Component-Resolved Diagnosis (CRD) of Type I Allergy with Recombinant Grass and Tree Pollen Allergens by Skin Testing

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The diagnosis of Type I allergy is based on the measurement of allergen-specific IgE antibodies and on provocation with allergens, most frequently conducted by skin testing. Both forms of diagnosis are currently performed with allergen extracts that are difficult to standardize regarding their allergen contents, and which contain additional undefined nonallergenic components. We report the expression in *Escherichia coli* and purification of some of the most relevant timothy grass- and birch pollen allergens. Recombinant timothy grass- (rPhl p 1, rPhl p 2, rPhl p 5) and birch pollen (rBet v 1, rBet v 2) allergens were purified and used for the measurement of allergen-specific IgE and IgG subclass responses as well as for skin prick testing in 55 pollen allergic patients and 10 nonatopic individuals. Results obtained showed that the recombinant allergens allowed *in vivo* allergy diagnosis in 52 of 54 of the grass pollen and in 35 of 36 of the birch pollen allergic patients. Positive skin reactions were observed almost exclusively in patients containing detectable allergen-specific IgE antibodies but not in the nonatopic group; however, sensitivity to a given allergen as measured by skin reactivity was weakly correlated with the levels of allergen-specific IgE. Our results demonstrate that recombinant allergens can be used for component-resolved skin test diagnosis (CRD) of the patients' allergen sensitization profile, whereas allergen extracts at best allow to identify allergen-containing sources. CRD may thus represent the basis for novel forms of patient-tailored immunotherapy. *Key words: IgE/recombinant allergens/skin testing/Type I allergy. J Invest Dermatol 113:830–837, 1999*

Patients suffering from Type I allergy produce IgE antibodies against *per se* innocous antigens/allergens (e.g., pollen, mite, animal hair/dander, mould, food proteins) (Casolaro *et al*, 1996; Kay, 1997). The immediate type symptoms of Type I allergy (allergic rhinitis, conjunctivitis, asthma, and anaphylaxis) result from the allergen-induced crosslink of effector cell-bound IgE antibodies and subsequent release of biologic mediators (Segal *et al*, 1979).

Diagnosis of Type I allergy requires a case history, detection of allergen-specific IgE antibodies in serum and other body fluids, as well as the demonstration of allergen-induced effector cell activation (Lockey and Buckantz 1998). The latter can be achieved by *in vitro* assays that are based on the measurement of effector cell products (histamine, leukotrienes) being released after antigen contact or by *in vivo* allergenprovocation (skin testing, nasal, oral, bronchial challenge) and recording of clinical symptoms. Serologic as well as effector-cell based allergy diagnosis is currently performed with allergen extracts that are prepared from the respective allergen sources (e.g., pollen, mite bodies/faeces, animal hair/dander, moulds, fruits, and vegetables). The allergen extracts contain, in addition to allergens, other nonallergenic, undefined components (Bousquet and Valenta, 1994). Current diagnosis of Type I allergy therefore only permits the identification of a given allergen source, but not of the molecular entities involved in the pathogenesis of the disease.

In order to reveal the precise molecular nature of allergens and to provide more specific tools for diagnosis and possibly therapy of Type I allergy, several groups have started to isolate and express cDNA coding for allergens (reviewed in Valenta and Kraft, 1995; Valenta *et al*, 1998). The concept of using single recombinant allergens to determine the patients sensitization profile was termed "component-resolved diagnosis-CRD". It should represent the basis for patient-tailored forms of immunotherapy, i.e., "component-resolved immunotherapy-CRIT" (Valenta *et al*, 1999).

We have previously isolated and expressed in *Escherichia coli* cDNA coding for three major timothy grass pollen allergens, Phl p 1 (Laffer *et al*, 1994), Phl p 2 (Dolecek *et al*, 1993), and Phl p 5 (Vrtala *et al*, 1993), as well as for two birch pollen allergens, Bet v 1 (Breiteneder *et al*, 1989) and Bet v 2 (Valenta *et al*, 1991a). The recombinant pollen allergens were shown to possess IgE-binding capacity comparable with their natural counterparts (Valenta *et al*, 1991b, 1992a), to induce specific histamine release from patients' basophils (Valenta *et al*, 1993; Vrtala *et al*, 1996a) and to stimulate allergen-specific T cells (Ebner *et al*, 1993; Schenk *et al*, 1995). The capacity of recombinant birch pollen allergens, rBet v 1 and rBet v 2, to induce immediate type skin reactions was also demonstrated (Menz *et al*, 1996; Pauli *et al*, 1996).

In this study we investigated whether skin test diagnosis of tree and grass pollen allergy can be performed with recombinant allergens. We purified a panel of three recombinant timothy grass

Manuscript received May 18, 1999; revised August 13, 1999; accepted for publication August 16, 1999.

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pollen allergens (rPhl p 1, rPhl p 2, rPhl p 5) and two recombinant birch pollen allergens (rBet v 1, rBet v 2) and investigated allergen-specific antibody (IgE-, IgG_{1-4}-subclass) responses as well as skin reactivity (skin prick test) in a population of 55 pollen allergic patients and 10 nonatopic individuals. Antibody responses and skin reactivity to purified recombinant allergens were compared with those to natural allergen extracts. Using recombinant allergens we also investigated whether skin sensitivity to a given molecule is associated with allergen-specific IgE antibody levels in serum. Based on the result that the five recombinant pollen allergens allowed serologic and skin test diagnosis of pollen allergy in almost all of the tested patients, we discuss the use of recombinant allergens for a novel form of *in vivo* allergy diagnosis (i.e., component-resolved skin test diagnosis), which permits to determine the patient's individual reactivity profile.

MATERIALS AND METHODS

Natural allergen extracts and purification of recombinant pollen allergens Timothy grass (*Phleum pratense*) and birch (*Betula verrucosa*) pollen were purchased (Allergon, Välinge, Sweden) and aqueous extracts were prepared by homogenization of 2 g pollen in 50 ml of distilled water containing 5 mM PMSF using a mechanical grinding device-ultraturrax (Ika, Stauffen, Germany) and further shaking at 4°C for 2 h. Pollen extracts were centrifuged at 10 000 × *g* in a SS34 rotor (Sorvall, Newtown, CT) and the clear supernatant was aliquoted in 1 ml portions, frozen, lyophilized, and stored at –20°C until use. Protein contents and quality were checked by SDS-PAGE analysis (Fling and Gregerson, 1986) and Coomassie Blue staining (Bradford, 1976).

Standardized SQ skin prick solutions (timothy grass, birch pollen, and histamine hydrochloride) (ALK, Horsholm, Denmark) were obtained from Epipharm (Linz, Austria).

Recombinant allergens The cDNA coding for the major timothy grass pollen allergens (Phl p 1, Phl p 2, and Phl p 5) and birch pollen allergens (Bet v 1 and Bet v 2) were inserted into plasmid pMW 172 (Way *et al*, 1990), a derivative of pRK 172 (McLeod *et al*, 1987). Plasmids were transformed into *E. coli* BL21 (DE3), derived from *E. coli* strain B (27) (Novagen, Maddison, WI). Recombinant pollen allergens, rPhl p 1, rPhl p 2, rPhl p 5, were expressed as nonfusion proteins in *E. coli* BL21 (DE3) and purified as described (Vrtala *et al*, 1996a). rBet v 1 and rBet v 2 were purified as described (Valenta *et al*, 1991a; Hoffmann-Sommergruber *et al*, 1997). Purified recombinant allergens were tested for their capacity to bind human IgE and to induce specific histamine release from allergic patients' basophils prior to *in vivo* application.

Demographic and clinical characterization of patients and control individuals The study group consisted of 55 pollen allergic patients (mean age 28.5 y; 19 women, 36 men) and 10 nonatopic individuals (mean age 29.4 y; eight women, two men) (**Table I**). One of the allergic individuals (#61: **Table I**) contained timothy grass pollen-specific IgE antibodies but reported no clinical symptoms. Individuals were characterized by case history (symptoms: rhinitis, conjunctivitis, asthma, gastrointestinal symptoms, urticaria, dermatitis; reactivity to known allergen sources), skin prick testing with birch and timothy grass pollen extract (ALK), determination of total and specific IgE antibodies using Pharmacia CAP system (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden). Fifteen patients had received grass pollen-specific immunotherapy and 25 patients had been treated with birch pollen-specific immunotherapy.

IgE-immunoblotting IgE reactivity to nitrocellulose-blotted natural pollen extracts was determined by immunoblotting (Valenta *et al*, 1992a,b). Proteins were separated by 12.5% SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany) by electroblotting (Towbin *et al*, 1979). Nitrocelluloses were blocked in buffer A (50 mM Naphosphate pH 7.5, 0.5% wt/vol BSA, 0.5% vol/vol Tween 20, 0.05% NaN_3) twice for 5 min and for 30 min and then incubated with 1:10 diluted sera at 4°C overnight. Nitrocelluloseblots were then washed three times in buffer A and exposed to 1:10 in buffer A diluted ^{125}I-labeled antihuman IgE antibodies (Pharmacia) at 4°C overnight, washed as above and visualized by autoradiography.

Study design The skin prick test study was performed out of the pollen season (January–February) in an Austrian population of 55 pollen allergic and 10 nonatopic individuals. Patients having severe asthma as defined by International Consensus, intercurrent infectious or neoplastic disease, AIDS, pregnant or lactating females and patients on beta-blocker were excluded from the study. Symptomatic patients taking antihistamines as their treatment were tested only if they could withdraw the drug for 5 d if it was cetirizine or loratidine and for 8 wk if it was astemizole. After written consent was obtained, the patients' case history was recorded and a blood sample was obtained by venipuncture of the antecubital vein. Skin prick tests were performed according to Dreborg (1989) on the patients' forearms with commercially available extracts (birch-, timothy grass pollen extract, histamine, sodium chloride) (ALK, Horsholm, Denmark) and with two concentrations (1, 10 μg per ml) of each of the purified recombinant allergens (rPhl p 1, rPhl p 2, rPhl p 5, rBet v 1, rBet v 2). Sterile prick lancettes (ALK) were used for prick testing. Reactions (wheal, flare) were recorded 20 min after prick testing by photography and by transferring the wheal area with a scotch tape to paper. Patients were asked to monitor the sites of prick testing for 48 h and to report late phase reactions. The study protocol was approved by the Austrian Ministry of Health and the local ethics committee.

Determination of total serum IgE levels and allergen-specific IgE and IgG subclass levels Blood samples were obtained on the study day before skin prick testing by venipuncture and sera were stored at –20°C until use. Total IgE levels (kU per liter) were determined by CAP RAST (Pharmacia) measurements. Allergen-specific IgE levels (kUA per liter) were measured by CAP RAST (timothy grass pollen extract, birch pollen extract, Pharmacia). Experimental Immuno CAPs containing recombinant timothy grass pollen (rPhl p 1, rPhl p 2, rPhl p 5) and recombinant birch pollen allergens (rBet v 1, rBet v 2) were prepared by Pharmacia & Upjohn, Diagnostics AB. Allergen-specific IgG_{1-4} subclass levels were measured by ELISA as described (Menz *et al*, 1996). ELISA plates (96 wells: Nunc, Roskilde, Denmark) were coated with timothy grass-, birch pollen extract (100 μg per ml) or purified recombinant allergens (5 μg per ml) dissolved in 0.1 M sodium carbonate buffer, pH 9.6 overnight at 4°C. Sera were diluted 1:50 for IgG subclasses and bound antibodies were detected with mouse monoclonal antihuman IgG_{1-4} antibodies (Pharmingen, San Diego, CA) and a peroxidase-coupled sheep antimouse Ig antiserum (Amersham, Buckinghamshire, U.K.). All determinations were carried out in duplicates. Results are displayed as mean optical density (OD) values.

Skin prick testing Skin prick tests were performed on the forearms of the individuals. Twenty microliter aliquots of recombinant allergens that were diluted in 0.9% sodium chloride (two concentrations: 10 μg per ml; 1 μg per ml) shortly before testing, were applied 3 cm apart and pricked with sterile prick lancets (ALK) according to Dreborg (1989). For control purposes, each individual was tested with commercially available allergen extracts (timothy grass pollen, birch pollen, histamine) (ALK) and with the 0.9% sodium chloride solution used for dilution of the recombinant allergens. Results were recorded 20 min after testing by photography. The wheal areas were transferred with a scotch tape to paper. Mean wheal diameters (Dm) were calculated from the maximal longitudinal (D) and the maximal transversal diameter (d) according to the formula [D + d]/2 = Dm. Patients were asked to monitor the sites of skin prick testing during the following 48 h and to report eventual late phase reactions.

The following parameters were compared with the two-tailed paired t test using Microsoft Excel: Dm (mm) of the wheals obtained with the 10 μg per ml concentrations of recombinant allergens, the allergen extracts and histamine; specific IgE levels (kUA per liter, mean OD values) for the recombinant allergens and allergen extracts. Correlations can be estimated as follows: >0.8, good; 0.6–0.8, medium; 0.25–0.6, weak; <0.25, no correlation.

RESULTS

SDS-PAGE analysis of natural pollen extracts *versus* purified recombinant pollen allergens SDS-PAGE analysis of natural birch and timothy grass pollen extracts indicates that they contain a variety of proteins that occur in varying amounts. For example, aqueous birch pollen extract contained large amounts of Bet v 1 (17 kDa) (Breiteneder *et al*, 1989), whereas Bet v 2 (14 kDa) (Valenta *et al*, 1991a) was not detected by protein staining and thus is not well represented in the extract (**Fig 1**). Timothy grass pollen extract consisted of a series of protein bands ranging from approximately 10–200 kDa. The comparison of the stained proteins (**Fig 1**) with the IgE immunoblots depicting the IgE-reactive components in the natural extracts (**Figs 2**, **3**) reveals that only a few extract components represent allergens.

Table I Demographic, clinical, and serologic characterization of tested individuals[a]

Ind.	Sex	Age	Sensitized to	Symptoms	CAP kUA per liter				SPT				CAP kUA per liter		aSPT				
					Tim	rP1	rP2	rP5	Tim	MD rP1	rP2	rP5	Birch	rB1	rB2	Birch	rB1	MD rB2	Hist
#1	f	26	g t pf	r c o	27.4	13.1	<0.3	30.4	8	5	0	8.5	11.2	11	<0.3	3	11.5	0	3
#2	f	30	g	r c	37.4	16.7	8.2	12.9	7	10.5	8	6	<0.3	<0.3	<0.3	0	0	0	6
#3	f	28	g t w	r c	17.5	6.8	<0.3	13.4	6	7.5	0	9	0.8	<0.3	0.4	4	2.5	3	6.5
#4	m	28	g t w	r c	7.3	2.4	1.1	2.6	8.5	5.5	0	5.5	<0.3	<0.3	<0.3	2	2	1	5
#5	f	22	g f	r a	8.3	5.7	2.5	<0.3	7	8	5.5	0	<0.3	<0.3	<0.3	0	0	0	6
#6	m	30	g t w an	r c a	87.2	26.8	14.1	42.5	15	6.5	14.5	11	41.6	33.4	12.2	7.5	5.5	4.5	7.5
#7	m	28	g t w mi	r c a	>100	39.0	37.7	19.6	5.5	6.5	6.5	7	11.1	<0.3	11.6	3.5	5	10	7
#8	m	29	g	r c	>100	>100	46.6	>100	8.5	7.5	7	5.5	7.1	<0.3	<0.3	0	0	0	6
#9	m	27	g t w mi an	r c	>100	49.7	12.2	81.1	17.5	5	8.5	11	>100	>100	11.2	8	11	7	8
#10	m	28	g w	r c	52.8	25.0	11.5	47.8	5	3.5	3	5	<0.3	<0.3	<0.3	0	0	0	4.5
#11	m	23	g mi an	r	2.0	1.9	<0.3	<0.3	3	8	0	0	<0.3	<0.3	<0.3	0	0	0	6.5
#12	m	28	g	r c a	19.4	9.4	<0.3	22.5	6	3	0	6.5	<0.3	<0.3	<0.3	0	0	0	5.5
#13	m	29	g t w mi	r c	1.9	0.6	<0.3	1.4	4.5	8	8.5	5	4.5	4.9	<0.3	9.5	11	0	6.5
#14	f	29	g t an	r c	24.1	14.2	1.0	18.5	9.5	5	0	8	60.4	55.0	<0.3	10.5	10.5	0	7
#15	m	32	g mi an	r c	67.7	27.1	20.0	18.4	8.5	5	4	9	<0.3	<0.3	<0.3	0	0	0	5.5
#16	m	23	g t an pf	r c o	>100	57.3	88.3	>100	20	7.5	11	9.5	61.0	44.4	<0.3	11	6	0	10
#17	m	30	g t w	r c	9.6	2.5	2.2	5.2	6	9	6	7	0.4	<0.3	<0.3	0	0	4.5	5.5
#18	m	41	g t w	r c	12.6	5.1	1.7	4.5	4.5	4	12	6	<0.3	<0.3	<0.3	5	0	1	5
#19	m	30	g t w	r c	>100	84.3	>100	>100	6.5	6.5	23.5	3.5	15.2	5.9	11.5	3.5	4	7.5	5.4
#20	f	24	g	t r c	13.2	3.3	1.9	7.5	13.5	11	9	13	<0.3	<0.3	<0.3	4	0	0	5
#21	f	26	g t w mi an	r c a	29.6	12.0	<0.3	30.3	11	5.5	0	9	7.4	7.8	<0.3	10	5.5	0	6
#22	f	24	g	t r c	19.0	18.9	1.8	4.0	6.5	6.5	0	10.5	1.1	0.5	<0.3	6	7	0	6.5
#23	m	33	g	r c	2.2	<0.3	<0.3	2.0	6.5	4	0	9.5	<0.3	<0.3	<0.3	0	0	0	5.5
#24	m	27	g an	r c	10.0	11.4	<0.3	<0.3	4.5	11.5	0	0	<0.3	<0.3	<0.3	0	0	0	5.5
#25	m	26	g t an pf	r c a d	>100	18.5	29.6	84.2	14	8.5	10.5	10	50.2	13.7	1.4	5	6	0	8
#26	f	24	g an ven	r c	93.8	99.7	13.7	<0.3	7	8	10.5	0	<0.3	<0.3	<0.3	0	0	0	6.5
#27	m	27	g t w	r c	48.0	14.9	6.6	28.9	15	10.5	11.5	12	13.8	14.9	<0.3	5	9	2.5	6
#28	m	24	g	r c	29.4	13.4	4.1	22.4	12	2.5	8	6	<0.3	<0.3	<0.3	0	0	0	5
#29	m	27	g	t r c	>100	65.9	43.4	>100	13	6	6	17.5	61.6	71.5	<0.3	7	8	0	7
#30	m	26	g mi an	r c a u	7.4	5.8	<0.3	<0.3	4.5	4.5	0	0	1.3	<0.3	1	2	0	0	5
#31	m	24	g t w	r c	95.2	28.4	16.9	41.6	8.5	8.5	12	8	2.6	<0.3	2.7	2.5	0	4	6
#32	m	25	g t w mi	r c d	11.1	5.8	<0.3	4.8	5	5.5	0	6	4.4	5.2	<0.3	6	4	1	6
#33	m	24	g t mi an pf	r c a o	3.2	3.4	<0.3	<0.3	5.5	6	0	0	8.5	8.9	<0.3	8.5	8.5	0	4.5
#34	m	24	g t mi an pf	r c	64.6	43.3	46.4	49.0	9.5	6.5	10.5	8	>100	>100	<0.3	7.5	8.5	0	6.5
#35	m	25	g w	r c	47.7	18.9	5.2	56.0	8	5	5	6.5	<0.3	<0.3	<0.3	0	0	0	6
#36	f	29	g t w mi an pf	r c	89.9	65.7	13.7	74.4	6.5	7	8	5	11.7	10.4	2.8	5.5	5.5	5.5	5
#37	f	47	g t an	r c a d	9.7	5.6	2.6	<0.3	4.5	5.5	0	0	5.2	1.9	<0.3	6	3.5	0	6
#38	m	34	g t w	r c	45.3	11.6	4.5	23.5	9	6	10.5	6.5	11.7	<0.3	7.2	4.5	0	7	5
#39	m	33	g	r c	11.0	1.8	4.5	5.6	5	5	5.5	5	0.5	<0.3	<0.3	0	0	0	5
#40	m	30	g	r c	29.3	8.1	<0.3	17.8	9	4	0	10	3.0	<0.3	1.8	3	0	5	5
#41	m	27	g	t r c	33.1	22.1	7.7	<0.3	5.5	5.5	14	0	3.0	3.2	<0.3	3.5	4	0	4.5
#42	f	48	g	t r c	56.1	36.1	19.7	4.3	6	8	13	8	77.0	66.9	<0.3	10.5	15	0	7.5
#43	f	22	g t mi an pf	r c a	38.0	12.1	9.8	20.1	11.5	5	10.5	8	51.7	70.5	<0.3	4	8	0	7.5
#44	f	25	g	t r c u	60.2	18.1	23.1	36.2	10.5	9.5	11.5	10	24.4	22.9	<0.3	4.5	7.5	0	3
#45	m	29	g t w	r c	36.8	23.0	1.9	20.8	8	11	0	7.5	3.7	<0.3	4.7	4.5	0	3.5	5.5
#46	f	26	g mi	r c	<0.3	<0.3	<0.3	<0.3	1.5	1.5	0	0	<0.3	<0.3	<0.3	0	0	0	5
#47	m	25	g t f pf	r c a	2.6	2.1	<0.3	<0.3	3.5	4.5	0	0	60.5	47.7	<0.3	4.5	4	0	8
#48	m	56	g	r c	27.5	6.4	6.0	11.2	7	5	9	6.5	<0.3	<0.3	<0.3	0	0	0	6.5
#49	f	33	g	t r c	>100	80.5	98.7	>100	9	5	9.5	4	43.3	48.0	<0.3	4.4	5.5	0	5
#50	f	23	g t w	r c a	>100	51.0	<0.3	52.9	9	6	0	6	33.6	<0.3	10.5	4.5	0	4.5	4
#51	m	25	g	t r c	>100	29.2	19.7	>100	0	2.5	2.5	2	3.2	1.6	<0.3	2.5	3	0	3
#52	f	24	g t w	r c a	1.5	<0.3	<0.3	<0.3	0	0	0	0	>100	84.9	<0.3	4	1.7	0	3.5
#53	m	30	t an	r c	<0.3	<0.3	<0.3	<0.3	0	0	0	0	1.8	1.6	<0.3	4	5.5	0	5.5
#54	f	33	0	0	<0.3	<0.3	<0.3	<0.3	0	0	0	0	<0.3	<0.3	<0.3	0	0	0	5
#55	f	32	0	0	<0.3	<0.3	<0.3	<0.3	0	0	0	0	<0.3	<0.3	<0.3	0	0	0	5
#56	f	27	0	0	<0.3	<0.3	<0.3	<0.3	0	0	0	0	<0.3	<0.3	<0.3	0	0	0	7
#57	f	26	0	0	<0.3	<0.3	<0.3	<0.3	0	0	0	0	<0.3	<0.3	<0.3	0	0	0	2.5
#58	f	23	g t mi	r	3.3	<0.3	<0.3	<0.3	0	0	0	0	2.1	<0.3	<0.3	0	0	0	6
#59	f	30	0	0	<0.3	<0.3	<0.3	<0.3	0	0	0	0	<0.3	<0.3	<0.3	0	0	0	3.5
#60	f	25	0	0	<0.3	<0.3	<0.3	<0.3	6	1	0	0	<0.3	<0.3	<0.3	0	0	0	6
#61	m	30	0	0	12.3	<0.3	<0.3	<0.3	0	0	0	0	2.8	<0.3	<0.3	0	0	0	4
#62	m	26	0	0	<0.3	<0.3	<0.3	<0.3	0	0	0	0	<0.3	<0.3	<0.3	0	0	0	6
#63	m	34	0	0	<0.3	<0.3	<0.3	<0.3	0	0	0	0	<0.3	<0.3	<0.3	0	0	0	4
#64	f	28	0	0	<0.3	<0.3	<0.3	<0.3	0	0	0	0	<0.3	<0.3	<0.3	0	0	0	4
#65	f	33	0	0	<0.3	<0.3	<0.3	<0.3	0	0	0	0	<0.3	<0.3	<0.3	0	0	0	5

[a]Serum IgE levels specific for timothy grass and birch pollen extract as well as recombinant timothy grass pollen (rP1: rPhl p 1; rP2: rPhl p 2; rP5: rPhl p 5) and birch pollen allergens (rB1: rBet v 1; rB2: rBet v 2) were determined by CAP RAST measurements. Wheal reactions to natural pollen extracts, histamine, and recombinant allergens (10 μg per ml) induced by skin prick testing (SPT) are shown as mean wheal diameters (MD) in mm. m, male; f, female; g, grass pollen; t, tree pollen; w, weed pollen; mi, mites; an, animal hair/dander; f, fungi, moulds; pf, plant-derived food; ven, bee venom; r, rhinitis; c, conjunctivitis; a, asthma; d, dermatitis; u, urticaria; o, oral symptoms; Tim, timothy grass pollen extract; Hist, histamine.

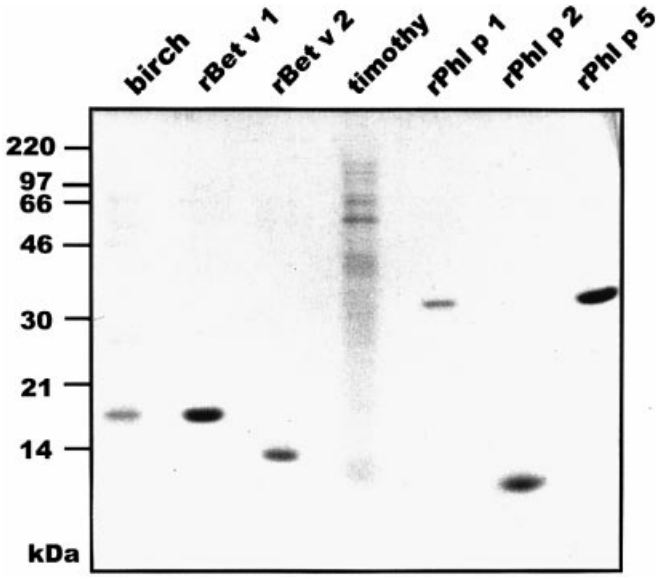


Figure 1. Coomassie blue-stained gel containing pollen extracts and purified recombinant pollen allergens. Birch pollen (birch) and timothy grass pollen extracts as well as recombinant birch (rBet v 1, rBet v 2) and timothy grass pollen allergens (rPhl p 1, rPhl p 2, rPhl p 5) were separated by SDS-PAGE and stained with Coomassie blue. Molecular weights (kDa) are indicated on the left margin.

We have expressed and purified some of the most relevant recombinant birch and timothy grass pollen allergens for skin testing: rBet v 1 represents the 17 kDa major birch pollen allergen that reacts with IgE antibodies of more than 90% of birch pollen allergic patients (**Fig 1**) (Breiteneder *et al*, 1989; Jarolim *et al*, 1989). rBet v 2, birch profilin is a 14 kDa protein that is recognized by fewer patients (10%) but is a highly cross-reactive allergen (**Fig 1**) (Valenta *et al*, 1991a, 1992b). rPhl p 1 and rPhl p 5 are the most frequently recognized timothy grass pollen allergens (**Fig 1**) (Laffer *et al*, 1994; Vrtala *et al*, 1993). Although of similar molecular weight, they represent immunologically independent entities and lack cross-reactivity. rPhl p 2 constitutes a low molecular weight (10 kDa) major timothy grass pollen allergen (**Fig 1**) (Dolecek *et al*, 1993). The purity of the recombinant allergens was determined by SDS-PAGE, isoelectric focusing, and silver staining, as well as by mass spectroscopy.

Western blot analysis with natural pollen extracts indicates different IgE reactivity profiles among pollen allergic patients Most of the timothy grass pollen allergic patients reacted with proteins of 30–35 kDa, 10–14 kDa, and 50–60 kDa in natural nitrocellulose-blotted timothy grass pollen extract (**Fig 2*A*, *B***). Their individual IgE reactivity profiles varied considerably, however; certain individuals (e.g., #4, 5, 11, 13, 24, 30, 32, 33, 37, 45, 47) reacted exclusively with the 30 kDa moieties containing group 1 and 5 allergens. Others exhibited IgE reactivity to components of all three molecular weight ranges (10–14 kDa, 30–35 kDa, 50–60 kDa) (e.g., sera #1, 3, 6–10, 15–21, 25, 27–29, 31, 34, 36, 38, 40, 42–44, 49–51). We also found patients who exclusively reacted with the 50–60 kDa proteins (#52, 58), presumably representing group 4 grass pollen allergens (Fischer *et al*, 1996). By IgE immunoblotting we were able to diagnose all 54 grass pollen allergic patients.

Most of the birch pollen allergic individuals displayed IgE reactivity to the major birch pollen allergen Bet v 1 at 17 kDa (**Fig 3*A*, *B***). The reactivity slightly below 17 kDa could not be unambiguously attributed to Bet v 1 isoforms (Swoboda *et al*, 1995) or Bet v 2. Certain patients (#58, 61) reacted exclusively with high molecular weight (40–80 kDa) components in birch pollen extract. Only 25 of 37 birch pollen allergic patients were detected by their IgE reactivity to nitrocellulose-blotted birch pollen extract.

In conclusion, IgE immunoblotting with natural pollen extracts allowed the detection of IgE antibodies specific for certain bands in blotted extracts and indicated that patients exhibit individual IgE reactivity profiles; however, IgE immunoblotting using natural allergen extracts did not provide quantitative information regarding allergen-specific IgE levels and did not allow the unambiguous identification of the recognized allergens.

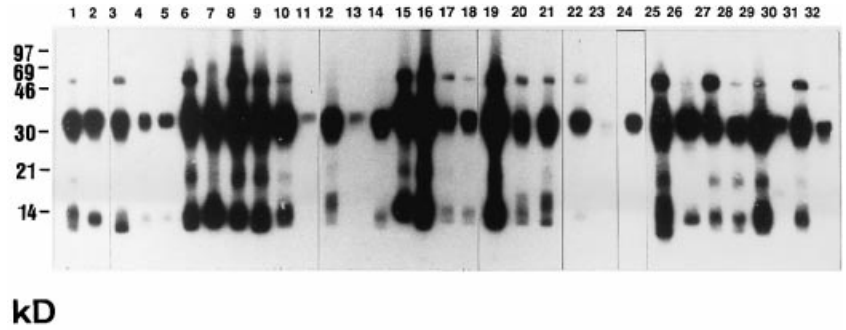
Component-resolved *in vitro* allergy diagnosis with recombinant pollen allergens: defining the patients' IgE reactivity profile and identification of allergens with high and low IgE binding capacity Using timothy grass pollen extract for quantitative serologic testing, all but one (#46: **Table I**) grass pollen allergic patients were diagnosed. The combination of three recombinant timothy grass pollen allergens (rPhl p 1, rPhl p 2, rPhl p 5) allowed diagnosis in all but three patients (sera #46, 52, 61: **Table I**). Ninety-four per cent of the sera were positive with rPhl p 1, 67% reacted with rPhl p 2, and 76% contained IgE anti-rPhl p 5. Although most grass pollen allergic patients displayed IgE reactivity to rPhl p 1, the quantitative individual IgE reactivity profiles showed great variability (**Table I**). Certain patients reacted exclusively with one recombinant allergen (e.g., #11, 24, 30, 33, 47: rPhl p 1; 23: rPhl p 5), whereas others contained IgE antibodies of varying levels to two or all three recombinant allergens.

Although only 37 patients had reported clinical symptoms in the birch pollen season, we found IgE antibodies to birch pollen extract in 39 of the 55 pollen allergic patients (**Table I**). Twenty-six sera contained IgE anti-rBet v 1 and 13 exhibited IgE reactivity to rBet v 2. A combination of rBet v 1 and rBet v 2 allowed detection of specific IgE antibodies in 34 individuals. Although most of the birch pollen allergic patients displayed IgE reactivity to rBet v 1 and most of the birch pollen-specific IgE was directed against rBet v 1, certain patients exhibited varying IgE reactivity profiles. Some sera contained IgE against rBet v 1 and rBet v 2 (e.g., #6, 9, 19, 25, 36) and colleagues reacted exclusively to rBet v 2 (e.g., #7, 30, 31, 38, 40, 45, 50) (**Table I**).

The quantitative analysis of the specific IgE levels indicated that certain recombinant allergens had a high IgE binding capacity that, in combination, frequently seemed to exceed that of natural extracts (**Table I**). Comparing the average IgE levels bound by the individual recombinant allergens, we found that certain of them had higher IgE binding capacity (**Table II**; mean IgE levels: rPhl p 1: 25 kUA per liter; rPhl p 5: 37.0 kUA per liter; rBet v 1: 32.2 kUA per liter) than others (**Table II**; mean IgE levels: rPhl p 2: 20.2 kUA per liler; rBet v 2: 6.1 kUA per liter).

Lack of association between allergen-specific IgE and IgG subclass responses Comparing IgE and $IgG_{1–4}$ subclass responses to purified recombinant pollen allergens, we noticed that IgE and IgG subclass responses showed great variability in the individual patients (**Tables I** and **II**, data not shown). Certain patients mounted high levels of allergen-specific IgE without IgG subclass reactivity (e.g., patient 29 contained high levels of rPhl p 1 and rPhl p 2-specific IgE but failed to mount significant $IgG_{1–4}$ responses to either of those) (**Tables I** and **II**, data not shown). Other individuals contained allergen-specific IgG subclass antibodies without allergen-specific IgE (e.g., patient 5 displayed IgG_2 and IgG_3 subclass reactivity to rPhl p 5 without accompanying IgE reactivity) and allergen-specific IgG subclass responses were found in the nonatopic group (e.g., individuals 54, 55, and 60 mounted IgG subclass responses to rBet v 2) (**Tables I** and **II**, data not shown).

Pollen allergic patients who had received specific immunotherapy contained higher levels of allergen-specific IgE and IgG4 antibodies Comparing the mean IgE and $IgG_{1–4}$ antibody levels specific for recombinant birch pollen allergens (rBet v 1, rBet v 2) and rPhl p 5, we found that patients who had received allergen-specific immunotherapy mounted stronger IgE responses than the untreated patients (**Table II**). Likewise we found for all five recombinant allergens that patients who had received immunotherapy contained higher mean allergen-specific IgG_4 anti-



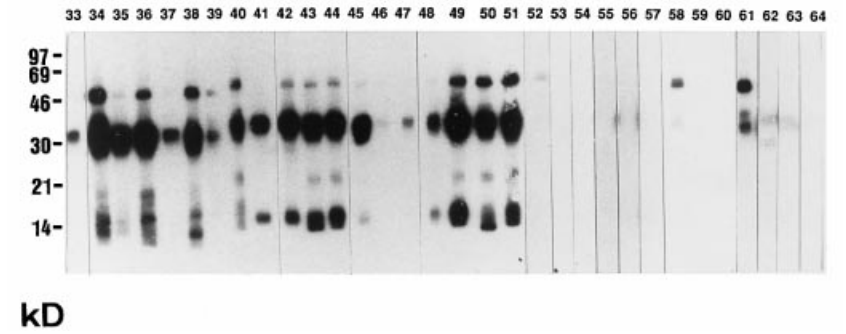
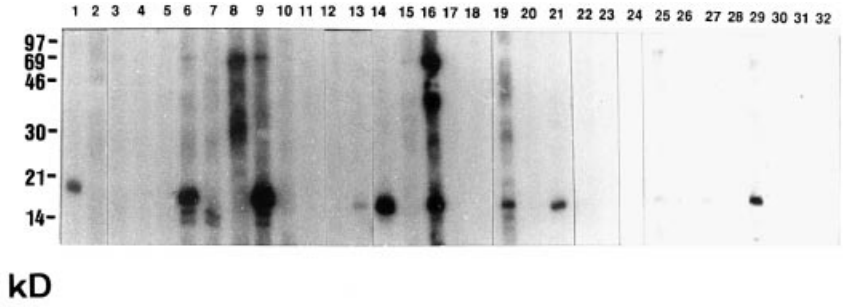


Figure 2. Serum IgE-reactivity to nitrocellulose blotted natural timothy grass pollen extract. Sera from grass pollen allergic patients (*lanes 1–52*, *58*, *61*), from an allergic patient without grass pollen allergy (*lane 53*), and from nonallergic individuals (*lanes 54–57*, *59–60*, *62–64*) were probed with nitrocellulose blotted timothy grass pollen extract. Bound IgE was detected with ^{125}I-labeled anti-human IgE antibodies and visualized by autoradiography. The molecular weights (kDa) are displayed on the left side.



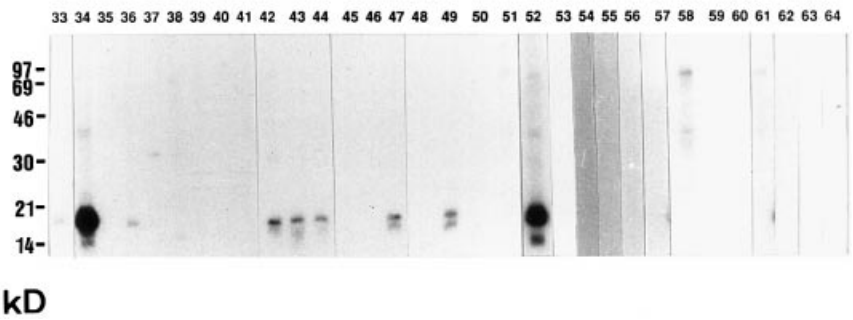


Figure 3. Serum IgE-reactivity to nitrocellulose blotted natural birch pollen extract. Sera from birch pollen allergic patients (*lanes 1*, *6*, *7*, *9*, *13*, *14*, *16–22*, *25*, *27*, *29*, *31–34*, *36–38*, *41–45*, *47*, *49–53*, *58*, *61*), from atopic individuals without birch pollen allergy (*lanes 2–5*, *8*, *10–12*, *15*, *23*, *24*, *26*, *28*, *30*, *35*, *39*, *40*, *46*, *48*), and from nonallergic individuals (*lanes 54–57*, *59–60*, *62–64*) were probed with nitrocellulose blotted birch pollen extract. Bound IgE was detected with ^{125}I-labeled anti-human IgE antibodies and visualized by autoradiography. The molecular weights (kDa) are displayed on the left side.

body levels (**Table II**). Similar results were obtained for IgG_2 and IgG_3 antibodies whereas the levels of allergen-specific IgG_1 tended to be low in the treated as well as untreated groups (**Table II**).

A panel of five recombinant pollen allergens allows *in vivo* diagnosis of grass and tree pollen allergy in most patients Using timothy grass pollen extract for skin prick testing, we diagnosed grass pollen allergy in 51 of the 54 grass pollen allergic patients, 52 of 54 exhibited skin reactivity to rPhl p 1, 32 of 54 reacted with rPhl p 2, and 41 of 54 with rPhl p 5. In three patients (**Table I**: #51, 52, 58) we found IgE reactivity to timothy grass pollen extract but no skin reactions, and one patient (**Table I**: #60) showed skin reactions without containing detectable IgE anti-timothy grass pollen extract. With the exception of one patient (#23: rPhl p 1), immediate type skin reactions to recombinant grass pollen allergens were exclusively observed in patients containing specific IgE antibodies to the very same allergen. No immediate type skin reactions to recombinant timothy grass pollen allergens were observed in the 10 nonatopic individuals. Positive skin reactions to birch pollen extract were found in all 37 of the pollen allergic patients. Whereas three patients who had reported immediate type symptoms during the birch pollen season (#17, 58, 61) were not diagnosed with birch pollen extract, positive skin reactions were found in two patients (**Table I**: #18, 20) who had not reported clinical symptoms during the birch pollen season. In three patients (**Table I**: #8, 17, 39) we found IgE antibodies to birch pollen extract without birch pollen-specific skin reactions. Thirty-five of 36 birch pollen allergic patients reacted either with

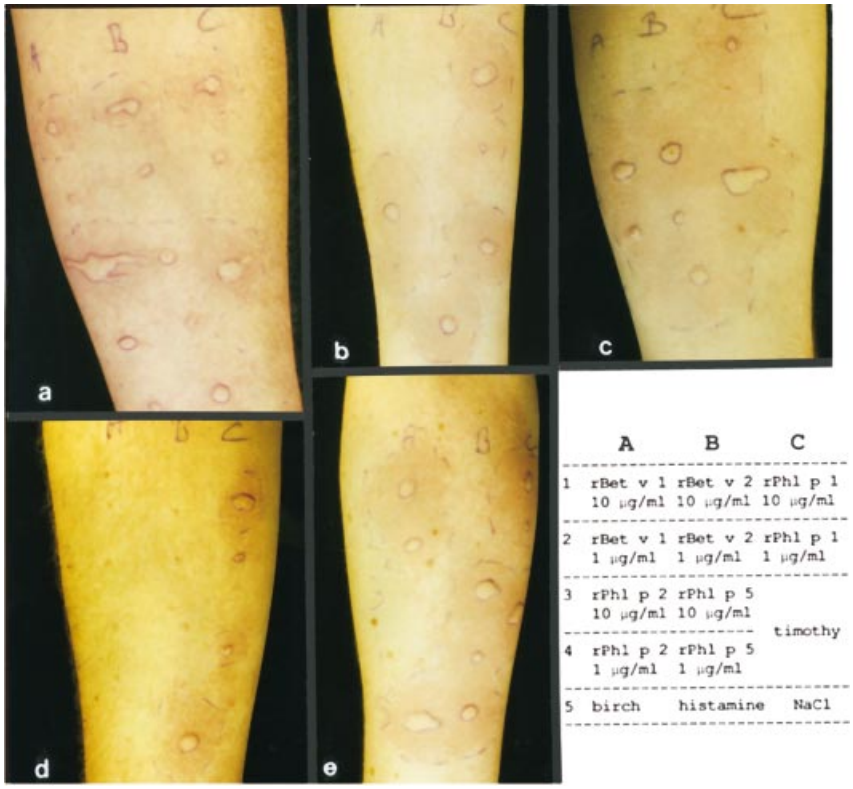


Figure 4. Skin prick activity of recombinant pollen allergens in representative patients. The wheal reactions of five representative patients (*a*, #19; *b*, #5; *c*, #28; *d*, #11; *e*, #21) were documented by photography. The dose of the allergens as well as the application order of allergens, histamine, and sodium chloride solution are displayed in the insert.

Table II. Mean IgE and IgG subclass reactivity as well as mean skin reactivity to recombinant timothy and birch pollen allergens[a]

	Phl p 1			Phl p 2			Phl p 5			Bet v 1			Bet v 2		
	T	H	N-H	T	H	N-H	T	H	N-H	T	H	N-H	T	H	N-H
	n = 49	n = 14	n = 35	n = 36	n = 11	n = 25	n = 41	n = 10	n = 31	n = 26	n = 22	n = 4	n = 13	n = 7	n = 6
CAP															
IgE	25.0	22.4	26.0	20.2	20.4	20.2	37.0	44.2	34.7	32.2	37.8	1.7	6.1	8.3	3.5
ELISA															
IgG_1	0.09	0.08	0.10	0.02	0.02	0.02	0.17	0.16	0.18	0.12	0.13	0.10	0.11	0.11	0.11
IgG_2	0.29	0.35	0.27	0.26	0.31	0.24	0.55	0.90	0.46	0.34	0.38	0.12	0.27	0.28	0.25
IgG_3	0.17	0.12	0.19	0.29	0.25	0.31	0.75	1.33	0.56	0.38	0.36	0.52	0.41	0.40	0.42
IgG_4	0.75	0.83	0.71	0.88	1.28	0.71	1.06	1.78	1.12	1.22	1.41	0.15	0.94	1.26	0.56
SPT	n = 52	n = 14	n = 38	n = 32	n = 10	n = 22	n = 41	n = 10	n = 31	n = 29	n = 23	n = 5	n = 15	n = 8	n = 8
MD	6.22	6.46	6.13	9.22	9.05	9.29	7.74	8.50	7.50	6.50	7.16	4.80	4.77	5.62	3.31

[a]The mean antibody reactivities (IgE: kUA per liter; IgG subclasses: mean OD) were calculated for all individuals (T) containing specific IgE antibodies and for the hyposensitized patients (H) and nonhyposensitized patients (N-H). Mean wheal diameters (MD) were calculated for those who mounted immediate type skin reactions to a particular allergen.

rBet v 1 or with rBet v 2 (rBet v 1, 29 patients; rBet v 2, 13 patients). Positive skin reactions to recombinant birch pollen allergens were observed only in patients who contained specific serum IgE antibodies, except in three patients (#4, rBet v 1; #17, 27, rBet v 2).

In summary, we found that a combination of three recombinant timothy grass pollen allergens allowed skin prick test diagnosis of 52 of 54 grass pollen allergic patients. Skin prick testing with two recombinant birch pollen allergens identified 35 of 36 birch pollen allergic patients.

Recombinant tree and grass pollen allergens allow component-resolved *in vivo* allergy diagnosis **Figure 4** exemplifies that grass and birch pollen allergic patients can exhibit different reactivity profiles to individual allergens that cannot be diagnosed with allergen extracts. Patients who exhibit immediate type skin reactions to timothy grass pollen extract can be sensitized to one (**Fig 4**, patient d), to two (**Fig 4**, patients b, c, e) or more (**Fig 4**, patient a) timothy grass pollen allergens. Likewise, birch pollen allergy can be due to sensitization against a single (**Fig 4**, patient e) or more (**Fig 4**, patient a) allergens. These results demonstrate that skin testing with purified recombinant allergens allows to determine the patient's individual sensitization profile, whereas skin test diagnosis with allergen extracts at best allows the identification of the allergen-containing source.

Comparison of skin prick results and allergen-specific IgE antibody levels Regardless of whether we tested with natural allergen extracts or purified recombinant allergens we found that the presence of allergen-specific IgE antibodies in serum was in almost all cases associated with a positive skin reaction to the corresponding allergen/allergen extract; however, when we compared the extent of skin sensitivity as determined by the mean wheal diameter elicited by the 10 µg per ml recombinant allergen

Table III. Correlations between allergen-specific IgE levels and skin reactivity

	IgE α timothy	IgE α rPhl p 1	IgE α rPhl p 2	IgE α rPhl p 5	IgE α IgE α birch	IgE α rBet v 1	IgE α rBet v 2
MD allergen	0.556747	0.351820	0.594301	0.420349	0.555301	0.432065	0.838625
MD histamine	0.298126	0.239615	0.278239	0.232458	0.408000	0.303811	0.210044

dilution with the IgE binding capacity (kUA per liter) we found weak correlations (**Table III**). The poor correlation of specific IgE levels and skin sensitivity is also exemplified by the following findings: less frequently recognized allergens with low IgE binding capacity frequently induced strong skin reactions (**Table I**, rBet v 2 patients #7, 19; **Table I**, rPhl p 2 patients #20, 38, 41). On the other hand, major allergens with high IgE binding capacity frequently induced mild skin reactions (**Table I**, rBet v 1 patient #52; **Table I**, rPhl p 5 patients #19, 49, 51). These results indicate the importance of biologic (provocation) testing (e.g., skin prick testing) for the evaluation of the allergenic activity of an allergen in a given patient.

DISCUSSION

We report the clinical evaluation of five recombinant pollen allergens in a skin prick test study performed in a group of 55 pollen allergic patients and 10 nonatopic individuals. Two recombinant birch pollen allergens, rBet v 1 (Breiteneder *et al*, 1989) and rBet v 2 (Valenta *et al*, 1991a), and three recombinant timothy grass pollen allergens, rPhl p 1 (Laffer *et al*, 1994), rPhl p 2 (Dolecek *et al*, 1993), and rPhl p 5 (Vrtala *et al*, 1993), were purified to homogeneity and used for the determination of allergen-specific IgE, IgG_{1-4} subclass responses as well as for skin prick testing. The panel of five recombinant allergens allowed diagnosis of Type I allergy in 52 of 54 grass pollen and in 35 of 36 birch pollen allergic patients. We observed no adverse effects and no late phase reactions in any of the tested patients. Furthermore no false positive reactions in the group of nonatopic individuals occurred. The specificity of recombinant allergen-based skin test diagnosis of Type I allergy is also evidenced by our finding that immediate type skin reactions were induced in all but four patients containing allergen-specific IgE antibodies. Also other authors noted that the use of recombinant allergens increased the specificity of diagnostic tests for bee venom allergy (Müller *et al*, 1997). The fact that a combination of only a few recombinant allergens permitted *in vivo* diagnosis of Type I allergy is in good agreement with previous serologic studies that indicated that birch and grass pollen allergy can be diagnosed with a panel of few purified recombinant or natural pollen allergens (Valenta *et al*, 1991*b*; Laffer *et al*, 1996; van Ree *et al*, 1998). Natural allergens (e.g., major birch pollen allergen, Bet v 1) can occur as isoforms with minor sequence variation but widely varying IgE binding capacity (Swoboda *et al*, 1995). Therefore, recombinant allergens to be used for *in vitro* and *in vivo* allergy diagnosis should contain most of the IgE epitopes present in natural allergen sources (Niederberger *et al*, 1998a, ,b). Although it may be necessary to add a few other recombinant birch and grass pollen allergens to the diagnostic panel evaluated in our study [e.g., Bet v 4 (Twardosz *et al*, 1997), Phl p 4 (Fischer *et al*, 1996)], our results indicate that it is possible to use defined recombinant allergens for *in vivo* diagnosis of Type I allergy. Whereas diagnosis with allergen extracts at best allows to identify an allergen-containing source, recombinant allergens permit to establish the patient's reactivity profile to defined molecular entities. Recently the latter concept has been termed "CRD: component-resolved diagnosis" and is suggested as a prerequisite for patient-tailored forms of immunotherapy (reviewed in Valenta *et al*, 1999).

When we used recombinant allergens for component-resolved *in vivo* (skin prick test) diagnosis we found that pollen allergic patients exhibited widely varying sensitization profiles to the individual recombinant allergens that could not be detected with allergen extracts. We thus believe that component-resolved *in vivo* diagnosis will represent a refined tool to monitor patients' sensitization profiles during the natural course of the disease or during allergen-specific immunotherapy to detect new sensitizations or changes of reactivities (Ball *et al*, 1999). Data from serologic analyses point out that allergen-contact enhances allergen-specific IgE antibody production (Henderson *et al*, 1975) and other reports indicate that patients can become sensitized to new allergenic components in the course of immunotherapy (van Ree *et al*, 1996, 1997; Ball *et al*, 1999). Using defined molecules for skin testing it may now also be possible to determine changes in sensitivity by quantitative skin testing.

Recombinant allergens will also be useful for measuring the levels of allergen-specific IgG subclass responses. Although we have no information regarding the details of previously administered immunotherapy, we found it of note that those study patients who had received immunotherapy, exhibited higher allergen-specific IgG_4, IgG_2, and IgG_3 levels. The fact that IgG subclass responses to a given allergen molecule were poorly associated with IgE responses to the very same allergen, indicates the involvement of different B cell clones in the development of IgE and IgG subclass reactivities and is in accordance with observations for other recombinant mite allergens/epitopes (Tame *et al*, 1996; Kobayashi *et al*, 1996).

Notably, we found that allergen-specific IgE levels and skin sensitivity were poorly correlated. This finding indicates that the IgE antibody binding capacity of a given allergen may not necessarily be associated with its potency to elicit allergic reactions. We noticed that frequently recognized major allergens (e.g., Bet v 1, Phl p 1, Phl p 5) with high IgE binding capacity that elicited strong antibody responses in animals (Vrtala *et al*, 1996b, 1998), sometimes induced weak skin reactions. By contrast, less frequently recognized allergens (e.g., Bet v 2, Phl p 2) that bound lower amounts of IgE and were less immunogenic in animals, were able to induce severe skin reactions. Several explanations for the weak correlation between allergen-specific serum IgE levels and skin reactivity are conceivable. They include the presence of blocking antibodies that may interfere differently with the allergen IgE interaction in *in vitro* and *in vivo* assays. It is equally well possible that IgE epitopes are presented in a different way during the *in vitro* and *in vivo* testing; however, the fact that allergens with low IgE binding capacity were able to induce severe skin reactions emphasises that it may be important to perform skin prick testing, and perhaps other provocation testing methods in addition to serology to determine the allergenic activity of allergen molecules in a given patient.

In summary, we demonstrate that a panel of recombinant pollen allergens can be used for component-resolved serologic as well as skin test diagnosis of tree and grass pollen allergy. The determination of the individual patient's sensitization profile by component-resolved diagnosis may allow the selection of the relevant allergens for component-resolved immunotherapy.

This study was supported by grant Y078GEN of the Austrian Science Foundation, by the ICP program of the Austrian Ministry of Science and Transport, and by a grant from Pharmacia-Upjohn Diagnostics AB, Uppsala, Sweden. All in vivo *studies were approved by the Austrian Ministry of Health and the ethics committee of the Medical Faculty, University of Vienna, Austria.*

REFERENCES

Ball T, Sperr WR, Valent P, *et al*: Induction of antibody responses to new B cell epitopes indicates vaccination character of allergen immunotherapy. *Eur J Immunol* 29:2026–2036, 1999

Bousquet J, Valenta R: *In vivo* and *in vitro* use of recombinant allergens. *ACI News* 6/2:54–59, 1994

Breiteneder H, Pettenburger K, Bito A, *et al*: The gene coding for the major birch pollen allergen, Bet v 1, is highly homologous to a pea disease resistance response gene. *EMBO J* 8:1935–1938, 1989

Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976

Casolaro S, Georas SN, Song Z, Ono SJ: Biology and genetics of atopic disease. *Curr Opin Immunol* 8:796–803, 1996

Dreborg S: The skin prick test in the diagnosis of atopic allergy. *J Am Acad Dermatol* 21:820–821, 1989

Dolecek C, Vrtala S, Laffer S, Steinberger P, Kraft D, Scheiner O, Valenta R: Molecular characterization of Phl, p. 2, a major timothy grass (*Phleum pratense*) pollen allergen. *FEBS Lett* 335:299–304, 1993

Ebner C, Szephalusi Z, Ferreira F, *et al*: Identification of multiple T-cell epitopes on Bet v 1, the major birch pollen allergen using T cell clones and overlapping peptides. *J Immunol* 2150:1047–1054, 1993

Fischer S, Grote M, Fahlbusch B, Müller WD, Kraft D, Valenta R: Characterization of Phl, p. 4, a major timothy grass (*Phleum pratense*) pollen allergen. *J Allergy Clin Immunol* 98:189–198, 1996

Fling SP, Gregerson DS: Peptide and protein molecular weight determination by electrophoresis using a high molarity Tris buffer system without urea. *Anal Biochem* 155:83–90, 1986

Henderson LL, Larson JB, Gleich GJ: Maximal rise in IgE antibody following ragweed pollination season. *J Allergy Clin Immunol* 55:10–15, 1975

Hoffmann-Sommergruber K, Susani M, Ferreira F, *et al*: High-level expression and purification of the major birch pollen allergen, Bet v 1. *Prot Expr Purif* 9:33–39, 1997

Jarolim E, Rumpold H, Endler AT, Ebner H, Breitenbach M, Scheiner O, Kraft D: IgE and IgG antibodies of patients with allergy to birch pollen as tools to define the allergen profile of *Betula verrucosa*. *Allergy* 44:385–395, 1989

Kay AB: *Allergy and Allergic Diseases*. Oxford: Blackwell Science, 1997

Kobayashi I, Sakiyama Y, Tame A, Kobayashi K, Matsumoto S: IgE and IgG_4 antibodies from patients with mite allergy recognize different epitopes of *Dermatophagoides pteronyssinus* group II antigen (Der, p. 2). *J Allergy Clin Immunol* 97:638–645, 1996

Laffer S, Valenta R, Vrtala S, *et al*: Complementary DNA cloning of the major allergen Phl, p. 1 from timothy grass (*Phleum pratense*); recombinant Phl, p. 1 inhibits IgE binding to group I allergens from eight different grass species. *J Allergy Clin Immunol* 94:689–698, 1994

Laffer S, Spitzauer S, Susani M, *et al*: Comparison of recombinant timothy grass pollen allergens with natural extract for diagnosis of grass pollen allergy in different populations. *J Allergy Clin Immunol* 98:625–658, 1996

Lockey RF, Buckantz SC, eds: *Allergen Immunotherapy*, 2nd edn. New York: Marcel Dekker, , 1998

McLeod M, Stein M, Beach D: The product of the mei^+ gene, expressed under the control of the mating-type locus, induces meiosis and sporulation in fission yeast. *EMBO J* 6:729–736, 1987

Menz G, Dolecek C, Schönheit-Kenn U, *et al*: Serological and skin-test diagnosis of birch pollen allergy with recombinant Bet v 1, the major birch pollen allergen. *Clin Exp Allergy* 26:50–60, 1996

Müller U, Fricker M, Wymann D, Blaser K, Crameri R: Increased specificity of diagnostic tests with recombinant major bee venom allergen phospholipase A2. *Clin Exp Allergy* 27:915–920, 1997

Niederberger V, Laffer S, Fröschl R, *et al*: IgE antibodies to recombinant pollen allergens (Phl, p. 1, Phl, p. 2, Phl, p. 5, and Bet v 2) account for a high percentage of grass pollen-specific IgE. *J Allergy Clin Immunol* 101:258–264, 1998a

Niederberger V, Pauli G, Grönlund H, *et al*: Recombinant birch pollen allergens (rBet v 1 and rBet v 2) contain most of the IgE epitopes present in birch, alder, hornbeam, hazel, and oak pollen: a quantitative IgE inhibition study with sera from different populations. *J Allergy Clin Immunol* 102:579–591, 1998b

Pauli G, Oster JP, Deviller P, *et al*: Skin testing with recombinant allergens rBet v 1 and birch profilin, rBet v 2: diagnostic value for birch pollen and associated allergies. *J Allergy Clin Immunol* 97:1100–1109, 1996

Schenk S, Breiteneder H, Susani M, *et al*: T cell epitopes of Phl, p. 1, major pollen allergen of timothy grass (*Phleum pratense*): evidence for crossreacting and non-crossreacting T cell epitopes within grass group I allergens. *J Allergy Clin Immunol* 96:986–996, 1995

Segal DM, Taurog JD, Metzger H: Dimeric immunoglobulin E serves as a unit signal for mast cell degranulation. *Proc Natl Acad Sci USA* 74: 2993–2997, 1977

Swoboda I, Jilek A, Ferreira F, *et al*: Isoforms of Bet v 1, the major birch pollen allergen, analyzed by liquid chromatography, mass spectrometry, and cDNA cloning. *J Biol Chem* 270:2607–2613, 1995

Tame A, Sakiyama Y, Kobayashi I, Terai I, Kobayashi K: Differences in titres of IgE, IgG4 and other IgG subclass anti-Der, p. 2 antibodies in allergic and non-allergic patients measured with recombinant allergen. *Clin Exp Allergy* 26:43–49, 1996

Towbin H, Staehelin T, Gordon T: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4354, 1979

Twardosz A, Hayek B, Seiberler S, *et al*: Molecular characterization, expression in *Escherichia coli*, and epitope analysis of a two EF-hand calcium-binding birch pollen allergen, Bet v 4. *Biochem Biophys Res Comm* 239:197–204, 1997

Valenta R, Duchêne M, Pettenburger K, *et al*: Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science* 253:557–560, 1991a

Valenta R, Duchêne M, Vrtala S, *et al*: Recombinant allergens for immunoblot diagnosis of tree pollen allergy. *J Allergy Clin Immunol* 88:889–894, 1991b

Valenta R, Vrtala S, Ebner C, Kraft D, Scheiner O: Diagnosis of grass pollen allergy with recombinant timothy grass (*Phleum pratense*) pollen allergens. *Int Arch Allergy Immunol* 97:287–294, 1992a

Valenta R, Duchêne M, Ebner C, *et al*: Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* 175:377–385, 1992b

Valenta R, Sperr WR, Ferreira F, *et al*: Induction of specific histamine release from basophils with purified natural and recombinant birch pollen allergens. *J Allergy Clin Immunol* 91:88–97, 1993

Valenta R, Kraft D: Recombinant allergens for diagnosis and therapy of allergic diseases. *Curr Opin Immunol* 7:751–756, 1995

Valenta R, Vrtala S, Laffer S, Spitzauer S, Kraft D: Recombinant allergens. *Allergy* 53:552–561, 1998

Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D, Grönlund H: The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD & CRIT). *Clin Exp Allergy* 29:896–904, 1999

van Ree R, Antonicelli L, Akkerdaas JH, Garritani MS, Aalberse RC, Bonifazi F: Possible induction of food allergy during mite immunotherapy. *Allergy* 51:108–113, 1996

van Ree R, van Leeuwen WA, Dieges PH, *et al*: Measurement of IgE antibodies against purified grass pollen allergens (Lol, p. 1, 2, 3, and 5) during immunotherapy. *Clin Exp Allergy* 27:68–74, 1997

van Ree R, van Leeuwen WA, Aalberse RC: How far can we simplify *in vitro* diagnostics for grass pollen allergy? A study with 17 whole pollen extracts and purified natural and recombinant major allergens. *J Allergy Clin Immunol* 102:184–190, 1998

Vrtala S, Sperr WR, Reimitzer I, *et al*: cDNA cloning of a major allergen from timothy grass (*Phleum pratense*) pollen; characterization of the recombinant Phl, p. 5 allergen. *J Immunol* 151:4773–4781, 1993

Vrtala S, Susani M, Sperr WR, *et al*: Immunologic characterization of purified recombinant timothy grass pollen (*Phleum pratense*) allergens (Phl, p. 1, Phl, p. 2, Phl, p. 5). *J Allergy Clin Immunol* 97:781–787, 1996a

Vrtala S, Mayer P, Ferreira F, Susani M, Sehon AH, Kraft D, Valenta R: Induction of IgE antibodies in mice and rhesus monkeys with recombinant birch pollen allergens; different allergenicity of Bet v 1 and Bet v 2. *J Allergy Clin Immunol* 98:913–921, 1996b

Vrtala S, Ball T, Spitzauer S, *et al*: 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. *J Immunol* 160:6137–6144, 1998

Way M, Pope B, Gooch J, Hawkins M, Weeds AG: Identification of a region in segment 1 of gelsolin critical for actin binding. *EMBO J* 9:4103–4109, 1990