

IgE binding to soluble and insoluble wheat flour proteins in atopic and non-atopic patients suffering from gastrointestinal symptoms after wheat ingestion

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Summary

Background The involvement of IgE-mediated hypersensitivity reactions in the genesis of gastrointestinal symptoms after ingestion of foods containing wheat has been rarely reported.

Objective To detect IgE specifically binding to wheat proteins in the sera of atopic and non-atopic patients suffering from gastrointestinal symptoms after ingestion of wheat and to evaluate the reliability of skin prick test and CAP in the diagnosis of food allergy to wheat.

Methods The sera of patients (10 atopic and 10 non-atopic) previously diagnosed as suffering from irritable bowel syndrome and complaining of symptoms after wheat ingestion were analysed by immunoblotting for IgE binding to water/salt-soluble and insoluble wheat flour proteins.

Results All the atopic patients and only one of the non-atopic patients were positive to wheat CAP. For the patients tested, skin prick test was positive for all the atopic patients and for only one of the non-atopic patients. However, immunoblotting experiments showed the presence of specific IgE to wheat proteins in all the patients. Ten out of 11 of the wheat CAP-positive patients had IgE binding to a soluble 16-kDa band, but the same band was recognized, in a slighter way, by only two out of nine of the wheat CAP-negative patients. Moreover, although almost all of the patients were negative in CAP testing with gluten, 19 out of 20 recognized protein bands belonging to the prolamin fraction.

Conclusions For the atopic patients the positivity to skin prick test and CAP to wheat was in accordance with the immunoblotting results and a food allergy to wheat could be diagnosed. In these patients a major allergen was a 16-kDa band corresponding to members of the cereal α -amylase/trypsin inhibitors protein family, the major allergens involved in baker's asthma. In the non-atopic patients the positive immunoblotting results contrasted with the responses of the allergologic tests, indicating that the allergenic wheat protein preparations currently used are of limited value in detecting specific IgE to wheat and that the fraction of irritable bowel syndrome (IBS) patients with food allergy may be larger than believed.

Keywords: atopy, food allergy, irritable bowel syndrome, wheat

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Introduction

Wheat (*Triticum aestivum*) is the staple food in the diets of most of the population. This cereal is particularly important in western countries because its flour has the unique property to give rise to a visco-elastic dough that can be transformed into leavened products and pasta. The proteins of the wheat flour and other related cereals are classically divided into four solubility classes [1]: the water soluble albumins (about 15% of the total protein), the salt (e.g. 0.5 M NaCl) soluble globulins (about 5% of the total protein), the water/salt insoluble monomeric gliadins (soluble in alcohol/water mixtures, about 40% of the total protein) and polymeric glutenin (soluble in diluted acetic acid, about 40% of the total protein), which is made up of high and low molecular weight subunits (HMW-GS and LMW-GS, respectively) linked together by disulphide (SS) bonds. While most of the components of the soluble protein fraction have a physiological function (enzymes, enzyme inhibitors), gliadins and glutenin, that are also known as prolamins and constitute the gluten proteins, are the storage proteins of the seed. A more recent classification that is based on the molecular characteristics rather than on solubility, divides the wheat storage proteins into high molecular weight prolamins (corresponding to HMW-GS, apparent molecular weight, Mr, around 100 kDa), S (sulphur)-poor prolamins, corresponding to ω -gliadins (Mr around 60 kDa) and S (sulphur)-rich prolamins, comprising LMW-GS plus α/β - and γ -gliadins (Mr 31–45 kDa) [2].

IgE-mediated allergy to wheat and other related cereals may develop after inhalation of flour particles or ingestion of foods prepared with these grains. Inhalation of cereal antigens by individuals handling wheat flour results in bakers' asthma [3,4]. In this condition the main IgE response is directed to wheat flour proteins soluble in water/salt solutions (albumins and globulins) [5,6], and in particular to proteins with Mr of 12–18 kDa, which have been shown to belong to the cereal α -amylase/trypsin inhibitor protein family [7,8]. In addition, IgE binding to water/salt insoluble gliadins has also been reported [9].

Hypersensitivity reactions after wheat ingestion have been reported by several authors [10–12] and include cereal-dependent exercise-induced anaphylaxis [13] and various cutaneous symptoms associated with atopic dermatitis (AD) [14]. Both wheat-allergic AD patients and those suffering from cereal-dependent exercise-induced anaphylaxis have been found to have IgE antibodies against gliadins and it has been suggested that peptides deriving from these proteins are important allergens in those patients [13–15].

Ingestion of foods containing wheat and other related cereals can induce gastrointestinal (GI) symptoms by either

immunological or non-immunological mechanisms [16]. Severe GI symptoms occur in coeliac disease, a non-IgE-mediated enteropathy caused by gluten proteins, but also as a result of an IgE-mediated hypersensitivity reaction to wheat proteins [17,18]. In other cases of wheat-induced GI symptoms, whose aetiology can not be established, a general intolerance to wheat is advocated [19].

In this paper we show that patients suffering from GI symptoms after ingestion of wheat and already diagnosed as affected by IBS, had IgE binding to soluble and insoluble wheat proteins, as demonstrated by immunoblotting. This suggests that the IBS symptoms reported by these patients were related to a food allergy to wheat. Moreover, we show that conventional methods used for the diagnosis of IgE-mediated hypersensitivity are inadequate for the allergological screening of patients showing adverse reactions to foods containing wheat. This could explain why IgE-mediated enteropathy from wheat has been rarely reported.

Materials and methods

Patient recruitment and clinical features of patients

Twenty patients who reported gastrointestinal symptoms (diarrhoea, abdominal pain and/or bloating) after ingestion of foods containing wheat were selected from a population suffering from irritable bowel syndrome (IBS) [20]. These patients were selected on the basis of the following criteria: (i) wheat was the food suspected to cause the GI symptoms, according to patients' histories; (ii) the symptoms disappeared with a wheat-free diet and appeared again immediately after the re-introduction of wheat-based foods, as proved by open oral challenge (see below); and (iii) absence of any documented GI disease, including coeliac disease. The patients underwent an intestinal permeability test in order to exclude the possibility that any intestinal damage was present. For each patient a detailed medical history was recorded, paying special attention to past or present allergies. Ten patients who were diagnosed as having bakers' asthma and not suffering from GI symptoms after wheat ingestion were also examined as positive controls. Serum samples from all patients were collected and stored at -20°C .

Oral challenge

Patients were asked to follow a strict wheat-free diet for 7 days before challenge. After this period all of them fully recovered from their GI symptoms.

Open challenge was performed as follows: 80 g of cooked pasta (prepared in the hospital kitchen) were administered to each patient. Patients were kept under

observation for 2 h before dismissal and invited to continue the elimination diet. Twenty-four hours after the challenge patients were examined for the appearance of GI symptoms.

In vitro IgE detection and skin prick tests

The detection of specific IgE to wheat flour and gluten was performed for all patients' sera with the ImmunoCAP-system (CAP-System-FEIA, Pharmacia Diagnostic, Uppsala, Sweden). The results were expressed as CAP scores from class 0–6, according to the manufacturer's instructions. Skin prick tests (SPT) were performed using commercial allergens to the common inhalants (Lofarma, Milan, Italy) and a wheat flour in-house extract prepared by suspending 1 g of flour in 5 mL of saline buffer. Histamine phosphate at 10 mg/mL and glycerol saline solution were used as positive and negative controls, respectively. Reading was taken at 15 min; wheal diameters equal to or larger than 3 mm were considered as positive in the absence of a reaction to the negative control.

Extraction of wheat proteins

Five grams of common (bread) wheat flour (variety San Pastore) were extracted with 50 mL of 0.5 M NaCl for 1 h at 4 °C. After centrifugation (12000 g, 20 min at 4 °C) the supernatant, containing the water/salt-soluble proteins, was collected and stored at –20 °C for subsequent use. The residue was washed with 100 mL of 0.5 M NaCl and centrifuged again. This procedure was repeated and a final wash with distilled water (100 mL) was carried out. The water/salt-soluble protein-free residue was then extracted overnight with 50 mL of 80 mM Tris-HCl, pH 8.0, 50% (v/v) propan-2-ol containing 20 mM dithiothreitol at room temperature (20°C) with continuous stirring. After centrifugation (as above) the supernatant, containing the salt-insoluble (prolamin) group of wheat proteins, was collected and stored at –20 °C.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli [21] in a Mini Protean II slab cell (Bio Rad, Milan, Italy). SDS-gels were prepared with a total polyacrylamide concentration of 15%. Molecular weight standard proteins (Bio Rad) were Phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

Before electrophoresis an aliquot of the salt-soluble protein solution was diluted with 0.25 volumes of a 0.5-M

Tris-HCl buffer, pH 7.2, containing 10% (w/v) SDS, 10% (v/v) 2-mercaptoethanol and 40% (w/v) glycerol and heated for 5 min in a boiling water bath.

Prolamins were precipitated from 1 mL of the propanol solution by addition of 9 mL of cold acetone. After standing at 4 °C for 15 min, the suspension was centrifuged and the supernatant discharged. The air-dried pellet was then solubilized with 2.5 mL of 0.1 M Tris-HCl, pH 7.2, containing 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol and 8% (w/v) glycerol and heated as above. Electrophoresis was run at 50 mA constant current until the tracking dye bromophenol blue reached the bottom of the gel. Gels were stained according to Koenig [22] or used for immunoblotting.

Immunoblotting

After SDS-PAGE separation, proteins were transferred onto nitrocellulose membranes by semidry electroblotting and membranes were probed with individual patients' sera as previously described [23]. Membranes were stained with Ponceau S (0.1% in 3% trichloroacetic acid) and bands marked with a pencil before de-staining with water. The position of the molecular weight standard proteins was marked with peroxidase according to the method of Eberini and Puglisi [24]. Membranes were blocked with TBS containing 0.05% Tween 20 (TBS-T) and 5% skim milk powder (M-TBS-T) for 4 h, and then incubated overnight with single patient sera diluted with 0.5 volumes of M-TBS-T. After washing five times with M-TBS-T, blots were incubated for 1 h with an antihuman IgE peroxidase-conjugate antibody (Sigma) diluted 1:6000 in M-TBS-T. After four washes in M-TBS-T and one with TBS, peroxidase activity was visualized by chemiluminescence using the SuperSignal detection kit (Pierce), following the manufacturer's instructions. The whole procedure was performed at room temperature.

Pooled sera from both healthy subjects and patients with allergy to bee venom were used as controls.

Results

We examined 20 patients affected by IBS in whom an adverse reaction to wheat was suspected on the basis of the results obtained with the elimination diet and with a successive oral challenge (Table 1). In fact, the patients fully recovered from their GI symptoms after a wheat-free diet and experienced the reappearance of the symptoms when challenged with wheat pasta (Table 2).

Of these patients, 10 showed the phenotypic characteristics of atopy, i.e. respiratory and/or cutaneous symptoms plus CAP/SPT positivities to more than one environmental allergen [25]. *In vitro* detection of specific IgE to wheat

Table 1. Allergological features of the patients

Patient	Age	Sex	Total IgE (kU/L)	History of atopy*	SPT Wheat†	CAP-FEIA Wheat	CAP-FEIA Gluten
1	29	M	265	R	Pos.	2	0
2	30	F	2713	RC, A, AD	Pos.	2	1
3	43	M	1053	RC, A	Pos.	3	0
4	60	M	492	RC, AD	ND	1	0
5	23	F	359	RC, A	Pos.	3	0
6	48	F	122	U	Pos.	1	0
7	29	F	114	RC, U	Pos.	1	0
8	30	F	470	R	ND	2	0
9	49	F	ND	A	Pos.	3	0
10	26	F	370	AD	Pos.	5	0
11	30	M	37	No	Neg.	1	0
12	25	F	2	No	Neg.	0	0
13	29	F	3	No	Neg.	0	0
14	60	F	12	No	Neg.	0	0
15	54	F	2118	No	Neg.	0	0
16	32	F	14	No	ND	0	0
17	30	F	42	No	ND	0	0
18	33	M	271	No	Neg.	0	0
19	28	F	323	No	Neg.	0	0
20	28	F	508	No	Pos.	0	0

*A = asthma; AD = atopic dermatitis; R = rhinitis; RC = rhinoconjunctivitis; U = recurrent urticaria. All the atopic patients, when tested for allergy to inhalants by CAP, were found to be positive to at least two substances.

†Pos. = positive reaction; Neg. = negative reaction; ND = not determined.

flour by CAP gave positive results in 11/20 (55%) patients, whereas it was negative in the remaining nine patients. The results with the CAP for wheat flour were in good agreement with those obtained by SPT. In addition, all the patients except one were negative in CAP testing for gluten (Table 1).

In the presence of similar GI clinical manifestations in both atopic and non-atopic patients we considered the possibility of false-negative results in wheat-specific IgE detection with routine laboratory tests. Immunoblotting experiments were then performed with single sera from the CAP-negative patients and the obtained results were compared with those of the CAP-positive ones. Both salt-soluble and salt-insoluble bread wheat flour proteins were considered. As expected, the SDS-PAGE protein patterns of the two solubility classes of wheat flour proteins were different (Fig. 1). The Mr of the major salt-soluble proteins ranged from about 60 to about 14 kDa. The salt-insoluble proteins were divided into the well-known three groups of polypeptides, i.e. HMW (Mr 92–100 kDa), S-poor (Mr around 45–60 kDa) and S-rich prolamins (Mr 31–45 kDa) [2] (Fig. 1).

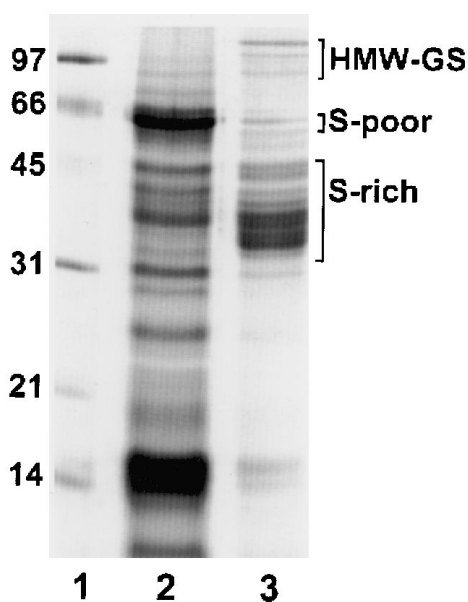
In immunoblotting experiments, all patients' sera were

shown to contain IgE against wheat flour proteins (Fig. 2). With the salt-soluble fraction (Fig. 2, top part), several protein bands were bound, single patients' sera giving different binding patterns. However, in some patterns the same bands were recognized, although with different intensity. In particular, the most frequent staining was obtained with a band of about 16 kDa that was recognized by 12/20 (60%) patients. It is interesting to note that the sera of all but one of the CAP-positive patients recognized the 16 kDa band, whereas among the sera of the nine CAP-negative ones, seven were not reactive to the same band and the other two showed only a faint binding to a band with a similar Mr (Fig. 2). Some other bands were recognized by both CAP-positive and CAP-negative patients (Fig. 2). All the patients suffering from bakers' asthma were positive to SPT and CAP with wheat (data not shown). Immunoblotting patterns representative of the results obtained with this group of patients are shown in Fig. 3. As expected [7,8], the presence of IgE binding to a 16-kDa protein was revealed in the sera of these patients.

The salt-insoluble wheat flour proteins were also bound by all but one of the patients. Most of them recognized bands belonging to the main groups of the wheat storage

Table 2. Gastrointestinal symptoms after challenging the patients with wheat-pasta

Patient	Symptoms
1	Abdominal pain, bloating, diarrhoea
2	Abdominal pain, bloating
3	Abdominal pain, diarrhoea
4	Abdominal pain, bloating
5	Abdominal pain, diarrhoea
6	Bloating, dyspepsia
7	Abdominal pain, bloating
8	Bloating, vomit
9	Abdominal pain, bloating
10	Abdominal pain, bloating, diarrhoea
11	Abdominal pain, diarrhoea
12	Abdominal pain, bloating, diarrhoea
13	Abdominal pain, bloating
14	Abdominal pain, bloating
15	Abdominal pain, bloating
16	Abdominal pain, bloating
17	Abdominal pain
18	Abdominal pain, bloating
19	Abdominal pain
20	Abdominal pain, bloating, diarrhoea

**Fig. 1.** SDS-PAGE analysis of water/salt soluble (lane 2) and insoluble (lane 3) common wheat flour proteins. HMW-GS, S-poor and S-rich indicate the high molecular weight, the sulphur-poor and the sulphur rich prolamins, respectively. Mr standard proteins are in lane 1.

proteins, i.e. HMW, S-rich and S-poor prolamins. In particular LMW glutenin subunit components with Mr around 42 kDa gave a strong reaction in 13/20 (65%) patients. As expected [9], also the sera of the patients suffering from bakers' asthma had IgE binding to some components of the prolamins fraction (Fig. 3).

Similar IgE immunoblotting experiments with pools of sera from both healthy subjects and patients suffering from wheat-unrelated allergy revealed no IgE binding to wheat proteins (not shown).

Discussion

In this study we have examined 20 patients suffering from GI symptoms after ingestion of wheat, without any gut disease. The history of atopy, the improvement of symptoms after an elimination diet and their re-appearance after a challenge with pasta, the detection of wheat-specific IgE by CAP and the SPT positivity, quickly indicated a food allergy to wheat proteins for 50% of the patients.

A food intolerance, rather than a food allergy, would have been diagnosed in the remaining patients with no past history of allergy nor specific IgE to wheat flour and gluten, as detected by CAP. Immunoblotting results, however, revealed that not only the CAP-positive, but also the CAP-negative, patients had IgE directed to soluble and insoluble wheat proteins. Therefore, an IgE-mediated reaction to wheat could be suspected to induce GI symptoms also in the CAP-negative patients.

These data, together with the positive results obtained with both the elimination diet and the open challenge with pasta, are in favour of the hypothesis that allergic reactions to food antigens may be involved in the genesis of symptoms in patients suffering from IBS [26]. In addition to the method of elimination diet and re-challenge, the double-blind placebo-controlled food challenge has been proposed as 'gold standard' for confirmation of food allergy [27]. However, this test, in the case of GI manifestation, is difficult to perform and interpret [28–30]. A specific mucosal provocation test would be necessary in order to fully confirm the allergological basis of the GI symptoms [28].

It is noteworthy that while all patients with a positive history of atopy were found to be CAP-positive, all but one of the non-atopic subjects were CAP-negative. These data indicate that a relationship could exist between atopic condition and CAP response.

For the non-atopic patients there is a discrepancy between the results of the SPT/CAP tests and those of immunoblotting. To explain this, three hypotheses can be made:

- The level of specific IgE in the circulation is low. Although sufficient for immunoblotting detection, the IgE

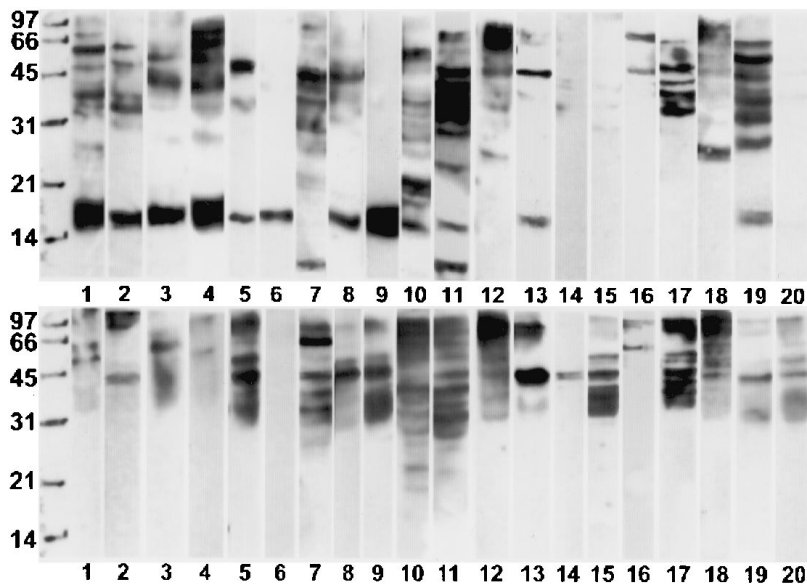


Fig. 2. Immunoblots showing the binding of IgE of single patient sera to the water/salt soluble (top part) and insoluble (bottom part) wheat flour proteins. Numbers correspond to patients listed in Tables 1 and 2. Mr standard proteins are on the left side.

level is beyond the detection limit of CAP and insufficient to induce the cutaneous reaction. In these patients the IgE production should be restricted to the gut [28].

- The proteins bound by the IgE of the non-atopic patients could be absent in the extracts used for SPT and CAP or their quantity could be too low for detecting IgE binding.

- The denatured state of the proteins after SDS-PAGE could have an effect on their ability to be bound by the IgE. Indeed, it was demonstrated that the reactivity of the IgE binding proteins from wheat flour is generally higher in the denatured state than in the native state [31].

In spite of the heterogeneity of the individual IgE binding patterns, it appears that more than 50% (12/20) of our patients had IgE that bound to a band with a Mr of about 16 kDa. According to its Mr and solubility properties,

this band can be identified as a member of the wheat flour α -amylase/trypsin inhibitors protein family. Members of this protein family from cereals have been characterized as the major allergens involved in bakers' asthma [7,8]. The results presented here would suggest that members of this protein family are major allergens also in the case of patients experiencing hypersensitivity GI reactions after ingestion of wheat proteins. James *et al.* [12], comparing the results obtained with atopic patients suffering from wheat allergy with those of other authors, concerning patients with baker's asthma, concluded that α -amylase inhibitors are relevant allergens after both ingestion and inhalation of wheat. Our data, however, show that IgE to the 16 kDa are present in almost all of the atopic patients, but only in 30% of the non-atopic ones and in these latter patients the IgE binding to this band seems to be quite faint. This is a clear indication that the cereal α -amylase inhibitors are major allergens in the atopic patients but not in the non-atopic ones.

Although all but one of the patients were negative in gluten CAP, IgE binding to water/salt-insoluble wheat proteins was present in the sera of all but one of the patients, as indicated by immunoblotting results. Sutton *et al.* [10] have identified the wheat glutenin fraction as an important allergen in determining IgE-mediated reactions after wheat ingestion. The insoluble gluten proteins, in fact, are likely to remain in the gut for a longer time compared with the soluble proteins [10] and to be converted, after proteolytic digestion, into soluble fragments that are able to reach the intestine with a size sufficient to stimulate antibody production [32]. Specific IgE to water/salt insoluble wheat proteins have been found in the sera of patients suffering from different forms of cereal allergy.

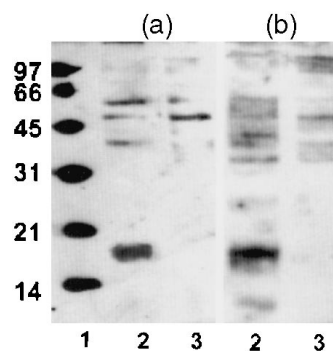


Fig. 3. Immunoblot showing the binding of IgE of the serum of two patients (a and b) suffering from bakers' asthma to the water/salt soluble (lanes 2) and insoluble (lanes 3) wheat flour proteins. Mr standard proteins are in lane 1.

In patients with baker's asthma, proteins belonging to gliadins and glutenin have been found to be important allergens, α (S-rich)- and ω (S-poor)-gliadins being the most allergenic components [9], and the data presented here confirm these previously reported results. Moreover, IgE antibodies against gliadins have been found also in the sera of wheat-allergic patients suffering from both atopic dermatitis [14] and cereal-dependent exercise-induced anaphylaxis [13], and Maruyama *et al.* [31] showed that the wheat proteins belonging to the insoluble fraction, and in particular glutenin, have a much higher reactivity with the IgE of allergic patients than soluble proteins.

Our results show that all the sera contained IgE binding to several proteins of the prolamins fraction and that LMW-GS components with molecular weights around 42 kDa were bound by the IgE present in the sera of most of the patients examined. Indeed, a recombinant LMW-GS with the same Mr has been previously shown to be a major allergen of the wheat flour [31]. The polyspecificity of the binding patterns is not surprising, because of the high degree of homology of the primary structure of the wheat storage proteins [33]. Moreover, some cross-reaction has been shown to occur also between the salt-soluble and salt-insoluble (prolamin) groups of wheat proteins [9]. In any case, the form in which the allergens actually interact with the organism should be considered.

Patients with baker's asthma, in fact, show a reaction to inhaled raw flour particles, whereas the GI symptoms occur after eating a food that has been previously heat-treated (cooked or baked) and then digested. In this context the modifications of the single flour proteins that can affect their immunological properties must be considered. For example, the activity of four members of the low Mr wheat α -amylase inhibitors protein family has been shown to be completely inhibited by both pepsin and heating at 100 °C [34], indicating a susceptibility of these protein molecules to such treatments. Moreover, wheat flour proteins have been shown to undergo heat-induced aggregation that results in a reduced digestibility [35], suggesting that, after baked products have been ingested, at least a fraction of the original flour proteins can reach the intestine in the form of relatively large cross-linked structures. Some interactions between sugars and proteins (glycation) also occur during heating as a result of Maillard-type reactions [32], which are known to generate molecular species with enhanced immunogenicity [36].

In vitro specific IgE detection and SPT are the procedures commonly used to indicate or exclude food-related IgE-mediated allergies. In this paper we have demonstrated that a high percentage of patients suffering from GI symptoms after ingestion of wheat and having IgE specific for some proteins of this food, as demonstrated by immunoblotting, are negative in both SPT and CAP testing

for wheat-specific IgE detection. Since the wheat CAP negative patients were those with no or low IgE binding to the 16 kDa protein, it appears that this diagnostic system is useful only for patients having levels of IgE to this protein sufficient to be detected by the CAP, i.e. people suffering from baker's asthma [7,8] and atopics with wheat allergy [12]. Moreover, although almost all of the patients examined had IgE binding to prolamin components, the CAP for gluten was found to be negative for all the patients except one.

A negative *in vitro* response is commonly considered very reliable in ruling out an IgE-mediated reaction to a particular food [37]. The results presented here, and those previously reported [28,38], show that a CAP-negative response can have no predictive value in some cases of food allergy and suggest that the fraction of patients with IBS caused by food allergy may be much larger than believed [39,40]. This makes necessary an effort to improve the feasibility of the routine diagnostic methods for detecting IgE to food components. In the case of wheat, the allergenic preparations currently used for the diagnosis of bakers' asthma appear to be of limited value when gastrointestinal symptoms develop after ingestion of wheat-based foods and a new preparation, specifically addressed to the diagnosis of food allergy to wheat, must be developed.

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