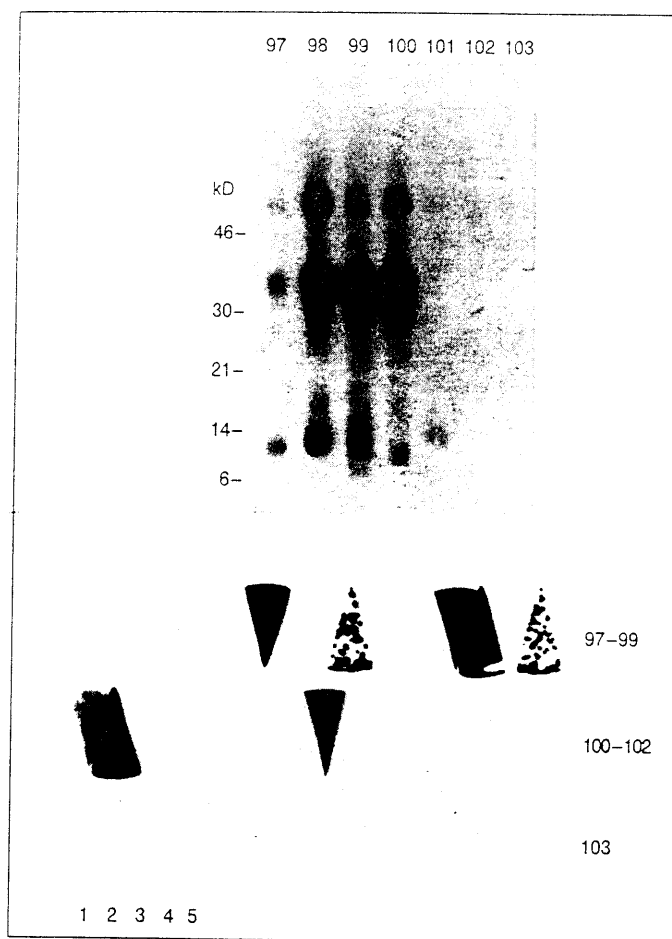


Fig. 2. Grass pollen-allergic patients were tested for IgE reactivity on IgE immunoblots containing natural *P. pratense* allergens (upper panel) and with nitrocellulose sectors (lower panel) containing recombinant *Phl p* I (1), *Phl p* V (2), profilin from timothy grass (3), clone 53 from timothy grass (4) and lambda gt11 without inserted cDNA (negative control) (5). Patients are numbered on the right side and were tested with the recombinant allergens as above from the left (1) to the right (5).

prominent protein bands on Coomassie-stained gels at molecular weights that correspond to the major allergens (fig. 2). The major allergenic components however can poorly be distinguished on IgE immunoblots because different allergens are found at nearly identical molecular weights. For example group I and group V [9, 20] allergens as well as group II/III allergens and profilin [6, 20, 22] possess very similar molecular weights. Clearly, such crude natural protein extracts cannot be used for a specific diagnosis of allergy against a particular allergen. In addition, natural grass pollen protein extracts used for RAST and skin prick tests contain a broad variety of allergens (group I [20], group V [9], group II/group III [20, 22], group IV [21] and group IX [12] allergens) that represent different

IgE-binding identities and can even be further distinguished into different isoforms [14]. Despite this puzzle, a variety of different grass pollen allergens, we tried to establish a diagnostic model of grass pollen allergy based on a reasonable number of well-defined recombinant allergens. For this purpose, we constructed an expression cDNA library from timothy grass pollen (*P. pratense*) and isolated by screening with serum IgE from grass pollen-allergic patients allergen encoding cDNAs. Different testing of these clones with sera from 30 different grass pollen-allergic patients allowed us to select four clones that represented different allergenic components. Different sequence analyses and comparison of the deduced amino acid sequences of these clones with already known sequences, as well as testing of these clones with different antibodies led to their identification as *Phl p* I, *Phl p* V and profilin from timothy grass [15]. The fourth clone so designated clone 53 represents an allergen with IgE-binding properties different from the above three and bound IgE from 65% of the tested grass pollen-allergic patients. No correlation was found between binding of patients' IgE to this recombinant protein and binding to group IV allergens on IgE immunoblots. This set of four recombinant timothy grass pollen allergens and control proteins from *E. coli* Y1090 infected with a non-recombinant phage lambda gt11 was then used to test well-documented grass pollen-allergic individuals. In addition, all these patients were also tested on IgE immunoblots with natural timothy grass pollen allergens. In all cases tested, we found that the patients displayed comparable IgE reactivity to natural as well as recombinant allergens. Moreover, we found that the three recombinant allergens (*Phl p* I, *Phl p* V and timothy grass profilin) could be sufficient for the diagnosis of grass pollen allergy. Using the three recombinant allergens, we were able to detect 97 out of 98 tested grass pollen-allergic patients. Since one patient with weak IgE reactivity to group IV allergens could not be diagnosed with the recombinant test system, the addition of a recombinant group IV allergen has to be considered. Clone 53 seems not to represent an essential component of such a test since we could not identify a single patient that displayed exclusive IgE reactivity to this protein whereas the panallergen profilin [6] should be included because several patients can be found who react exclusively with this allergen. Since profilins of different plant species share IgE epitopes, they are even useful for the diagnosis of tree [2], weed [6] and food [24] allergy and may allow to identify patients with multivalent allergy to distantly related plants (pollen and plant-derived food). Low representation of profilin in total grass pollen extracts that are used

for RAST tests may be the reason why patient 101 could not be identified as grass pollen-allergic individual by the RAST technique although IgE binding to profilin from timothy grass was observed.

Patients 39, 40, 45 and 76 seemed to react more strongly with immunoblots containing crude timothy grass pollen extracts than with the recombinant proteins, which might be due to the lack of the one or other component in the recombinant test system. Further improvement of the recombinant test system may be expected by including additional recombinant timothy grass pollen allergens or by purification of the recombinant proteins. The results obtained with the recombinant allergens however indicate that recombinant techniques may improve in vitro allergy tests by precise characterization of the allergens evoking an IgE response in man. The possibility to define a limited panel of recombinant allergens for each patient may also

allow the setup of new therapeutic concepts based on the use of defined allergens for immunotherapy. The data we obtained so far from the comparison of recombinant and natural tree pollen allergens concerning their IgE-binding capacity [2] and their ability to release histamine from basophils of allergic patients via cross-linking of high affinity Fc ϵ receptors [6] may encourage the use of these molecules to improve future diagnosis and probably therapy of allergic diseases.

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