

CLINICAL RESEARCH

The Gluten Response in Children With Celiac Disease Is Directed Toward Multiple Gliadin and Glutenin Peptides

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See editorial on page 2072.

Background & Aims: Gluten (GLU)-specific T-cell responses in HLA-DQ2 positive adult celiac disease (CD) patients are directed to an immunodominant α -gliadin (GLIA) peptide that requires deamidation for T-cell recognition. The aim of the current study was to determine which GLU peptide(s) are involved early in disease. **Methods:** We have characterized the GLU-specific T-cell response in HLA-DQ2 positive children with recent onset CD. **Results:** We found that 50% of these patients do not respond to the α -GLIA peptide but to a diverse set of GLIA and glutenin (GLT) peptides, including 6 novel epitopes. Moreover, individual patients respond to distinct (combinations of) GLU peptides. T-cell cross-reactivity toward homologous GLIA and GLT peptides was observed, which might play a role in the initial spreading of the GLU-specific T-cell response. Although all pediatric patients displayed deamidation-dependent responses, deamidation-independent responses were found in the majority of patients as well. Finally, T-cell responses to 3 of these novel GLU peptides were found in adult CD patients. **Conclusions:** The diversity of the GLU-specific T-cell response is far greater than was previously appreciated. Both adult and young CD patients can respond to a diverse repertoire of GLU peptides. The observation that T-cell responses to 3 of the novel peptides are independent of deamidation indicates that T-cell responses can be initiated toward native GLU peptides. The possibility that deamidation drives the GLU response toward immunodominant T-cell stimulatory peptides after disease initiation is discussed.

Celiac disease (CD) is the most common food-sensitive enteropathy in humans, caused by a permanent intolerance for the dietary gluten (GLU) and is considered to be a T cell-mediated disease.¹⁻³ The large majority of patients express the HLA-DQ2 [DQ(α 1*0501, β 1*02)] and/or -DQ8 [DQ(α 1*03, β 1*0302)] mole-

cules⁴ and GLU-specific, HLA-DQ2 and/or DQ8-restricted T-cells are present at the site of the lesion in the gut.^{5,6} Peptide binding to HLA-DQ2 and HLA-DQ8 molecules is most efficient when negatively charged amino acids are present at anchor positions in the peptide.⁷⁻⁹ Yet GLU contains very few negatively charged amino acids, which makes GLU-derived peptides low affinity ligands for HLA-DQ2 and -DQ8. This paradox has been solved by the finding that the enzyme tissue transglutaminase (tTG), the target of the endomysium-specific antibodies in CD patients,¹⁰ can modify GLU peptides by conversion of glutamine residues into glutamic acid (termed deamidation¹¹), which introduces the negative charges favored for binding. Recent work has led to the identification of 5 GLU peptides that are recognized by small intestinal T cells from CD patients and has revealed that the T-cell response to these peptides is usually enhanced or even dependent on deamidation.¹²⁻¹⁸ Importantly, this work has also indicated that the response to GLU appears to focus on only a limited number of GLU peptides. All HLA-DQ8 patients examined were found to respond to a unique GLIA peptide¹⁶ and 2 independent studies showed that all HLA-DQ2 patients respond to a particular α -GLIA peptide.^{12,13} Obviously, such a limited response would greatly facilitate tolerance induction protocols as an alternative treatment for CD or the development of safer food products. However, these studies have been carried out with adult patients^{12,13,15,16,18} and the observed immunodominance may thus reflect an advanced stage in the development of the GLU-specific T-cell response and may not be indicative for the initiation of the disease. In

Abbreviations used in this paper: CD, celiac disease; GLIA, gliadin; GLT, glutenin; GLU, gluten; TCC, T-cell clone; tTG, tissue transglutaminase.

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an attempt to resolve this issue, we have now investigated the heterogeneity and specificity of the GLU-specific T-cell response in children with recent onset CD.

Materials and Methods

Children With CD

Twenty-six white CD patients were included in the present study. Their age at diagnosis (first small bowel biopsy) was between 1 and 12 years old (average age 4.0 years \pm 2.5; 1 year old, 4 patients; 2 years old, 3 patients; 3 years old, 9 patients; 4 years old, 6 patients; 6 years old, 2 patients; 9 years old, 1 patient; 12 years old, 1 patient). In addition, biopsies of 4 adult CD patients were obtained. Only DQ2 (DQA1*0501/DQB1*02) positive patients with a confirmed diagnosis of CD have been included. The parents of all young patients and the adult patients gave informed consent to the study, which was approved by the hospital ethics committees.

Isolation of GLU-Specific T-Cell Line and Clones

Polyclonal GLU-specific T-cell lines from the patients were generated from small intestinal biopsies of the patients as described.¹⁶ In short, biopsies were treated with dithiothreitol to remove epithelial cells and subsequently cultured with either the trypsin/pepsin treated GLU preparation or with a τ TG/trypsin/pepsin treated GLU (Fluka, Ulm, Germany) preparation for 5 days. Subsequently interleukin (IL)-2 was added. Restimulation was performed with autologous peripheral blood mononuclear cell (PBMC) if available, otherwise with HLA-DQ2-matched PBMC, (τ TG-)GLU, and IL-2. The cells were tested for specificity and frozen until further use. In proliferation assays in which matched and mismatched APC were used it was found that the T-cell lines and/or clones responded to stimulation with GLU preparation in the presence of HLA-DQ2 positive APC only. Moreover, the response could be blocked with HLA-DQ-specific antibodies.

Antigens and Peptides

A pepsin/trypsin digest of GLU was prepared as described.¹⁶ GLU was obtained from Fluka (Ulm, Germany). Peptides were synthesized by standard Fmoc chemistry on a SyroII peptide synthesizer (MultiSynTech GmbH, Witten, Germany). The integrity of the peptides was checked by reverse-phase high pressure liquid chromatography and mass spectrometry. τ TG treatment was performed by incubating the peptides (500 μ g/mL) with τ TG (100 μ g/mL; Sigma, T-5398) in 50 mmol/L triethylamine-acetate pH 6.5, 2 mmol/L CaCl₂ at 37°C for 4 hours.

T-Cell Proliferation Assays

Proliferation assays were performed in duplicate in 150 μ L RPMI1640 (Gibco, Breda, The Netherlands) supplemented with 10% human serum in 96-well flat-bottomed plates (Falcon, Lelystad, The Netherlands) using 10⁴ T-cells stimulated with 10⁵ irradiated HLA-DQ2-matched allogeneic

PBMCs (3000 RAD) in the presence or absence of antigen (1–10 μ g/mL). After 48 hours at 37°C, cultures were pulsed with 0.5 μ Ci of ³H-thymidine and harvested 18 hours thereafter.

HPLC Purification of the Pepsin/Trypsin Digest of GLU

Approximately 1 mg of an enzymatic digest of GLU was fractionated via micro-rpHPLC (SMART system, column C2/C18, sc 2.1/10; Pharmacia, Uppsala, Sweden) using an acetonitrile gradient from 0 to 70% (2%/min, flow rate 100 μ L/min, containing 0.1% trifluoroacetic acid). The second dimension of fractionation by rpHPLC was performed with a gradient of 0.5% acetonitrile per minute, and in a third round trifluoroacetic acid was replaced with 0.1% heptafluorobutyric acid.

Mass Spectrometry

Electrospray ionization mass spectrometry was performed on the most abundant peaks present in the bioactive HPLC fraction using a Q-TOF hybrid mass spectrometer (Micromass, Manchester, England) as described.¹⁶ Briefly, precursors were selected with the quadrupole and fragments were collected with high efficiency with the orthogonal time of flight mass spectrometer. The collision gas applied was argon (pressure 4 \times 10⁻⁵ mbar) and the collision voltage approximately 30 V.

Database Searching

The program PeptideSearch was used for sequence elucidation. Database similarity searches were done based on the newly identified GLU peptide sequences by FASTA searches (accessible at <http://alpha10.bioch.virginia.edu/fasta>) in a selected subset of wheat proteins from the Swiss Prot databank.

Results

Establishment of GLU-Specific T-Cell Lines From Pediatric CD Patients

T-cell biopsies were collected from young patients that were suspected of CD as indicated by typical clinical symptoms and/or a positive anti-endomysium test. Individual biopsies were cultured with either a trypsin/pepsin digest of GLU (termed GLU hereafter) or the same preparation that had additionally been treated with τ TG (termed τ TG-GLU hereafter). After 5 days, IL-2 was added and cultures that showed evidence of T-cell proliferation were expanded and tested for specificity in a proliferation assay using the 2 GLU preparations and HLA-DQ2 matched antigen-presenting cells. Altogether 28 GLU reactive T-cell lines were obtained from 25 patients (Figure 1). Fifteen T-cell lines were acquired after primary stimulation of the biopsies with GLU (Figure 1A). Of these, 4 responded to GLU, while the

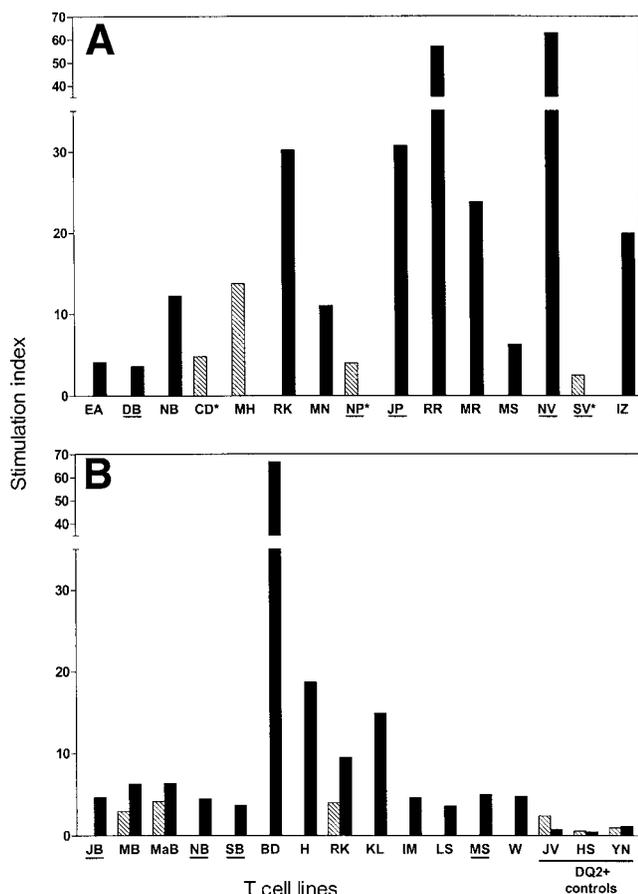


Figure 1. GLU-specific responses of T-cell lines from pediatric CD patients. GLU recognition by small intestinal T-cell lines generated after initial GLU challenge with (A) GLU and (B) with tTG-GLU. The T-cell lines were tested against the GLU (hatched symbols) and tTG-GLU (closed symbols) preparations. (B) Three T-cell lines isolated from small intestinal biopsies of HLA-DQ2+ controls are shown. Responses were considered positive when the GLU-specific stimulation was 3 times above background: SI ≥ 3. The T-cell lines that were used for cloning are underlined. *Tested against GLU only.

remainder responded to tTG-GLU (Figure 1A). Thirteen T-cell lines were acquired after primary stimulation of the biopsies with tTG-GLU, all of which responded to stimulation with tTG-GLU, whereas 3 also responded to GLU (Figure 1B). In addition, 3 T-cell lines were generated from HLA-DQ2 positive children that do not have CD. These T-cell lines did not respond to either GLU or tTG-GLU (Figure 1B). Also, we have never observed response to either GLU or tTG-GLU in T-cell lines from intestinal biopsies of non-HLA-DQ2 individuals (not shown).

Specificity of T-Cell Clones From CD Patients

From 9 of the GLU-specific T-cell lines (indicated in Figure 1), T-cell clones (TCCs) were generated and tested for reactivity against GLU and tTG-GLU (Table 1). Three patterns of reactivity were observed: (1) TCCs that did not respond to GLU but did respond to tTG-GLU (Table 1, tTG-GLU only); (2) TCCs that responded to both GLU and tTG-GLU (Table 1, GLU & tTG-GLU); and (3) TCCs that did respond to GLU but not to tTG-GLU (Table 1, GLU only). In 8 of 9 patients tTG-dependent clonal T-cell responses were found, whereas in 7 patients specific responses to nondeamidated GLU were also observed (Table 1). Together, the results indicate that in these patients a large proportion of the GLU-specific responses are directed to deamidated GLU but that responses to nondeamidated GLU are also common.

Characterization of Novel GLU Epitopes

Next the TCCs clones were tested against peptides corresponding to 3 known HLA-DQ2 restricted

Table 1. Gluten-Specific T-Cell Clones Derived From Polyclonal Gluten-Specific T-Cell Lines of Children With Celiac Disease

| Patient | Age (yr) | HLA typing | No. of T-cell clones ^a | No. of T-cell clones responding to | | |
|---------|----------|--------------|-----------------------------------|------------------------------------|-----------------------|-------------|
| | | | | tTG-gluten only ^b | Gluten and tTG-gluten | Gluten only |
| DB | 6.1 | DQ2, DR3 | 11 | 5 ^c | 2 | 4 |
| JB | 3.1 | DQ2, DR3 DR7 | 10 | 8 | 1 | 1 |
| NB | 4.0 | DQ2, DR3 | 32 | 32 | – | – |
| SB | 4.0 | DQ2, DR3 | 28 | 24 | 4 | – |
| NP | 3.8 | DQ2, DR3 | 1 | – | 1 | – |
| JP | 1.2 | DQ2, DR3 | 34 | 14 | 10 | 10 |
| MS | 4.3 | DQ2, DR3 | 13 | 12 | 1 | – |
| NV | 1.2 | DQ2, DR3 DR7 | 18 | 18 | – | – |
| SV | 2.4 | DQ2, DR3 DR7 | 37 | 2 | 32 | 3 |

^a Number of clones generated from polyclonal gluten reactive T-cell line.

^btTG treatment of gluten differentially affects clonal T-cell responses to gluten:

tTG-gluten only: T-cell clones that respond to tTG-gluten only.

tTG-gluten and gluten: T-cell clones that respond to gluten and tTG-gluten, tTG treatment of gluten often enhances T-cell reactivity.

gluten only: T-cell clones that respond to gluten only.

^cNumber of T-cell clones exhibiting the gluten-specific reactivity indicated. NB and SB are identical twins, the other patients are unrelated.

Table 2. Amino Acid Sequence of Novel T-Cell Stimulatory Gluten Peptides

| Designation | Characterized peptide | Minimal epitope | T-cell clone |
|-----------------------------|-----------------------------------|-----------------|--------------|
| Glia- α 20 (93-106) | PFRPQQYPQPQPQ | nd ^a | JB20 |
| Glia- γ 30 (222-236) | VQGQGIIQPQQPAQL | IIQPQQPAQ | SV30 |
| Glt-156 (40-59) | QQQQPPFSQQQQSPFSQQQQ | PFSQQQQSPF | MS156 |
| Glt17 (46-60) | QPPFSQQQQQPLPQ | PFSQQQQQ | NV17 |
| Glu-21 | QPQPFPQQSEQSQPFQPQPF | QSEQSQPFQPQ | SV21 |
| Glu-5 | QQXSQPQXPQQQXPQQPQQF ^b | QXPQQPQQF | JP437 |

NOTE. The amino acid sequence of 4 of the novel gluten epitopes could be matched with protein sequences from databases, and are named after the origin of the peptide: Glia- α , Glia- γ , and Glt, for α -gliadin, γ -gliadin, and glutenin molecules respectively. The remaining 2 gluten epitopes are indicated with Glu. The amino acid sequence of the characterized peptides, the minimal epitopes required for T-cell stimulation, and the designation of the T-cell clones (TCC) used to characterize the peptides, are indicated. The glutamine residues that are specifically deamidated by treatment with tTG are underlined. While the GLT17 and Glt156 peptides share a large degree of sequence homology, e.g., the sequence QQQPPFSQQQQ, they are distinct since the C-terminal flanking sequence is important for T-cell recognition.

^a Not determined.

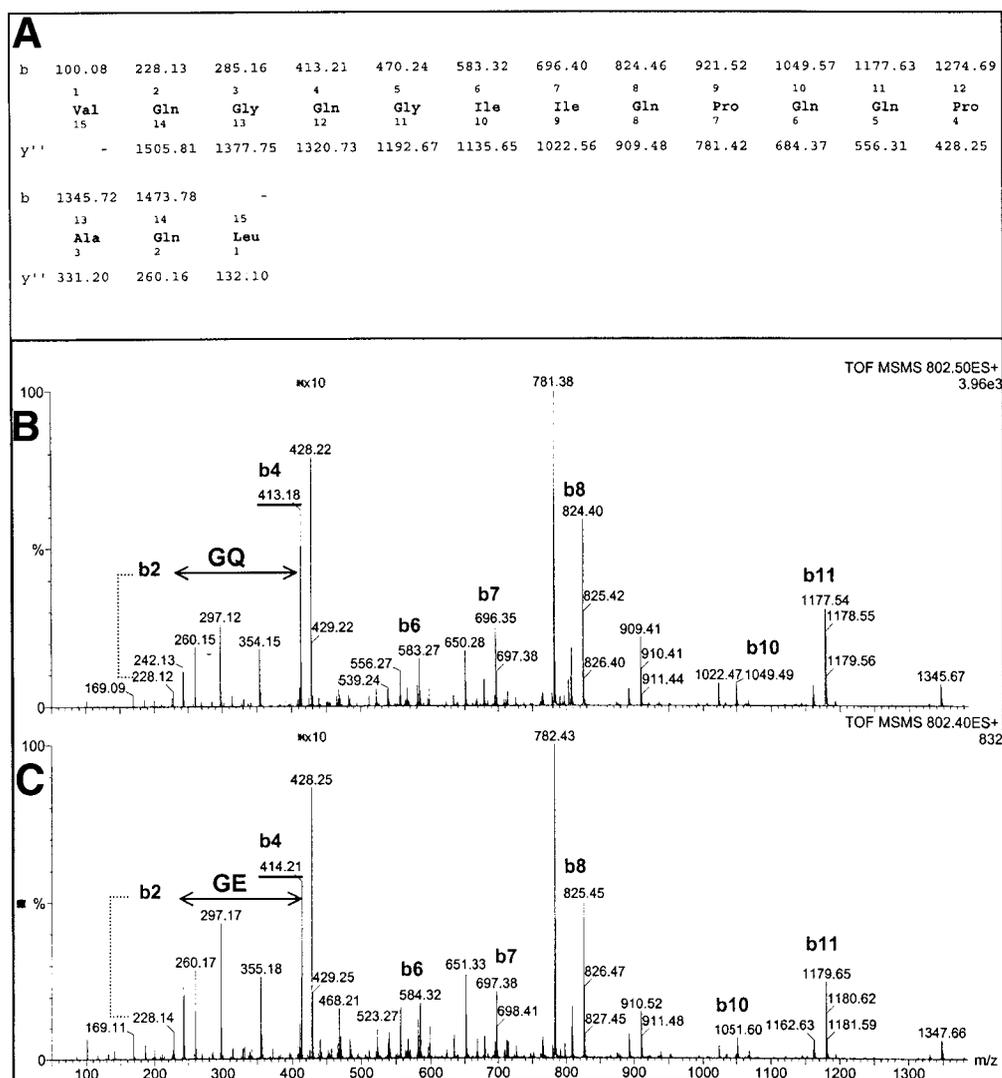
^b X is isoleucine or leucine.

T-cell stimulatory GLIA-derived peptides, GLIA- α 2(62-75) PQPQLPYPQPQLPY, GLIA- α 9(57-68) QLQPF-PQPQLPY, and GLIA- γ 1(138-153) QPQQPQQSF-PQQQRPF.^{12,13,15} This analysis indicated that the large majority of the TCCs did not respond to these peptides (not shown), and were thus likely to be reactive toward yet unidentified GLU peptides. To characterize these novel peptides we have used 2 different methods. First, we purified and characterized T-cell stimulatory GLU epitopes from pepsin/trypsin digests of (tTG-) GLU as described previously.^{16,18} In this procedure the GLU digests were fractionated by rpHPLC and the fractions tested for T-cell stimulatory activity. Subsequently, the identity of the T-cell stimulatory peptides was determined by mass spectrometry as described.^{16,18} This method led to the characterization of 3 novel T-cell stimulatory peptides: GLIA- γ 30(222-236), GLU-21, and GLU-5 (Table 2). Second, we tested the TCCs against a set of 250 synthetic GLU peptides, representing GLIA and GLT sequences, divided in 50 pools of 5 peptides each. A representative example of this procedure is given in Figure 2, e.g., TCC JB20 responded to 5 of 50 peptide pools (Figure 2). Analysis of the sequence of the peptides present in those pools indicated that the sequence PQQYPQPQPQ was present in all the T-cell stimulatory pools and thus likely responsible for the T-cell stimulatory activity (Figure 2). Subsequent testing of the individual peptides confirmed this and identified the GLIA- α 20 peptide as the epitope for TCC JB20 (Figure 2). This method has led to the identification of 3 additional novel T-cell stimulatory peptides: GLIA- α 20(93-106), GLT-156(40-59), and GLT-17(46-60) (Table 2).

| Pools | Peptide sequences | cpm |
|---------------------------|--|------------------|
| Pool 8 +tTG | QIKQQILQQQLIFCMDVVLQ QNPSQQQPQEQLVQQQQFL LQQQLIFCMDVVLQQHNIAHG QPQEQLVPLVQQQQLGQQQPF LPYSQPQPF <u>PQQYPQPQPQ</u> | 2460 \pm 1670 |
| Pool 10 +tTG | QPF <u>PQQYPQPQPQ</u> YSQPQQ QPYPQPQPQYSQPQQPISQQQ QFLGQQQPFPQPQPQPQPF PLVQQQQLGQQQPFPQPQPY HNVVHAILHQQQQQQEQKQ | 43061 \pm 5102 |
| Pool 16 +tTG | VQQQQFUGQQQPFPQPQP FPPQPYPQPQPFPFSQQP PQXQPQYQQQPQPISQ QQPQQF <u>PQQYPQPQPQ</u> LGQQQPFPQP | 51025 \pm 569 |
| Pool 19 +tTG | YQPQYF <u>PQQYPQPQPQ</u> FPPQPYPQPQPFPFSQQP FPQPQPFPPLPYQPYQF FPPLPYQPYPQPQPQ LQLQFPQPQPFPPLPY | 33286 \pm 4532 |
| Pool 22 +tTG | FPPQPYPQPQPFPFSQQP QEQLVPLVQQQQLGQQQPF FSPQPYPQLQFPQPQPQ YPQPQPFPFSQQQPYPQLQ YQPQYF <u>PQQYPQPQPQ</u> | 28438 \pm 4306 |
| Pool 32 +tTG | QQQPGYYTSPQQPGQEQ YPTSPQQPGQEQSGQAQ SGEGSFQPSQEN QQPGQEQSGQAQQSGQW GQGSFRPSQQNPQAQ | 151 \pm 13 |
| Glia- α 20 +tTG | PFRPQQYPQPQPQ | 32640 \pm 1407 |

Figure 2. Identification of the GLIA epitope GLIA- α 20. The TCC JB20 responded to 5 of 50 peptide-pools, each containing 5 tTG treated GLIA and/or GLT peptides. A single sequence was present in the stimulatory pools but not in nonstimulatory pools, e.g., pool 32. A newly synthesized version of this peptide, termed GLIA- α 20, was recognized by the TCC. Cpm represents incorporated ³H-thymidine values (the negative control was 312 \pm 324 cpm). Some peptides were synthesized with a mixture of 2 amino acids at particular amino acid position. This is indicated as follows: U = P + L; X = P + Q; Y = P + S; Z = P + R.

Figure 3. Mass spectral analysis of deamidation of the GLIA- γ 30 epitope. (A) Predicted ion fragment masses of the GLIA- γ 30 epitope based on amino acid sequence. (B) Observed fragments of the GLIA- γ 30 epitope, b-ions are indicated according to panel A. (C) Observed fragments of the GLIA- γ 30 epitope after deamidation by tTG. The mass difference between the b-ions 228 and 413 in panel B corresponds to the sequence GQ. This mass difference has increased 1 dalton in panel C (b-ions 228 and 414), which corresponds to the sequence GE and indicates a Q to E conversion at position 4. Similarly, the Q at position 10 is converted into an E by tTG treatment as indicated by the 2 dalton shift of the b10-ion from 1049 to 1051.



Characteristics of the Novel GLU Epitopes

For 4 of 6 novel peptides deamidation by tTG either enabled or enhanced the T-cell stimulatory activity (see below). Therefore, the effect of tTG treatment was determined by mass spectral analysis of the original peptides and the tTG treated peptides. A representative example of this procedure is shown for the GLIA- γ 30 epitope: VQGQGIIQPQQAQL (Figure 3). tTG treatment led to deamidation of 2 glutamine residues at positions p4 and p10, resulting in the sequence VQGE-GIIQPEQAQL (Figure 3). The complete analysis of the 6 novel T-cell epitopes indicated that the glutamine residues underlined in Table 2 are deamidated by tTG.

To identify the minimal peptide sequence required for the induction of T-cell stimulation, N- and C-terminal truncation variants of the peptides were synthesized and tested for their T-cell stimulatory activity as described previously.^{16,18} The minimal core sequences required are shown in Table 2.

Clonal Analysis of T-Cell Responses to Novel GLU Peptides Demonstrates 3 Modes of Responses

Subsequently we tested the response of the TCCs to the identified peptides in deamidated and nondeamidated form (Figure 4). The T-cell response to GLT-17, GLT-156, and the GLIA- α 20 peptides required prior deamidation (Figure 4, top). In contrast, the response of TCC SV30 to the GLIA- γ 30 peptide was found to be largely indifferent to deamidation (Figure 4, bottom). Moreover, while the response toward the GLU-5 peptide was strongly enhanced by deamidation in the case of TCC JP437, it was much less influenced by deamidation in the case of TCC NP27 (Figure 4, bottom). Finally, deamidation abolished the response of TCC SV21 toward the GLU-21 peptide (Figure 4, bottom). Thus, in agreement with the results shown in Table 1, the effect of deamidation by tTG on GLU-specific T-cell stimulation is heterogeneous and can be positive, neutral, and negative.

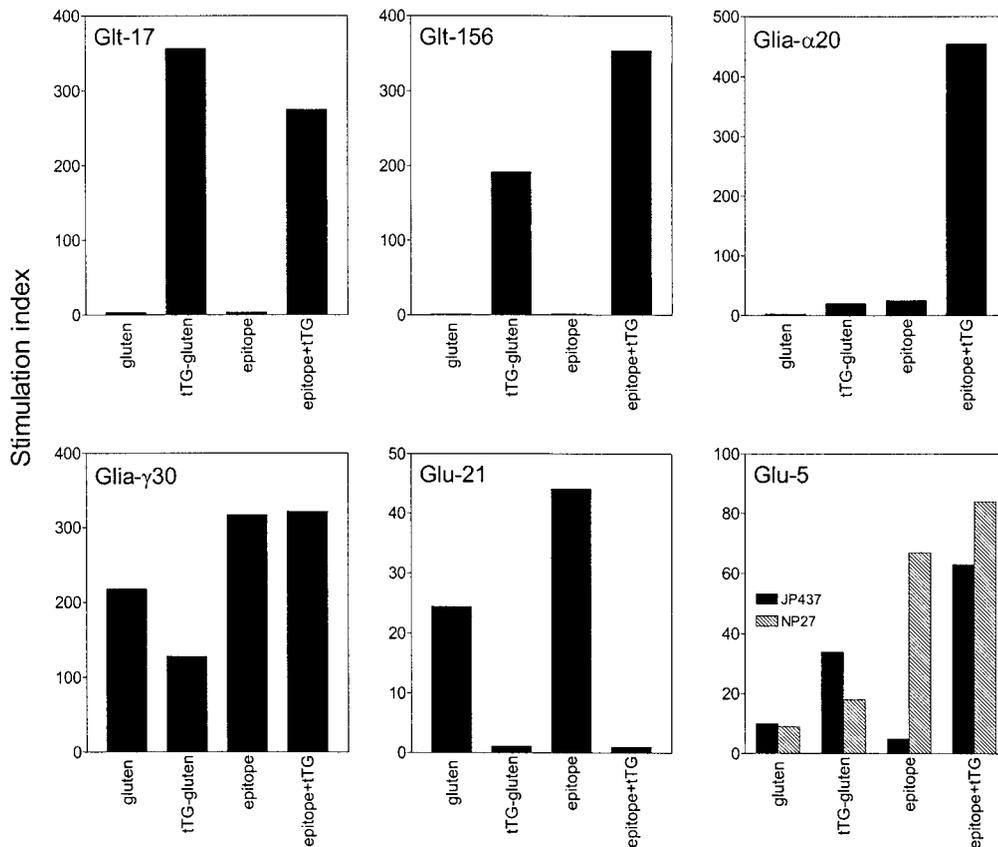


Figure 4. Recognition of the novel DQ2 epitopes. The effect of deamidation on recognition of the new GLU epitopes by TCCs shows 3 major patterns. tTG treatment is required for T-cell recognition of the GLU epitopes GLT-17(46-60) by TCC NV17, GLT-56(40-59) by TCC MS156, and GLIA-α20(93-106) by TCC JB20. Equal or enhanced responses after specific deamidation by tTG are found for epitopes GLIA-γ30(222-236) (TCC SV30), and GLU-5 (TCC JP437 and NP27). In the third pattern, the T-cell reaction against the GLU-21 epitope by TCC SV21 is blocked by deamidation of the peptide.

T-Cell Reactivity Toward Naturally Occurring Variant GLU Sequences

Homology searches in a dedicated GLIA/GLT database indicated that the identified GLIA peptides represent relatively rare sequences (not shown). In contrast, many natural variants of the GLT sequences were found. A search with the GLT sequence QQPPFSQQQQ yielded 95 hits in the GLIA/GLT database, representing 34 homologous but distinct sequences (not shown). Although all of these were GLT sequences, 2 were also present in GLIA molecules. Synthetic peptides corresponding to 8 of these sequences (6 from glutenins, 2 from glutenins/gliadins) were synthesized and tested for T-cell stimulatory activity (Figure 5). Five of these peptides stimulated the GLT-156 reactive TCC MS156, whereas the TCC NV17 responded to 7 peptides. Thus, the response of these GLT-specific TCCs is highly promiscuous and directed to multiple GLT homologous peptides. Strikingly, while each clone exhibited a unique reactivity pattern, both clones responded to stimulation with GLT and GLIA-derived homologues (Figure 5).

Heterogeneity in Pediatric T-Cell Responses Toward GLU Peptides

Subsequently the GLU-specific TCCs and T-cell lines of the children were tested against the previously

characterized HLA-DQ2 restricted GLU peptides,^{12,13,15} as well as against the peptides reported in the present study. In addition, we tested 4 GLU-specific T-cell lines isolated from small intestinal biopsies of adult celiac disease (CD) patients against these peptides. The results are summarized in Figure 6. For the children, large heterogeneity in the specificity of the GLU-specific T-cells was observed. Although responses to some peptides

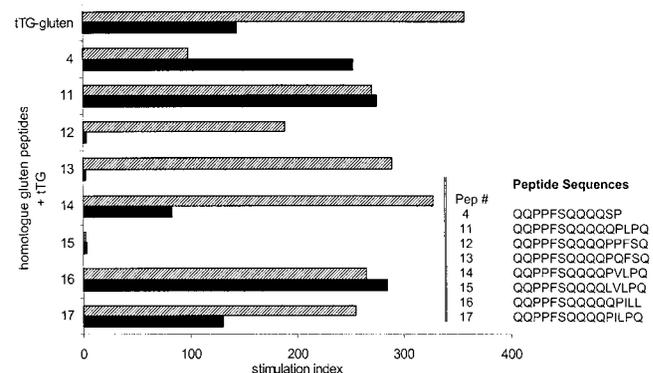


Figure 5. Responses of 2 TCCs against homologue peptides. Response of TCCs TCC NV17 (hatched symbols) and TCC MS156 (closed symbols) to GLT/GLIA homologue peptides. TCC NV17 responds to all peptides except peptide 15, whereas TCC MS156 does not recognize peptides 12, 13, and 15. All peptides are GLT-derived sequences, whereas peptides 11 and 14 also match with GLIA sequences.

| Epitopes | Novel DQ2 gluten epitopes | | | | | | Glia- γ 1 | Glia- α 2 | Glia- α 9 |
|----------|---------------------------|-------------------|--------|---------|-------|--------|------------------|------------------|------------------|
| | Glia- α 20 | Glia- γ 30 | Glt-17 | Glt-156 | Glu-5 | Glu-21 | | | |
| LP | | | | | | | | | |
| JB | tTG | | | | | | tTG | | |
| JP | | | | | | | | | |
| MB | | | | | | | | | |
| SV | | | | | | no tTG | tTG | | |
| NP | | | | tTG | | | | tTG | |
| NV | | | tTG | tTG | | | | | |
| MS | | | tTG | tTG | | | tTG | | |
| RR | | | tTG | tTG | | | tTG | tTG | |
| KL | | | | | | | | tTG | tTG |
| DB | tTG | | | | | | | tTG | |
| SB | | | | | | | | tTG | tTG |
| NB | | | | | | | tTG | tTG | tTG |
| BD | | | | | | | | tTG | tTG |
| LS | | | | | | | | tTG | tTG |
| MaB | | | | | | | tTG | | |
| Adult1 | | | | | | | | | |
| Adult2 | | | | | | | | | |
| Adult3 | | | | | | | | tTG | tTG |
| Adult4 | tTG | | | | | | tTG | tTG | tTG |

Figure 6. Overview of the T-cell responses against the known DQ2 epitopes. TCCs and lines of young and adult patients were tested against the DQ2 epitopes characterized in the present study and the previously published epitopes Glia- α 2(62-75) PQPQLPYQPQLPY, Glia- α 9(57-68) QLQPFQPQLPY, and Glia- γ 1 (138-153) PQQPQSF-PQQRP. The black boxes represent stimulation of the T cells by the epitope. The effect of deamidation of the epitope on the T-cell response is indicated in the individual boxes. tTG indicates requirement of deamidation for recognition, and no tTG indicates blocking of the response by deamidation of the peptide. The remaining responses are (largely) indifferent to deamidation.

were found in 1 patient only, responses to other peptides were found in various patients, and these may represent more immunodominant peptides. Examples are the GLU-5, GLIA- γ 30, GLIA- α 2, and GLIA- α 9. Strong immunodominance, however, could not be assigned to any of these peptides. In the adults, responses were found against the α 2- and α 9-GLIA peptides and to 3 of the novel GLU peptides: GLIA- α 20, GLIA- γ 30, and GLU-5. In this small panel of adults, no responses were found against the GLT peptides. Altogether, these results indicate a highly diverse T-cell response against GLU peptides in CD patients.

Discussion

It is generally accepted that CD is caused by uncontrolled T-cell responses to GLU peptides that are presented by HLA-DQ2 and/or -DQ8 molecules. In recent years, 5 GLU peptides have been identified that stimulate TCCs derived from small intestinal biopsies of

CD patients.^{12,13,15,16,18} An important breakthrough has been the demonstration that deamidation of the GLU peptides by the enzyme tTG is either required for, or enhances, T-cell recognition of 4 of these peptides.¹²⁻¹⁹ The conversion of glutamine into glutamic acid by deamidation generates negative charges in GLU peptides that facilitate binding to HLA-DQ2 and -DQ8 molecules, thus providing a molecular basis for the well-established association between CD and HLA-DQ2/8.²⁻⁶

However, 2 major issues remain unsolved. First, all the studies so far have investigated the GLU-specific T-cell response in adult patients. It is unclear whether the identified GLU peptides are also involved in T-cell activation earlier in the disease process. Second, it is not known whether deamidation of GLU peptides is required for the breaking of oral tolerance or that it merely enhances T-cell reactivity toward GLU.

To investigate these matters, we have performed an extensive investigation of the GLU-specific response in children with recent onset CD. We provide evidence that the T-cell response in these young patients is heterogeneous and directed to a diverse set of GLU peptides that are derived from both GLIA and GLT molecules. Although some patients respond to one set of peptides, others respond to (partially) different sets of peptides. Moreover, while responses to deamidated peptides were observed in all the young patients, responses to both nondeamidated and deamidated peptides were detected in T-cell lines of 9 of 16 patients. With the panel of T clones we found deamidation independent responses in 7 of 9 patients, which is a common phenomenon in these young patients.

We detected responses to the α 2- and/or α 9-GLIA peptides in 8 of 16 young patients and 2 of 4 adults. This seems in contrast to the reported immunodominant responses to these peptides in adults in 2 previous studies.^{12,13} However, in the previous studies not all adult patients responded to the α 2- and/or α 9-GLIA peptides and sometimes only very weak responses were found. The results indicate that also in adults the GLU-specific T-cell response can be directed to multiple GLU peptides.

GLT-specific T-cell responses were frequent in the pediatric patients but not observed in the 4 adults. These GLT-specific T-cell responses are directed to a repetitive sequence of which many homologues exist, including 2 T-cell stimulatory homologues in GLIA. This may indicate that because of cross-reactivity T-cell responses may spread from GLT to GLIA and vice versa. In the youngest patient in the present study, only T-cell responses to these GLT homologues were observed. Collectively, these results suggest that responses against GLT might play an

important role in young CD patients. How this relates to disease initiation remains to be investigated.

Previously, it has been shown that particular GLU peptides can be recognized in the context of both the disease-associated DQ2-dimer ($\alpha 1^*0501$, $\beta 1^*0201$) and the nondisease-associated DQ2-dimer ($\alpha 1^*0201$, $\beta 1^*0202$).¹⁹ We have made similar observations for the HLA-DQ2 restricted GLU peptides identified in the present study (not shown) with one notable exception. Three types of TCCs were isolated from patient SV, an HLA-DR3/7,DQ2 positive child that expresses both DQ2 dimers. These TCCs were found to be reactive to the GLIA- $\gamma 1$, GLIA- $\gamma 30$, and GLU-21 epitopes (Table 2, Figure 6). Although the responses to the GLIA- $\gamma 1$ and GLIA- $\gamma 30$ were restricted by the disease-associated HLA-DQ2 dimer, the GLU-21 epitope was only recognized in the context of the nondisease-associated HLA-DQ2 dimer (not shown). These results indicate that GLU-specific responses may spread from disease-associated to nondisease-associated DQ2-dimers in patients that express both DQ2-dimers.

In summary, our results clearly show that in CD patients T-cell responses can be directed against a series of immunogenic GLU peptides. In half of the pediatric patients, no responses to the $\alpha 2$ - and/or $\alpha 9$ -GLIA peptides were found, an indication that T-cell responses to other GLU peptides can lead to pathogenesis. Moreover, the results show that responses to nondeamidated peptides are frequently found in children, and to a lesser extent in adults, suggesting that native GLU peptides are immunogenic in CD patients.

Based on previous studies¹²⁻¹⁹ and our present work, we propose the following model for the development of the GLU-specific T-cell response: CD patients can start an immune response to a large set of immunogenic GLU peptides derived from GLIA and GLT molecules. Although deamidation is not a prerequisite for the initiation of GLU responses, it is likely to occur because of the presence of endogenous tTG in the lamina propria, and this could facilitate the development of a response to multiple GLU peptides. Cross-reactivity between GLT and GLIA homologues may further contribute to the development of a diverse and spreading T-cell response. When disease progression results in tissue damage and the release of cytoplasmic tTG, progressive deamidation of GLU will occur, enhancing the GLU-specific response to the strongest DQ-binding peptides. Conversely, the response to GLU peptides that are not recognized in deamidated form is suppressed. Finally, the response focuses on those peptides that combine strong DQ-binding capacity and potent T-cell stimulatory activity. We are of the opinion that this hypothesis explains the

current data. However, we cannot exclude the possibility that other explanations are more valid. Longitudinal studies, following the specificity of the GLU response in patients in time, will ultimately be required to test this hypothesis. Irrespective of this, our present results indicate that CD patients are capable of responding to a large array of GLU peptides. Our findings raise the number of identified T-cell stimulatory GLU peptides to 11 and show that many antigenic homologues of GLT peptides exist.¹⁸ Based on this result, we estimate that GLU may contain up to 50 T-cell antigenic peptide sequences. Although this may complicate efforts to reintroduce oral tolerance to GLU, the identification of the toxic GLU peptides provides new opportunities to screen and develop safer food products. This may help to prevent disease development in individuals at risk and would provide safer food alternatives for patients. In view of the observation that poor compliance with a GLU-free diet leads to a significant increase in mortality, such food products are likely to become more in demand in the near future.²⁰

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Friedenwald of the Friedenwald Award



Julius Friedenwald (1866–1941) was born into a medically prominent Baltimore family. His father Aaron was a member of the faculty of the Baltimore College of Physicians and Surgeons, which in 1915 merged to form the medical school of the University of Maryland. Brothers Edgar, Harry, and Jonas all became prominent Baltimore physicians. Julius helped establish the subspecialty of gastroenterology in the United States. In 1908, he served as president of the AGA. The Julius Friedenwald medal is awarded annually by the AGA in recognition of distinguished lifetime service to gastroenterology. The first medal, in 1941, was awarded to Walter B. Cannon.

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