BASIC–ALIMENTARY TRACT

Characterization of Cereal Toxicity for Celiac Disease Patients Based on Protein Homology in Grains

L. WILLEMIJN VADER,* DARIUSZ T. STEPNIAK,* EVELIEN M. BUNNIK,* YVONNE M. C. KOOY,* WILLEKE DE HAAN,* JAN WOUTER DRIJFHOUT,* PETER A. VAN VEELEN,* and FRITS KONING*
*Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands

See editorial on page 1264.

Background & Aims: **Celiac disease is caused by T-cell responses to wheat gluten–derived peptides. The presence of such peptides in other widely consumed grains, however, has hardly been studied.** ***Methods:*** **We have performed homology searches to identify regions with sequence similarity to T-cell stimulatory gluten peptides in the available gluten sequences: the hordeins of barley, secalins of rye, and avenins of oats. The identified peptides were tested for T-cell stimulatory properties.** ***Results:*** **With 1 exception, no identical matches with T-cell stimulatory gluten peptides were found in the other grains. However, less stringent searches identified 11 homologous sequences in hordeins, secalins, and avenins located in regions similar to those in the original gluten proteins. Seven of these 11 peptides were recognized by gluten-specific T-cell lines and/or clones from patients with celiac disease. Comparison of T-cell stimulatory sequences with homologous but non–T-cell stimulatory sequences indicated key amino acids that on substitution either completely or partially abrogated the T-cell stimulatory activity of the gluten peptides. Finally, we show that single nucleotide substitutions in gluten genes will suffice to induce these effects.** ***Conclusions:*** **These results show that the disease-inducing properties of barley and rye can in part be explained by T-cell cross-reactivity against gluten-, secalin-, and hordein-derived peptides. Moreover, the results provide a first step toward a rational strategy for gluten detoxification via targeted mutagenesis at the genetic level.**

Celiac disease is a permanent intolerance for cereal proteins present in the daily diet.[1] So far, studies have focused on the toxicity of wheat, in particular the gliadin and glutenin molecules.[2–11] These storage proteins contain high percentages of proline residues (20%) and glutamine residues (38%). The latter serve as the nitrogen source for germinating seeds. Other grains contain similar storage proteins, called the hordeins, secalins, and avenins in barley, rye, and oats, respectively. Clinical studies indicate that barley and rye cause similar symptoms as wheat, whereas oats is considered safe for most patients with celiac disease.[12,13] The main difference in composition between oats and the other grains is the lower amount of proline residues (10%) present in avenin proteins.[14] Furthermore, oats contains a relatively low content of storage proteins (approximately 10% of the total grain protein compared with 40%–50% in wheat, barley, and rye).[15]

The symptoms of celiac disease, comprising diarrhea, weight loss, and fatigue, originate from a chronic inflammation in the small intestine of patients in response to ingestion of the cereal proteins. Susceptibility to celiac disease is strongly associated with HLA-DQ2 (α0501, β0201) and to a lesser extent with HLA-DQ8[16] as well as the presence of $CD4^+$ T cells in the small intestine of patients that recognize gluten in the context of HLA-DQ2 or HLA-DQ8.[4,17] These gluten-specific T-cell responses were found to be enhanced by the influence of tissue transglutaminase (tTG).[8,18] The enzyme converts particular glutamine residues in gluten peptides into glutamic acid, which results in higher affinity of these peptides for HLA-DQ2 or HLA-DQ8 because negative charges are preferred at anchor positions in the peptide-binding groove of this molecule.[8,19–21] A large number of T-cell stimulatory peptides were characterized in gluten proteins in past years.[2–5,7,22,23]

Recently, we have described that the specificity of tTG in deamidation of gluten is largely dependent on the presence of proline residues flanking the glutamine residues. Good target sites for deamidation are represented in the motifs QXP, QXXF(YWIL), and QXPF(YWIL),

Abbreviation used in this paper: tTG, transglutaminase.

0016-5085/03/$30.00
doi:10.1053/S0016-5085(03)01204-6

whereas the presence of a proline residue inhibits deamidation in the motifs QP and QXXP.[2] These rules for deamidation were combined with the peptide-binding motif of HLA-DQ2, which enabled us to predict novel epitopes in gluten protein databases.[2] One of the identified epitopes is also present in the hordeins of barley and the secalins of rye but not in the avenins of oats. So far, this is the only known T-cell stimulatory peptide for patients with celiac disease derived from grains other than wheat. Because the presence of a large number of T-cell stimulatory peptides in gluten has been established, the question arises whether a similar repertoire of T-cell stimulatory peptides exists in barley, rye, and/or oats. This has been investigated in the present study.

Materials and Methods

Database Searches

Databases of gliadins and glutenins (wheat) and hordeins (barley), secalins (rye), and avenins (oats) were composed by combining the proteins listed in the Swiss Prot, Swiss new, Pir, Sptrembl, Remtrembl, Tremblnew, Refseqprotein, Owl, Kabatp, Pdbseq, and Exprot databanks. The program FASTA was used to align protein sequences of each database. For epitope alignments, the minimal sequences needed for T-cell recognition were used.[3,7] Selected hordein, secalin, and avenin sequences were prepared as synthetic peptides of 14–amino acid residues.

Synthetic Peptides and Deamidation

Peptides were synthesized by standard Fmoc chemistry on a SyroII peptide synthesizer. The integrity of the peptides was checked by reverse-phase high-performance liquid chromatography and mass spectrometry. tTG treatment was performed by incubating the peptides (500 μg/mL) with tTG (100 μg/mL; T-5398; Sigma, Zwijndrecht, the Netherlands) in 50 mmol/L triethylamine acetate, pH 6.5, and 2 mmol/L $CaCl_2$ at 37°C for 4 hours.

Mass Spectrometry

Electrospray ionization mass spectrometry was performed on the synthetic peptides before and after tTG treatment using a Q-TOF mass spectrometer (Micromass, Manchester, England). Overall conversion was determined by the deamidation-induced mass shift as described previously.[18] In tandem mass spectrometry (MS/MS) experiments, precursor ions were selected with the quadrupole window set to 3 dalton. The collision gas applied was argon (pressure, 4×10^{-5} mbar) and the collision voltage approximately 30 V. The conversions were assigned to particular glutamine residues by comparison of the fragmentation spectra of tTG-treated and nontreated peptides.

T-Cell Proliferation Assays

Proliferation assays were performed in triplicate in 150 μL RPMI 1640 (Gibco, Breda, the Netherlands) supplemented with 10% human serum in 96-well flat-bottom plates (Falcon, Lelystad, the Netherlands) using 10^4 gluten-specific T cells stimulated with 10^5 irradiated HLA-DQ2–matched allogeneic peripheral blood mononuclear cells (3000 rad) in the presence or absence of antigen (1–10 μg/mL). After 48 hours at 37°C, cultures were pulsed with 0.5 μCi of ^{3}H-thymidine and harvested 18 hours thereafter.

Production of Interferon Gamma

Cytokine secretion by T cells was determined in culture supernatants that were collected from T-cell proliferation tests on day 2 of cultures. Cytokines were detected by using the Human Th1/Th2 Cytokine Cytometric Bead Array Kit (BD PharMingen, Alphen aan de Rijn, the Netherlands) and a FACS Calibur flow cytometer (Becton Dickinson, Alphen aan de Rijn, the Netherlands).

Enzymatic Degradation of Gluten, Hordein, Secalin, and Avenin Peptides

The synthetic epitope peptides were digested with the following enzymes: pepsin (P-6887), leucine aminopeptidase (L-5006), carboxypeptidase A (C-0261), elastase (E-1250), and dipeptidyl aminopeptidase IV (D-7052) (all from Sigma). The original producer's protocols were followed. Immediately after digestion, the samples were analyzed by matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) using a Voyager De-Pro mass spectrometer (Applied Biosystems, Nieuwekerk aan de Ijssel, the Netherlands).

Results

Matching of Gliadin Epitopes in Gluten

In patients with celiac disease, T-cell reactivity is found against a large panel of epitopes derived from α/β-gliadin, γ-gliadin, and glutenin proteins.[2–7,22,23] To define the immunogenicity of the different gliadin molecules, we have performed database searches with gliadin epitopes to locate the epitopes in these proteins.[7] We observed that individual gliadin molecules contain a variable number of toxic sequences. In 4 different α/β-gliadin molecules, for example, a variable number of epitopes was found, ranging from one glia-α9 epitope in gda0 to one glia-α9 and 3 glia-α2 epitopes in the gda9 molecule (Figure 1*A*). The epitopes share the same starting position at residue 77 in the gliadin protein and are extended with 1, 2, or 3 glia-α2 epitope sequences to the C-terminal side. The gliadin molecule gda9 that contains 4 epitopes can thus be expected to raise the strongest T-cell response.

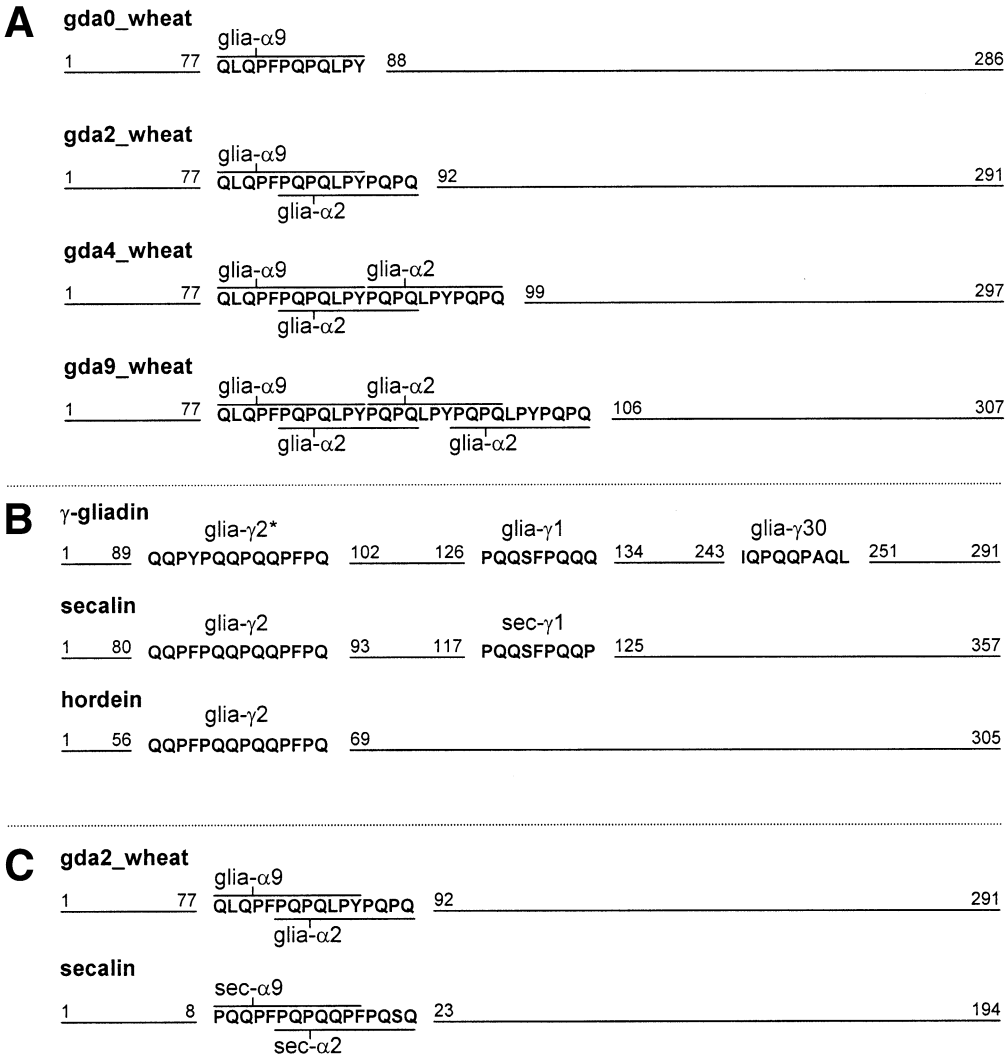

Figure 1. Alignments of gliadin, secalin, and hordein proteins. (*A*) The presence and location of the glia-α9 peptides in gliadin molecules. The accession numbers of the represented gliadin molecules are gda0_wheat (P02863), Gda2_wheat (P04722), Gda4_wheat (P04724), and Gda9_wheat (P18573). (*B*) The presence and location of gliadin homologues in secalins and hordeins. The glia-γ2 epitope present in γ-gliadin (P06659), secalin (S18236), and hordein (P17990) aligns in the same region in these proteins. Moreover, a secalin homologue of the glia-γ1 epitope is present at the same relative position. (*C*) Clustered glia-α2 and glia-α9 homologues are present at the N-terminus of a secalin molecule (A23277). *A glia-γ2 homologue that is equally recognized by gluten-specific T cells.[2]

Matching of Gluten Epitopes in Rye, Barley, and Oats

Subsequently, we performed similar searches with the gluten epitope sequences in hordein, secalin, and avenin protein databases to define the toxicity of barley, rye, and oats. We reported previously that the glia-γ2 epitope is also present in hordein and secalin.[2] In the present alignments, we found that the matched glia-γ2 epitopes are positioned in similar regions of the γ-gliadin, secalin, and hordein proteins (Figure 1*B*). The presence of a tyrosine residue in gliadin instead of a phenylalanine in hordein and secalin does not affect T-cell recognition of this epitope.[2] In addition, the secalin protein contains a sequence (sec-γ1) that differs only one amino acid from the glia-γ1 peptide present in the gliadin molecule at the same relative position (Figure 1*B*). No other near-identical matches with gluten epitopes were found.

Less-strict alignments of gluten protein sequences with the hordein, secalin, and avenin sequences allowed us to assign regions in hordein, secalin, and avenin that share sequence homology with immunogenic regions in wheat proteins. Comparison of gliadin and secalin sequences indicates similar clustering of T-cell epitopes in the N-terminal regions of these proteins (Figure 1*C*).

We selected hordein and secalin peptides on the basis of compliance with previously defined motifs (QXP, QXXF[YWIL], and QXPF[YWIL]) that determine the specificity of deamidation and are critical for HLA-DQ peptide binding characteristics of the gluten epitopes.[2] Moreover, in these alignments, several amino acid dissimilarities were accepted, including the replacement of tyrosine with phenylalanine, the replacement of glutamine for a proline, and the replacement of glutamine with glutamic acid (in the case of avenins) because these changes are frequently found in these proteins. These

Table 1. Gluten Epitopes and the Homologous Peptide Sequences in Hordein, Secalin, and Avenin

Gluten epitopes and homologue peptides		No. of patients responding
Designation	Sequence	
Glia-α2	QPF**PQPQLPYPQ**PQLPY	6/8
Hor-α2	QQFPQPQQPFPQQP	4/8
Sec-α2	QPFPQPQQPFPQSQ	3/8
Glia-α9	QLQ**PFPQPQLPY**PQ	6/8
Hor-α9	PQQPFPQPQQPFRQ	4/8
Sec-α9	PQQPFPQPQQPFPQ	3/8
Av-α9^A	QYQPYPEQQEPFVQ	3/8
Av-α9^B	QYQPYPEQQQPFVQ	2/8
Glia-α20	PQP**FRPQQPYPQ**PQPQ	3/8
Hor-α20	QQPFPPQQPFPQQP	0/8
Glia-γ1	PQQ**PQQSFPQQQ**RPF	2/8
Hor-γ1	PFPPQQAFPQQPPF	0/8
Sec-γ1	PQQPQQSFPQQPQR	1/8
Glia-γ2	**QQPFPQQPQQPFPQ**	1/8
Av-γ2^A	QQPFVQQQQQPFVQ	0/8
Av-γ2^B	QQPFVQQQQPFVQQ	1/8

NOTE. The fragments of the epitope sequences that were used for database searches and the homologue sequences that align are indicated in *bold,* the elongated residues are indicated in *gray.* The glutamine residues deamidated by tTG are *underlined.* Abbreviations are used to describe the origin of the homologue peptide, hordein (Hor), secalin (Sec), and avenin (Av). T-cell lines and clones derived from 8 patients were tested. The number of patients responding to the individual peptides is indicated.

less-strict alignments resulted in the identification of 4 hordein, 3 secalin, and 4 avenin peptides that align with the minimal epitopes of the glia-α2, glia-α9, glia-α20, glia-γ1, and glia-γ2 epitopes (Table 1).

T-Cell Stimulatory Properties of Homologue Peptides

To test the functional relevance of the homologue peptides, these were synthesized as 14-mer peptides according to the original hordein, secalin, or avenin sequences identified (Table 1). Because T-cell recognition of the original gliadin peptides is dependent on deamidation by tTG, we first established the deamidation pattern of the homologue peptides (Table 1). The results indicate that the original deamidation pattern is preserved in the hordein, secalin, and avenin homologue peptides of the glia-α2 and glia-α9 epitopes. However, identical deamidation patterns were not observed in any of the other homologue peptides. This is due to the influence of amino acid changes in residues flanking the target Q residues, in particular the introduction or replacement of a proline residue.[2] The latter effect is most prominently seen in the avenin homologues of the glia-γ2 peptides where the replacement of a proline for a glutamine results in a stretch of deamidated glutamine residues (Table 1).

The relevance of these homologue peptides for patients with celiac disease was established by testing their T-cell stimulatory capacity. Gluten-specific T-cell lines and clones were selected from 8 patients with celiac disease specific for the different gluten epitopes. In T-cell proliferation assays, the responses against the original gluten epitopes were compared with the responses against the homologue peptides. Whereas none of the nondeamidated homologue peptides were recognized by gluten-specific T-cell lines and clones (not shown), several deamidated peptides induced T-cell proliferation and interferon gamma production. T-cell stimulation of a polyclonal gluten-specific T-cell line was obtained with the secalin homologues of the glia-α2 and glia-α9 epitopes and the hordein homologue of the glia-α9 (Figure 2*A* and *B*). We also observed moderate T-cell stimulation with the avenin homologue of the glia-α9 epitope (Figure 2*A*). The induction of T-cell proliferation by the homologue peptides is mirrored by interferon gamma secretion in all cases (Figure 2*A* and *B* and not shown). The glia-α20, glia-γ1, and glia-γ2 homologue peptides failed to induce T-cell proliferation in 2 T-cell lines that displayed strong reactivity with the original gluten epitope (not shown). The lack of reactivity of these homologue peptides can be explained by changed deamidation patterns and introduction or absence of proline residues at several positions.

The pattern of T-cell recognition by the T-cell lines was subsequently confirmed by stimulation of additional gluten-specific T-cell lines and clones of 7 patients (Figure 2*C* and *D*). In a second polyclonal gluten-specific T-cell line, reactivity was observed against 5 peptides (Figure 2*C*). Moreover, the secalin-α2, secalin-α9, and hordein-α9 were recognized by glia-α2/glia-α9 specific T-cell clones of 2 patients. The avenin-α9 peptide, however, failed to stimulate the gluten-specific T-cell clones. In total, cross-reactivity between gluten and hordein, secalin, and/or avenin peptides was observed in T-cell lines and/or T-cell clones derived from 5 patients. In 3 patients, no cross-reactivity was found (Figure 2 and Table 1 and not shown).

Characterization of Natural Homologues of the Glutenin-17 Epitope That Lack T-Cell Stimulatory Capacity

We have previously reported that glutenin epitopes have a large number of natural homologues in glutenin proteins.[3,5] We have now selected a variant of the glutenin-17 epitope that is not recognized by 2 independently derived glutenin-17 specific T-cell clones due to a single substitution of a proline residue at relative position P8 with a leucine residue (Figure 3). Analysis of

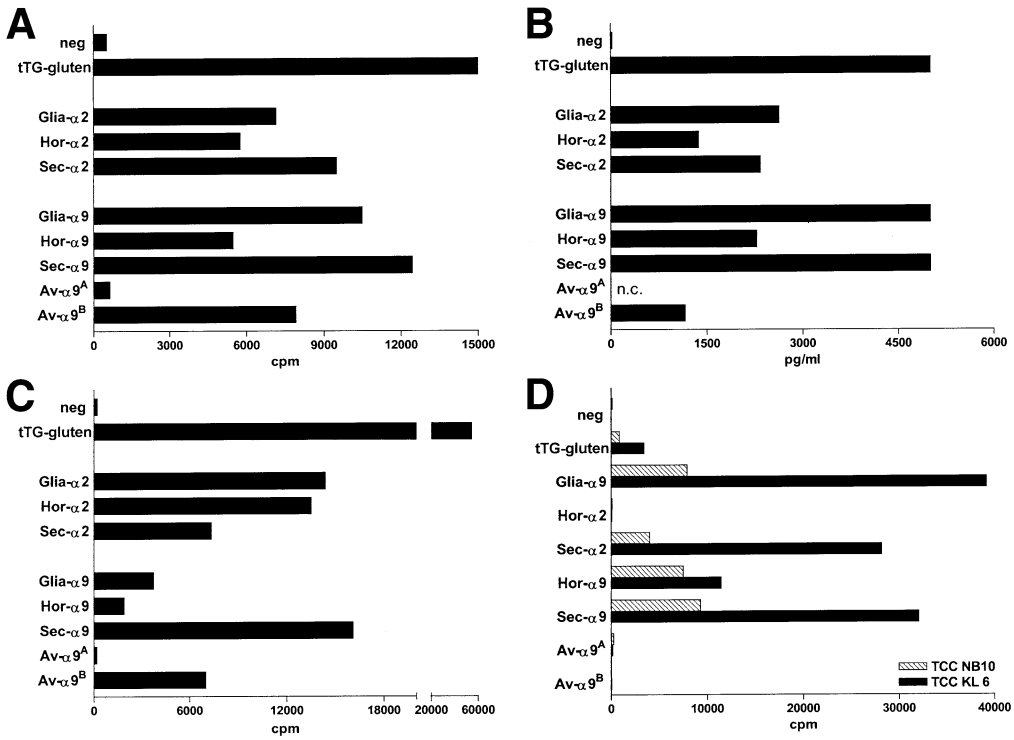

Figure 2. Stimulation of gluten-specific T cells by hordein, secalin, and avenin peptides. (*A*) and (*C*) Proliferative response of polyclonal T-cell lines derived from 2 patients with celiac disease against hordein, secalin, and avenin peptides. (*B*) Interferon gamma production by a T-cell line from patient 1. (*D*) Proliferative response of T-cell clones derived from 2 other patients with celiac disease against hordein, secalin, and avenin peptides.

the deamidated peptides shows that the specific deamidation of the glutamine residues at positions P4 and P6 in the glutenin-17 epitope (PFSEQEQPV) is lost in the homologue peptide, in which all 4 glutamine residues are deamidated (PFSEEEELV, Figure 3). A variant of the T-cell stimulatory peptide was synthesized that incorporates the a-selective deamidation pattern in the homologue peptide with 4 glutamic acid residues in the core sequence (PFSEEEEPV). This modification also led to abrogation of the T-cell response (Figure 3), confirming the negative effect of a-specific deamidation on T-cell recognition. Moreover, a synthetic peptide variant in which the proline at P8 is replaced by a leucine but that does contain glutamic acid residues at the correct relative positions P4 and P6 as in the original epitope (PFSEQEQLV) could not induce T-cell recognition (Figure 3). The effect of the substitution of the proline is therefore 2-fold: the resulting a-selective deamidation and conformational changes both cause abrogation of the T-cell response.

Targeted Mutations in T-Cell Stimulatory Gluten Peptides

Next we tested a strategy to destroy T-cell stimulatory properties of gluten peptides by minimal amino acid changes. We selected the glia-α9 epitope (QLQPFPQPQLPY) that is frequently recognized by gluten-specific T cells of patients with celiac disease. The epitope is recognized by the T cells when the C-terminal glutamine residue (relative position P6, underlined) is deamidated by tTG. The other glutamine residues in this peptide are not deamidated by tTG due to the presence of proline residues at the position Q + 1.[2] Analogous to avenin protein se-

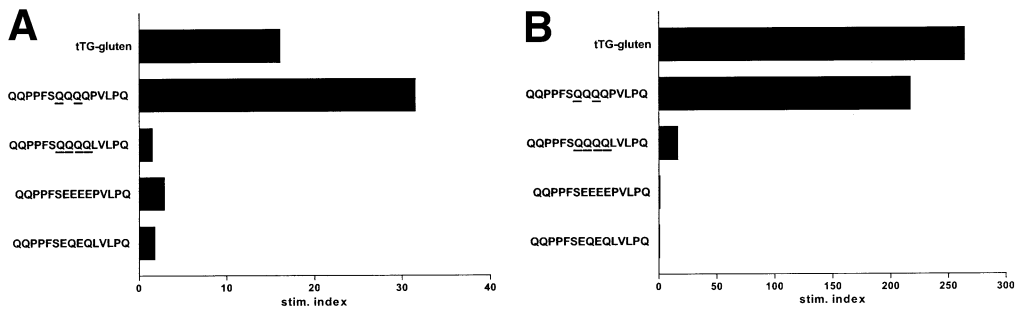

Figure 3. Stimulation of gluten-specific T cells by modified gluten epitopes. Stimulation of T-cell clones (*A*) NV17 and (*B*) Sim156 by the glutenin-17 epitope (QQPPFSQQQQPVLPQ), a natural homologue (QQPPFSQQQQLVLPQ), and 2 modified variant peptides (QQPPFSEQEQLVLPQ and QQPPFSEE EEPVLPQ). Deamidation of the glutamine residues in the peptides by tTG is indicated with an *underlined Q*.

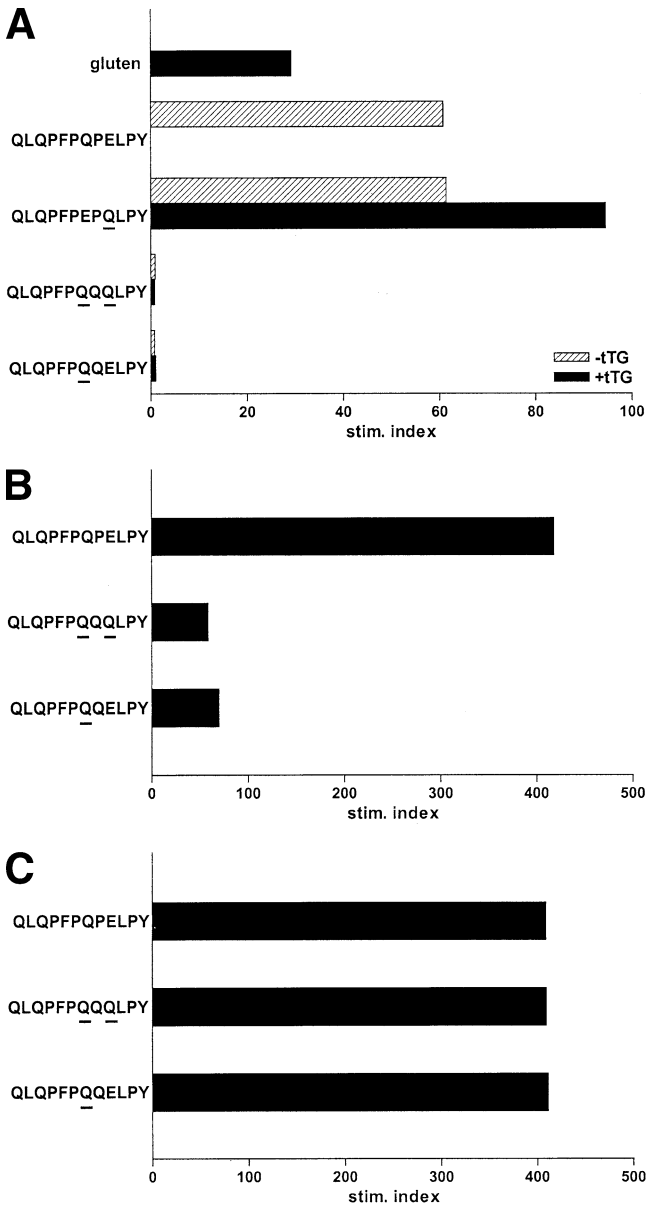

Figure 4. Stimulation of gluten-specific T-cell clones with gluten homologue peptides. (*A–C*) Stimulation of T-cell clones NB10, L6, and L9, respectively, by the deamidated glia-α9 epitope (QLQPFPQPELPY) and variant peptides both before and after treatment with tTG. Deamidation of the glutamine residues in the peptides by tTG is indicated with an *underlined Q*.

quences that lack T-cell stimulatory capacity, we have introduced amino acid changes at 3 positions, a Q to E substitution at P4, a P to Q substitution at P5, and a P to Q substitution at P5 in combination with a Q to E substitution at P6, to resemble the original deamidation pattern. The modified peptides were tested both as the native peptide and deamidated by tTG in T-cell proliferation assays with T-cell clones that recognize the glia-α9 epitope (Figure 4). Specific deamidation of the glutamine residues in the peptides was determined by mass spectrometry (Figure 4). The introduction of a glutamic acid at the relative position P4 instead of a glutamine residue generated a peptide with higher T-cell stimulatory capacity (Figure 4*A*). The peptide induced equal recognition in the native form compared with the deamidated glia-α9 epitope, whereas the deamidated peptide enhanced this T-cell response (Figure 4*A*). Introduction of a negative charge at relative position P4 is therefore not suitable for elimination of the T-cell stimulatory capacity of this epitope.

The second replacement, however, substitution of the proline at relative position P5 with a glutamine residue, did affect the T-cell stimulatory properties of the peptide, but the magnitude of the effect depended on the T-cell clone tested (Figure 4). Whereas a complete abrogation of T-cell recognition was observed for one clone (Figure 4*A*), in another clone the substitution led to an approximately 4-fold reduction of T-cell responses (Figure 4*B*) and in a third no effect was observed (Figure 4*C*). Also, the other version of this peptide, with a glutamine at P5 and a glutamic acid at P6, the latter identical to the deamidated glia-α9 epitope, induced similar effects on the T-cell clones tested.

Thus, a proline-to-glutamine substitution in this peptide only partially eliminates the T-cell stimulatory capacity.

Codon Usage Coding for Proline Residues in Gluten Proteins

Our present study and previous work shows that proline residues in gluten epitopes are often crucial for T-cell recognition.[2] We show that the substitution of proline for glutamine and leucine can lead to abrogation of the T-cell stimulatory capacity of a gliadin and a glutenin-derived peptide, respectively. At the DNA level, the conversion of a proline residue into a glutamine residue can be achieved by the mutation of a single nucleotide (Table 2). Two codons encoding for proline, CCG and CCA, can be transformed into a codon coding for a glutamine by substitution of the middle cytosine for an adenine. The same mutation in the 2 other codons coding for proline (CCT, CCC) results in a codon that encodes a histidine, which would be less favorable in the modification of gluten proteins. We have therefore determined the codon usage for prolines in 3 classes of gluten proteins, an α-gliadin molecule containing the glia-α9 epitope, a γ-gliadin molecule containing the glia-γ2 epitope, and a glutenin molecule containing the glutenin-17 epitope (Table 3). The codon frequency for prolines in these proteins shows a predominant usage of CCA. A single nucleotide substitution yields CAA, the codon that encodes a glutamine residue (Tables 2–4). These gluten genes thus contain numerous sites where

Table 2. Codons Coding for Leucine, Proline, Glutamine, and Histidine in Gluten Proteins

Codons coding for			
Leucine	Proline	Glutamine	Histidine
Ctg	ccg	cag	—
Cta	cca	caa	—
Ctt	cct	—	cat
Ctc	ccc	—	cac

site-directed mutagenesis would eliminate proline residues by substitution with glutamine residues.

The examples of mutations shown in this study can indeed be achieved by substitutions of one nucleotide (Tables 2–4). The replacement of the proline in the glia-α9 epitope by a glutamine, which results in reduced T-cell stimulatory properties (Figure 4), can be achieved by substitution of the middle cytosine in the codon CCG with an adenosine (Table 4). Similarly, the difference between the T-cell stimulatory glutenin epitope and the non–T-cell stimulatory homologue peptide that contains a leucine (codon CTA) instead of a proline (codon CCA) is caused by a cytosine-to-thymidine substitution (Table 4).

Discussion

The presence of a large number of T-cell stimulatory peptides in gluten has been established.[2–7,22,23] This raises the question whether a similar repertoire of T-cell stimulatory peptides exists in barley, rye, and/or oats. In the present study, we have searched for such peptides based on the assumption that gluten-specific T cells might cross-react with homologous peptides in barley, rye, and/or oats. We have therefore compared T-cell stimulatory wheat-derived sequences with barley-, rye-, and oats-derived sequences present in databases. Apart from the previously described glia-γ2 epitope,[2] none of the other T-cell stimulatory gluten peptides had an identical match in the hordeins, secalins, or avenins. Less stringent searches, however, readily identified peptides with sequence similarity. Several of these peptides stimulated gluten-specific T cells. To our knowledge, this is the first demonstration that T-cell cross-reactivity between gluten peptides and related peptides in the hordeins and secalins can be related to the toxicity of barley and rye for patients with celiac disease. Obviously, T-cell reactivity against additional peptides that are exclusively present in hordeins and/or secalins could aggravate the T-cell response on ingestion of these grains by patients. The demonstration of the existence of such epitopes in other grains requires further investigation.

Comparison of the hordein and secalin peptides characterized in the present study shows that T-cell recognition is influenced by the nature of the sequences flanking the 9–amino acid core of the peptides. The distinct recognition of the secalin homologue peptide of the glia-α2 peptide, but not of the hordein homologue peptide, for example, is due to amino acid differences outside the core of the peptides.

Whereas the identified secalin and hordein peptides had high sequence identity with the gluten peptides, more pronounced differences were present in the avenin homologues. A general lack of proline residues in the core of the avenin peptides and the presence of glutamic acid residues instead of glutamine residues are the most common differences observed. Moreover, the presence of glutamic acid in the avenin peptides at positions that are not HLA-DQ2 anchor positions could have an adverse effect on the binding properties of these peptides for HLA-DQ2. The consequence of the absence of proline residues in avenin proteins for presentation by HLA-DQ2 and T-cell recognition is manifold. First, we find a selective deamidation of the peptides by tTG, which can be explained by the effect of proline residues on the specificity of the enzyme.[2] This leads to the unfavorable introduction of negative charges for binding and presentation by HLA-DQ2. Second, a proline residue also directly affects the peptide-binding properties, because it introduces a bulge and rigid conformation in the peptide structure. Moreover, the relative position P6 in the peptide-binding groove of HLA-DQ2 functions as an anchor that prefers the binding of a proline residue.[20,21] Finally, proline residues are known to confer resistance against protein degradation by proteases of the gastrointestinal tract. Consequently, the characterized gluten epitopes localize in proline-rich regions of the gluten proteins.[22] The lack of proline residues in avenin molecules thus results in higher susceptibility of the oats proteins for degradation by proteases in the gastrointestinal tract. In fact, we have studied the degradation of the identified hordein, secalin, and avenin peptides by gastrointestinal tract enzymes and found that the only peptides that are

Table 3. Codon Usage for Proline Residues in Gluten Proteins

	Codon usage in gluten proteins		
Proline	α/β-gliadin	γ-gliadin	Glutenin
ccg	4	1	1
cca	31	32	23
cct	5	4	7
ccc	1	9	2

Accession numbers for the represented proteins: α/β-gliadin (gi: 1304263), γ-gliadin (gi: 15148397), and glutenin (gi: 17425205 and gi: 1857649).

Table 4. Single Nucleotide Substitutions in Gluten Genes Result in Gluten Peptides With Diminished (Glia-alpha 9) and Absent T Cell Stimulatory Properties (glt-17)

glia-α9		→	Mutated	glia-α9	glt-17		→	Homologue	glt-17
P	cca		P	cca	P	cca		P	cca
F	ttt		F	ttt	F	ttt		F	ttt
P	ccg		P	ccg	S	tca		S	tcg
Q	cag		Q	cag	Q	cag		Q	cag
P	ccg	→	Q	cag	Q	caa		Q	caa
Q	caa		Q	caa	Q	caa		Q	caa
L	cta		L	cta	Q	caa		Q	caa
P	cca		P	cca	P	cca	→	L	cta
Y	tat		Y	tat	V	gtt		V	gtt

clearly sensitive to breakdown are the avenin Av-γ2 peptides that are cleaved by elastase (results not shown). Thus, the rapid degradation of potential harmful avenin peptides may help to prevent the initiation of an immune response against oats in the small intestine. Notably, the treatment of gluten with an enzyme specific for proline-rich sequences has recently been suggested to destroy the toxic properties of gluten.[24]

The observed differences between the protein composition of cereals and the resulting effect on stimulation of gluten-specific T cells led us to test a strategy for destroying the T-cell stimulatory capacity of peptides in cereal proteins. First, we studied a natural homologue of a glutenin epitope that fails to induce a T-cell response due to the presence of a leucine residue instead of a proline residue in the T-cell stimulatory epitope (Figure 3*A*). The results show that the presence of the proline in the T-cell stimulatory peptide causes selective deamidation and a distinct conformation of the peptide, which are both required for T-cell recognition.

We also specifically modified the glia-α9 epitope that is recognized by most patients with celiac disease.[6,7] Although the substitution of the proline residue in the core of the peptide could reduce and even abrogate T-cell recognition, this was not the case for all T-cell clones tested. Additional substitutions will thus be required to completely abolish the T-cell stimulatory capacity of such peptides. Moreover, at present, we cannot exclude the possibility that the modified gluten peptides will be immunogenic and may thus cause novel T-cell responses.

Our results indicate that the unique composition of cereal proteins that contain high amounts of glutamine and proline residues is the basis of the toxicity of wheat, barley, and rye for patients with celiac disease. However, this study shows differences in the immunogenicity of individual gluten proteins (Figure 1*A*). Strategies to detoxify wheat and other cereal proteins should therefore first select these less-immunogenic gluten molecules and subsequently focus on the elimination of residual T-cell stimulatory sequences. We show that the substitution of a proline residue with a glutamine residue can destroy the T-cell stimulatory capacity of a gluten peptide. Proline and glutamine are the 2 most abundant amino acids in gluten, and the comparison of gluten sequences with those of other grains shows that proline-to-glutamine substitutions are frequently found (Table 1 and not shown). The effects of such amino acid substitutions on the unique properties of gluten may thus be acceptable. In practice, this substitution can be accomplished by mutagenesis of a single nucleotide in the codon coding for a proline (Tables 2–4). The codon usage for proline residues in immunogenic wheat proteins thus makes such an approach feasible.

In conclusion, we have characterized novel T-cell stimulatory sequences in barley and rye on the basis of T-cell cross-reactivity with gluten proteins. Such cross-reactive T-cell responses are likely to play a role in the observed toxicity of these cereals for patients with celiac disease. Moreover, we show that subtle changes in gluten genes would eliminate some of the T-cell stimulatory properties of gluten molecules. Whether this will be applicable to the generation of safer wheat strains remains to be determined.

References

1. Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). Gastroenterology 1992;102:330–354.
2. Vader LW, De Ru A, van de Wal Y, Kooy YM, Benckhuijsen W, Mearin ML, Drijfhout JW, van Veelen P, Koning F. Specificity of tissue transglutaminase explains cereal toxicity in celiac disease. J Exp Med 2002;195:643–649.
3. Vader W, Kooy Y, van Veelen P, De Ru A, Harris D, Benckhuijsen W, Pena S, Mearin L, Drijfhout JW, Koning F. The gluten response in children with celiac disease is directed toward multiple gliadin and glutenin peptides. Gastroenterology 2002;122:1729–1737.
4. van de Wal Y, Kooy YM, van Veelen PA, Pena SA, Mearin LM, Molberg O, Lundin KE, Sollid LM, Mutis T, Benckhuijsen WE, Drijfhout JW, Koning F. Small intestinal T cells of celiac disease

patients recognize a natural pepsin fragment of gliadin. Proc Natl Acad Sci U S A 1998;95:10050–10054.
5. van de Wal Y, Kooy YM, van Veelen P, Vader W, August SA, Drijfhout JW, Pena SA, Koning F. Glutenin is involved in the gluten-driven mucosal T cell response. Eur J Immunol 1999;29: 3133–3139.
6. Anderson RP, Degano P, Godkin AJ, Jewell DP, Hill AV. In vivo antigen challenge in celiac disease identifies a single transglutaminase-modified peptide as the dominant A-gliadin T-cell epitope. Nat Med 2000;6:337–342.
7. Arentz-Hansen H, Korner R, Molberg O, Quarsten H, Vader W, Kooy YM, Lundin KE, Koning F, Roepstorff P, Sollid LM, McAdam SN. The intestinal T cell response to alpha-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. J Exp Med 2000;191:603–612.
8. Molberg O, McAdam SN, Korner R, Quarsten H, Kristiansen C, Madsen L, Fugger L, Scott H, Noren O, Roepstorff P, Lundin KE, Sjostrom H, Sollid LM. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. Nat Med 1998;4:713–717.
9. Maiuri L, Picarelli A, Boirivant M, Coletta S, Mazzilli MC, De Vincenzi M, Londei M, Auricchio S. Definition of the initial immunologic modifications upon in vitro gliadin challenge in the small intestine of celiac patients. Gastroenterology 1996;110:1368–1378.
10. Sturgess R, Day P, Ellis HJ, Lundin KE, Gjertsen HA, Kontakou M, Ciclitira PJ. Wheat peptide challenge in coeliac disease. Lancet 1994;343:758–761.
11. Wieser H. Relation between gliadin structure and coeliac toxicity. Acta Paediatr Suppl 1996;412:3–9.
12. Hoffenberg EJ, Haas J, Drescher A, Barnhurst R, Osberg I, Bao F, Eisenbarth G. A trial of oats in children with newly diagnosed celiac disease. J Pediatr 2000;137:361–366.
13. Janatuinen EK, Kemppainen TA, Julkunen RJ, Kosma VM, Maki M, Heikkinen M, Uusitupa MI. No harm from five year ingestion of oats in coeliac disease. Gut 2002;50:332–335.
14. Wieser H, Seilmeier W, Eggert M, Belitz HD. Tryptophan content of cereal proteins. Z Lebensm Unters Forsch 1983;177:457–460.
15. Shewry PR. Plant storage proteins. Biol Rev Camb Philos Soc 1995;70:375–426.
16. Sollid LM, Markussen G, Ek J, Gjerde H, Vartdal F, Thorsby E. Evidence for a primary association of celiac disease to a particular HLA-DQ alpha/beta heterodimer. J Exp Med 1989;169:345–350.
17. Lundin KE, Scott H, Hansen T, Paulsen G, Halstensen TS, Fausa O, Thorsby E, Sollid LM. Gliadin-specific, HLA-DQ(alpha 1*0501,beta 1*0201) restricted T cells isolated from the small intestinal mucosa of celiac disease patients. J Exp Med 1993; 178:187–196.
18. van de Wal Y, Kooy Y, van Veelen P, Pena S, Mearin L, Papadopoulos G, Koning F. Selective deamidation by tissue transglutaminase strongly enhances gliadin-specific T cell reactivity. J Immunol 1998;161:1585–1588.
19. Kwok WW, Domeier ML, Raymond FC, Byers P, Nepom GT. Allele-specific motifs characterize HLA-DQ interactions with a diabetes-associated peptide derived from glutamic acid decarboxylase. J Immunol 1996;156:2171–2177.
20. van de Wal Y, Kooy YMC, Drijfhout JW, Amons R, Koning F. Peptide binding characteristics of the coeliac disease-associated DQ(alpha1*0501, beta1*0201) molecule. Immunogenetics 1996;44:246–253.
21. Vartdal F, Johansen BH, Friede T, Thorpe CJ, Stevanovic S, Eriksen JE, Sletten K, Thorsby E, Rammensee HG, Sollid LM. The peptide binding motif of the disease associated HLA-DQ (alpha 1* 0501, beta 1* 0201) molecule. Eur J Immunol 1996;26: 2764–2772.
22. Arentz-Hansen H, McAdam SN, Molberg O, Fleckenstein B, Lundin KE, Jorgensen TJ, Jung G, Roepstorff P, Sollid LM. Celiac lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline residues. Gastroenterology 2002;123:803–809.
23. Sjostrom H, Lundin KE, Molberg O, Korner R, McAdam SN, Anthonsen D, Quarsten H, Noren O, Roepstorff P, Thorsby E, Sollid LM. Identification of a gliadin T-cell epitope in coeliac disease: general importance of gliadin deamidation for intestinal T-cell recognition. Scand J Immunol 1998;48:111–115.
24. Shan L, Molberg O, Parrot I, Hausch F, Filiz F, Gray GM, Sollid LM, Khosla C. Structural basis for gluten intolerance in celiac sprue. Science 2002;297:2275–2279.

Received October 8, 2002. Accepted June 19, 2003.

Address requests for reprints to: Frits Koning, Ph.D., Department of Immunohematology and Blood Transfusion, E3-Q, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands. e-mail: f.koning@lumc.nl; fax: (31) 71 5216751.

Supported by grants from the European Community (BHM4-CT98-3087 and QLK1-2000-00657) and the Stimuleringsfonds Voedingsonderzoek LUMC.

The authors thank Drs. R. R. P. de Vries and R. Offringa for critical reading of the manuscript, A. de Ru for mass spectrometric analysis, and W. Benckhuijsen for peptide synthesis.