

Original article

Micro-arrayed wheat seed and grass pollen allergens for component-resolved diagnosis

Background: Wheat is a potent allergen source and can cause baker's asthma, food and pollen allergy. The aim of the study was to develop an allergen micro-array for differential diagnosis of baker's asthma, wheat-induced food allergy and grass pollen allergy.

Methods: We analysed the immunoglobulin-E reactivity profiles of patients suffering from baker's asthma, wheat-induced food allergy and grass pollen allergy to micro-arrayed recombinant wheat flour allergens and grass pollen allergens and compared these results with clinical results and diagnostic tests based on crude wheat flour, wheat pollen and grass pollen allergen extracts.

Results: We identified recombinant wheat flour allergens, which are specifically recognized by patients suffering from baker's asthma, but not from patients with food allergy to wheat or pollen allergy. rPhl p 1 and rPhl p 5 were identified as marker allergens specific for grass pollen allergy. They can be used to replace grass pollen extracts for allergy diagnosis and to identify grass pollen allergic patients among patients suffering from baker's asthma and wheat-induced food allergy. Profilin was identified as a cross-reactive allergen recognized by patients suffering from baker's asthma, food and pollen allergy.

Conclusions: Our results indicate that it will be possible to design serological tests based on micro-arrayed recombinant wheat seed and grass pollen allergens for the discrimination of baker's asthma, wheat-induced food allergy and grass pollen allergy.

C. Constantin¹, S. Quirce²,
M. Poorafshar³, A. Touraev⁴,
B. Niggemann⁵, A. Mari⁶, C. Ebner⁷,
H. Akerström³, E. Heberle-Bors⁴,
M. Nystrand³, R. Valenta¹

¹Division of Immunopathology, Department of Pathophysiology, Center of Physiology and Pathophysiology, Medical University of Vienna, Vienna, Austria; ²Allergy Department Hospital La Paz and CIBRES, Madrid, Spain; ³Phadia AB, Uppsala, Sweden; ⁴Max F. Perutz Laboratories, University of Vienna, Vienna, Austria; ⁵Pediatric Allergy and Pneumology, DRK Kliniken Berlin, Berlin, Germany; ⁶Center for Clinical and Experimental Allergy, IDI-IRCCS, Rome, Italy; ⁷Allergieambulatorium Reumannplatz, Vienna, Austria

Key words: Baker's asthma; grass pollen allergy; micro-array; recombinant allergens; wheat food allergy.

Rudolf Valenta
Christian Doppler Laboratory for Allergy Research
Division of Immunopathology
Department of Pathophysiology
Center of Physiology and Pathophysiology
Medical University of Vienna
Vienna General Hospital
Waehringer Guertel 18-20
1090 Vienna
Austria

Accepted for publication 1 December 2008

Triticum aestivum (bread wheat) can cause three distinct immunoglobulin-E (IgE)-mediated allergies, respiratory allergy caused by inhalation of wheat flour, food allergy by ingestion of wheat products and wheat (i.e. grass) pollen allergy. Respiratory allergy to wheat flour is one of the most frequent causes of occupational asthma. Approximately 1–10% of bakery workers are affected by baker's asthma (1). Wheat-induced food allergy is common and affects up to 8% of young children (≤ 3 years) and 2% of adolescents and adults (2–5). *Triticum aestivum* is an important member of the grass

family (6). Up to 40% of all allergic individuals show IgE reactivity to grass pollen allergens (7). Several studies have reported cross-reactivity between wheat flour and grass pollen due to common IgE epitopes in wheat flour and grass pollen proteins (8–10). Furthermore, diagnosis based on wheat flour extract does not allow discriminating between patients suffering from respiratory allergy and those suffering from food allergy to wheat. Therefore, precise diagnosis still relies on specific inhalation challenge in case of respiratory allergy to wheat flour (11) and double-blind, placebo-controlled food challenge (DBPCFC) in the case of suspected food allergy (12). To improve IgE-serological tests for the discrimination between patients suffering from baker's asthma, wheat-induced food allergy and grass pollen allergy, we have

Abbreviations: DBPCFC, double-blind, placebo-controlled food challenge; FI, fluorescence intensity; IgE, immunoglobulin E; Phl p, *Phleum pratense*.

developed an allergen micro-array on the basis of purified allergen molecules. We investigated the IgE reactivity profile to recombinant wheat allergens, timothy grass pollen allergens and compared the test results with clinical results and serological tests based on allergen extracts. The results from the micro-arrays show that recombinant wheat allergens can be identified, which are highly specific for baker's asthma, whereas others represent cross-reactive allergens in pollen and food allergy. The IgE reactivity profile of patients to the recombinant wheat and grass pollen allergens may allow differentiating between baker's asthma, food allergy to wheat and grass pollen allergy.

Materials and methods

Patients and sera

Sera were obtained from Spanish patients suffering from baker's asthma (B1–B22), Austrian (F1–F20) and German patients (F21–F32) suffering from wheat-induced food allergy and Austrian patients (G1–G17) suffering from grass pollen allergy. Patients were selected according to a positive case history, specific inhalation challenge tests for confirmation of a clinically relevant sensitization in case of baker's asthma (11) and DBPCFC in infant patients with food allergy to wheat. Sera from the patients have been analysed for total serum IgE levels and IgE specific for wheat flour and timothy

grass pollen by the CAP-FEIA System (Phadia, Uppsala, Sweden). Demographic, clinical and serological data of the patients are summarized in Tables 1–3. For control purposes, sera from non-allergic individuals were included in all experiments.

Biological materials

Recombinant wheat proteins #10, #37, #38, #112, #123 and #126 were obtained from cDNAs that had been isolated from a cDNA library by screening with sera from baker's asthma patients by expression in *Escherichia coli* as described (13). Recombinant protein #10 is a serine proteinase inhibitor (accession no. gi|122065237) (14), #37 is a thioredoxin H (accession no. EU584496), #38 is a glutathione transferase (accession no. EU584497), #112 is a 1-Cys-peroxyredoxin (accession no. EU584498), #123 is a profilin (accession no. EU584499) and #126 is a dehydrin (accession no. EU584500) according to homology with sequences deposited in the NCBI database (Constantin C & Valenta R, unpublished data, sequences submitted to the database).

Wheat pollen was purchased from Allergon (Vällinge, Sweden) and recombinant *Phleum pratense* 1 (Phl p 1, Phl p 5, Phl p 7, Phl p 12) were from BIOMAY (Vienna, Austria).

Protein extract

Triticum aestivum pollen (500 mg) was extracted at 4°C over night in 5 ml PBS, 2 mM EDTA, 1 mM PMSF. After centrifugation for 1 h at 13 000 g 4°C, the protein concentration of the supernatant was determined with a Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and aliquots were stored at –20°C until use.

Table 1. Demographic, clinical and serological characteristics of patients suffering from respiratory allergy to wheat

Patient	Gender	Age	IgE kUA/l		Total IgE kU/l	Job	Years exp.	Symptoms	Other allergies	PC ₂₀ mg/ml
			Wheat flour	<i>Phleum pratense</i>						
B1	M	35	74.2	5.25	163	Confectioner	20	A, RC	g, k, w	0.29
B2	M	29	18	53.5	271	Confectioner	12	A, RC	g, k, me, p	2
B3	M	36	1.66	<0.35	353	Baker	8	A, RC	pe	0.31
B4	M	39	25.8	0.73	1387	Baker–confectioner	20	A, RC, U	g	0.25
B5	M	54	0.35	<0.35	30.1	Baker	42	A, RC	n.k.	n.d.
B6	M	35	24.3	4.67	416	Baker	12	A, RC	g	0.25
B7	M	28	2.58	<0.35	248	Baker	14	A, RC	c, d, h, hdm	0.25
B8	M	27	7.86	>100	1773	Baker	2.6	A, RC	g, k, p, me, n	0.47
B9	M	24	2.35	>100	204	Baker	18	A, RC	c, d, g, h, hdm, k, p, n	0.09
B10	M	60	3.44	<0.35	73.3	Confectioner	46	A, RC	hdm, m	0.44
B11	M	22	3	<0.35	2509	Baker	5	A, RC, U	k	3.38
B12	M	26	>100	>100	321	Baker–confectioner	5	A, RC	a, ch, g, k, me, p, b	0.5
B13	M	54	2.48	<0.35	480	Confectioner	25	A, RC	none	0.12
B14	M	60	31.8	<0.35	629	Baker	13	A	c, cr, d	0.15
B15	M	27	5.06	42.2	278	Confectioner	10	A, RC	c, cr, d, ew, g	0.079
B16	F	42	3.71	2.15	271	Pizza	12	A, RC, U	g	0.187
B17	M	54	1.68	<0.35	79.2	Confectioner	38	A	hdm	16
B18	M	59	<0.35	<0.35	673	Baker–confectioner	22	A, RC	n.k.	0.23
B19	M	26	74.6	57.4	17.7	Confectioner	16	A, RC	g	1.16
B20	M	43	13.8	11	538	Baker	19	A	g, o	0.25
B21	F	34	2.05	<0.35	229	Confectioner	2	A	hdm, o	0.5
B22	F	41	1.77	<0.35	23.4	Cook/pastry maker	1	A	o	0.23

M, male; F, female; kUA/l, kilounit antigen per litre; n.k., not known; A, asthma; RC, rhinoconjunctivitis; U, urticaria; a, avocado; b, banana; c, cat; ch, chestnut; cr, cockroach; d, dog; ew, egg white; g, grass pollen; h, horse; hdm, house dust mite; k, kiwi; me, melon; m, molds; n, nuts; o, olive pollen; p, peach; pe, peanut; w, walnut; PC₂₀, methacholine inhalation challenge (positive if <16mg/ml).

Table 2. Demographic, clinical and serological characteristics of patients suffering from food allergy to wheat

Patient	Gender	Age	IgE kUA/l		Total IgE kU/l	Symptoms	DBPCFC	Other allergies
			Wheat flour	<i>Phleum pratense</i> IgE kUA/l				
F1	F	56	0.74	<0.35	820	U	n.d.	s
F2	F	50	0.76	0.35	275	Ab, Cj, Di, P	n.d.	hdm
F3	F	49	>100	0.65	1794	No symptoms-diet	n.d.	hdm, n, sh
F4	F	72	5.99	<0.35	325	E, D, P	n.d.	he, n, s
F5	F	11	2.15	3.28	11	A, C, G, Ri	n.d.	g, n, s, o
F6	F	40	1.9	<0.35	19.3	C, Cj, G, S	n.d.	n.k.
F7	M	24	5.76	<0.35	111	C, Cj, G, N, R	n.d.	n.k.
F8	F	21	1.22	25.8	94.8	C, N, G, P, R, RC	n.d.	g
F9	F	5	1.93	34.3	453	G, RC	n.d.	a, b, cm, g, n
F10	F	29	1.55	10.4	101	De, Ri, RC	n.d.	c, b, g, p
F11	F	45	5.72	1.12	30.1	A, C, D, blisters on tongue	n.d.	n, s, r
F12	M	7	2.8	>100	<2000	B, G, RC	n.d.	a, b, g
F13	F	28	1.27	>100	616	En, RC	n.d.	a, cm, l, m
F14	M	50	7.57	22.6	450	A, C, D, G, N, RC	n.d.	b, g
F15	F	61	3.97	5.12	44.1	Di, F	n.d.	f, n
F16	F	43	1.03	58.3	338	C, G, RC	n.d.	b, h, n, s
F17	F	19	20.5	<0.35	949	A, C, D, G, R	n.d.	n.k.
F18	M	12	0.81	1.47	769	A, G, RC	n.d.	he, w
F19	F	65	2.11	<0.35	152	No symptoms-diet	n.d.	n, sea
F20	M	27	6.11	67.7	974	C, Cj, D, Ri, RC, in the past A	n.d.	ca, he, ce, sp
F21	F	9	6.47	>100	1233.5	E, Ri	+	n.k.
F22	M	0.5	7.39	<0.35	65.2.E'	E, U, wheezing	+	n.k.
F23	F	1	33.5	<0.35	201	Redness, itching	+	cm, he
F24	F	1	10	n.d.	25.5	U, itching	+	cm, he, s
F25	M	1	18.6	n.d.	1343	U	+	s
F26	M	1	35.1	0.83	325	U, Ri, itching	+	s
F27	F	1	4.27	<0.35	38.4	U, Ri, redness	+	n.k.
F28	F	0.5	21.3	<0.35	1511	U	+	cm
F29	F	1	5.21	n.d.	1718	AD, G, U	+	cm
F30	M	1	61.4	0.69	876	Redness, itching	+	cm
F31	M	1	52	0.38	254	Ri, U	+	cm, he
F32	F	0.5	94.4	1.9	1524	De, Ri	Open challenge	n.k.

M, male; F, female; n.k., not known; n.d., not done; kUA/l, kilounit antigen per litre; A, asthma; Ab, abdominalgia; AD, atopic dermatitis; B, bronchitis; C, cough; Cj, conjunctivitis; D, dyspnoea; De, dermatitis; Di, diarrhoea; E, eczema; ex, exanthema; F, flatulence; G, gastro-intestinal symptoms; N, nasal congestion; P, pruritus; R, rhinorrhoea; RC, rhinoconjunctivitis; Ri, rhinitis; S, sore throat; U, urticaria; DBPCFC, double-blind placebo-controlled food challenge; a, artemisia; b, birch pollen; c, cat; ca, carrot; cm, cow's milk; f, formaldehyde; g, grass pollen; h, hazelnut; he, hens egg; hdm, house dust mite; l, latex; m, malt; n, nuts; o, orange; p, plum; r, rice; s, soybean; ce, celery; sea, seafood; sh, shrimps; sp, spices; w, wasp.

Allergen micro-array analysis

Wheat pollen extract, 1–1.5 ng/spot, recombinant and purified wheat proteins and recombinant grass pollen allergens, 0.1–0.15 ng/spot, were spotted using a Nano Plotter NP2 (Gesellschaft für Silizium-Mikrosysteme mbH, Großberkmannsdorf, Germany) on nitrocellulose membranes that were attached to microscope glass slides as described (15). Purified human IgE was used as a position marker and was spotted at similar concentrations as purified and recombinant proteins (16). The spotted micro-arrays were pre-washed with 30 µl assay buffer (phosphate buffer, pH 7.5) and incubated with 30 µl undiluted sera from allergic patients or controls. After washing with 30 µl assay buffer, bound IgE antibodies were detected with 20 µl fluorophore-conjugated anti-IgE antibodies and fluorescence intensities (FI) were measured at a wavelength of 635 nm (GenePix 4000B Axon Instruments; Molecular Devices, Sunnyvale, CA, USA). The cut off level was set to FI = 300 based on values obtained with spotted human serum albumin, which was used as a negative control.

Results

Characterization of patients

Patients with respiratory allergy to wheat. The group of patients with respiratory allergy to wheat consisted of 22 persons (three women; 19 men; mean age: 39 years; range: 22–60 years) with occupational exposure to wheat flour. All baker's asthma patients suffered from asthma due to inhalation of wheat flour and 77% complained of symptoms of rhinoconjunctivitis. A sensitization to other respiratory allergen sources (e.g. cat, cockroach, dog, grass pollen, horse, house dust mites, moulds and/or olive pollen) was found in 70% of the patients and 50% of the patients suffered also from grass pollen allergy (Table 1). Interestingly, we did not find baker's asthma patients with

Table 3. Demographic, clinical and serological characteristics of patients suffering from grass pollen allergy

Patient	Gender	Age	IgE kUA/l		Total IgE kU/l	Symptoms	Other allergies
			<i>Phleum pratense</i>	Wheat flour			
G1	M	22	37.8	1.13	529	D, RC, U	c, b, d, hdm, s
G2	M	30	44.6	0.52	290	D, RC	b, a, hdm, d
G3	F	25	59.8	<0.35	1004	RC	b, d, hdm, h, ce
G4	M	n.k.	25.9	3.62	140	RC	a, b
G5	F	22	37.2	<0.35	566	R	b, c, hdm
G6	F	22	22.4	<0.35	77.4	RC	hdm, r
G7	M	24	30.8	<0.35	60.8	n.k.	n.k.
G8	M	35	9.92	<0.35	88.8	D	b
G9	M	36	20.7	0.45	128	R	ap, b, hdm
G10	F	22	>100	4.13	>5000	RC	b, c, hdm, rye
G11	M	41	49.3	4.01	>2000	A, CJ	a, b, c, hdm
G12	M	37	n.d.	1.11	243	n.k.	n.k.
G13	M	28	39.3	1.06	144	RC	b, a
G14	F	27	>100	<0.35	260	A, RC	n.k.
G15	M	39	>100	11.1	401	n.k.	n.k.
G16	M	54	>100	9.93	1528	A, CJ	n.k.
G17	M	45	n.d.	1.76	175	RC	d, hdm, s, p

M, male; F, female; n.k., not known; n.d., not done; kUA/l, kilounit antigen per litre; A, asthma; CJ, conjunctivitis; D, dyspnoea; RC, rhinoconjunctivitis; R, rhinitis; U, urticaria; a, artemisia; ap, apple; b, birch pollen; c, cat; d, dog; h, hazelnut; hdm, house dust mite; r, rabbit; s, soybean; ce, celery; p, potato.

cereal-induced food allergy, but some of them (36%) experienced oral allergy syndrome with mostly fruits and nuts.

Patients with wheat-induced food allergy. This group comprised 32 individuals (22 women; 10 men; mean age: 23 years; range: 0.5–65 years) (Table 2). We found that 34% are sensitized to other respiratory allergen sources (e.g. birch pollen, grass pollen, house dust mites and mugwort pollen) and 66% were also sensitized to other food allergens (e.g. carrot, cow's milk, hazelnut, hen's egg, malt, nuts, orange, plum, rice, soybean, celery, seafood, shrimps and/or spices) (Table 2). An IgE-mediated sensitization to grass pollen according to CAP was found in 56% of these patients ($n = 18$) but only for 7, evidence for grass pollen allergy was found in the case record forms. The symptoms of the grass pollen-positive patients varied from respiratory symptoms (e.g. asthma, bronchitis, cough, conjunctivitis, dyspnoea, nasal congestion, rhinorrhoea, rhinoconjunctivitis) to cutaneous symptoms (e.g. eczema, pruritus and urticaria) (Table 2).

Grass pollen allergic patients. This group consisted of 17 patients (five females; 12 males; mean age: 13 years; range: 0.5–65 years). Seventy-one per cent of these patients also suffered from allergy to other respiratory allergen sources (e.g. birch pollen, cat, dog, house dust mites, rabbit and mugwort pollen) (Table 3). According to serology, 65% exhibited IgE reactivity to wheat flour and 23% contained IgE against other food allergens. However, only one patient suffered from urticaria and no symptoms of

clinically relevant food allergy could be recorded for these patients. Respiratory symptoms such as asthma, conjunctivitis, dyspnoea, rhinoconjunctivitis and rhinitis dominated in the grass pollen allergic patients.

Composition of the allergen array

Six recombinant wheat seed allergens designated as #10, #37, #38, #112, #123 and #126, wheat pollen extract, recombinant timothy grass pollen allergens (rPhl p 1, rPhl p 5, rPhl p 7 and rPhl p 12) (17–21) and IgE were spotted onto nitrocellulose-coated glass slides (Fig. 1A).

Representative images of allergen micro-arrays after incubation with sera are shown in Fig. 1B,C. When the chip was probed with serum from a nonallergic person, no reactivity to any allergen could be detected and only the spotted IgE markers reacted with the anti-IgE conjugate (Fig. 1B). Figure 1C show representative images of micro-array chips incubated with sera from a baker's asthma patient (1), a patient suffering from wheat-induced food allergy (2) and a grass pollen allergic patient (3). The baker's asthma patient (1: Table 1: B4) shows strong IgE reactivity to recombinant wheat allergens #10 and weak reactivities to #126 and #112. For the food allergic patient (2: Table 2: F20), strong IgE binding to recombinant profilin from wheat #123 and timothy grass pollen rPhl p 12 and weak binding to wheat pollen extract and rPhl p 1 were observed. The image of the chip from a grass pollen allergic patient (3: G16) shows strong signals to rPhl p 5 and rPhl p 12 and weaker signals with wheat profilin #123, wheat pollen extract and rPhl p 1.

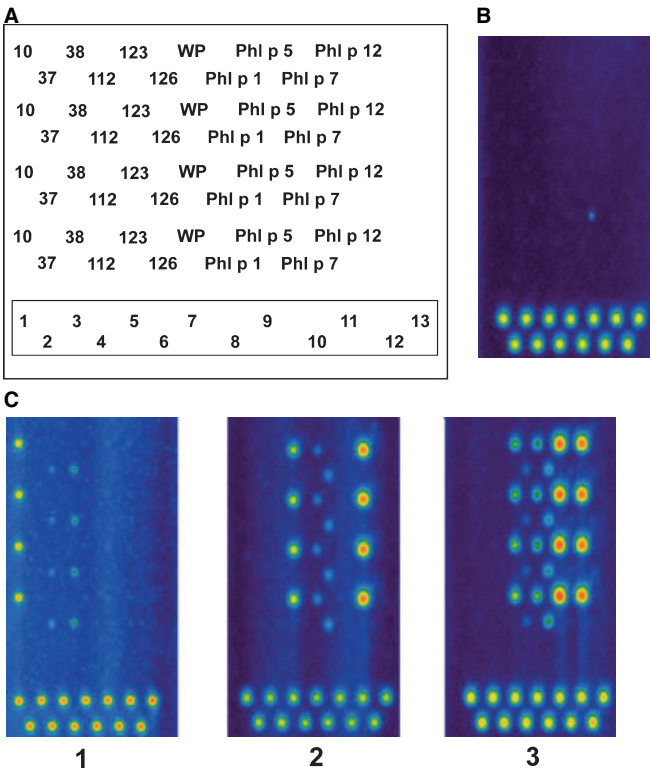


Figure 1. Allergen micro-array. (A) Application scheme of micro-arrayed proteins and wheat pollen extract. Recombinant wheat proteins are designated as: 10, 37, 38, 112, 123, 126; WP: wheat pollen extract; recombinant timothy grass pollen allergens: Phl p 1, Phl p 5, Phl p 7 and Phl p 12. Numbers in the box at the bottom indicate position markers. (B) and (C) Images of micro-arrays after incubation with serum and detection of IgE-reactive spots with fluorophore-conjugated anti-IgE antibodies. (B) Image after incubation with serum from a non allergic individual. (C) Images after incubation with serum from a representative patient suffering from baker's asthma (1: B4), wheat induced food allergy (2: F20), grass pollen allergy (3: G16). Dots on the bottom of the slides indicate position markers which are purified IgE antibodies detected with fluorophore-conjugated anti-IgE antibodies.

Identification of wheat seed allergens specifically recognized by IgE antibodies from baker's asthma patients

Ninety-one per cent of the baker's asthma patients were positive in the wheat flour CAP (Table 1). Using spotted wheat allergen molecules, the IgE reactivity profile for 64% of the baker's asthma patients could be established. Allergens #126 (32%), #10 (27%), #123 (23%) and #112 (18%) were the most frequently detected components, whereas #37 and #38 reacted only with 4.5% of the sera (Fig. 2A). Interestingly, the two patients who were negative in the wheat flour CAP (B5, B18: Table 1) reacted with allergen #126, the dehydrin. Fifty per cent of the baker's asthma patients showed IgE reactivity to timothy grass pollen extract. All but two patients (#B6: Phl p 12 positive; #B16: grass pollen positive) were also

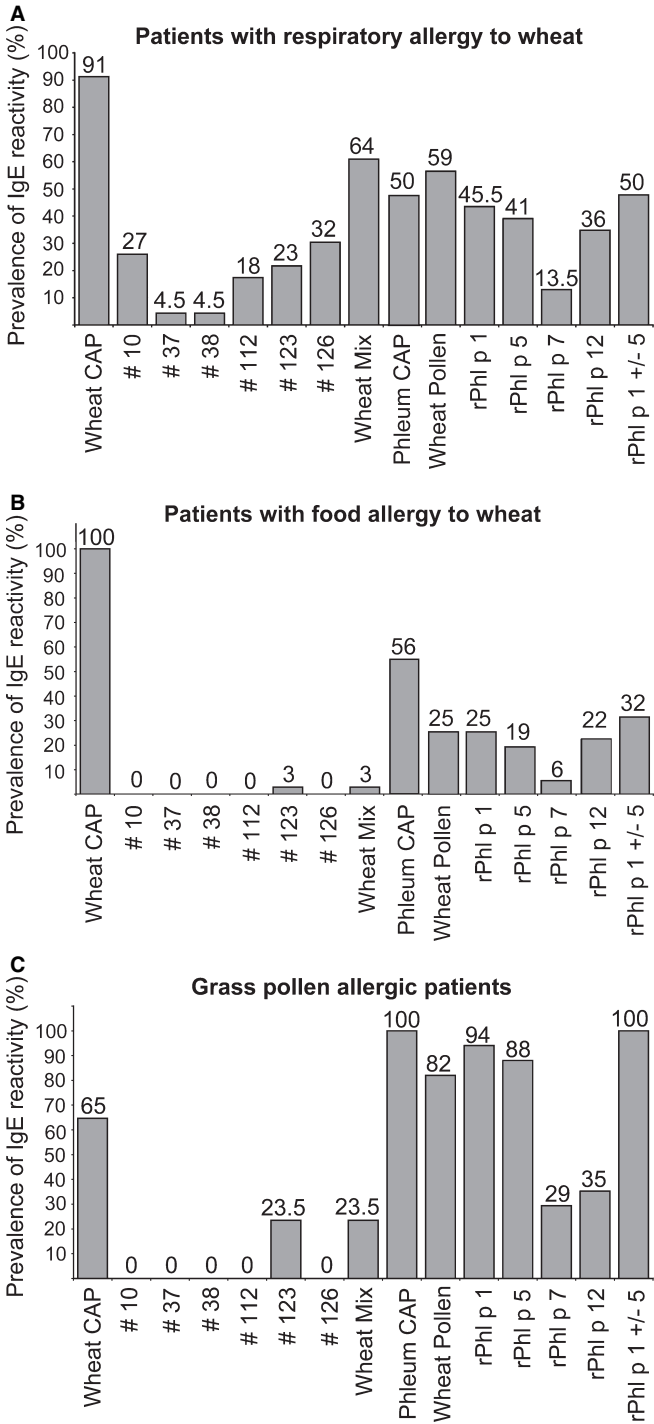


Figure 2. Prevalence of IgE reactivity to wheat seed proteins, wheat pollen extract and grass pollen allergens. Percentages of patients with IgE reactivity are shown for baker's asthma (A) ($n = 22$), food allergy to wheat (B) ($n = 32$) and grass pollen allergy (C) ($n = 17$) at the y-axis. The x-axis shows the tested recombinant wheat proteins #10, #37, #38, #112, #123 and #126, wheat pollen extract, recombinant grass pollen allergens Phl p 1, Phl p 5, Phl p 7 and Phl p 12, a 'Wheat Mix' comprising all recombinant wheat proteins, wheat flour and timothy grass CAP used to measure IgE reactivity.

diagnosed with a combination of spotted rPhl p 1 and rPhl p 5. Recombinant Phl p 12 (36%) was always stronger and more often recognized than wheat profilin #123 (23%). The cross-reactive pollen allergen rPhl p 7 reacted with 13.5% of the sera.

Each of the patients with food allergy to wheat were positive in the wheat flour CAP, but did not react with the recombinant wheat proteins #10, #37, #38, #112 and #126 (Fig. 2B). The patient who was positive to the cross-reactive timothy grass pollen profilin, had also a positive signal to wheat profilin #123 in the micro-array.

Fifty-six per cent of patients with wheat-induced food allergy showed IgE reactivity to the Phleum CAP, but only 25% were positive to wheat pollen extract on the chip and 32% were detected with the combination of spotted rPhl p 1 and rPhl p 5. Spotted rPhl p 1 and rPhl p 5 alone were recognized by 25% and 19% of the sera respectively, the cross-reactive allergens rPhl p 7 and rPhl p 12 reacted with 6% and 22% of the sera respectively.

Recombinant Phl p 1 and rPhl p 5 are diagnostic marker allergens for grass pollen allergy

Each of the 17 grass pollen allergic patients was positive when a combination of rPhl p 1 and rPhl p 5 was used in the micro-array and to the Phleum CAP (Fig. 2C). These findings are in accordance with previously published data suggesting that grass pollen allergy can be diagnosed by IgE reactivity to rPhl p 1 and rPhl p 5 (22, 23).

Sixty-five per cent of grass pollen allergic patients were positive in the wheat flour CAP, but none of these patients exhibited clinical symptoms (i.e. respiratory or food allergy) to wheat seed products. Out of the recombinant wheat proteins tested in the micro-array, only recombinant wheat profilin #123 was recognized by 23.5% of the patients (Fig. 2C). Each of these patients was also positive to the cross-reactive timothy grass pollen profilin rPhl p 12. rPhl p 12 was always stronger and more frequently (35%) recognized than #123, wheat profilin (23.5%). All patients suffering from grass pollen allergy were positive when tested with the timothy grass pollen CAP and in the combination of spotted rPhl p 1 and rPhl p 5. Recombinant Phl p 1 alone was more often recognized (94%) than rPhl p 5 (88%) by patients with grass pollen allergy. Wheat pollen extract reacted with 82% of the sera and cross-reactive rPhl p 7 with 29%.

Association of clinical allergy with allergen extract- and recombinant allergen-based IgE serology

From the 22 patients with respiratory symptoms to wheat (Table 1), 91% could be diagnosed by IgE serology with wheat flour extract and 64% when the mix of the so far isolated six recombinant wheat allergens was used in the micro-array (Fig. 2A). Among this patients group, all patients (i.e. 50%) with grass pollen sensitization were

diagnosed with a combination of micro-arrayed rPhl p 1 and rPhl p 5 or when timothy grass pollen extract was used (Fig. 2A).

Each of the 32 patients with wheat-induced food allergy was diagnosed using wheat flour extract, whereas five of the recombinant wheat allergens (#10, #37, #38, #112, #126) showed no IgE reactivity and thus seemed to be specifically recognized by patients with respiratory wheat allergy. Only profilin showed IgE reactivity with 3% of the patients. For six (i.e. 19%) of the patients with wheat-induced food allergy, evidence for a clinically relevant grass pollen allergy was found in the case record forms. Each of these patients was diagnosed with a combination of micro-arrayed rPhl p 1 and rPhl p 5. The IgE serology with timothy grass pollen extract indicated that 56% of the patients with wheat-induced food allergy also had grass pollen sensitization, but this result did not match the percentage of patients with clinical symptoms of grass pollen allergy or clinical data regarding grass pollen allergy were not available. Positive serological test results without evidence for clinical sensitization were found mainly for patients with timothy grass pollen-specific IgE levels lower than 5 kUA/l.

Each of the 17 patients with grass pollen allergy was diagnosed with a combination of micro-arrayed Phl p 1 and Phl p 5 or timothy grass pollen extract. Although none of these patients had clinically relevant symptoms of respiratory or food allergy to wheat, 65% gave false positive test results with wheat extract (Fig. 2C).

Discussion

Allergens from wheat (*T. aestivum*) can mediate baker's asthma, food allergy and grass pollen allergy. Here, we investigated the usefulness of micro-arrayed recombinant wheat seed allergens and recombinant grass pollen allergens for the differential diagnosis of respiratory allergy to wheat flour, wheat-induced food allergy and grass pollen allergy. We compared IgE antibody reactivities to traditional allergen extracts and micro-arrayed recombinant allergens in three groups of clinically well-characterized patients.

The panel of six recombinant wheat seed allergens had been obtained by IgE immunoscreening of wheat seed cDNA library using sera from patients with baker's asthma. Of the six wheat seed allergens described by us, only thioredoxin H has been identified earlier as wheat allergen together with an alpha amylase inhibitor, gliadins, a serine carboxypeptidase, a leucine-rich repeat protein, agglutinin, a putative high mobility group protein and a Phl p 1 homologue (24). Other earlier described wheat allergens comprise alpha amylase inhibitor, omega 5 gliadin, members of the alpha amylase/trypsin inhibitor family, glutenin, lipid transfer protein, peroxidase, serpin, a lipid binding protein and glycogen/starch synthase (25–28).

Using four of the recombinant allergens isolated by us, we were able to differentiate between respiratory forms of wheat allergy and food allergy to wheat, which indicates that patients suffering from baker's asthma are sensitized to allergens, which are different from those recognized by patients suffering from food allergy to wheat. The allergen panel is not yet complete and also other allergens may be involved in baker's asthma because only 64% of the patients suffering from respiratory allergies to wheat were diagnosed, but our results indicate that it may be possible to develop recombinant allergen-based tests for the discrimination of the two types of wheat-induced allergies.

Except profilin, which occurs as cross-reactive structure in grass pollen and wheat seeds, the other five recombinant wheat seed allergens were specifically recognized by patients suffering from allergies to wheat seed products, but not by grass pollen allergic patients. The recombinant wheat seed allergens should therefore also be useful to discriminate pollen and seed-induced forms of allergies. The latter aspect seems to be of particular importance because we found that half of the wheat food allergic patients with positive IgE reactivity to crude timothy grass pollen extract showed no evidence for a clinically relevant grass pollen allergy. Moreover, 65% of the patients with grass pollen allergy exhibited false positive IgE test results to crude wheat seed extracts, but none of these patients suffered from clinically relevant respiratory or food allergy to wheat seed products. These findings are in accordance with former studies, which have shown that a CAP-FEIA positive result to wheat flour extract does not correlate with clinical symptoms (9). Our results thus indicate that *in vitro* diagnosis of allergy to wheat may be improved by using micro-arrayed recombinant wheat seed allergens.

In agreement with former investigations, we found that the clinical condition of grass pollen allergy could be readily diagnosed with recombinant timothy grass pollen marker allergens Phl p 1 and Phl p 5, which therefore can be confirmed as marker allergens for a genuine sensitization to grass pollen (22, 23, 29). Moreover, it was possible to identify with micro-arrayed recombinant grass pollen allergens (e.g. Phl p 1, Phl p 5) those patients among the patients with wheat-induced food allergy and bakers asthma, who suffered also from grass pollen-related symptoms.

In addition to the species-specific marker allergens rPhl p 1 and rPhl p 5, the cross-reactive allergens rPhl p 7 and rPhl p 12 were included in the micro-array. We observed that all patients positive to wheat seed profilin also were positive to timothy grass pollen profilin (rPhl p 12), which can be explained by the high cross-reactivity between profilins from different allergen sources (30).

In conclusion, our study demonstrates that micro-arrays based on recombinant wheat seed allergens and grass pollen allergens may be useful to discriminate among three different clinical conditions of allergy caused by grasses, i.e. wheat flour-induced respiratory allergy, food allergy to wheat seeds and grass pollen allergy. These tests provide fast results regarding the IgE reactivities against large numbers of allergen molecules based on only minute amounts of serum and thus offer a large number of diagnostic informations to the allergologist, which should facilitate diagnosis and therapy of allergies.

Acknowledgments

This study was supported by grant F1815 of the Austrian Science Fund (FWF), a research grant from Phadia, Uppsala, Sweden and by the Christian Doppler Association, Vienna, Austria.

References

1. Baur X, Degens PO, Sander I. Baker's asthma: still among the most frequent occupational respiratory disorders. *J Allergy Clin Immunol* 1998;**102**: 984–997.
2. Bock SA. Prospective appraisal of complaints of adverse reactions to foods in children during the first 3 years of life. *Pediatrics* 1987;**79**:683–688.
3. Bock SA, Sampson HA. Food allergy in infancy. *Pediatr Clin North Am* 1994;**41**:1047–1067.
4. Jansen JJ, Kardinaal AF, Huijbers G, Vlieg-Boerstra BJ, Martens BP, Ockhuizen T. Prevalence of food allergy and intolerance in the adult Dutch population. *J Allergy Clin Immunol* 1994;**93**:446–456.
5. Sampson HA. Food allergy. Part 1: Immunopathogenesis and clinical disorders. *J Allergy Clin Immunol* 1999;**103**:717–728.
6. Baldo BA, Sutton R, Wrigley CW. Grass allergens, with particular reference to cereals. *Prog Allergy* 1982;**30**: 1–66.
7. Freidhoff LR, Ehrlich-Kautzky E, Grant JH, Meyers DA, Marsh DG. A study of the human immune response to *Lolium perenne* (rye) pollen and its components, Lol p I and Lol p II (rye I and rye II). I. Prevalence of reactivity to the allergens and correlations among skin test, IgE antibody, and IgG antibody data. *J Allergy Clin Immunol* 1986;**78**:1190–1201.
8. Donovan GR, Baldo BA. Crossreactivity of IgE antibodies from sera of subjects allergic to both ryegrass pollen and wheat endosperm proteins: evidence for common allergenic determinants. *Clin Exp Allergy* 1990;**20**:501–509.
9. Jones SM, Magnolfi CF, Cooke SK, Sampson HA. Immunologic cross-reactivity among cereal grains and grasses in children with food hypersensitivity. *J Allergy Clin Immunol* 1995;**96**:341–351.
10. Sander I, Raulf-Heimsoth M, Duser M, Flägel A, Czuppon AB, Baur X. Differentiation between cosensitization and cross-reactivity in wheat flour and grass pollen-sensitized subjects. *Int Arch Allergy Immunol* 1997;**112**:378–385.

11. Quirce S, Fernandez-Nieto M, Escudero C, Cuesta J, de Las Heras M, Sastre J. Bronchial responsiveness to bakery-derived allergens is strongly dependent on specific skin sensitivity. *Allergy* 2006;**61**:1202–1208.
12. Bock SA, Sampson HA, Atkins FM, Zeiger RS, Lehrer S, Sachs M et al. Double-blind, placebo-controlled food challenge (DBPCFC) as an office procedure: a manual. *J Allergy Clin Immunol* 1988;**82**:986–997.
13. Vrtala S, Fischer S, Grote M, Vangelista L, Pastore A, Sperr WR et al. Molecular, immunological, and structural characterization of Phl p 6, a major allergen and P-particle-associated protein from Timothy grass (*Phleum pratense*) pollen. *J Immunol* 1999;**163**:5489–5496.
14. Constantin C, Quirce S, Grote M, Touraev A, Swoboda I, Stoecklinger A et al. Molecular and immunological characterization of a wheat serine proteinase inhibitor as a novel allergen in baker's asthma. *J Immunol* 2008;**180**:7451–7460.
15. Nystrand M. A multiplexed immunoassay for the rapid detection of specific IgE in allergy diagnosis. *IVDT* 2006;**61**: (Technical Note).
16. Nilsson K, Bennich H, Johansson SG, Ponten J. Established immunoglobulin producing myeloma (IgE) and lymphoblastoid (IgG) cell lines from an IgE myeloma patient. *Clin Exp Immunol* 1970;**7**:477–489.
17. Laffer S, Valenta R, Vrtala S, Susani M, van Ree R, Kraft D et al. Complementary DNA cloning of the major allergen Phl p I from timothy grass (*Phleum pratense*); recombinant Phl p I inhibits IgE binding to group I allergens from eight different grass species. *J Allergy Clin Immunol* 1994;**94**:689–698.
18. Vrtala S, Sperr WR, Reimtz I, van Ree R, Laffer S, Muller WD et al. cDNA cloning of a major allergen from timothy grass (*Phleum pratense*) pollen; characterization of the recombinant Phl pV allergen. *J Immunol* 1993;**151**:4773–4781.
19. Niederberger V, Hayek B, Vrtala S, Laffer S, Twardosz A, Vangelista L et al. Calcium-dependent immunoglobulin E recognition of the apo- and calcium-bound form of a cross-reactive two EF-hand timothy grass pollen allergen, Phl p 7. *FASEB J* 1999;**13**:843–856.
20. Valenta R, Ball T, Vrtala S, Duchene M, Kraft D, Scheiner O. cDNA cloning and expression of timothy grass (*Phleum pratense*) pollen profilin in *Escherichia coli*: comparison with birch pollen profilin. *Biochem Biophys Res Comm* 1994;**199**:106–118.
21. Sampson HA. Food allergy. Part 2: diagnosis and management. *J Allergy Clin Immunol* 1999;**103**:981–989.
22. Kazemi-Shirazi L, Niederberger V, Linhart B, Lidholm J, Kraft D, Valenta R. Recombinant marker allergens: diagnostic gatekeepers for the treatment of allergy. *Int Arch Allergy Immunol* 2002;**127**:259–268.
23. Andersson K, Lidholm J. Characteristics and immunobiology of grass pollen allergens. *Int Arch Allergy Immunol* 2003;**130**:87–107.
24. Weichel M, Vergoossen NJ, Bonomi S, Scibilia J, Ortolani C, Ballmer-Weber BK et al. Screening the allergenic repertoires of wheat and maize with sera from double-blind, placebo-controlled food challenge positive patients. *Allergy* 2006;**61**:128–135.
25. James JM, Sixbey JP, Helm RM, Bannon GA, Burks AW. Wheat alpha-amylase inhibitor: a second route of allergic sensitization. *J Allergy Clin Immunol* 1997;**99**:239–244.
26. Palosuo K, Varjonen E, Kekki OM, Klemola T, Kalkkinen N, Alenius H et al. Wheat omega-5 gliadin is a major allergen in children with immediate allergy to ingested wheat. *J Allergy Clin Immunol* 2001;**108**:634–638.
27. Simonato B, De Lazzari F, Pasini G, Polato F, Giannattasio M, Gemignani C et al. IgE binding to soluble and insoluble wheat flour proteins in atopic and non-atopic patients suffering from gastrointestinal symptoms after wheat ingestion. *Clin Exp Allergy* 2001;**31**:1771–1778.
28. Pastorello EA, Farioli L, Conti A, Pravettoni V, Bonomi S, Iametti S et al. Wheat IgE-mediated food allergy in European patients: alpha-amylase inhibitors, lipid transfer proteins and low-molecular-weight glutenins. Allergenic molecules recognized by double-blind, placebo-controlled food challenge. *Int Arch Allergy Immunol* 2007;**144**:10–22.
29. Laffer S, Spitzauer S, Susani M, Pairleitner H, Schweiger C, Gronlund H 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* 1996;**98**:652–658.
30. Radauer C, Willeroider M, Fuchs H, Hoffmann-Sommergruber K, Thalhammer J, Ferreira F et al. Cross-reactive and species-specific immunoglobulin E epitopes of plant profilins: an experimental and structure-based analysis. *Clin Exp Allergy* 2006;**36**:920–929.