

Celiac Disease: Antibody Recognition against Native and Selectively Deamidated Gliadin Peptides

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Background: Selective deamidation of glutamine residues by tissue transglutaminase (tTG) turns gliadin peptides into stronger activators of T cells from celiac disease (CD) patients. We examined the possibility that these modified peptides could be more specific epitopes for circulating antibodies than are native peptides.

Methods: Two native synthetic peptides and their respective modified sequences were used as antigens for ELISA assays: peptide-1, with residues 56–75 of α -type gliadin; and peptide-2, with residues 134–153 of γ -type gliadin. We examined 40 CD patients [31 not being treated with a gluten-free diet (GFD) and 9 being treated with a GFD] and 30 non-CD patients.

Results: An enhanced response against deamidated peptides was observed in 4 (IgA) and 22 (IgG) of 31 untreated CD patients for peptide-1 and in 25 (IgA) and 29 (IgG) patients for peptide-2. Higher anti-gliadin antibody and anti-tTG IgA concentrations correlated with increased IgA reactivity to modified peptides. Among the nine treated CD patients, eight also displayed an improved IgG signal for the deamidated sequence. Deamidation of peptides did not increase the reactivity of non-CD sera.

Conclusions: Selective deamidation specifically increases circulating antibody recognition of gliadin peptides in CD patients. This suggests that deamidated gliadin peptides are more specific CD B-cell epitopes

than native peptides; this finding may be relevant for designing improved diagnostic tests.

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Celiac disease (CD)³ is an enteropathy triggered in susceptible individuals by ingestion of wheat prolamins (gliadins) and related proteins from other cereals. CD is strongly associated with the HLA-DQ2 and -DQ8 haplotypes and is considered a T-cell-mediated disorder. High concentrations of anti-gliadin and autoantibodies to endomysium are found in nontreated CD patients. Tissue transglutaminase (tTG; EC 2.3.2.13) has been identified (1) as the major target autoantigen of endomysium antibodies. High tTG activity has been detected in the small intestine mucosa of CD patients.

tTG, a calcium-dependent enzyme, catalyzes the replacement by primary amines (cross-linking) as well as the hydrolysis (deamidation) of amide groups of protein- and peptide-bound glutamine residues. Gliadin, a glutamine-rich protein, is a specific substrate for the enzyme. Molberg et al. (2) provided evidence of a new role of tTG in CD. They were able to show that the enzyme selectively modifies gliadin peptides that are recognized by gut-derived T cells from CD patients. They found that the peptide QQLPQPQQPQQSFPQQQRPF, which includes residues 134–153 from the γ -type gliadin, was enzymatically deamidated, following an ordered and progressive pattern, giving the peptide QQLPQPEQPQQSFPEQERPF, which is deamidated at positions 140, 148, and 150. This substitution of glutamine by glutamic acid enhances both peptide binding to DQ2 and activation of DQ2-restricted, transfecting T cells specific for gliadin. A link could be established between the disease, viewed as a T-cell-

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³ Nonstandard abbreviations: CD, celiac disease; tTG, tissue transglutaminase; AGA, anti-gliadin antibody; GFD, gluten-free diet; and TBS-BSA, Tris-buffered saline containing bovine serum albumin.

mediated disorder, and the role of tTG. It was seen as a localized mucosal effect because only gliadin-specific DQ2-restricted T cells isolated from intestinal CD lesions were specifically activated by enzymatically treated peptides.

Anderson et al. (3) identified a peptide modified in one position by transglutaminase as the dominant A-gliadin epitope in T cells from the peripheral blood of CD patients. This peptide contains residues 56–75 of A-gliadin (QLQPFQPQLPYQPQS) and is enzymatically deamidated at position 65 to yield the peptide QLQPF-PQPELPYPQPQS. It should be noted that in these experiments, specific reactive T cells were isolated from peripheral blood after gluten challenge.

Our aim was to investigate whether these deamidated peptides are CD antigen epitopes that are more specific for circulating antibodies than native peptides. ELISA assays using both native and modified peptides were carried out, and the IgA and IgG isotypes in sera from CD patients, healthy controls, and non-CD controls were analyzed. Anti-gliadin antibodies (AGAs) and anti-tTG IgA were also assessed.

Patients and Methods

PEPTIDES

Synthetic peptides were purchased from the Service of Peptide Synthesis of the University of Barcelona (Spain). The synthetic crude peptides were purified by reversed-phase liquid chromatography to give satisfactory materials (~90% homogeneity by analytical HPLC) with the correct amino acid analyses and mass spectra. The peptides were designated as follows.

Peptide-1. Peptide-1 contained residues 57–73 of A-gliadin. The native peptide was designated N-1, and the peptide with a glutamine-to-glutamic acid substitution at Q⁶⁵ was designated D-1:

N-1: Q L Q P F P Q P Q L P Y P Q P Q S
D-1: Q L Q P F P Q P E L P Y P Q P Q S

Peptide-2. Peptide-2 contained residues 138–153 of γ -gliadin. The native peptide was designated N-2, and the peptide with glutamine-to-glutamic acid substitutions at residues 140, 148, and 150 was designated D-2:

N-2: Q P Q Q P Q Q S F P Q Q Q R P F
D-2: Q P E Q P Q Q S F P E Q E R P F

PATIENTS

Serum samples were collected and provided by the Laboratory of the Dr. Orlando Alassia Children's Hospital, Santa Fe. Studies were approved by this Institution's Responsible Committee. CD patients were diagnosed by the Gastroenterology Service according to the following criteria: (a) clinical evaluation; (b) laboratory analyses, including intestine absorption/excretion function and AGAs; (c) jejunal biopsy revealing grade III–IV enteropathy according to Marsh (4); and (d) favorable evolution

with a gluten-free diet (GFD). Subjects studied were divided into the following groups: group A, CD patients not being treated with a GFD (n = 31); group B, CD patients being treated with a GFD (n = 9); and group C, non-CD controls (n = 30; 10 with nonspecific diarrhea or parasitosis, and 20 healthy or with nongastrointestinal disease). The age range of the participants was 7 months to 14 years, and the mean age was 3 years. The CD patients were 60% females and 40% males, and the controls were 50% females and 50% males.

ELISA

Costar flat-bottomed microplates (high binding capacity) were used for all assays. Antigen adsorption was accomplished by incubation for 2 h at 37 °C and overnight at 4 °C with 50 μ L of the corresponding antigen solution: (a) tTG adsorption solution [0.005 g/L guinea pig tTG (Sigma) in 50 mmol/L Tris (pH 7.5), 20 mmol/L CaCl₂]; (b) peptide adsorption solution [0.020 g/L peptide in 100 mmol/L carbonate-bicarbonate (pH 9.6)]; (c) gliadin adsorption solution [0.02 g/L gliadin (Sigma) in 100 mmol/L carbonate-bicarbonate (pH 9.6), containing 3 mol/L urea]. Residual sites were blocked by incubation for 1 h at 37 °C with 150 μ L of Tris-buffered saline containing bovine serum albumin [TBS-BSA; 50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 10 g/L bovine serum albumin].

Serum samples were diluted 1:100 in TBS-BSA, and 50 μ L/well was incubated for 1 h at 37 °C. Peroxidase-labeled α - or γ -chain-specific immunoconjugates (Sigma) diluted in TBS-BSA (50 μ L/well) were incubated for 30 min at 37 °C. Between each step, wells were washed six times with TBS containing 1 mL/L Tween-20. Color was developed by the addition of 100 μ L/well of 100 mg/L 3,3',5,5'-tetramethylbenzidine–0.15 mL/L H₂O₂ in 100 mmol/L citric-acetic buffer (pH 6) and incubation for 30 min at 37 °C. The reaction was stopped by the addition of 100 μ L of 1 mol/L sulfuric acid, and color was read at 450 nm in an ELISA microplate reader (Emax Model; Molecular Devices). Two replicates were used for each serum sample.

Positive and negative controls (pools of five CD and five non-CD sera, respectively) and buffer blanks were included in each assay.

A cutoff absorbance value was defined for each antibody as the upper limit of the confidence interval ($P = 0.01$) calculated from data obtained for the non-CD control group. Among positive serum samples, antibody concentrations were considered as low or high according to whether the absorbance values were lower or higher than twofold the cutoff value.

To represent the relative absorbance difference between each modified and native sequence, the parameter designated $r\Delta A$ was calculated as: $r\Delta A = (AD - AN)/AN$, where AN and AD are the absorbances of the native and deamidated sequences, respectively; $r\Delta A-1$ and $r\Delta A-2$ refer to differences calculated for peptides-1 and -2.

STATISTICS

Peptides absorbances showed gaussian distribution in all groups of patients. Thus the confidence intervals for differences between mean absorbances obtained for each peptide and group of patients were calculated using the Student *t*-test (two-tailed, two independent groups). The parameters $r\Delta A-1$ and $r\Delta A-2$ did not show gaussian distribution; thus, for statistical description of values from different patients groups, we present medians and the values of the first and third quartiles (Q1 and Q3). For comparison, the Mann-Whitney, nonparametric, unpaired two-tailed test was applied (5). The statistical program SPSS 10.0 for Windows was used.

Results

Twenty-nine sera from the group of CD patients not being treated with a GFD were positive for AGAs and anti-tTG IgA, showing different antibody concentrations, and 2 sera from the same group were shown to be negative for both AGAs and anti-tTG IgA. Of the group of CD patients being treated with a GFD, six sera were negative for both antigens, whereas three were positive (two of the three were suspected of not adhering to a GFD). Of 30 non-CD control patients, 28 were AGA- and anti-tTG-negative and 2 were identified as low AGA-positive.

The mean absorbance values, with 95% confidence intervals, obtained for both the native and deamidated peptides in each group of patients are shown in Fig. 1. Regarding the reactivity of the native peptides, we observed that the mean absorbance values were moderate for both peptides and isotypes in CD patients not on a GFD and low in CD patients on a GFD as well as in the control groups. Comparison of the mean absorbances between sera of CD patients not on the GFD and non-CD controls showed significant differences ($P = 0.01$) between both groups for native peptide-1 (N-1) for both isotypes, whereas for native peptide-2 (N-2), this difference was observed only for IgA.

Glutamine modification significantly ($P = 0.001$) enhanced the mean IgG absorbance of peptide-1 in sera from nontreated CD patients. This modification turned peptide-2 into a strongly reactive CD epitope for both isotypes, and significantly ($P < 0.001$) higher mean absorbances were detected in patients not on a GFD; however, the same effect was observed only for IgG isotype among CD patients on a GFD. The non-CD controls showed no increase in mean absorbances for the modified sequences, and the bias was decreased.

The number of sera that were positive to each peptide and isotype among the three groups of patients are shown in Table 1. The results are correlated with subgroups of sera showing different AGA and anti-tTG IgA concentrations. Within the nontreated CD patient group A, subgroup A(1) includes sera showing high antibody concentrations for one or both antigens, whereas subgroup A(2) includes sera showing low or negative absorbance values. Within treated CD patient group B, subgroup B(1) in-

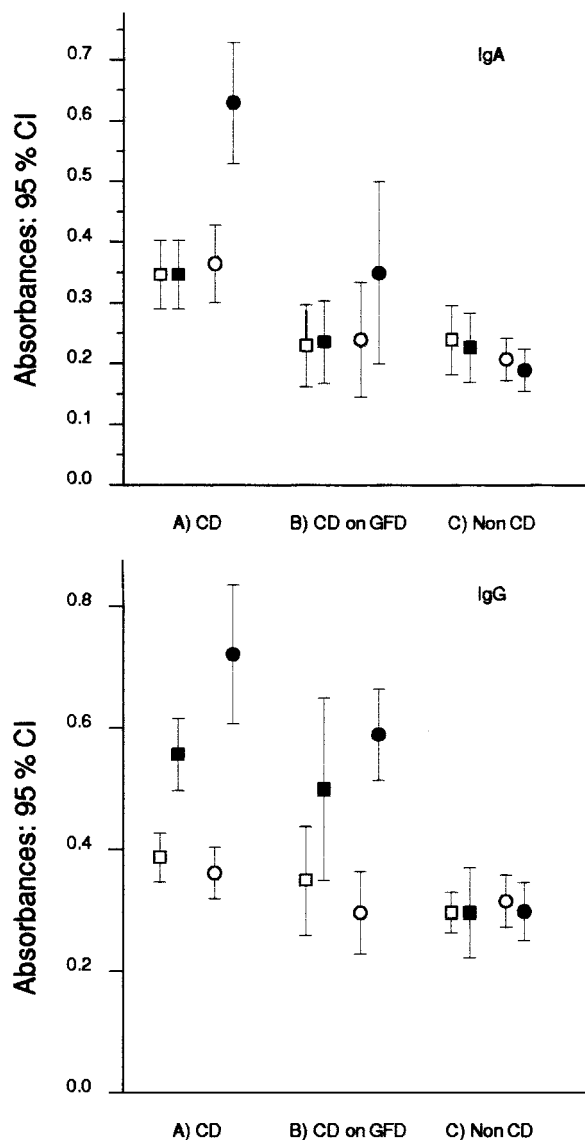


Fig. 1. Mean IgA and IgG reactivities (as absorbance) against native and deamidated peptides.

Shown are the mean absorbances, with 95% confidence intervals (bars), obtained for each peptide and group of patients. Peptides: □, native peptide-1; ■, deamidated peptide-1; ○, native peptide-2; ●, deamidated peptide-2. Groups of patients: A, nontreated CD patients ($n = 31$); B, CD patients being treated with GFD ($n = 9$); C, non-CD controls ($n = 30$).

cludes sera showing a positive reaction, and subgroup B(2) includes sera negative for both antigens. When we compared the reactivity between native peptides, we observed that native peptide-1 (N-1) showed a higher ratio of positivity (positives/total) than native peptide-2 (N-2; Table 1) among CD patients; this suggests that the former is a more sensitive CD epitope than the latter in the native form. Deamidation increased the number of positive sera among the treated and nontreated groups of CD patients, in particular for peptide-2 for both isotypes and for peptide-1 only for the IgG isotype.

To denote this increase in reactivity, the parameters

Table 1. Number of sera that were IgA- and IgG-positive against peptides in the different groups of patients.^a

	IgA				IgG			
	N-1 ^b	D-1	N-2	D-2	N-1	D-1	N-2	D-2
Group A ^c								
Subgroups ^d								
A(1)	19/24	19/24	16/24	24/24	14/24	19/24	11/24	22/24
A(2)	4/7	4/7	3/7	5/7	3/5	7/7	2/7	6/7
Total group A	23/31	23/31	19/31	29/31	17/31	26/31	13/31	28/31
Group B								
Subgroups ^d								
B(1)	1/3	2/3	2/3	2/3	1/3	3/3	0/3	3/3
B(2)	1/6	1/6	1/6	2/6	4/6	5/6	2/6	5/6
Total group B	2/9	3/9	3/9	5/9	5/9	8/9	2/9	8/9
Group C								
Subgroups ^e								
C(I)	2/20	2/20	2/20	2/20	3/20	3/20	2/20	2/20
C(II)	2/10	2/10	2/10	2/10	2/10	2/10	2/10	2/10
Total group C	4/30	4/30	4/30	4/30	5/30	5/30	4/30	4/30

^a Table shows positivity ratios (positives/total) against each peptide and immunoglobulin class.

^b N-1, N-2, D-1, and D-2: native and deamidated peptides 1 and 2, respectively.

^c Groups: (A), CD patients not being treated with GFD; (B), CD patients being treated with GFD; (C), non-CD controls.

^d Subgroups within groups A and B: subgroups (1), higher IgA AGA and anti-tTG concentrations than subgroups (2), as described in text.

^e Subgroups within group C: (I), healthy and nonrelated disease controls; (II), disease-related controls.

r Δ A-1 and r Δ A-2 (relative difference in absorbance between each native and deamidated sequence, as described in *Patients and Methods*) were calculated for each serum, and data obtained for CD patients were compared with those for non-CD controls. Significant differences ($P < 0.001$) were found between the medians for the non-treated CD patients and the non-CD controls, corresponding to the parameters r Δ A-1 and r Δ A-2 (both isotypes). The same difference was observed in comparison data obtained from treated CD patients and non-CD controls with regard to IgG isotype. The medians of these parameters corresponding to IgG response, calculated from data for nontreated CD patients, were as follows: r Δ A-1 = 0.4 (Q1–Q3, 0.25–0.7); r Δ A-2 = 0.85 (Q1–Q3, 0.35–1.3). In the same group of patients, the results for the IgA isotype were as follows: r Δ A-1 = 0 (Q1–Q3, –0.02 to 0.25); r Δ A-2 = 0.5 (Q1–Q3, 0.2–1.3).

The median values obtained for both r Δ A-2 and r Δ A-1 (both isotypes) were negative or null among non-CD patients, with only three sera showing positive, although values were < 0.2 for one isotype. A cutoff value of 0.2 (i.e., a $> 20\%$ increase in absorbance between the native and deamidated peptides) was chosen, and sera showing a r Δ A > 0.2 were considered as positive for this parameter. These results are shown in Table 2. Glutamine modification enhanced the IgG reactivity of peptide-1 in 24 of 31 samples from patients not on a GFD, but the same effect was detected in only 4 of 31 patients from this group for the IgA isotype. The difference in reactivity shown by the native and deamidated sequences of peptide-2 must be highlighted; for the IgA isotype, 26 of 31 sera from the nontreated CD patients showed a significant increase. For

the IgG isotype, the same happened in 29 of 31 CD sera. All 31 CD patients not being treated with GFD showed a r Δ A-2 > 0.2 for at least one isotype, whereas 26 patients showed a r Δ A-2 > 0.2 for both isotypes. Of the five patients from this group who showed a nonsignificant increase in absorbance for the IgA isotype against deamidated peptide-2, only one (who had high absorbance values against native and deamidated sequences) dis-

Table 2. Number of sera that showed significant increases in IgA and IgG reactivities against deamidated sequences in each group of patients.^a

	IgA		IgG	
	r Δ A-1 ^b	r Δ A-2	r Δ A-1	r Δ A-2
Group A ^c				
Subgroups				
A(1)	4/24	23/24	17/24	23/24
A(2)	0/7	3/7	7/7	6/7
Total group A	4/31	26/31	24/31	29/31
Group B				
Subgroup				
B(1)	1/3	2/3	3/3	3/3
B(2)	0/6	2/6	5/6	5/6
Total group B	1/9	4/9	8/9	8/9
Group C				
Total	0/30	0/30	0/30	0/30

^a Table shows the number of sera that were considered positive for the parameters designated as r Δ A-1 and r Δ A-2 (positives/total) in each group and subgroup of patients.

^b r Δ A-1 and r Δ A-2, as defined in text.

^c Groups and subgroups are the same as in Table 1.

played a relative decrease in absorbance for the deamidated peptide. Moreover, two sera from the same group, which gave IgG absorbance values below the cutoff value for this peptide, displayed a $r\Delta A-2 > 0.2$. In the group of CD patients being treated with GFD, eight of nine sera were positive for both parameters in the case of IgG.

Mean IgA reactivities against both peptides were higher for the subgroups of CD sera with high AGA and anti-tTG IgA concentrations (subgroups A1 and B1), whereas these values decreased as one or both antibodies became lower (subgroups A2 and B2; data not shown). It can be seen in Table 1 that the number of positives/total obtained for IgA-class anti-peptide antibodies and for $r\Delta A-1$ and -2 also decreased from subgroups 1 to subgroups 2. This shows that there exists a correlation with IgA isotype in the responses against those antigens and peptides; these responses were also correlated with the parameters $r\Delta A-1$ and -2 . A high reactivity to deamidated peptides and a high relative difference in absorbance were shown for the IgG isotype in CD patients (both treated and nontreated), irrespective of AGA and anti-tTG IgA concentrations. This is particularly interesting in the case of IgA-deficient patients. An IgA-deficient CD patient showing type III-V enteropathy (i.e., destructive hypoplastic lesion), negative to AGA, anti-tTG, and anti-peptide IgA, was IgG-positive to native and deamidated peptides and showed a highly increased response to deamidated sequences.

Healthy and non-CD control sera showed negative or low (4 of 30) reactivity against both native and deamidated peptides. None of these sera (including those that gave positive reactions against one or both peptides) showed a significant increase in absorbance for the modified sequence. This allowed us to suggest that these differences in reactivity are more specific markers than the actual absorbance values obtained for each peptide.

Discussion

Our work supports the results of a recent report by Osman et al. (6), who were able to identify the hexapeptide QXQPPF (X being P, Q, and L) as a relevant epitope for IgA and IgG. This hexapeptide overlaps with residues 57–62 from peptide-1 (both native and deamidated), with $X = L$, and it is not present in peptide-2. Native peptide-1 was more easily recognized than native peptide-2, probably because of the epitope already mentioned. These results correlate with those obtained from a previous work (7), in which we found that the α -gliadin peptide (residues 31–55; LGQQQPFPPQQYPQPQPFPSQQPY), which includes the relevant hexapeptide twice, was a highly specific and sensitive antigen for AGA evaluation by ELISA.

Osman et al. (6) also reported that the PEQ tripeptide was present in sequences from a phage library that had been specifically recognized by CD sera. This PEQ tripeptide is present twice within deamidated peptide-2, whereas the only glutamic residue of peptide-1 belongs to

the PEL tripeptide. We were able to show that the deamidation process turns peptide-2 into a relevant epitope for IgG and IgA, whereas the same happened only for IgG in peptide-1. The same authors also reported that deamidation of hexapeptides QPQQPF and QQQPFP from γ -gliadin, which released QPEQPF and QEPPFP, increased CD serum antibody binding. The alignment of the hexapeptide sequence QPEQPF with that of the deamidated peptide-2 shows that they share the pentapeptide QPEQP. Not only do our results show an improved antibody recognition toward peptide-2, but they also highlight the importance of this selectively deamidated structural motif.

The most interesting result was that selective deamidation greatly increased peptide reactivity, particularly that of peptide-2, for both isotypes in CD sera, but not in control sera. This differential reactivity was noted even at peptide absorbance values considered as low for CD sera, but this was not so for those few non-CD control sera showing higher absorbances.

It is already known that both AGAs and anti-tTG IgA antibodies are more specific CD serologic markers than IgG. Our results suggest that the AGA and anti-tTG IgA responses correlate with that of parameter $r\Delta A-2$ for IgA. We also found a specific IgG differential response to deamidated peptides that could be observed even at low IgA antibody concentrations. We therefore conclude that the differential responses of both IgA and IgG to deamidated peptides could be helpful in diagnosis. It would be interesting to broaden the scope of the screening to include more samples and other specifically deamidated peptides.

The presence of these specific circulating antibodies leave some questions open: Why are they elicited in CD patients? How can these results be integrated with previous findings? Which is the origin of these circulating antibodies? Because a prevalence of B cells specific for deamidated epitopes has not yet been shown to exist, two main reasons can be argued to explain the generation of these antibodies in patients' digestive tracts: (a) preferential activation of B cells presenting deamidated peptides, as a consequence of the existence (8) of T cells that recognize these deamidated epitopes in CD mucosa; and (b) a concentration of modified peptides higher than that in healthy individuals as a result of demonstrated increased enzymatic activity in the mucosa of CD patients (8–10). These two factors may lead one to predict local production of antibodies against deamidated peptides, although not necessarily a systemic production, considering that mucosal and systemic responses are independent of one another (11). However, we detected these antibodies in peripheral blood, and evidence of circulating antibodies originating in the digestive tract against alimentary antigens has been found in immune and viral pathologies (12, 13), CD, and dermatitis herpetiformis (14–16). It is therefore possible that, under pathologic

conditions, an increased fraction of these locally produced antibodies could contribute to the circulatory pool.

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