

ORIGINAL ARTICLE

Immunoglobulin-E-binding epitopes of wheat allergens in patients with food allergy to wheat and in mice experimentally sensitized to wheat proteins

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Summary

Background At present, B cell epitopes involved in food allergy to wheat are known only for a few allergens and a few categories of patients.

Objective To characterize the epitopes of different wheat kernel allergens: α -, γ , ω 2, and ω 5-gliadin, a low-molecular-weight (LMW) glutenin subunit, and a lipid transfer protein (LTP1) recognized by allergic patients and by sensitized mice and provide further understanding of the role of structure in determining allergic response.

Methods Sera were obtained from 39 patients suffering from food allergy to wheat. BALB/c mice were sensitized to gliadins or LTP1 by intraperitoneal immunizations. Continuous epitopes bound by IgE were delineated by the Pepscan technique. The response to reduced, alkylated LTP1 was compared with that of the native form to evaluate the importance of protein folding on IgE reactivity.

Results Few continuous epitopes of LTP1 reacted with IgE from allergic patients and mice, but one of them was common to several patients and sensitized mice. The unfolded protein was not recognized by either patient or mouse IgE, emphasizing the major role of LTP1 folding and discontinuous epitopes in IgE-binding. In contrast, many continuous epitopes were detected by patient and mouse IgE especially for an ω 5-gliadin, which is an unstructured protein, and to a lesser extent, for the other gliadins and a LMW-glutenin subunit.

Conclusion and Clinical Relevance The conformation of LTP1 appeared to have a strong impact on the type of IgE-binding epitopes elicited by this protein in both man and mouse. The responses in mice sensitized to gliadins or LTP1 were sufficiently comparable with the human response in terms of IgE-binding epitopes to provide support for the use of the mouse model in further investigations.

Keywords food allergy, gliadins, IgE-binding epitopes, LTP, mouse model, wheat

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Introduction

Characterization of the IgE-binding epitopes of food allergens is important for understanding the molecular mechanisms underlying food allergies and for the development of new tools for diagnosis, prognosis, and treatment. The regions of proteins or peptides bound by IgE correspond to those likely to be involved in the sensitization phase of allergy, possibly due to resistance to enzyme

digestion and intestinal translocation. For several food allergens, e.g. egg, milk, and peanuts, close relationships have been highlighted between IgE-binding to particular continuous/sequential epitopes and the persistence of allergy or the clinical reactivity of patients [1–5]. In the case of food allergy to wheat, we previously observed differences in the recognition of gliadin epitopes between patients according to the severity of their symptoms [6]. Matsuo et al. [7] have used the characterization of wheat

protein epitopes to propose a diagnostic tool for exercise-induced anaphylaxis (EIA) due to wheat that combined epitopes from ω 5-gliadins and high-molecular-weight (HMW) glutenin subunits.

Various techniques have been developed for B cell-epitope mapping. The Spot or Pepscan technique has been used for several food or respiratory allergens [8]. More recently, peptide micro-arrays were applied to analysis of the epitopes of peanut and milk allergens [4, 9]. These techniques are assumed to identify sequential/continuous epitopes on proteins as they employ synthetic short peptides (usually from 10 to 20 amino acids). Phage display or site-directed mutagenesis of recombinant allergens have been developed to elucidate the discontinuous (conformational) epitopes of proteins [10]. Differentiating between continuous and discontinuous epitopes results mainly from the experimental techniques used for epitope localization. In structural terms, this distinction is, however, not trivial insofar as discontinuous epitopes often display several stretches of a few contiguous residues that might be considered as continuous epitopes [11]. High-resolution, three-dimensional structures are valuable for determining the structures of possible discontinuous epitopes, but are available for only a few wheat allergens, one being lipid transfer protein (LTP), which has been shown to be a highly-ordered compact protein. No such three-dimensional structures have been obtained for any gluten protein (gliadin monomers or glutenin subunits) of the wheat kernel.

In this study, we have focused on LTP1 and types of gluten proteins that have either one or two sequence domains. The ω -gliadins consist of a single repetitive domain, rich in glutamine and proline residues. The domains of ω 2- and ω 5 gliadins are made up of different, slightly diverged types of repeats, but they both appear to assume mainly a flexible, largely unstructured form in solution [12]. The ω -gliadins usually have no cysteines and hence no disulphide bonds. The α -gliadins, γ -gliadins, and the low-molecular-weight (LMW)-glutenin subunits are composed of two major domains: (1) an N-terminal, repetitive domain that is similar to the single domain of the ω -gliadins, being rich in glutamine and proline residues and probably unstructured and flexible in solution (although with a different motif predominant in the repeats); and (2) a non-repetitive, C-terminal domain that includes either three or four intramolecular disulphide bonds, which link specific cysteine residues. Accordingly, the C-terminal domain is presumably a somewhat structured domain that is almost certainly more compact than the single domain of ω -gliadins [12, 13]. The LMW-glutenin subunit has two cysteine residues that form intermolecular disulphide bonds along with the six cysteines that form three intramolecular disulphide bonds [14]. For the repetitive domains of the gliadins and the LMW-glutenin subunit, the Pepscan technique in which overlapping peptide sequences are scanned should be appropriate for an

initial and rapid search for epitopes. Even though LTPs are compact proteins, resistant to digestion, the Pepscan approach seems to have been used successfully in the epitope analysis of LTPs found in fruits [15, 16].

Mouse models of allergy to wheat proteins have been described recently, but the epitope patterns of the allergens were not determined [17–19]. In our laboratory, mice were sensitized with total gliadins to produce IgE antibodies that recognized the different gliadin proteins: α , γ , ω 2, and ω 5 [18], and in another study, a group of mice sensitized with the purified wheat LTP1 produced satisfactory levels of specific IgE [19]. The relevance of this mouse model needs to be supported by determination of the IgE-binding epitopes involved.

Consequently, we here describe characterization of the IgE-binding epitopes of different wheat allergens representatives of the main gliadin classes (α , γ , ω 2, and ω 5), a LMW-glutenin subunit, and LTP1 for IgE from patients suffering from wheat allergy and for IgE from mice sensitized to particular wheat proteins. The present study extended previous data [6] obtained on 11 patients with food allergy to wheat to a larger group of 39 patients and to LTP1, a non-gluten protein allergen. It also permitted us to compare epitopes reacting with IgE of wheat allergic patients with those of sensitized mice to find out whether IgE antibodies elicited in mice by intraperitoneal (i.p.) injection are representative of human antibodies resulting from wheat allergens encountered by other pathways (such as ingestion).

Materials and methods

Gliadin and glutenin extraction and purification

Gliadins were extracted from wheat flour (cultivar Hardi), using a sequential procedure [18]. The different gliadin classes (α , γ , ω 2, and ω 5) and LMW-glutenin subunits were prepared by several steps of chromatography [20, 21].

Preparation and characterization of the different lipid transfer protein 1 forms

Purification. LTP1 from wheat was purified by ion exchange and RP-HPLC and its purity and identity were controlled by SDS-PAGE under reducing conditions and by mass spectrometry [22].

Reduction and alkylation. A total of 1 mg of protein was solubilized in 500 μ L of Tris-HCl 0.5 M, pH 8.5, containing guanidine-HCl 6 M and EDTA 0.01 M. Reduction was performed by adding 0.1 M dithiothreitol. After 2 h at 50 °C under nitrogen, alkylation was carried out with iodoacetic acid in 0.5 M NaOH. The reduced and alkylated proteins were fractionated by RP-HPLC on a C18 nucleosyl 5 μ m, 300 Å

bonded silica column (25×0.46 cm) as described for semi-preparative RP-HPLC except that the flow rate was 1 mL/min and detection was performed at 220 nm.

Synchrotron radiation circular dichroism (SRCD). For SRCD, freeze-dried proteins were diluted in Tris buffer 50 mM pH 7.5 with 50 mM NaF. Tris buffers were prepared from a Tris-HCl/Tris-base mixture to produce 1 M stock solution. Measurements were carried out on the DISCO beamline at Soleil synchrotron (Gif-sur-Yvette, France). Calcium fluoride circular cuvettes (Hellma, Jena, Germany) [23] with 34 µm path-length were used. Two protein concentrations were used: 1 g/L (purified LTP1) and 2 g/L (reduced and alkylated LTP1). Protein concentration was determined using the appropriate absorbance coefficient at 280 nm. For each protein concentration, three scans from 170 to 280 nm with 1 nm intervals, were performed. Three consecutive scans of the baseline (using the sample buffer) were obtained in the same manner. For data processing, spectra of buffer were subtracted from those of corresponding samples. The 260–270 nm region was set to zero, and resulting spectra were calibrated with CSA (d-10-camphorsulfonic acid) using the CDtool software [24]. Secondary structure determination was performed using ContinLL programs [25] in Dichroweb [26], and using SP175 as reference set. SP175 contains the largest set (72) of soluble proteins to date. Secondary structures were grouped in four different types: α -helix, strands, turns, and unordered structures.

Characteristics of patients

Sera were obtained from the Service of Clinical Immunology and Allergology, Nancy hospital. They were from 39 patients with food allergy to wheat, 17 children and 22 adults, and from six non-atopic subjects. Patients with different symptoms [atopic eczema dermatitis syndrome (AEDS), laryngeal angio oedema, anaphylactic shock (AS), urticaria (U), or EIA] after wheat ingestion were included in the study. Sera were sampled from patients during prick-in-prick (PIP) tests to natural wheat flour. Wheat allergy was confirmed 1 month later by a positive double-blind placebo-controlled food challenge (DBPCFC) after a wheat-free diet of 3 weeks or by an evident positive effect of avoidance diet when DBPCFC could not be performed for ethical reasons. DBPCFC corresponded to the consumption every 20 min of increasing doses of wheat flour included in apple sauce or of placebo (corn or potato starch) [6]. Blood collection, PIP, and DBPCFC were performed with informed consent of the patients or their parents, and after ethical committee approval (authorization number DGS2007–0066). Table 1 summarizes clinical features of patients (age, symptoms, PIP reaction, DBPCFC reactive dose) and their IgE reactivity against purified wheat proteins (α -, γ -, ω 2-, and ω 5-gliadins,

LMW-glutenins, and LTP1) determined by ELISA [27] or immunoblotting [28]. Each serum was used for epitope mapping of one or several allergens depending on its reactivity or on the volumes available.

Sensitization of BALB/c mice with gliadins or lipid transfer protein 1

Three-week-old female BALB/cJ mice from the Centre d'Élevage René Janvier were fed with a semisynthetic diet free of plant proteins. Mice were housed in filtered cages under classical specific pathogen free husbandry conditions and were acclimated for 2–3 weeks before immunizations. All experiments were performed according to the European Community rules of animal care and with Permission 91–368 and B 44–502 of the French Veterinary Services. The i.p. immunizations with 10 µg of gliadin extract or LTP1 adsorbed onto alum (Alhydrogel 3%, Superfos Biosector A/S, Frederikssund, Denmark) were performed on days 0, 10, 20, and 30 (0.2 mL per mouse). Each group comprised six to eight mice. The efficient sensitization of mice was evidenced by the production of anti-gliadin or anti-LTP1-specific IgE or IgG1. A challenge was performed by intranasal administration of allergens and elicited an allergic reaction in mice demonstrated by a Th2 cytokine profile and eosinophil influx in bronchoalveolar lavage fluids (BALF) [18, 19]. Mouse sera from each group were pooled for Pepscan analysis.

Fluorimetric ELISA (F-ELISA) was performed for determination of LTP1-, gliadin-, and glutenin-specific IgE.

The determination of human and mouse IgE specific for purified wheat gliadins, glutenin subunits and LTP1 in its native or reduced form was performed as described by Bodinier et al [27].

Immunoglobulin-E-binding to solid-phase synthetic peptides (Pepscan)

Decapeptides, overlapping by eight amino acids, spanned the sequences (excluding the signal peptide) of an α -gliadin (accession number P04725), a γ -gliadin (P08453), a ω 2-gliadin (Q9FUW7), a ω 5-gliadin (sequence obtained from EST contigs), a LMW-glutenin subunit (Q9ZNY0), and of LTP1 (P24296). The method was described by Battais et al. [6]. Anti-human IgE (P0295; Dako, Glostrup, Denmark) and anti-mouse IgE (A90-15P; Bethyl) antibodies labelled with peroxidase were used to reveal human or mouse IgE binding to peptides. Chemiluminescent substrate (SuperSignal West Dura Extended Duration Substrate; Pierce, Rockford, IL, USA) was added and luminescence was measured by a CCD camera (Luminescent Image Analyzer LAS 3000; Fujifilm, Tokyo, Japan).

Table 1. Clinical features of patients and their IgE reactivity against purified wheat proteins

Patient serum no	Age (year)	Symptoms	PIP (mm/ wheat flour)	DBPCFC reactive dose (mg protein equivalent)	IgE-binding proteins (specific IgE concentration in ng/mL IgE or binding intensity+/++)
Children					
18	2	AEDS	9	1000	LMWG (+), LTP (+)
25	4	AEDS	4	1000	α (19), ω 5(2), LTP (149), LMWG (12)
27	5	AEDS	11	290	α (+)
33	4	AEDS	8,5	480	α (53), ω 2(++), α (+) ω 5(20), LTP (45), LMWG (25)
41	3	AS	13	NT*	α (160), ω 2(++), ω 5(51), LTP (68), LMWG (113)
50	2	AEDS	10	1000	α (++), ω 2(+), ω 5(++), LTP (14)
56	2	AEDS	2	1560	α (10), ω 2(16), ω 5(9), LMWG (15)
65	3	AEDS	15	97,5	α (50), ω 5(1), LTP (60), LMWG (4)
68	6	AEDS	8,5	78	α (94), ω 2(33), ω 5(37), LTP (71), LMWG (115)
116	3	AEDS	pos	500	LTP (96)
249	6	AEDS	8,5	117	α (16), LTP (15)
254	11	AEDS AS	4	1200	α (51), LMWG (24), LTP (25)
257	4	AEDS, AS	9	117	α (10), LTP (12)
327	4	AEDS	8,5	750	α (160), ω 2(7), LTP (67), LMWG (7)
336	4	AEDS	9,5	LT	α (96), ω 2(18), ω 5(15), LTP (93), LMWG (38)
404	9	AEDS	0	7110	α (39), LMWG (51), LTP (131)
451	4	AEDS	15,5	1000	α (104), ω 2(150), ω 5(62), LTP (143), LMWG (139)
Adults					
3	65	U	6	9000	α (1), ω 5(105), LMWG [†]
4	56	U	2	5000	α (1), ω 2(1), ω 5(34), LMWG (1), LTP (1)
6	21	U	0	1000	ω 5(1)
15	29	AEDS	4,5	2000	LTP++
17	25	AEDS	0	5000	LTP+
20	71	AS	9,5	3000	ω 5(114), LMWG (++)
31	37	EIA	11,5	NT	ω 5(53), LMWG [†]
45	50	U	3	4200 [‡]	ω 5(20), LMWG [†]
52	42	U	2	NT*	ω 5(38), LMWG [†]
58	44	EIA	2	1500	α (1), ω 2(1), ω 5(112), LMWG (7)
59	27	EIA	0	6600	ω 5(14), LMWG [†]
60	24	EIA	2,5	8150 [‡]	ω 5(28), LMWG (1)
61	41	EIA	6	8600	α (15), ω 2(4), ω 5(102), LMWG (3)
63	31	EIA	1,5	11 240 [‡]	LTP (+), LMWG (+)
66	38	EIA	5	12350	ω 5(9)
71	54	EIA	3	15 300	ω 5(6), LMWG (+)
80	49	EIA	12	NT*	ω 5(16), LMWG [†]
82	34	AEDS	pos	1800	LTP (1)
85	54	EIA	0	8400	α (10), ω 5(25)
289	20	AEDS	NT	NT	LTP (25)
382	34	U, LAO	5	1800	LTP (76)
500	18	EIA	3	NT [‡]	LTP (26)

*DBPCFC not performed or discontinued for ethical reason because of severe symptoms.

[†]Positive response in immunoblotting.

[‡]Positive effect of avoidance diet.

AEDS, atopic eczema dermatitis syndrome; LAO, laryngeal angio oedema; AS, anaphylactic shock; U, urticaria; EIA, exercise-induced anaphylaxis; PIP, prick-in-prick tests; DBPCFC, double-blind placebo-controlled food challenge; α , γ , ω 2, ω 5, α , γ , ω 2, ω 5-gliadins; LMWG, LMW glutenins; LTP, lipid transfer protein; specific IgE concentrations in ng/mL or binding intensity in ELISA are indicated in parenthesis; pos, positive; NT, not tested; LT, labial test.

A semiquantitative evaluation of IgE-binding was carried out with the Multi-Gauge v3.0 software (Fujifilm). For each serum, the spot detected with the highest intensity

was taken as reference. IgE-bindings to peptides were then classified as strongly positive (>60%) or positive (20–60%) according to this reference and binding under

20% was ignored. Five of the six control sera were totally negative and one displayed a positive response for one LMW-glutenin, one α -gliadin and two LTP1 peptides.

Results

Identification of immunoglobulin-E-binding epitopes on wheat lipid transfer protein 1 in human and mouse contexts

Human sera. Sera from 15 patients (seven children and eight adults) possessing IgE specific for LTP1 (Table 1) were analysed. Only five of the patient sera exhibited IgE binding to the LTP membrane (Fig. 1). The peptide ³⁷QARSQSDRQS⁴⁶ was bound by IgE from four patients (25, 33, 60, and 82) with a positive, but weak, intensity. This peptide corresponded to the loop linking the helix α 2 to the first part of α 3. The peptide ²⁴GQCCDGVKNL³³ belonging to the α 2-helix was detected with a strong intensity by a single serum (63). A third peptide equivalent to the C-terminal end of LTP1 ⁸¹ISLNIDCSRV⁹⁰ was detected only by serum 25.

Mouse sera. Intranasal administration of allergens elicited an allergic reaction in the sensitized mice demonstrated by a Th2 cytokine profile and eosinophil influx in BAL [19]. Pepscan analysis of pooled sera from the LTP1 sensitized mice detected two peptides found in the LTP1 sequence: P37-46 and a mouse-specific epitope, ⁵³GIARGIHNLN⁶², localized in the loop between the α 3 and α 4 helices. Few continuous epitopes were detected by IgE from allergic patients and mice, but one of them (P37-46) was common to several patients and mice.

Influence of lipid transfer protein 1 structure on human and mouse immunoglobulin-E recognition. To evaluate the importance of LTP1 structure on human and mouse IgE binding, the purified LTP1 was reduced and alkylated. The secondary structures of the native and reduced/alkylated LTP were analysed by SRCD. The CD spectrum of the native LTP1 showed a double minimum at 210 and 220 nm, characteristic of α -helical structures (Fig. 2). In contrast, the CD spectrum of the reduced and alkylated

(a)

Epitope sequence and position	Human		Mice
	Patient sera	Intensity of IgE binding	Intensity of IgE binding
²⁴ GQCCDGVKNL ³³	63	++	0
³⁷ QARSQSDRQS ⁴⁶	25, 33, 60, 82	+	+
⁵³ GIARGIHNLN ⁶²		0	++
⁸¹ ISLNIDCSRV ⁹⁰	25	+	0

Pepscan negative patient sera: 18, 41, 50, 65, 68, 15, 17, 289, 382, 500

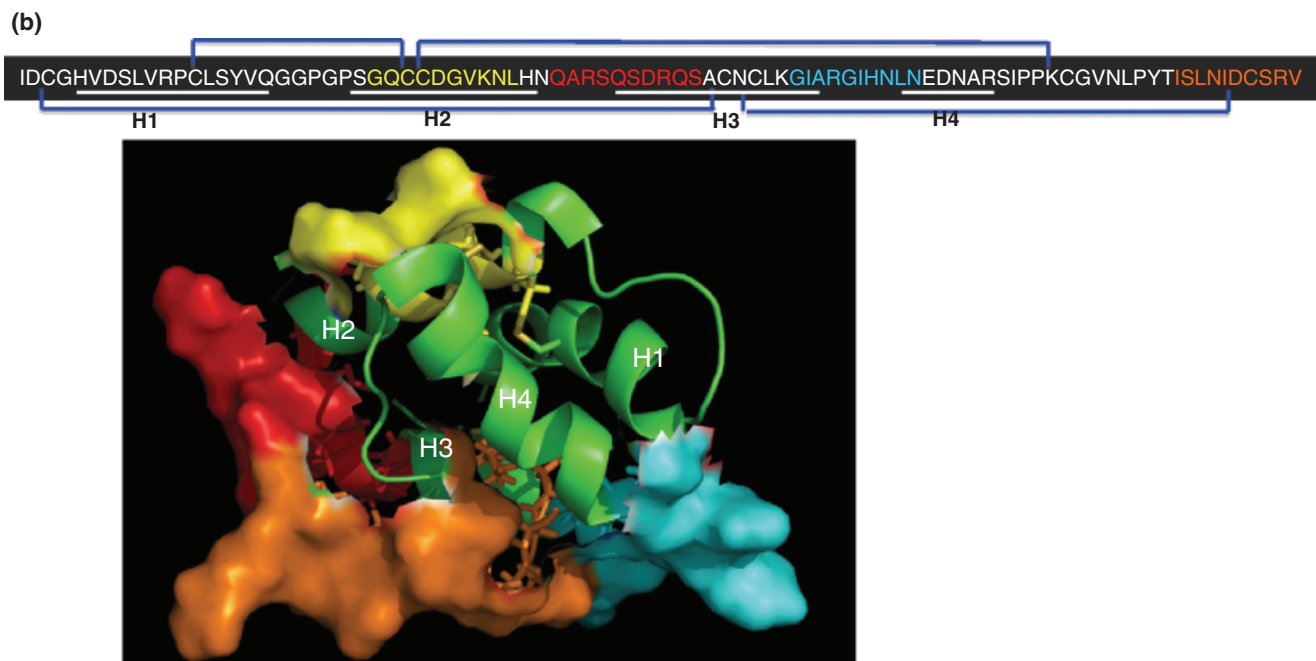


Fig. 1. (a) Continuous epitopes on LTP1 bound by IgE from allergic patients and sensitized mice. (b) Position of epitopes mapped on the LTP1 sequence and 3D structure. Underlined (H1 ... H4): positions of α -helix; blue lines: positions of disulphide bridges.

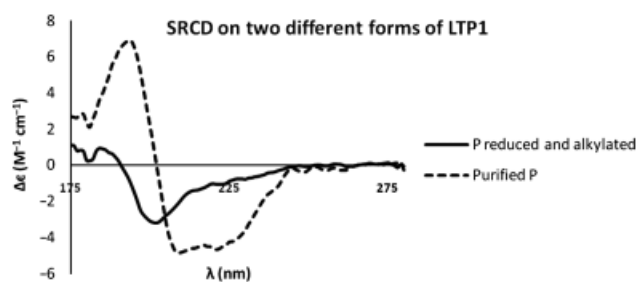


Fig. 2. CD spectra obtained by synchrotron radiation circular dichroism (SRCD) of the native lipid transfer protein 1 (LTP1) (Purified P: dotted line) and reduced and alkylated LTP1 (P reduced and alkylated: solid line).

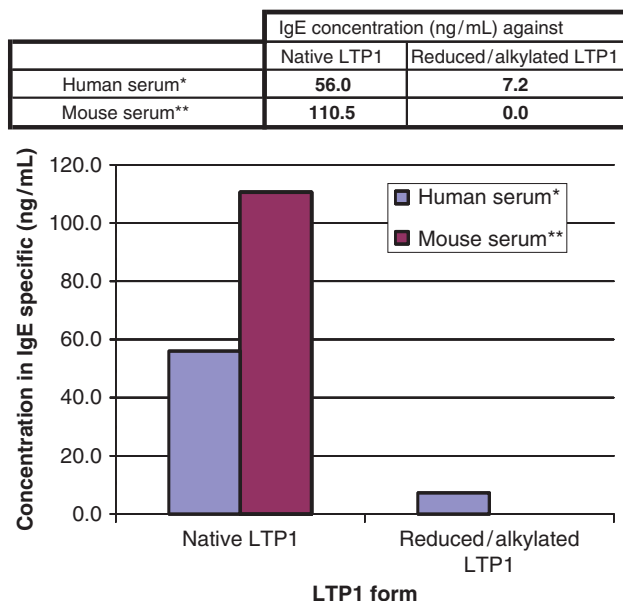


Fig. 3. Mean reactivity of patient and mouse IgE against the native and reduced and alkylated lipid transfer protein 1 (LTP1) in ELISA. *IgE reactivity of 15 patient sera (numbers 18, 33, 41, 50, 68, 116, 249, 254, 257, 327, 336, 404, 451, 382, 500). **Reactivity of pooled sera from seven mice.

form was very different with a strong negative band around 205 nm and a shoulder between 230 and 240 nm, spectral features of unfolded proteins. Reduction and alkylation of the wheat LTP1 strongly modified the protein secondary structure contents with a drastic reduction of helix content (15% vs. 47%) and an increase of β strands (25% vs. 6%) and unordered structures (43% vs. 32%).

The recognition of the two LTP1 forms (native and unfolded) by human and mouse IgE was also analysed by ELISA. Sera from 15 patients possessing LTP-specific IgE (13 children and two adults) were used (Fig. 3). The reduced and alkylated LTP1 was very poorly recognized by patient IgE and this was true of the mouse IgE, which also failed to recognize the denatured protein when the mice had been stimulated by i.p. injection of native, unreduced LTP1. These results confirmed the importance of the 3D structure of LTP1 for its recognition by human and mouse IgE and

indicated the major importance of discontinuous epitopes for this wheat allergen.

Identification of gliadin and glutenin epitopes recognized by immunoglobulin-E from patients and mice

Epitopes detected by human immunoglobulin-E on gliadins and glutenins (Table 2 – Fig. 4). Twenty-three sera from patients with food allergy to wheat and six sera from non-allergic patients were used to identify IgE-binding epitopes on α -, ω 2-, ω 5-gliadins and a LMW-glutenin subunit. Depending on their IgE-binding profiles in ELISA or in some cases in immunoblotting (Table 1) and on volumes available, these sera were then used for mapping experiments on one or more allergens.

ω 5-gliadin. Twenty sera were used to map the epitopes of ω 5-gliadin, and 15 of them (75%), mostly from adults, displayed positive responses by Pepscan analysis (Table 2). As previously observed [6], numerous ω 5-gliadin peptides were strongly bound by IgE (Table 2). Many of our serum samples recognized the same epitopes as those described by Battais et al. [6] and Matsuo et al. [29] (QQIPQQQ, QQFPQQQ, QQSPEQQ, QQLPQQQ, and QQYPQQQ), the dominant epitope in the present work being the one with I (Ile) in the third position. These results confirmed the consensus IgE-binding motif of ω 5-gliadin being QQX₁PX₂QQ (X₁ being I, L, F, S, or Y and X₂ Q, and E) not only for patients with EIA, but also for some patients having the symptoms of U and AS.

ω 2-gliadin. Eight of the 10 sera (80%) displayed positive responses for ω 2-gliadin peptides. The PQQPFP motif, which is predominant in the diverged repeating sequences of ω 2-gliadin [30] was present in several decapeptides only detected by the sera from children, whereas two decapeptides containing the motif QQPIP and the peptide QQSEQIIPQQ were detected only by the sera from adults. However, there were other peptides that were recognized by both adults and children (Table 2).

α -gliadin. Seven sera among the 12 tested (60%) detected synthetic peptides from the α -gliadin (Table 2). No immunodominant epitope (i.e. bound by at least 50% of the patient's sera) was obvious. In the repetitive domain, the sequence ²⁷QQQFPGQQQ³⁶ recognized by three sera was very close to the ω 5-gliadin consensus epitope. Four epitopes in the non-repetitive domain contained a cysteine or were in the vicinity of a cysteine. As shown in Fig. 4, these cysteines are involved in intramolecular disulphide bonds [31]. Therefore, these apparently continuous epitopes from the non-repetitive domain might be brought close together by the conformational structure of this domain of α -gliadin to participate in discontinuous epitopes.

Table 2. Sequences of continuous epitopes bound by patient and mouse IgE on the sequences of ω 5-, ω 2- and α -gliadins and bound by patient IgE on a LMW glutenin subunit

ω5-gliadin: Pepsan-negative patients sera: 33, 50, 56, 6, 85																				
ω5-gliadin																				
Pepsan-positive patients																				
Children					Adults															
68	41	3	4	45	52	20	31	58	59	60	61	66	71	80						
AEDS	AS	U	U	U	U	AS	AIE	AIE	AIE	AIE	AIE	AIE	AIE	AIE						
Peptide																				
18–27	+				+															
26–35		+			+															
30–39			++	+																
42–51	+				++	+			+											
46–55		+			++	++			++			+		+						
56–64		++	++	++	++	++			++	++		+	++							
75–84				+	+	+	++				++									
100–109		+			+	++					++	+								++
111–120																			+	
116–125																			+	
120–129					+	+														+
148–157		+			++	+	+					+								+
152–161					++	++														+
156–165		+			++	++			+											+
159–168					++	++						++								
163–172					++	++														
166–175																				
170–179		+			++	+			+		+									+
174–183		+			++	+	++													+
184–93		+		++	++	++				+										+
186–195		+		+	+	+			+	+										+
198–207		+			+	+														+
208–217						+														
211–220																				
230–239		+		+	++	+	++	+			++									+
251–260					++	++					++									+
268–277		+			++	++		+		+	++	+								+
274–283		+			+	+					++	+								+
310–319		+			+	+					++	+								+

002-gliadin: Pepscan-negative patient sera: 50, 56

ω2-gliadin	Pepsan-positive patients									
	Position	Peptide	Children			Adults				
			AEDS	68	41	Adults				
						AEDS	3	U	58	EIA
5-14	NPSNKEIQSP		+							
13-22	SPQQSFYQQ			+						
43-52	SQQPFPTQQ		++							
47-56	FPTPQQQFPE			+						
49-58	TPQQQFPEQS									
51-60	QQQFPEQSQQ			++						
71-80	IQPQQPFPPQ		++	+						
99-108	PQQPFPTQQ		++							
105-114	QTQSFPLQP									
107-116	QQSFPLQPQQ			++						
135-144	QQSEQIIPQQ									
143-152	QQLQQPFPLQ		++							
151-160	LQFQQPFPPQ		++	+						
161-170	PQQPFPPQFPQQ		++							
169-178	QQPIPVQPPQQ	++								
193-202	QPQQLFPELQ									
195-204	QQLFPELQQP									
199-208	PELQQPIPPQ									
207-216	QQPQQPFPLQ		++							
215-224	LQPQQPFPPQ		++	+						
228-237	FPQQPQQSFP									

α -gliadin: Pepscan-negative patient sera: 33, 50, 56, 65, 4

α-gliadin	Pepscan-positive patients										
	Children					Adults					Control
	27	68	41	3	31	58	61	H12			
Position	Peptide	AEDS	AEDS	AS	U	EIA	EIA	EIA	Mice		
1–10	VRVPVQLQP		+								
19–28	QEQVPLVQQQ							++	++		
23–32	PLVQQQQFPG								++		
25–34	VQQQQFPGQQ								++		
27–36	QQQFPGQQQQ			++	+			+	++		
29–38	QFPGQQQQFP								++		
31–40	PGQQQQFPQP								+		
33–42	QQQQFPPQQP								+		
39–48	PQQPYQPQPQ								+		
47–56	QPFSQQPYL								+		
49–58	FPSQQPYLQL								++		
51–60	SQQPYLQLQP								++		
55–64	YLQLQFPQPQ		+						++		
84–93	YPQQQPFQYLQ								+		
118–127	QILQQILQQQ		++						++		
161–170	CQQLLOIPEQ	+						+			
165–174	LOIPEQSQCC	+									
192–101	QEKKQQLQQQ	+									
224–233	SFQQPQQQYP		++			+			+		
265–274	LALQTLPAMC	++									
277–286	YIPPHCSITI	+									

LMW glutenin subunit: Pepscan-negative patient sera: 6, 59, 60

LMW glutenin	Pepscan-positive patients											Control
	Children					Adults						
	33	68	45	4	52	20	63	66	71	80	85	
Position	AEDS	AEDS	U	U	U	AS	EIA	EIA	EIA	EIA	EIA	H12
37-46					+	+			++	+		
39-48		++		+	+				++	+	+	
47-56					++							
211-220												
231-240	++					+						+
263-272				+						++	++	
269-278			+	+			++	+		++	++	

AEDS, atopic eczema dermatitis syndrome; U, urticaria; EIA, exercise-induced anaphylaxis.

Common epitopes between human and mice are indicated in bold characters and by shading background. On ω 2-gliadin, consensus motifs for children are underlined.

ω5 gliadin :

SRLSPRGKELHTPQEFQFP**QQQQFPQPQQFPQQQIPQQHQIPQQPQQFPQQQQFLQ**
QQQIPQQQIPQQHQIPQQPQQLPQQQFPQQQQFPQQHQ
 PKQF**QQQQFPQQQFPQQQFPQQEFQQQQFPQQQ**IAQQPQQLPQQQQIPQQP**QQFP**
QQQQFPQQQSPQQQFPQQQFPQQQQLPQQQFPQPQQIPQQQQIPQQPQQFPQQQ
FPQQQFPQQQEFQQQFPQQQFHQQQLPQQQFPQQQFPQQQFPQQQFPQQQL
 TQQQF**RPQQSP**EQQQFPQQQFPQQPPQQFPQQQFPPIPYPPQQSQEPS**PYQQYPQQ**
QPSGSDVISISGL

ω2 gliadin :

ARELNPSNKLQ**SPQQSFSYQQ**QFPQQPYPPQPYPSQQPY**SPQQFPPTQQQFP**EQSQQPFTQP
 QQPT**IPQQQFP**QQPQQPQQFPQPQQFPWQP**QQPFPQTQQSFPLQ**PPQFPQQPQQFPQP
 QLFP**QQSEQIIP**QQQLQQ**FPFLQPQQFPQQPQQFPQPQQIPVQPQQSFPQQSQSQSPFAQ**
QLLFPELQQIPQQPQQFPFLQPQQFPQQPQQFPQQPQQSFPQQPQQPYQQPYGSSLTISIG
 GQ

α gliadin :

VRVPVQLQPQNPSQQQP**QEQVPLVQQQQFPGQQQQFP**QQPYPPQPPFSQQPY**LQLQFP**QQ
 PFPQLPYPPQSFPPQQPY**QQQPQYLQ**PPQPPISQQQAQQQQQQQQQQQQ**QILQQILQQQL**
 P**CR**DVVLQQHNIHASSQVLQQSTYQLLQQ**CC**QQL**LQIPEQSQ**CQAIHNVAHAIMHQQQQQEQK
 QQLQQQQQQQQQLQQQQQQQQQPPSSQ**SFQQPQQQYP**SSQVSFQPSQLNPQAQGSVQPQQL
 QFAEIRNLALQTL**PAMC**NVYIP**PHCSTTI**APFGISGTN

LMW glutenins :

SHIPGLERPSQQQLPQQTLSSH**HQQQPIQQQPQQFPQQQP**C**S**QQQQQPPLS
 QQQQPPFSQQQQPPFSQQQQPVLPQQPSFSQQQLPPFSQQQQPPFSQQQQPVLPQQPSFSQQQL
 PPFSQQLPFSQQQQPVLPQQPPFSQQQLPPFSQQQQPVLPQQPPFSQQQQQPILPQQ
 PPFSQQQPVLQQQIPFVHPSILQQLNP**CK**VFLQQ**QCS**PVAMPQSLARSQMLQSS**CH**VMQQQ**C**
CQQLPQIPQQSRYE**AIR**AIIVYSIILQEQQQVQGSIQTQQQPQQLGQ**C**VSQPQQSQSQQLGQQPQ
 QQQLAQGTFLQPHQIAQLELMTSIALRTLPTM**C**NVNVPLYRTTTRVPFGVGTGVGAY

Fig. 4. Main, continuous epitopes bound by patient and mouse IgE for the sequences of α-, ω2- and ω5-gliadins and bound by patient IgE for a low-molecular-weight (LMW)-glutenin subunit. Blue lines indicate the positions of intramolecular disulphide bridges in the α-gliadin and the LMW-glutenin subunit sequences. Bold characters: human epitopes. Underlined sequences: mouse epitopes.

Peptides ¹¹⁸QILQQILQQQ¹²⁷(LIPC) and ²⁶⁵LALQTL**PAMC**²⁷⁴ might be brought close together by the formation of the first two disulphide bonds that involve the Cys–Cys motif, the first linking cysteines 131 and 160 and the second, cysteines 161 and 274. Peptides ¹⁶⁵LQIPEQSQ**CQ**¹⁷⁴ and ²⁷⁷YIP**PHCSTTI**²⁸⁶ contained, respectively, the fourth and sixth cysteine residues in the protein molecule that link to form the third disulphide bond.

low-molecular-weight-glutenin subunit. The Pepscan analysis of the LMW-glutenin subunit gave positive results for 12 sera of the 15 analysed (80%) and revealed 6 different IgE-binding epitopes. Only one of the epitopes, recognized by six sera (³⁹QQPIQQQPQ⁴⁸) was immunodominant. The sequence following p39–48, ⁴⁷QQFPQQQ**PCS**⁵⁶, was detected by two sera and contained a cysteine involved in an intermolecular disulphide

bond in native glutenin (unreduced). Other epitopes were localized in the non-repetitive domain (Fig. 4). One of them (²³¹QCSPVAMPQS²⁴⁰) contained a cysteine implicated in the second intramolecular disulphide bond while another was in the vicinity of a cysteine implicated in the formation of the third intramolecular disulphide bond (CQQ²⁶³LPQIPQQSRYEAIRAI²⁷⁸); these peptides might then also be part of discontinuous epitopes.

Adults vs. Children. Differences in Pepscan results between children and adults were observed (Table 2). Few of the sera from children were tested on ω 5-gliadin peptides as this allergen is bound mainly by adult IgE, but three (33, 50, 56) of the five sera from children analysed were negative despite medium concentrations in ω 5-gliadin-specific IgE (Table 1). In contrast, most samples of sera from adults recognized many decapeptides of ω 5-gliadin (only two of the 15 tested were negative). Although ω 5- and ω 2-gliadins have the same organisation composed entirely of repetitive sequences, for adult sera, many fewer epitopes were found for ω 2-gliadin than for ω 5-gliadin. Some ω 2-gliadin epitopes appeared specifically detected by IgE from children and others by adult IgE. For α -gliadin: four of seven children had sera that were negative when analysed in Pepscan despite medium to high concentrations of α -gliadin-specific IgE (Table 1), whereas four of five adults had sera that were positive in the Pepscan analysis despite low concentrations of α -gliadin-specific IgE. The α -gliadin epitopes for the sera from children were clustered more in the non-repetitive domain than in the repetitive domain whereas the adult sera recognized mainly epitopes in the repetitive domain.

Gliadin epitopes detected by mouse immunoglobulin-E – Comparison with the human response. A pool of sera from seven mice sensitized with 10 μ g of total gliadins [18] was used to identify IgE-binding epitopes for α -, γ -, ω 2-, and ω 5-gliadins (Table 2 – Fig. 4).

For the ω 5-gliadin sequence, mouse IgE reacted very similarly to human IgE with numerous peptides detected. Three of the human epitopes were bound by mouse IgE (QQFPQQQ, QQLPQQQ, and QQSPQQQ) and the peptide QQLPQQ was recognized with the highest intensity. However, the dominant sequence QQIPQQQQ bound by patient IgE was not bound by mouse IgE. In contrast, mouse IgE specifically detected other peptides (QQEFPQQQ, QQQFPQQEFP).

The responses of mouse IgE to ω 2-gliadin peptides were of low intensity. Two epitopic zones were common to human and mouse IgE (43–60 and 105–116); these zones contained notably the peptides FFTPQQQFPE and QQSFPLQPQQ. The QQPFP motif or the QQIP motif recognized by IgE from children and adults, respectively, were not detected by mouse IgE while the epitope ¹⁹⁵QQLFPELQ²⁰² was mouse specific.

Mouse IgE reacted strongly with several α -gliadin peptides. Four IgE-binding epitopes were common to mice and

wheat allergic patients: three were in the repetitive domain (²³PLVQQQ²⁸, ²⁷QQQFPGQQQ³⁶, ⁵⁵YLQLQP⁶⁰) and one localized in the C-terminal fragment (²²⁴SFQQPQQQYP²³³). A large fragment composed of overlapping epitopes (²³PLVQQQFPGQQQFPPQQPYQPQPFSSQQPYLQLQP⁶⁰) and the peptide ⁸⁴YPQQQPQYLQ⁹³ of the repetitive domain were specifically recognized with a high intensity by mouse IgE.

For the γ -gliadin sequence, mouse IgE detected two groups of repetitive peptides (not shown) containing either the PQQPF or the SFPQ motif, both of them being also present in patient epitopes according to an earlier study [6]. Another repeated motif was recognized specifically and with a high intensity by mouse IgE (PQQTFP).

Discussion

The proteins of wheat endosperm constitute a complex mixture. Although the gluten proteins predominate quantitatively and qualitatively, corresponding to about 70 components differentiated by the sequence in a single cultivar with most having allergic potential in humans, other wheat endosperm proteins, sometimes minor from a quantitative aspect, are also significant allergens. In order to provide a better understanding of wheat grain protein allergenicity at the molecular level, we have compared several gluten proteins, including the notably allergenic ω 5-gliadin, with a wheat LTP1, recognized as an important human allergen across many plant species, by means of the Pepscan technique. Although this approach seemed likely to give information primarily about continuous epitopes, we were also able to infer the presence of possible discontinuous epitopes by making use of information about disulphide linkages, as discussed below. Furthermore, because the availability of samples of suitable human sera is often limited in number and volume, we have examined the suitability of a mouse model in which the IgE response to purified wheat protein fractions was induced by i.p. injection of the proteins. Pepscan results for BALB/c mice were compared with the equivalent results obtained for human sera.

For the wheat LTP1 (also known as Tri a 14), the Pepscan technique revealed only a few peptides bound by human or mouse IgE and these generally showed low intensity. In the case of human sera, patients had encountered LTP1 as a food component, whereas the mice had been injected i.p. with unreduced LTP1. Only 30% of the human sera containing LTP1-specific IgE reacted with a few of the decapeptides. The lack of continuous epitopes in LTP1 found by the Pepscan approach was supported by the very weak recognition by the patient and mouse IgE of unfolded (reduced and alkylated) LTP1 protein. The critical role of protein structure and disulphide bonds on IgE binding has been reported for other food allergens, such as a 2S peanut albumin (Ara h 2) [32, 33]. These results suggest that use of

short synthetic peptides for epitope identification is not ideal for proteins like LTP1 that are small and have very compact structure, which evidently favours discontinuous epitopes. A discontinuous epitope has been identified for wheat and peach LTPs by the combination of peptide phage display and three-dimensional modelling. This epitope bound by IgE from peach-allergic patients and patients with baker's asthma involves two regions (residues 36–43 and 70–79) of Pru p 3 and Tri a 14 [10, 34].

Although low signal was obtained, the Pepscan analysis (Fig. 1) of LTP1 highlighted an epitope, ³⁷QARSQSDRQS⁴⁶, that was common to four patients with food allergy and also reacted to by the sensitized mice. A part of this sequence was also identified on Tri a 14 by Pepscan analysis (³²KNLHNQARSQ⁴¹) and phage display (first part of the discontinuous epitope N36 R39 S40 S42 D43 ...) in baker's asthma [34]. Therefore, it appears that this epitope involved in food allergy to wheat is similar to one recognized by IgE in baker's asthma [34], another type of wheat allergy, but in which sensitization to wheat occurs via the respiratory tract. Detection of this epitope by mouse IgE after intraperitoneal injection, demonstrates that this epitope can access the immune system by yet a third route. The weak recognition of this sequence by both human and mouse IgE in our study may be the consequence of this sequence being part of the larger discontinuous epitope that was defined for baker's asthma and peach allergy [10, 34].

The LTP syndromes including wheat and serological cross-reactions between LTPs derived from several plants have been described [34–37]. The present work confirms the presence of common epitopic regions between wheat and fruit LTPs, notably the region 37–46 reported for several LTPs from *Rosaceae* (Pru p 3, Mal d3, Pru d 3 and Pru ar 3) [10, 15, 16]. The C-terminal peptide 81–90 detected here by a single serum was not reported yet on Tri a 14 for baker's asthma, but has been mapped as an IgE-binding region of fruit LTP [16, 34]. To the best of our knowledge, the allergic patients in this study with specific IgE to wheat LTP1 are not allergic to fruit. Consequently, we speculate that the highly conserved 3D structure of plant LTPs seems to give rise to the same epitopic zones even if the overall sequence homology between wheat and fruit LTP is weak and does not always lead to serological or clinical cross-reactions. However, distinct epitopic regions also exist. The wheat LTP1 epitope 53–62 detected here by mouse IgE and by IgE from patients with baker's asthma [34] was not obtained for fruit LTP, while the epitopic region 11–25 reported on fruit LTP [16, 34] was not found for wheat LTP1.

The Pepscan approach yielded many more positive sequences for the epitope mapping of gliadins and a LMW-glutenin subunit. Sixty to eighty percent of patient sera with specific IgE reacted with some decapeptides in the Pepscan analysis. Differences were, however, evident between prolamins classes and were also dependent upon the characteristics of allergic patients. Most of the Pepscan sera

that reacted positively with ω 5-gliadin peptides were from adult patients suffering from severe symptoms (U, anaphylaxis or EIA). Although only five patients were children, the sera from these children showed either weak or no responses in the Pepscan analysis. Therefore, as observed by Beyer et al. [38] for the intact ω 5-gliadin protein, ω 5-gliadin peptides may not be suitable for diagnosis of wheat allergy in childhood in contrast to the situation for adults, where extensive epitopes were found. Differences in the type of peptide detected for ω 2-gliadin were observed between children and adults as well, with the sera of children appearing to react to more epitopes on the ω 2-gliadin than adults (Table 2). Some differences between children and adults were also in evidence for α -gliadin, with higher proportion of adult sera than child sera positive in Pepscan despite lower concentrations of α -gliadin-specific IgE. In the Pepscan analysis of α -gliadin, two of the serum samples from children showed peptides that included, or were close to, a cysteine residue and might participate in discontinuous epitopes. This was not the case for sera from adults or mice. The IgE-binding epitopes of ω 5-gliadin were repeated many times and covered a large part of the protein sequence (Fig. 4). This might be expected as the ω 5-gliadin consists almost entirely of repeated sequences that are either identical or similar to one another. These repeated epitopic sequences may promote very efficient mast cell degranulation and contribute to the development of severe symptoms in these patients. The number and intensity of epitopes found for ω 5-gliadin with adult sera appeared to be somewhat greater than was the case for the ω 2-gliadin. The main difference between these two ω -gliadins is in the type of repeating sequence motif exhibited; the key repeating motif in ω 5 is close to QQQXP, where X is F, I, or L, and a notable repeating motif in ω 2-gliadins is close to QQPFP. The high proportion of proline residues in both types make it likely that the digestive enzymes of humans will be restricted in their ability to degrade these proteins so that quite large peptides are likely to persist in the small intestine. Both types of ω -gliadin appear to be for the most part intrinsically unstructured proteins [12] although perhaps with some turns (β or γ) superimposed on the flexible polypeptide chains. The persistence of large fragments, repeating sequences, and the ability of the flexible chain to adapt to receptor sites might be factors contributing to the allergenicity of these proteins and the continuous nature of the epitopes involved. In contrast, the compact, stable structure of LTP1, which is also resistant to digestion, seems likely to be a factor in the predominance of discontinuous epitopes for this allergenic protein.

The sequence of a LMW-glutenin subunit was mapped for the first time with the Pepscan technique and one dominant epitope, P39–48, was detected. Other peptides from the non-repetitive C-terminal domain were bound by patient sera. The presence of IgE-binding epitopes in

this domain non-related to ω 5-gliadins was also demonstrated by Snegaroff et al. [39] using recombinant subunits and domains of LMW-glutenins.

The determination of some similar continuous epitopes for α -, ω 2-, ω 5-gliadins and a LMW-glutenin subunit gives rise to several possibilities for serological cross-reactions between all these proteins. The epitope $^{27}\text{QQQFPGQQQ}^{36}$ of α -gliadin might certainly lead to cross-reactions with ω 5-gliadins. Similarly, epitopes $^{39}\text{QQPIQQQPQQ}^{48}$ and $^{47}\text{QQFPQQQPCS}^{56}$ might be responsible for cross-reactions between some LMW-glutenin subunits and ω 5-gliadins. Cross-reactions might also occur for some patients between ω 2 and ω 5-gliadins via the sequences QQFPQ, QQQFPEQ, or QQFPQ.

It is worthy to note that the same epitopes of ω 5-gliadin found for Japanese patients were evident for French patients, both groups having the same consensus motif $\text{QQX}_1\text{PX}_2\text{QQ}$ (X_1 being L, F, S or and X_2 Q, E or G). For ω 2-gliadin, the main epitope bound by IgE from children (PQQFPQ) was similar to the sequence found in Japanese patients allergic to wheat with AEDS [40]. However, the peptides evidenced here were different from LMW-glutenin epitopes described for Japanese patients with AEDS. It may be noted, however, that the Japanese IgE-binding epitopes were obtained from a chymotryptic hydrolysate of gluten and not by the Pepscan technique [41].

The mouse model of sensitization to wheat proteins was evaluated in this study for the ability of the mice to produce IgE that could mimic human IgE in binding characteristics. In mice sensitized by intraperitoneal injections of the purified LTP1, two epitopes (37–46 and 53–62) were identified by Pepscan. The first one was also bound by IgE from our patients with food allergy to wheat whereas the second was not. However, the second matches an epitope (52–61) identified for patients with baker's asthma by Tordesillas et al. [34]. Moreover, LTP1 folding was highly important both for human and mouse IgE-binding. These results point out interesting similarities between patient response and that of mice in terms of allergy to LTP1. Mice sensitized with total gliadins also showed correspondence in the resulting epitopes with those of allergic patients. For example, the response of mouse IgE to the ω 5-gliadin sequence was similar to that of adult patients allergic to wheat. And for α -gliadin, mouse and human IgE recognized similar epitopic sequences. However, a major difference is the specific and strong binding of regions 31–60 and 82–93 by mouse IgE. This first fragment (31–60) contains a peptide sequence (31–49) that is close to a

peptide active in celiac disease, another pathology linked to wheat. Response of mouse IgE is also close to that of human IgE for γ - and ω 2-gliadin peptides. The mouse model of sensitization to gliadins, despite some divergences, appears to be useful as a substitute for humans in some studies. This is consistent with previous data showing similarities between the peanut or egg protein epitopes recognized by mouse and human IgE [42, 43]. Although mouse sensitizations were obtained by intraperitoneal injections, they are reasonably representative of an IgE response induced in allergic patients by the oral route as already observed by Adel-Patient et al. [44] for a milk allergen. Comparison of different entry routes of ovalbumin in BALB/c mice showed heterogeneity in epitopes, fewer epitopes being obtained by intraperitoneal injections compared with oral and subcutaneous routes. However, the epitopes found by intraperitoneal injections were the immunodominant ones [42].

Clinical relevance

For the wheat allergen LTP1 this work evidenced a strong influence of protein conformation and sequence on the type of IgE-binding epitopes, regardless of whether human or mice were studied, or the route of entry for the allergen. Indeed, the very compact 3D structure of LTP1 is not so readily broken down in the digestive tract to small peptides because it is resistant to digestive enzymes; this evidently favours the development of IgE directed to conformational epitopes. We contrast LTP1, which appears to have predominantly discontinuous epitopes with the ω -gliadins, which seem to elicit mostly continuous epitopes, both in humans and mice, presumably as a consequence of the ω -gliadins having largely unordered and flexible polypeptide chains. The α -gliadins appear to have both types of epitopes, continuous in the repeating sequence domain and discontinuous in the unique sequence domain. Also, our work confirms the existence of differences between children and adults in the allergic response to gliadins.

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