Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY

EAACI

Allergy

ORIGINAL ARTICLE EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Allergy to deamidated gluten in patients tolerant to wheat: specific epitopes linked to deamidation

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To cite this article: Denery-Papini S, Bodinier M, Larré C, Brossard C, Pineau F, Triballeau S, Pietri M, Battais F, Mothes T, Paty E, Moneret-Vautrin D-A. Allergy to deamidated gluten in patients tolerant to wheat: specific epitopes linked to deamidation. *Allergy* 2012; **67:** 1023–1032.

Keywords
deamidation; food allergy; gliadins. IgE-binding epitopes; wheat.

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Accepted for publication 15 May 2012

DOI:10.1111/j.1398-9995.2012.02860.x

Edited by: Reto Crameri

Abstract

Background: Gluten proteins can be modified by deamidation to enhance their solubility and technological applications. However, severe allergic reactions have been reported after the consumption of food products containing deamidated gluten (DG) in subjects tolerant to wheat. This work aimed to characterize allergen profiles for these patients in comparison with those of patients allergic to wheat and to identify IgE-binding epitopes.

Methods: Sera were obtained from 15 patients allergic to DG and from nine patients allergic to wheat proteins (WP). IgE-binding profiles were characterized both in ELISA and in a humanized rat basophilic leukaemia (RBL) cell model. Epitopes were mapped on γ- and ω2-gliadin sequences by Pepscan, and effect of glutamine/glutamic acid substitutions was studied.

Results: Compared to the heterogeneous pattern of allergens detected by IgE from patients allergic to WP, responses of patients allergic to DG were homogeneous. In ELISA, all the sera displayed IgE binding to deamidated γ- and ω2-gliadins and deamidated total gliadins, frequently with high concentrations. These modified proteins induced RBL degranulation with most of the sera from DG-allergic patients. A consensus epitope was found on native γ- and ω2-gliadins (QPQQPFPQ); it was repeated several times in their sequences. The substitution of two or three glutamines of this epitope into glutamic acid at positions Q3 or Q4 and Q8 (QPEEPFPE) increased its recognition the best.

Conclusion: Allergy to DG is a separate entity from wheat allergy. It can be evidenced by strong IgE binding to deamidated gliadins or peptides of the type QPEEPFPE.

Gluten proteins (gliadins and glutenin subunits) play a major role in bread making. Their amino acid sequences are remarkable, because they contain a high amount of proline (about 30%) and glutamine (about 40%), and repetitive sequences. Four classes of gliadins are separated (α-, γ-, ω2 and ω5) according to their sequences and electrophoretic mobility. The ω2- and ω5-gliadins only contain a single repetitive domain (1), while the α- and γ-gliadins also contain a non-repetitive C-terminal domain including intramolecular disulphide bonds (2). Viscoelasticity and insolubility of gluten proteins are the basis of their functionality, but also limit their use. Diversification of gluten applications was achieved through the creation of water-soluble or water-dispersible products. Deamidation, one of the methods for this purpose, may be obtained with either acid or alkali treatment (3). Removing the amide group from some of the many glutamine residues to form corresponding glutamic acid reduces gluten protein aggregation (hydrogen bonding) and increases the potential charge. Several of such gluten-derived products can be found in the food industry, for example isolated wheat proteins (WP), used as a food emulsifier, that are acidic deamidated WP with enhanced protein solubility (4).

In 2003, Leduc et al. (5) described the first case of food allergy to a wheat isolate, without allergy to native wheat

flour. The symptoms were severe, angioedema with generalized urticaria, and occurred after consumption of meat-based products. Since then, 14 cases of severe allergic reactions to deamidated gluten (DG) in patients tolerant to wheat have been reported in France through the Allergy Vigilance Network (www.allergyvigilance.org). A solution of wheat isolate for skin prick test was proposed in order to help the diagnosis of allergy to DG (6). However, a differential diagnosis between a specific allergy to DG and an allergy to native WP is not always easy, as the isolate fraction contains not only modified wheat allergens but also some native allergens involved in WP allergy. Moreover, a double-blind, placebo-controlled food challenge with native and modified WP may not be possible notably because of symptom severity. Unlike allergy to WP, no study was yet carried out to identify allergens and epitopes bound by IgE in DG allergy. A previous work (7) on gliadin epitope mapping included the serum of only one patient allergic to DG. In coeliac disease, influence of deamidation on the recognition of B-cell and T-cell epitopes of gliadins is well known; this has never been studied in the context of food allergy to DG. However, an epitope-based method might be useful to allow a specific diagnosis of allergy to DG.

Our work aimed to study the IgE binding of patients allergic to DG towards different wheat protein fractions, both in ELISA and in a model of basophil activation, and to compare their allergen profiles with those of patients allergic to WP. IgE-binding epitopes were also mapped on gliadins for the patients allergic to DG.

Material and methods

Gliadin and glutenin subunit purification

Gliadins were extracted from wheat flour (cultivar Hardi) using a sequential procedure (8). Briefly, the albumins/globulins (water-/salt-soluble proteins from wheat flour) were extracted from the flour with a saline buffer; after several washings, the flour was suspended in 70% ethanol for 1 h at room temperature; after centrifugation, the supernatant, that is the total gliadins, was collected. The gliadin classes (α-, γ, ω2 and ω5) were then prepared by ion-exchange chromatography and reverse-phase HPLC (9–11). Low-molecular-weight (LMW) glutenin subunits were obtained by the procedure of Verbruggen et al. (12). After pre-extraction of gliadins, glutenin subunits were solubilized from flour at 60° C with 50% propanol containing 1% dithiothreitol. Low-molecular-weight subunits were then precipitated with a final concentration of 85% propanol.

Preparation of deamidated wheat protein fractions

Purified α-, γ-, ω2- and ω5-gliadins, total gliadins and LMW glutenin subunits were deamidated by acidic treatment (13). Briefly, 20mg of proteins was dispersed in 1 ml of 0.1N hydrochloric acid. The temperature was increased to 90°C and maintained for 1 h. The reaction was stopped by neutralization with 0.1N NaOH. After dialysis against water and centrifugation to recover the supernatant, the fractions were freeze-dried. Deamidation was controlled by native PAGE (without SDS) in a gel at 10% acrylamide in comparison with native fractions. As gliadins and glutenin subunits contain very few charged residues, their migration is much reduced in the absence of SDS, whereas the negative charges induced by deamidation allow the protein migration; the deamidation percentage was calculated by determining the level of Glu and Gln and was in the range of 36–51% for the different fractions (13).

Studied population

Fifteen patients (five children and ten adults) tolerant to wheat but allergic to DG were recruited (Table 1). They experienced acute urticaria (U), exercise-induced anaphylaxis (EIA) or anaphylactic shock (AS). Repetitive episodes occurred. Attention was drawn to DG, a common feature of different food products that had elicited allergy: sausages, pork pie, meats with bread crumbs, reconstituted meats, ready-cooked vegetable dishes, industry-processed soups or industrial cakes and cookies. In this group, patients 30, 34 and 91 also had contact urticaria with cosmetics containing wheat hydrolysates (creams, eye-liners, shampoo). Nine patients allergic to wheat (six children and three adults) were also tested. They had atopic eczema dermatitis syndrome (AEDS), AS, U or EIA (Table 1). Sera from non-atopic patients without any past or present history of atopic diseases, and negative prick test to 12 common aero-allergens were used as controls.

Skin prick tests

Prick-in-prick tests (PIP) were carried out to natural wheat flour, natural gluten and DG (isolate from ALK-Abello, Courbevoie, France). Patient 91 was tested to two cosmetics (containing wheat hydrolysate). All subjects had a positive control to codeine phosphate and negative control to saline. A wheal diameter of 2.5 mm was considered positive.

Oral challenge

Oral challenge was performed after at least 3 weeks of strict avoidance of wheat and DG. The double-blind placebo-controlled food challenge (DBPCFC) procedure was used. The placebo mixture was maize or potato starch included in stewed apple. The vehicle was stewed apple. Raw wheat flour and raw DG were tested. Deamidated gluten (isolate) was obtained (GemtecTM functional WP; Manildra group, Auburn, New South Wales, Australia). Increasing doses were given at 20-min interval. The starting dose was generally 1 g of wheat in the case of suspected wheat allergy and 10 g in wheat-tolerant subjects. Two DBPCFCs to wheat were coupled to a 30-min exercise on a treadmill. For gluten isolate, the two-first doses were 100 mg and then 400 mg (cases 30, 34 and 352). For cases 91 and 390, the scale was 40, 100 and 300 mg (cumulated dose of 440 mg). Criteria of positivity were objective symptoms in all cases except case 390, but in

Table 1 Clinical characteristics of patients allergic to deamidated gluten (DG) and patients allergic to native wheat proteins (WP). Patients' reactivity towards native proteins (wheat or gluten) or deamidated proteins (isolate) was tested by skin prick test, CAP® Phadia and oral food challenge

Patients' information				Skin prick test (mm)			Specific IgE (KUI/L)		DBPCFC with wheat or isolate result, maximal or reactive dose (g) and symptoms			
Code	Sexe	Age	Symptoms	Wheat	Gluten	Isolate	Gluten	Isolate	Wheat	Symptoms	Isolate	Symptoms
Patients allergic to DG												
30	F	24	U, contact U	–	2.5	7	3.1	nd	Neg 30g		Pos 2 g	Generalized erythema, rhinitis, laryngeal pruritus, cough
34	F	18	EIA, contact U	–	–	8.5	2.9	nd	Pos 27 g + effort	Decrease of PFR, dyspnea	Pos 1.5 g	Abdominal pain, asthma
78	F	Adult	EIA				Lost information					
88	F	28	EIA	–	–	10	3.9	8.4	nd		nd	
91	F	46	EIA, contact U	–	2.5	8.5	0	nd	Neg 37.5 g + effort		Pos 0.44 g	Laryngeal pruritus, tongue irritation, palate pruritus
137	F	38	U	nd	nd	nd	7.7	78.4	nd		nd	
275	F	26	U	nd	nd	nd	4	29.2	nd		nd	
276	M	Child	U	–	–	10	3.4	100	nd		nd	
280	F	38	AS	–	7	17	15.3	57.1	Neg 31 g		Pos 1 g	Palpebral and hand angioedema, urticaria (2 h)
281	F	25	U	nd	nd	nd	4.4	100	nd		nd	
285	F	10	AS	–	nd	6	1.32	nd	Neg		nd	
299	M	8	AS	–	nd	5.5	nd	nd	Neg		nd	
352	M	12	EIA	–	–	4.5	0.4	nd	Neg 70 g + effort		Pos 2.2 g	Rhinitis, conjunctivitis, cough decrease of PFR
390	M	10	U	–	3	5	nd	nd	nd		Pos 0.44 g	Laryngeal pruritus (1 h30)
497	F	24	U	nd	nd	6.5	0.42	nd	nd		nd	
Patients allergic to WP												
9	M	1.5	AEDS	7	3	nd	40	nd	Pos 5 g	Rash on atopic dermatitis	nd	
32	M	1	AEDS	4	8.5	nd	16.5	nd	Pos 4 g	Rash on atopic dermatitis	nd	
35	M	35	U	–	–	nd	0	nd	Pos 30 g		nd	
36	M	79	U	–	10	nd	0	nd	nd		nd	
111	F	12	AS	4	5	nd	21.4	nd	Pos 25 g		nd	
120	F	3	AEDS	5	4	nd	0	nd	Pos 5 g		nd	
302	M	1	U	6	5	nd	>100	nd	nd		nd	
324	M	11	U	6.5	3.5	nd	nd	nd	Pos 10 g	Sneezing, urticaria	Neg 0.95 g	
834	M	37	EIA	3	6.5	5	9.4	nd	nd		nd	

AEDS, atopic eczema dermatitis syndrome; AS, anaphylactic shock; EIA, exercise-induced anaphylaxis; U, urticaria; DBPCFC, double-blind, placebo-controlled food challenge; nd, not determined; –, negative prick tests. Results of food challenges: neg, negative; pos, positive; PFR, peak flow rate; WP, wheat proteins.

this case laryngeal pruritus lasted 1 h30 and besides, placebo challenge was asymptomatic. When the oral challenge was not performed, the final diagnosis was set on both criteria: a convincing history incriminating either wheat or gluten isolate and a total recovery after the specific avoidance. 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).

Analysis of IgE reactivity towards native and deamidated fractions by ELISA

Sera were analysed for specific IgE by fluorimetric ELISA (14). Concentrations of specific IgE were calculated from the adjusted standard human IgE curves provided that the fluorescence exceeded the quantification limit. The mean quantification limit calculated from 16 experiments was 0.24 ± 0.16 ng/ml. All determinations were carried out in duplicate and are expressed as mean values. For two sera (34 and 299), enough volume was available to perform eight replicates, resulting in about 10% variation. Matched data per serum were submitted to repeated-measures one-way ANOVA and subsequent Tukey's multiple-comparisons test with level of significance set at 0.05 using GRAPHPAD PRISM version 5.02 (GraphPad Software, Inc., San Diego, CA, USA).

Inhibition experiments were carried out using the same methodology except that plates were coated with the deamidated ω2-gliadin fraction (1 μg/ml) and sera were incubated during 4 h in the presence of inhibitors: ω2-gliadins native or deamidated (concentration range of 1.25–0.0125 nmol/ml) or synthetic peptides LQPQQPFPQQ and LQPEEPFPEQ (concentration range of 95–2.9 nmol/ml).

Analysis of biological activity of native and deamidated fractions with humanized rat basophilic leukaemia (RBL) cells

The RBL degranulation test was carried out with RBL cells expressing the human FcεRI α-, β and γ-chains (clone SX38 P4B7) as described (14). IgE-sensitized cells were stimulated in Tyrode buffer containing 50% deuterium oxide with proteins (0.02–0.2–2 μg/ml) or 1 μg/ml anti-human IgE (positive control). Rat basophilic leukaemia degranulation was measured in duplicate and expressed as a percentage of total β-hexosaminidase release. A positive threshold of 5% of degranulation was calculated by analysing 27 control sera (non-atopic subjects or grass pollen allergy) and corresponded to the mean + 2 SD.

Epitope mapping on gliadins with solid-phase synthetic peptides (Pepscan)

IgE-binding epitopes were searched among sequences of γ-[P08453] and ω_2-gliadins [Q9FUW7] with decapeptides overlapping by eight amino acids as described (7, 15). Critical amino acids and effect of substitutions of glutamine into glutamic acid were analysed. Peptides were prepared by automated spot synthesis and covalently fixed by their C-terminal end on a cellulose membrane (Abimed, Lanenfeld, Germany). After washing 5 min with ethanol and three times 5 min with a Tris 50 mM buffer, pH 8.0, containing 0.05% Tween 20 (v/v) (TBS/T), membranes were blocked with 2.5% (w/v) skimmed dried milk and 5% (w/v) sucrose in TBS/T for 1 h, washed in TBS-T and incubated overnight with sera from patients diluted in blocking buffer. After further washing, anti-human IgE antibodies labelled with peroxidase (P0295; Dako, Glostrup, Denmark) diluted 1/10 000 in blocking buffer were added for 1 h and washed. Membranes were then incubated with a chemiluminescent substrate (SuperSignal West Dura Extended Duration Substrate; Pierce, Rockford, IL, USA). IgE binding was revealed by chemiluminescence with a CCD camera (Luminescent Image Analyzer LAS 3000; Fujifilm, Tokyo, Japan). A semi-quantitative evaluation of IgE binding was carried out with the MULTI GAUGE version 3.0 software (Fujifilm). For each serum, the spot detected with the highest intensity was taken as a reference. IgE bindings to peptides were then classified according to this reference. Non-atopic control sera displayed no binding to the peptides; the same was observed for a serum from an allergic patient (total IgE about 5000 ng/ml) not sensitized to wheat.

Results

Characterization of deamidated gliadins

Modified fractions migrated in electrophoresis without SDS, showing the effectiveness of the deamidation reaction (Data S1). These fractions displayed a large migration area in relation to the heterogeneity of the deamidation process. Their percentages of deamidation were in the range of 36% (±12)–51% (±15) as described (13).

Comparison of IgE-binding profiles in ELISA between patients allergic to DG and those allergic to WP

IgE binding to native gluten fractions

IgE responses for patients allergic to DG were homogeneous (Fig. 1A), all but one patients presenting IgE binding to ω2- or γ-gliadins. No sera displayed IgE binding to ω5-gliadins nor to the albumin/globulin extract (water/salt-soluble proteins from flour – not shown), and few responses were observed towards α-gliadins and LMW glutenin subunits. Responses of patients allergic to WP were heterogeneous (Fig. 1B), with IgE binding to different fractions according to sera and, for some of them, high concentrations in IgE specific for albumins/globulins (not shown).

IgE binding to deamidated gluten fractions

All the sera of patients allergic to DG displayed IgE binding to deamidated γ- and ω2-gliadins as well as to deamidated total gliadins, and the majority of them showed very high concentrations in IgE specific for these fractions (Fig. 1C). Most of these sera also showed IgE binding to deamidated α- and ω5-gliadins and LMW glutenin subunits. The IgE

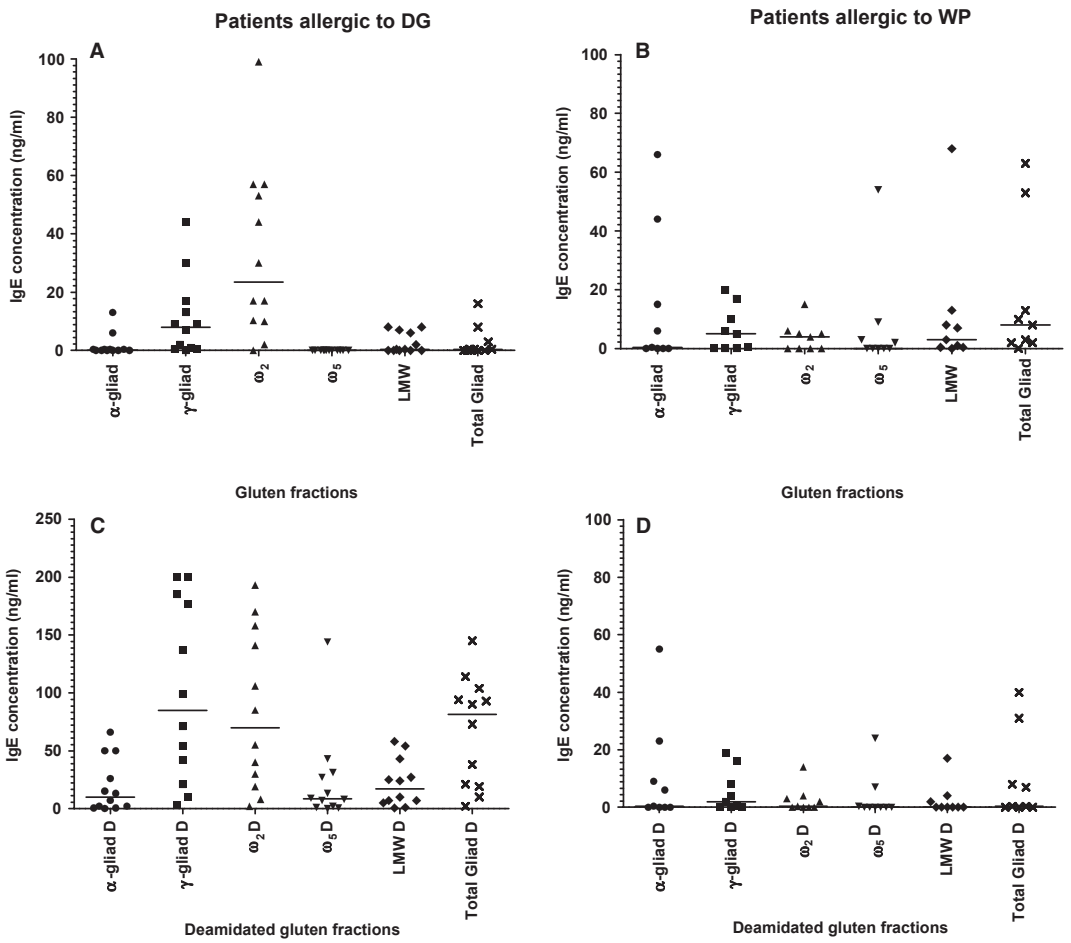

Figure 1 IgE reactivity profiles in ELISA of sera from patients allergic to deamidated gluten (DG: A, C) and patients allergic to native wheat proteins (WP: B, D). Concentrations of specific IgE towards native (A, B) and deamidated (C, D) gluten fractions are expressed in ng/ml. Bars stand for median concentrations. α-gliad, γ-gliad, ω2, ω5 and total gliad: α, γ, ω2, ω5 and total gliadins; LMW: low molecular weight glutenin subunits – α-gliad D, γ-gliad D, ω2 D, ω5 D and total gliad D: deamidated α, γ, ω2, ω5 and total gliadins; LMW D: deamidated LMW glutenin subunits.

responses towards DG proteins from patients allergic to WP were unchanged or decreased compared to responses towards native proteins (Fig. 1D).

Comparison of IgE reactivity in the two groups of patients by a statistical analysis

One-way ANOVA of matched observations revealed insignificant differences between means for IgE reactivity of sera from WP patients towards native gluten proteins ($P = 0.42$), deamidated proteins ($P = 0.28$) or both types ($P = 0.20$). However, significant differences were observed ($P < 0.0001$) for sera from DG patients. For these patients, IgE reactivity of sera was significantly higher (1) towards native ω2-gliadins than towards the other native fractions, (2) towards deamidated ω2-, γ- and total gliadins than for the other deamidated fractions and (3) towards deamidated ω2-, γ- and total gliadins than for their respective native fraction.

Triggering potential in the RBL assay of native and deamidated γ-, ω2- and total gliadins for patients allergic to DG or to WP

As shown in Table 2, degranulation was observed for nine of 12 sera from patients allergic to DG in the presence of deamidated total gliadins; the degranulation percentage was often strong (>25%). Lower or no degranulation was measured with native gliadins. Degranulation was elicited by native and deamidated ω2-gliadins for eight sera (seven with a response $\geq 25\%$). Yet, for most of these sera, the deamidation of ω2-gliadins increased the response intensity or decreased the reactivity threshold (Data S2). No degranulation was induced by the native γ-gliadins for these 12 sera, while the deamidated γ-gliadins triggered the reaction with half of them.

Only five sera from patients allergic to WP out of nine displayed positive responses in the degranulation test.

Table 2 Degranulation percentages obtained in a RBL test with sera from patients allergic to deamidated gluten (DG) or patients allergic to native wheat proteins (WP) in presence of native or deamidated antigens

Serum	Tot gliad	Tot gliaD	ω2-gliad	ω2-gliadD	γ-gliad	γ-gliadD
Patients allergic to deamidated gluten						
30	36	57	25	28	0	25
34	0	20	47	68	0	0
78	0	0	0	0	0	0
88	27	23	0	0	0	0
91	0	0	0	0	0	0
137	0	37	56	89	0	37
280	12	47	31	39	0	21
285	13	34	36	49	0	41
299	29	51	28	33	0	31
352	17	47	37	37	0	31
390	0	0	0	0	0	0
497	0	13	10	10	0	0
Patients allergic to native wheat proteins						
9	0	0	0	0	0	0
32	0	0	0	0	0	0
35	8	7	12	7	8	8
36	6	0	0	0	0	0
111	0	0	0	0	0	0
120	17	15	11	6	10	13
302	42	36	0	0	8	8
324	0	0	0	0	0	0
834	0	0	0	0	5	0

RBL, rat basophilic leukaemia.
Tot gliad, ω2-gliad, γ-gliad: total, ω2 and γ-gliadins.
Tot gliadD, ω2-gliadD, γ-gliadD: deamidated total, ω2 and γ-gliadins.
Colors indicate the range of degranulation percentages: □ <threshold; □ 5% < × < 25%; ■ 25% < × < 50%; ■ >50%.

Percentages of degranulation were generally low, and no clear differences were detectable between deamidated and native proteins.

Continuous epitopes detected by IgE from patients allergic to DG

As γ- and ω2-gliadins were the native WP the most frequently bound by IgE from patients allergic to DG, IgE-binding epitopes were mapped among the entire sequences of these proteins. Sera from 12 patients were used, and eight of them detected several peptides (Data S3) distributed along the sequences of both proteins. The sequences detected with the highest intensity and by the larger number of sera were as follows: LQPQQPFPQQ and QQPQQPFPQQ present on both γ- and ω2-gliadins, QQPQQPFPQT, QQPQQPFPQL on γ-gliadin and IQPQQPFPQQ on ω2-gliadin. Remarkably, all these sequences contained the octapeptide QPQQPFPQ. This consensus native sequence was chosen to investigate the critical amino acids for IgE binding, first by scanning the peptide sequence by alanine and then by shortening its sequence by removing the N- or C-terminal residues (Table 3). Three alanine sequential replacements on positions Q4, P5 and F6 eliminated the octapeptide recognition for more than 50% of the sera. Deletion of the first residue in N-terminal or of the two residues from the C-terminal end (P7Q8) abolished the peptide recognition by all sera.

To address the effect of deamidation, the four glutamine residues were sequentially or simultaneously substituted with glutamic acids at their different positions (Table 4). The substitution of a single Gln of the consensus peptide into Glu induced its recognition by additional sera or increased recognition in other cases. Simultaneous substitution of two Gln generally increased the response intensities. Substitutions of three of the four Gln enhanced again the response, and the peptide detected with the highest intensity was QPEEPFPE with substitutions at positions Q3, Q4 and Q8. Replacement of all Gln into Glu did not increase the intensities more (except for one serum) and even abolished the recognition for one serum.

Inhibition experiments were carried out with four sera from DG-allergic patients to check the specificity of the response towards deamidated epitopes. The peptide LQPEEPFPEQ was used in comparison with the corresponding sequence in native proteins LQPQQPFPQQ; native or deamidated ω2-gliadins were used as controls. Inhibition of IgE binding to the deamidated ω2-gliadin coated on the plate was observed for the peptide LQPEEPFPEQ with the four sera, whereas no inhibition was observed with the native sequence (Fig. 2).

Discussion

Allergic reactions to food containing DG or cosmetics containing wheat hydrolysates have been reported (5, 16–18). In this work, we examined sera from patients who developed severe symptoms of food allergy to gluten proteins modified by deamidation. To elucidate which molecular structures were involved among the complexity of wheat flour proteins, a range of purified proteins in native and deamidated forms was used as well as synthetic peptides, to study the effect of different levels and positions of Glu/Gln substitutions.

In food allergy to native wheat flour, several allergens are known among the albumin/globulin and the gluten fractions (19), and IgE-binding profiles are heterogeneous (10). The IgE-binding profile from patients allergic to DG was completely homogeneous with frequent IgE binding to γ- and ω2-gliadin fractions in their native state. Despite ω5-gliadin is known as a major allergen in cases of EIA caused by wheat, no IgE specific for ω5-gliadins could be detected in DG-allergic patients with symptoms of EIA. The deamidated γ- and ω2-gliadins appeared to be the dominant allergens for patients allergic to DG, as all sera displayed IgE specific for these fractions, mostly with high concentrations (>50 ng/ml IgE); lower reactivity to other DG fractions was probably due to cross-reactions because of sequence homologies. This result was confirmed in a functional assay using RBL cells.

Native γ-gliadin was unable to elicit degranulation of RBL cells for DG-allergic patients, whereas native ω2-gliadin did

Table 3 Effect of alanine scanning and truncation on the recognition of the peptide QPQQPFPQ in Pepscan by the sera from patients allergic to deamidated gluten

Patients	30	78	137	275	276	280	281	299	Number of positive sera
Peptides									
Q P Q Q P F P Q	+	+	+	+	+	+	+	+	8
A P Q Q P F P Q	+	0	0	+	+	+	+	0	5
Q **A** Q Q P F P Q	+	0	0	0	+	+	+	+	5
Q P **A** Q P F P Q	+	+	0	0	0	+	+	0	4
Q P Q **A** P F P Q	0	0	0	0	0	+	+	0	2
Q P Q Q **A** F P Q	+	+	0	0	0	0	+	0	3
Q P Q Q P **A** P Q	0	0	0	0	0	0	0	0	0
Q P Q Q P F **A** Q	+	+	0	0	0	+	+	0	4
Q P Q Q P F P **A**	+	0	0	0	+	+	+	0	4
P Q Q P F P Q	0	0	0	0	0	0	0	0	0
Q P Q Q P F P	+	+	+	0	0	0	+	0	4
Q P Q Q P F	0	0	0	0	0	0	0	0	0

+: positive response; 0: response suppression; gray shade: response suppression for all sera.

Table 4 Effects of substitution of Gln/Glu at different positions on the recognition of the peptide QPQQPFPQ in Pepscan by the sera from patients allergic to deamidated gluten

Serum 30	Patients	30	34	78	88	137	275	276	280	281	285	299	352
	Peptides												
	Q P Q Q P F P Q	(+)	0	(+)	0	(+)	+	++	(+)	+++	0	(+)	0
	E P Q Q P F P Q	(+)	(+)	(+)	0	(+)	+++	++	0	++	(+)	(+)	0
	Q P **E** Q P F P Q	(+)	(+)	++	0	+	0	+	(+)	++	(+)	(+)	(+)
	Q P Q **E** P F P Q	+	(+)	+	0	+	0	++	0	++	(+)	(+)	+
	Q P Q Q P F P **E**	+	(+)	++	0	+	0	+	(+)	++	(+)	(+)	(+)
	E P **E** Q P F P Q	(+)	+	+	0	+	nd	nd	(+)	nd	(+)	(+)	(+)
	E P Q **E** P F P Q	+	(+)	(+)	0	++	0	++	0	++	(+)	(+)	+
	E P Q Q P F P **E**	(+)	(+)	(+)	0	+	0	+	(+)	++	(+)	(+)	(+)
	Q P **E E** P F P Q	+	+	+	0	+	nd	nd	(+)	nd	+	(+)	+
	Q P **E** Q P F P **E**	+	++	+++	0	+	++	+++	(+)	+++	(+)	(+)	+
	Q P Q **E** P F P **E**	++	+	++	0	+	nd	nd	(+)	nd	+	(+)	++
	E P **E E** P F P Q	++	+	0	0	+	nd	nd	(+)	nd	+	+	+
	E P Q **E** P F P **E**	++	+	0	0	+	nd	nd	(+)	nd	++	+	++
	E P **E** Q P F P **E**	++	++	(+)	0	+	nd	nd	+	nd	++	++	+
	Q P **E E** P F P **E**	+++	+++	+++	0	+++	nd	nd	+++	nd	+++	++	+++
	E P **E E** P F P **E**	+++	+++	0	0	+++	nd	nd	+++	nd	+++	+++	+++

The response intensity for each serum is indicated as follows: taking as reference, the spot detected with the highest intensity: 0: no response, (+): <20%; +: 20–40%; ++: 45–70%; +++: 70–100%; nd, not determined because of lack of volume.
Example of the results obtained by Pepscan with serum 30.

with most tested sera. A similar result was observed *in vivo* by skin prick tests performed on six of the DG-allergic patients. Native ω2-gliadin provoked a clear positive reaction in these six patients, while only two of them reacted weakly with the native γ-gliadin (data not shown). Deamidated ω2-gliadin still elicited higher degranulation percentages than deamidated γ-gliadin in the RBL assay.

The Pepscan analysis on these two allergens and on the substitution library revealed many peptides bound with high intensity by most sera, indicating the ability of IgE from DG-allergic patients to detect continuous epitopes. Remarkably, all the Pepscan-positive sera detected similar peptides of the γ- and ω2-gliadins, and all these peptides contained a common sequence QPQQPFPQ. It is very close to the typical repeat units of ω2 (PQQPFPQQ) and of γ-gliadins (QPQQPFP) and absent on α- or ω5-gliadins or glutenin subunits. The substitution of some Gln into Glu increased the peptide recognition and induced its detection by additional sera. When substituting two or three of the four Gln into Glu, the positions that increased the response intensity the most were Q3 or Q4 and Q8. The peptide detected with the highest intensity was QPEEPFPE. Peptide truncation and

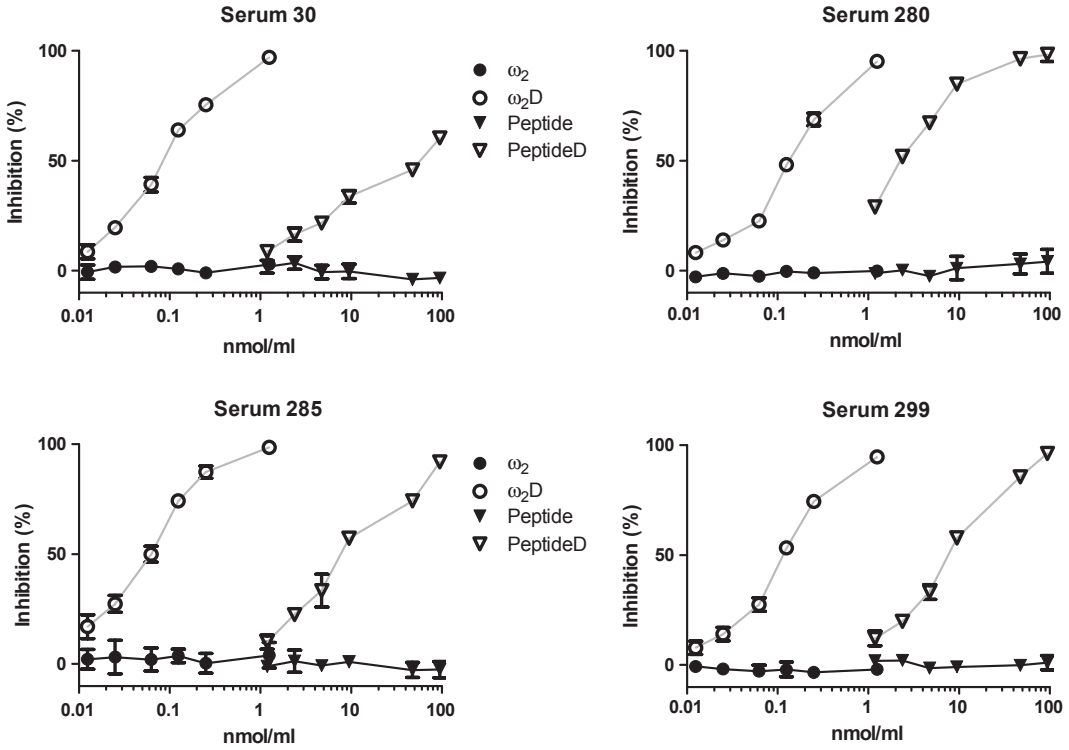

Figure 2 Inhibition of IgE-binding to the deamidated ω2-gliadin in ELISA, for four patients allergic to deamidated gluten. Inhibitors were the native (close symbols) and deamidated (open symbols) ω2-gliadins or a native peptide (LQPQQPFPQQ) and a deamidated peptide (peptide D: LQPEEPFPEQ).

alanine replacements indicated that the first position could not be deleted nor the Phe in the sixth position substituted.

As observed for the allergen profile, the epitope profile was completely homogeneous for this group of patients allergic to DG. This is an unusual observation; in the case of WP-allergic patients, interpatient variability was much greater even though dominant epitopes could be found (on ω5-gliadin for example) (15, 20). When comparing epitopes bound on ω2-gliadins in patients allergic to WP and DG, it is interesting to note that the (Q)PQQPFP(Q) sequence was also bound by IgE from some children (but not from adults) allergic to WP (15).

The difference between ω2- and γ-gliadins in their ability to elicit RBL activation is not easy to understand. The number of repetitions of the QPQQPFPQ sequence is almost the same in both proteins (eight or nine repetitions), and similar deamidation percentages were obtained. An explanation may come from the presence of a folded non-repetitive domain in the γ-gliadin which may hamper the accessibility of antibodies to the repeated domain containing all IgE-binding epitopes.

Most of the DG-allergic patients possessed IgE directed towards the native γ- or ω2-gliadins or epitopes, despite the fact that they could eat normal wheat products. This may be explained either by cross-reactions between native and deamidated sequences or by the partial deamidation of products (usually 20–50% of deamidation according to manufacturers), leading to possible sensitization to non-deamidated portions of the proteins.

Deamidation of gluten proteins can also be achieved by transglutaminases (TG) (21). In coeliac disease, selective deamidation by tissue TG greatly enhances peptide binding in the context of both B- and T-cell epitopes (22–26). Even though α-gliadins are considered to be the dominant T-cell-stimulating proteins, peptides from γ- and ω2-gliadins were also reported as T-cell-stimulating epitopes (27). Some homologies exist between the epitope QPQQPFPQ deamidated at some positions reported in this study and several peptides found as relevant IgA-/IgG-binding sequences (PLQPEQPFP) (28) or T-cell epitopes (QPQQPFPQPQQPFPWQP…) (27) in coeliac disease.

The involvement of ω2- and γ-gliadins was also shown in immediate hypersensitivity to hydrolysed wheat proteins (HWP) present in certain foods and cosmetics (29). In this pathology, there was no evidence of chemical modification of protein sequences. Unmodified epitopes seemed primarily involved in the context of high MW entities originating from partial hydrolysis and re-aggregation of the fragments (30). In our case, no hydrolysis induced by deamidation could be observed.

Allergies to DG or HWP and EIA dependent on wheat, all result in severe symptoms: generalized urticaria or anaphylaxis. This may be related to the recognition of ω-gliadins even though two different ω-gliadins, ω5 and ω2, are bound

by patient IgE with a high specificity in the three pathologies. ω-Gliadins possess a particular sequence organization in a unique repeated domain and are assumed to have an unordered and flexible form. There may be adequate spacing and accessibility of repeated epitopes, allowing very efficient mast cell activation.

A common aspect of these three pathologies is the modification of the exposition to allergens. Hydrolysed wheat proteins and DG have an enhanced solubility compared to WP; exercise (or aspirin) increases intestinal barrier permeability and crossing of gliadin peptides (31). The combination of different factors (solubility or increased crossing, repetition or assemblies of epitopes, their easy accessibility) may boost the sensitization and elicitation steps of the allergic reaction.

Similar observation was made in a mouse model in which we compared native and deamidated gliadins for their ability to sensitize Balb/c mice (13). Like in human patients and despite different sensitization routes (oral and intraperitoneal), deamidated gliadins were much more efficient in sensitizing mice and induced a more severe elicitation phase.

In conclusion, DG allergy is a separate entity, characterized by a homogeneous IgE response. This entity is a direct consequence of present technologies applied to industrial food products. Deamidated ω2-gliadins or the dominant IgE-binding epitope QPEEPFPE could be used as tools for the diagnosis of this new allergy.

Author contribution

S. Denery-Papini designed the study, participated in the interpretation of results and wrote the manuscript; M. Bodinier, C. Larré, C. Brossard and DA. Moneret-Vautrin contributed to the conception of the work and drafts of the paper; F. Battais, E. Paty and DA Moneret-Vautrin took part in patient's recruitment, diagnosis and acquisition of clinical data and helped to revise the manuscript. F. Pineau performed and analysed the ELISA and Pepscan tests; S. Triballeau carried out the RBL assays and some ELISA and formatted the data; M. Pietri and T. Mothes completed the Pepscan assay and helped in the manuscript revision. All authors concur with the submission.

Conflict of interest

Authors have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Migration of native and deamidated fractions in native-PAGE.

Data S2. Degranluation percentage in the RBL assay as a function of protein concentration for sera of four patients allergic to DG.

Data S3. Peptides detected by the sera from DG allergic patients on the sequences of γ and ω2-gliadins by Pepscan.

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