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Skin-prick test and RAST responses to cereals in children with atopic dermatitis. Characterization of IgE-binding components in wheat and oats by an immunoblotting method

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Summary

Background Hypersensitivity to cereals may occur via inhalation or ingestion. Although cereals are essential in the daily nutrition, only little information is available of the allergens causing symptoms in patients with atopic dermatitis (AD).

Objective The purpose of the present study was to analyse the IgE immune-response to various cereals and specific cereal fractions of wheat and oats in children with severe AD and correlate the results with challenge studies.

Methods Skin-prick tests (SPT) with a NaCl suspension of wheat, oats, rice, corn, millet and buckwheat and the ethanol soluble gliadin fraction of wheat were performed to 34 wheat/oats challenge positive or negative children with AD. Simultaneously serum total IgE and specific IgE antibody radioallergosorbent test (RAST), levels to wheat, oats and gluten were determined. In addition serum samples of these 34 AD patients and five age matched controls were analysed with IgE immunoblotting using neutral and acidic protein extracts of wheat and oats.

Results From the 34 AD children 33 were SPT positive with wheat and 18 with oats. Positive RAST to wheat and oats could be detected in 32 and 30 samples respectively. From the oral wheat challenge positive children 12/14 appeared positive with gliadin SPT and revealed positive RAST to gluten, but each of the wheat challenge negative were negative in SPT with gliadin. In immunoblotting using neutral and acidic fractions of cereals the IgE binding with sera of challenge positive children showed the most intensive staining, but no correlation was found between different staining patterns and the clinical wheat sensitivity. The 26, 38 and 69 kDa bands in wheat and the 46 and 66 kDa in oats could be classified as major IgE binding proteins of these cereals (> 50% of the sera were positive). SPT with rice, corn, millet or buckwheat was positive in 16/34 patients.

Conclusion Intensive IgE staining to neutral/acidic soluble proteins in wheat and oats was seen, with major IgE binding to 26, 38 and 69 kDa proteins in wheat and 46 and 66 kDa in oats, but no specific IgE staining patterns correlating with clinical cereal sensitivity were found. The strong association between the positive oral wheat challenge and the positive SPT with the ethanol soluble gliadin suggests that also gliadin is an important allergen in wheat-allergic children with AD. The allergens in rice, corn, millet and buckwheat should be better studied before they can be recommended as alternatives for cereal allergic children.

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Introduction

Cereal flours are known to cause baker's asthma/rhinitis by inhalation and the strongest immunoglobulin E (IgE) antibody responses have been observed with albumins and globulins, the proteins soluble in neutral solutions [1,2], although some studies have also shown reactivity to proteins soluble in ethanol or acidic solvents [3]. Recently Pfeil *et al.* [4] and Sanchez-Monge *et al.* [5] have demonstrated with immunoblotting method that the major allergens in neutral fractions of wheat and barley flours appeared mostly in the low molecular weight regions under 20 kDa with sera of baker's asthma patients. On ingestion cereals are known to cause coeliac disease (CD) and dermatitis herpetiformis (DH). Several studies have shown that the ethanol soluble gliadin fraction is the most harmful antigen [6,7] resulting in an IgA and IgG immune-response in these two diseases [8,9].

Although cereals are essential in our daily nutrition and they are often suspected to cause deterioration of atopic dermatitis (AD), especially in children, little information is available of the allergenic components eliciting symptoms in AD patients. Hoffman [10] showed in his study with atopic children mainly with asthma that IgE antibodies measured by radioallergosorbent test (RAST) were bound by the albumin and globulin fractions, less by gliadin and gluten. Sutton *et al.* [11] used sera of 20 paediatric patients with asthma and/or eczema with high RAST scores to wheat and found out that the globulins and glutenins were able to bind more IgE than the gliadins and albumins.

In the present study we have used the sensitive immunoblotting method to analyse sera from 34 wheat/oats challenge positive or negative children with AD to investigate the allergenic components in wheat and oats. The SPT responses to other cereals were measured simultaneously. Five non-atopic control sera were included for comparison in the immunoblotting experiments.

Materials and methods

Patients

The sera were obtained from 34 children attending the

Skin and Allergy Hospital of Helsinki University. They had been sent to the hospital because of severe and extensive AD with intense itching and suspicion of food allergy. In addition to dermatitis, 12 had asthma, six had allergic rhinitis and four both asthma and allergic rhinitis. The diagnosis of AD was established by pediatricians or dermatologists according to the criteria of Hanifin and Rajka [12]. Four patients were under 1 year of age, 20 between 1 and 5 years and 10 between 5 and 11 years. From the patients 19 were males and 15 females. Each patient was found allergic to one or more food products confirmed by positive challenge tests performed and followed up at the hospital. Before the challenges the children were kept on a diet eliminating the suspected foods for at least 2 weeks. They were also treated with topical hydrocortisone. The dermatitis was at most mild when the challenges were performed and the results were evaluated by the treating physician. The challenge tests with cereals were carried out in an open way starting with the suspected cereal as a drop of porridge cooked in water applied on intact skin on the volar side of the wrist and on the upper lip. Development of weal(s) on the test site was considered positive. If the skin test was negative the offending cereal was given orally with doses of 1, 5 and 10 g. Two doses were given during the day and the challenge was continued for three consecutive days up to 10 g twice a day. It was stopped immediately if clear symptoms appeared. During oral challenges five children had developed symptoms within 2 h. These included local urticaria (Nos. 5 and 29, Table 1), itching in the mouth and throat besides local urticaria (Nos. 4 and 6) and generalized urticaria and rhinitis (No. 11). The delayed reactions, which occurred several hours to 3 days after starting the challenge, were seen in 10 children. Eight children had developed erythema and pruritus causing intensive scratching (Nos. 1–3, 8–9, 12, 17, 34), one had developed morbilliform rash (No. 7) and one child had abdominal pain followed by pruritus of the skin (No. 26). Six children had developed several weals on skin contact site (Nos. 10, 13–15, 18, 27) and three of them (Nos. 13, 15, 18) had experienced wheezing from wheat flour. These six patients were not orally challenged. In SPT 17 patients had been tested with inhalant allergens and 13 were positive to tree pollens (76%), six to grass pollens (35%) and nine (53%) to animal dander (cat and/or dog). At the time of the present study 20 were

Table 1.

No	Age/sex	Symptom	Skin-prick test			RAST kU/L			Total IgE kU/L
			Wheat	Oats	Gliadin	Wheat	Oats	Gluten	
1	3y 10m /M	AD	++++ *O	+++ *O	+++	66.0	28.0	57.7	3000
2	2y 4m /M	AD,A	+++ *O	+++ *O	++	96.0	71.0	> 100	16000
3	1y 1m /M	AD	+++ *O	-	*O	6.0	1.4	4.5	600
4	11y /M	AD,A,R	++ *O	-	ND	14.0	1.7	10.4	140
5	3y 6m /F	AD	+++ *O	++	++	18.0	2.1	17.5	130
6	6y /F	AD	+++ *O	+++	+++	34.0	2.4	16.7	56
7	5y 8m /F	AD,A,R	+++ *O	++	++	3.0	1.9	1.7	270
8	6y 5m /F	AD,R	++++ *O	-	-	4.6	1.0	3.5	200
9	1y 6m /F	AD	++++ *O	+++	+++	23.0	2.2	6.0	47
10	2y 7m /F	AD	++ *	++	++	5.0	1.5	2.8	47
11	2y 6m /M	AD,A	++ *O	-	+++	64.0	17.0	15.3	730
12	3y 9m /M	AD,A,R	++++ *O	++++	+++	32.0	8.7	13.6	1500
13	2y 7m /F	AD	++++ *	-	++++	20.0	1.4	7.4	53
14	1y 4m /M	AD	+++ *	++	++	44.0	3.5	10.2	430
15	5y 1m /F	AD,A	++ *	-	++	4.1	3.9	2.0	1200
16	2y 6m /F	AD	++	-	-	0.3	0.1	0.0	42
17	6y 4m /F	AD	+++ *O	++	-	14.0	9.4	10.6	2000
18	3y 11m /M	AD,A	++++ *	++++	+++	13.0	1.0	8.2	110
19	5y 9m /M	AD	++	++	-	3.0	2.1	0.0	280
20	1y 3m /F	AD	+++	-	-	22.0	1.8	7.3	130
21	9m /M	AD	++	-	-	3.5	1.1	2.1	340
22	7y 6m /M	AD,R	+++	+++	-	2.9	3.5	0.4	610
23	3y 3m /M	AD	-	-	-	0.4	0.5	0.0	170
24	5y 4m /F	AD,A	++	-	-	19.0	17.0	3.0	2500
25	7y 1m /M	AD,A,R	++	++	-	4.6	4.0	0.5	3200
26	2y 4m /M	AD	++ *O	-	++	3.1	0.5	0.5	89
27	2y 3m /M	AD,A	++++ *	++ *O	++++	44.0	16.0	11.8	990
28	4y 7m /M	AD,A	++	+++	-	7.0	8.0	1.8	1100
29	3y 7m /M	AD,A	+++ *O	++++	++	68.0	10.0	21.1	120
30	11m /M	AD	+++	-	++	5.6	1.6	1.6	320
31	7m /F	AD	+++	-	++	21.0	0.5	2.1	81
32	1y 7m /F	AD	++	-	++	13.0	1.5	2.9	210
33	1y 5m /F	AD	+++	-	++	13.0	0.7	5.4	140
34	5m /M	AD	+++ *O	+++ *O	+++	40.0	8.0	69.9	260

y. years. m. months. M. Male. F. Female. AD. Atopic dermatitis. A. Asthma. R. Rhinitis. ND. Not done. O. Positive oral challenge.
* Positive challenge result.

allergic to cow's milk and 26 to egg, avoiding them in their diet.

The study protocol was approved by the Ethical Committee of the Skin and Allergy Hospital of Helsinki University.

Preparation of cereal antigens

Neutral/acidic extracts Commercial grains of wheat and oats were milled to produce the flour. To obtain the neutral and acidic protein fractions the flour samples

were extracted with potassium phosphate buffer, p.H. 7.0, containing NaCl or with sodium acetate buffer, p.H. 3.8 respectively as previously described in detail [13]. The protein content of each sample was determined according to Lowry *et al.* [14] and equal amounts of neutral and acidic extracts of wheat and oats were pooled for the immunoblotting analysis.

NaCl suspensions Commercial grains of wheat, rye, barley, oats as well as rice, corn, millet and buckwheat were milled to produce the flour. Each cereal flour was

suspended to 1 : 10 v/v with 0.9% NaCl overnight to get the SPT suspensions.

Purified gliadin A total of 10 mg of commercial gliadin (BDH, British Drug Houses, Poole, UK) was dissolved in a mixture of 2.5 mL of 96% ethanol and 2.5 mL of albumin diluent and centrifuged at 10 000 rpm for 30 min. Then the supernatant was suspended with equal amount of 85% glycerol (containing phenol) and sterile filtrated through 0.22 µm Millipore filter to get the 1 mg/mL test solution used in SPT.

SPT

All the patients underwent SPT with the NaCl suspensions of wheat, rye, barley and oats as well as rice, corn, millet and buckwheat. Purified gliadin in ethanol was tested with the 1 mg/mL test solution. SPT with commercial inhalant allergens, including grass, birch and alder pollens, cat and dog dander (ALK, Allergologisk Laboratorium, Copenhagen, Denmark) had been carried out earlier in patients with asthma and/or rhinitis and when inhalant allergy was suspected. Each patient had been tested also with cow's milk and egg (ALK, Copenhagen, Denmark). A weal with a diameter of 3 mm and more than half of that of the weal caused by histamine hydrochloride (10 mg/mL) was regarded positive, provided that the vehicle control appeared negative and was graded as follows: ++ half of the size, +++ equal to, or ++++ twice as large as the positive control.

Laboratory tests

Blood samples were taken during the same occasion to be analysed for specific IgE antibodies to wheat, rye, barley, oats and gluten (RAST, Pharmacia, Uppsala, Sweden) and serum total IgE (CAP RIA, Pharmacia, Uppsala, Sweden). RAST > 0.5 kU/L was regarded positive. Anti-gliadin IgA and IgG antibodies were evaluated from 31 patients with commercial solid phase enzyme immuno-metric assay (Gluten EIA, Pharmacia, Uppsala, Sweden), in which positive value for IgA was regarded > 10 AU (arbitrary units) and for IgG > 85 AU. Anti-reticulin antibodies were measured from 14 patients by an indirect immunofluorescence method [15]. Titres > 5 were regarded positive.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Pooled acidic and neutral protein extracts of wheat and oats and molecular weight markers (Rainbow, Amersham, Buckinghamshire, UK) were separated by SDS-

PAGE according to Laemmli [16]. Equal amounts of cereal proteins were loaded into the slots (1.5 mg/122 mm slot) and were separated in a large 5–17% gradient gel under non-reducing conditions. The proteins were then transferred to nitro-cellulose sheets (Schleicher & Schnull, 0.2 µm, Dassel, Germany) in a transphor apparatus (LKB, Bromma, Sweden) according to Towbin *et al.* [17]. The protein bands on gel were stained with Coomassie brilliant blue. After transfer the nitrocellulose sheet was cut into strips for incubation with the patients' sera. A reference strip (incubated in buffer only) and five non-atopic control sera were included as controls in the assay.

The nitrocellulose strips were then incubated at room temperature overnight with each patient sera diluted 1 : 4 with phosphate buffered saline (PBS) — 0.5% Tween. Then the strips were washed three times for 10 min with 0.9% NaCl–0.5% Tween and thereafter they were incubated for 4 h at RT with ¹²⁵I-labelled anti-human IgE (Cap RAST, Pharmacia, Uppsala, Sweden) diluted 1 : 10 with PBS–0.5% Tween. The ¹²⁵I-labelled bands were detected by a sensitive autoradiography method using x-omatic film (Kodak, USA), an intensifying screen and storage at –70° C.

Results

Patients

The characteristics of the patients, who were skin-prick tested with various cereals and whose sera were used in RAST and total IgE determinations and in immunoblotting experiments are presented in Table 1.

The reactivity with wheat Table 1 shows the SPT and RAST results as well as the total IgE values of the patients. The children's numbers in Table 1 refer to the numbers in Fig. 1A, which shows the immunoblotting patterns of the patients' sera against wheat.

All the patients, except one, reacted to the NaCl wheat suspension in SPT and they also had elevated wheat RAST values. The patients who were challenge positive tended to have large SPT reactions, although 8/9 patients who were challenge negative still displayed positive SPT with the NaCl suspension of wheat and they also had elevated wheat RAST values (Table 1). Positive SPT reactions to gliadin were seen in 22/23 cases; in all of the six with positive skin contact challenge and in 12 out of 14 with positive oral wheat challenge (Table 2). None of those who were wheat challenge negative displayed positive SPT with gliadin. The positive gliadin SPT result correlated also with the gluten RAST, although among

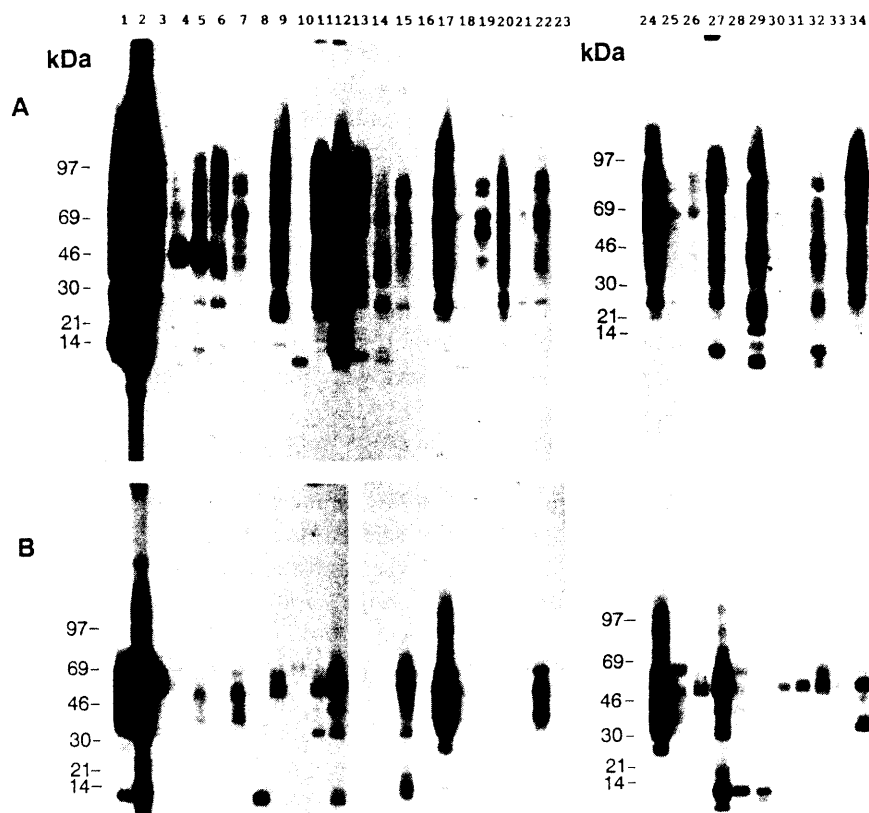


Fig. 1. IgE immunoblots against wheat and oats with sera from 34 atopic children. A. Wheat, lanes 1–15, 17, 18, 26, 27, 29 and 34 represent immunoblots of the wheat challenge positive patients. B. Oats, lanes 1–3, 27 and 34 represent immunoblots of the oats challenge positive patients.

11 patients with negative gliadin SPT six appeared positive in gluten RAST, but four of these six patients were wheat challenge negative (Table 1).

The immunoblotting analysis revealed IgE stained bands in 29/34 studied sera and the most intensively stained blots were seen in patients with high total or cereal specific IgE values. Altogether 20 various IgE stained bands were seen. The most frequent staining was obtained with the 26 and 69 kDa bands which both were positive in 26 patients (89%), followed by the 38, 24 and 79 kDa bands (62%, 48% and 41% of the samples were positive respectively). Bands at 8, 11, 22 and 82 kDa were found in eight patients (27%) and bands at 30, 40 and 58 kDa in 7 (24%). No variation was noted in the binding patterns between patients with pure dermatitis compared with those with concomitant

asthma and/or rhinitis or between wheat challenge positive or negative patients. The IgE staining pattern was found more intense with the serum samples obtained from children with positive gliadin SPT or gluten RAST. Intensively stained bands were also found in four sera from patients who were challenge negative (Fig. 1A), two of them had asthma or rhinitis besides AD and were allergic to tree and/or grass pollens. Some faint staining was seen also in control sera and the reference strip 26, 69 and 79 kDa bands in extensively long autoradiography. These bands were easily distinguishable from the specific bands.

From the results with wheat it can be concluded that the 26, 38 and 69 kDa bands could be classified as major IgE binding proteins (> 50% of the samples were positive). Besides the neutral soluble proteins, the ethanol

Table 2. Comparison of results of SPT with gliadin and wheat challenge

		Oral wheat challenge	
		+	–
SPT gliadin	+	12	0
	–	2	9

Table 3. Comparison of results of gluten RAST and wheat challenge

		Oral wheat challenge	
		+	–
RAST gluten (> 5.5 kU/L)	+	14	4
	–	1	5

and partly also acidic soluble gliadin was found to be an important wheat allergen in children with AD.

The reactivity with oats The SPT and RAST results for oats are presented in Table 1 and the individual IgE immunoblots in Fig. 1B. There were 18 positive SPT reactions with oats but only five of these children were oats challenge positive. Two patients were negative in SPT but had high specific RAST value (Nos. 11 and 24). The others with negative SPT tended to have only slightly positive RAST values compared with those with positive SPT. The SPT with oats was positive in 4/5 challenge positive patients and in 14/29 challenge negative cases.

In immunoblotting the binding patterns with oats were more variable than with wheat and 28/34 sera were found to be positive. Intensively stained bands were observed at molecular weight regions between 30 and 69 kDa as also at 8 and 10 kDa. Altogether 14 various IgE stained bands could be detected. The 46 kDa band was strongly stained by 19 sera (67%), followed by the 66 and 30 kDa bands (53% and 35% respectively). Sera of five patients were seen to bind to 84 and 92 kDa bands (17%) (Fig. 1B). Some weak staining occurred at 30, 46 and 66 kDa bands also in the non-atopic control sera and the reference strip.

Thus, in oats the 46 and 66 kDa bands were stained by > 50% of the sera and could be classified as major IgE binding proteins. Fewer IgE stained bands were found than with wheat suggesting oats flour to have less allergenic properties than wheat flour.

SPT with other cereals The SPT results with rye and barley were always seen to be positive when positive reactions with wheat was seen and the corresponding RAST results correlated well with the results of SPT. The SPT results with rice, corn, millet and buckwheat are presented in Fig. 2. Positive reactions were seen to at least one of these cereals in 16 patients (47%) and buckwheat was most often responsible for these reac-

tions (13/16 positive). Positive results to these four cereals were seen simultaneously with wheat. Nine of those patients who were positive with rice, corn, millet or buckwheat had also symptoms of asthma and/or rhinitis besides atopic dermatitis.

Other laboratory tests Measures for anti-gliadin IgA and IgG antibodies were taken from 31 of the patients and 14 of these were also evaluated for the presence of anti-reticulin antibodies. None of the patients had elevated anti-reticulin antibodies, but one patient had an elevated anti-gliadin IgG antibody level (value 104 AU, normal value < 85 AU). He was negative for anti-gliadin IgA and anti-reticulin antibodies and he did not have any gastrointestinal symptoms suggesting for CD (patient no. 12).

Discussion

Many previous studies have shown that food hypersensitivity plays an important role in children with AD and that appropriate diagnosis and allergen avoidance, e.g. elimination diets result in clinical improvement [18,19]. Cereals belong to the group of foods, among egg, milk, fish, peanut and soy, which cause most frequently allergic reactions [20]. SPT and RAST are the most often used diagnostic tools. The value of these assays has been questioned especially when cereal allergy is suspected [21] and several studies have suggested that commercial extracts may lack the relevant allergens to screen for food allergy [22,23,24].

In the present study we have evaluated the IgE antibody response against various cereals by SPT and RAST and characterized the allergenic fractions in neutral and acidic extracts of wheat and oats in 34 highly atopic children using the sensitive immunoblotting method.

In 33/34 patients the NaCl suspension of wheat elicited a positive SPT reaction which correlated well with the corresponding RAST. All the patients who reacted with wheat were also positive with rye and barley, which can

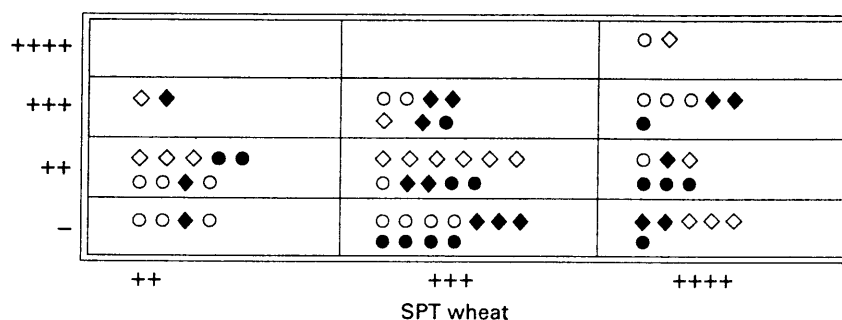


Fig. 2. SPT results with (○) rice, (◆) corn, (●) millet and (◇) buckwheat in 16 wheat SPT positive children.

be explained by the fact that these three species are closely related grasses and all belong to the tribe Triticeae from the Gramineae family [8,25]. Oats are not so closely related to wheat than rye and barley belonging to the tribe Aveneae. It showed less IgE binding bands than wheat and seems to have less allergenic properties and is often the first of these cereals to which the previously allergic patients lose their hypersensitivity. The cereal allergic children of our study were also frequently SPT positive with rice, corn, millet and buckwheat. This may be due to the crossreactivity or may reflect true sensitization, as these cereals are recommended as alternatives for patients with allergy to wheat and its close relatives. Rice allergy is common in countries where it is widely used and it may crossreact with wheat, rye, corn and millet [26]. Buckwheat, which actually is not a member of the grass family but a herbaceous species, has been found to elicit IgE antibody response in atopic patients [27].

It is evident that the crude NaCl flour suspensions which we used in the SPT are mixtures of different allergens of the cereals containing mainly the neutral soluble proteins, albumins and globulins, but also some of the glutenins and gliadins. They elicited positive reactions even in the 7/8 wheat and 14/29 oats challenge negative patients. Thus these extracts revealed also IgE response which did not correlate with the clinical cereal sensitivity. On the other hand, 12/14 of the oral wheat challenge positive and all six wheat skin contact positive patients had a positive reaction in gliadin SPT and the result correlated well with gluten RAST. Earlier this association has been rather overlooked. Goldstein *et al.* [28] described a single case with gastrointestinal and pulmonary symptoms caused by wheat with a strong reaction to gliadin. In their studies Sutton *et al.* [11] found in 20 children with asthma and/or eczema that the neutral soluble globulins and the ethanol soluble gliadins gave the highest RAST uptakes with pooled sera, but testing with individual sera the globulins and glutenins bound IgE most often. However, no correlation with provocation studies with cereals was examined.

In our study four patients (Nos. 30–33) who were allergic to cow's milk were completely cleared from AD when also cereals (wheat, rye, barley and oats) were withdrawn from the diet, including breast-feeding mothers. The patients were challenged with oats with negative result. Wheat was not yet challenged and was not introduced in their diet either. They all displayed positive SPT results with wheat and were clearly positive in corresponding RAST, gliadin SPT as well as gluten RAST. Two of these (Nos. 30 and 31) and patient No 34 could have been sensitized through breast-feeding because cereal products had not yet been given to them.

Our immunoblotting analysis was carried out with

pooled neutral and acidic soluble fractions of wheat and oats. There were many protein bands detected both by the sera of challenge positive or negative children. The results were in line with our previous IgE immunoblotting study with 35 adult AD patients [13], in which we found the most frequent binding with wheat at 26, 79 and 69 kDa bands, compared with the 26 and 69 kDa bands in this study. The 66 kDa protein in oats was recognized by 84% of the adult AD sera and among children the most frequent staining was seen to 46 and 66 kDa bands. The adult patients exhibited more polyspecific binding patterns than was found with atopic children. Although, in the present study none of the different immunoblotting staining patterns were connected with wheat provocation, it was noted that the most intensive IgE staining to wheat was seen with sera of provocation positive children.

The antigens used in the immunoblotting experiments contained the neutral soluble albumins and globulins and also the acidic soluble glutenins and gliadins. Thus at least a part of the immunoblotting staining might be due to the glutenin and gliadin fractions, since they gave simultaneously many clearly positive results when measured by SPT and RAST. Gliadin has until now been mainly linked to celiac disease. In baker's asthma the most prominent allergens have been described in the neutral soluble extracts of wheat and barley with a molecular weight under 20 kDa [1,4] and in the study by Sanchez-Monge *et al.* [5] the carbohydrate moieties of proteins from wheat and barley seemed to be essential in IgE binding capacity. Thus the neutral soluble fractions, albumins and globulins, were evidently the allergens which were readily soluble through the mucous membranes of the respiratory tract of baker's asthma patients.

Crossreactivity of IgE antibodies between wheat flour and ryegrass pollen allergens has been reported [29], although some studies using sera from young atopic children have shown poor correlation between cereals and grass pollens [10,11]. However, this crossreactivity suggests that both sources must be regarded as potentially involved in the sensitization of the patients and could have accounted for some of the binding in our immunoblotting studies, especially in older children with concomitant asthma and/or rhinitis.

It could be suggested from the results of our study, that in AD also the least soluble glutenins and gliadins are important when ingested cereals are suspected causes of dermatitis. Further IgE-immunoblotting studies, especially with the ethanol soluble fractions, are in progress to characterize these allergens and to investigate their role in the pathogenesis of AD.

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