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Natural Variation in Toxicity of Wheat: Potential for Selection of Nontoxic Varieties for Celiac Disease Patients

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See editorial on page 1111.

Background & Aims: Celiac disease (CD) is an intestinal disorder caused by T-cell responses to peptides derived from the gluten proteins present in wheat. Such peptides have been found both in the gliadin and glutenin proteins in gluten. The only cure for CD is a lifelong gluten-free diet. It is unknown, however, if all wheat varieties are equally harmful for patients. We investigated whether wheat varieties exist with a natural low number of T-cell–stimulatory epitopes. **Methods:** Gluten proteins present in public databases were analyzed for the presence of T-cell–stimulatory sequences. In addition, wheat accessions from diploid (AA, SS/BB, and DD genomes), tetraploid (AABB), and hexaploid (AABBDD) *Triticum* species were tested for the presence of T-cell–stimulatory epitopes in gliadins and glutenins by both T-cell and monoclonal antibody–based assays. **Results:** The database analysis readily identified gluten proteins that lack 1 or more of the known T-cell–stimulatory sequences. Moreover, both the T-cell– and antibody-based assays showed that a large variation exists in the amount of T-cell–stimulatory peptides present in the wheat accessions. **Conclusions:** Sufficient genetic variation is present to endeavor the selection of wheat accessions that contain low amounts of T-cell–stimulatory sequences. Such materials may be used to select and breed wheat varieties suitable for consumption by CD patients, contributing to a well-balanced diet and an increase in their quality of life. Such varieties also may be useful for disease prevention in individuals at risk.

Celiac disease (CD) is a small intestinal disorder caused by the ingestion of gluten proteins present in food. CD is considered to be a T-cell–mediated disease and the molecular basis is well understood. In the upper part of the gastrointestinal tract gluten proteins are digested by enzymes into peptides of variable length. In the small intestine these peptides are deamidated by the enzyme tissue transglutaminase.[1–8] Subsequently, these peptides can bind to HLA-DQ molecules expressed by specialized antigen-presenting cells and be recognized by gluten-specific $CD4^+$ T cells.[9,10] On activation, these cells multiply and release inflammatory cytokines that cause intestinal damage and flattening of the intestinal epithelium. This results in diarrhea, poor absorption of nutrients,[11–13] malnutrition, abdominal pain, and retardation of growth, some of the characteristic symptoms associated with CD. The only cure for patients with CD is a strict lifelong gluten-free diet. The omission of gluten from the diet is difficult and often results in a shortage of vitamins. Another problem for patients who adhere to a gluten-free diet is that many naturally gluten-free products are contaminated with gluten because of its widespread use in the food industry.

Wheat gluten is a group of proteins that can be partitioned into 2 protein families: the glutenins and the gliadins. The glutenins can be subdivided further into high molecular weight (HMW) and low molecular weight (LMW) glutenins and the gliadins can be divided into α-, γ-, and ω-gliadins.[14] At present, many gluten-derived T-cell–stimulatory peptides are known and they originate from the α- and γ-gliadins, and the HMW and LMW glutenins.[6,10,15–18] Homologue sequences are found in the secalins of rye, the hordeins of barley, and the avenins of oats. Gluten and gluten-like molecules thus contain many immunogenic peptides. Moreover, the unique food-industrial properties of gluten are in part related to a very high proline content that renders gluten

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Abbreviations used in this paper: CD, celiac disease; HMW, high molecular weight; IFN-γ, interferon-γ; LMW, low molecular weight; mAb, monoclonal antibody.

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relatively resistant to enzymatic degradation in the gastrointestinal tract.[19–21] Hence, many of the immunogenic gluten peptides are likely to survive for extended periods in the intestine, increasing the probability of triggering a T-cell response. Thus, the unique properties of gluten are linked tightly to their disease-inducing potential in CD patients.

Each wheat plant contains a few (HMW) to several hundred (α-gliadins) copies of gliadin and glutenin genes,[22] but it is unknown whether all these genes encode proteins that are equally harmful for patients. The first indication that heterogeneity may exist in this respect came from our observation that not all α-gliadins contained equal copy numbers of a particular T-cell–stimulatory peptide.[23] Moreover, the genus *Triticum* harbors several species, of which *Triticum aestivum* is the most widely used in the food industry. Thousands of *Triticum* accessions are available in genebanks. The oldest material is diploid and contains either the AA, BB/SS, or DD genome. These grass-like species have a very low seed yield and drop their seed easily. Because of natural hybridization between some of these species the tetraploid *Triticum* species with the AABB genotype (pasta wheat) originated. Finally, through spontaneous hybridization between tetraploid and diploid species around 4000 BC, hexaploid wheat (AABBDD) species were formed. Further breeding over past centuries led to an increase in gluten content, enhanced crop yield, and other properties that are important for the application of hexaploid bread wheat in food production. The existence of thousands of different *Triticum* accessions that in all probability will have a very different gluten protein composition raised the question of whether all *Triticum* accessions are equally toxic for CD patients. Recently, 2 assays have become available that allow a rational screening of wheat and food products for the presence of T-cell–stimulatory gluten sequences. In the first assay, gluten-specific T-cell clones that have been isolated from intestinal biopsy specimens of CD patients are used. In the second assay, monoclonal antibodies specific for T-cell–stimulatory gluten peptides are used (this study).[24]

Here we have used these assays to screen wheat accessions of various origins and genetic make-up for the presence of T-cell–stimulatory epitopes. The results indicate that there are considerable differences in the levels of T-cell–stimulatory epitopes present among wheat accessions. Together with the observation that individual gluten molecules contain highly variable numbers of T-cell–stimulatory sequences, these results indicate that through rational screening and breeding strategies, wheat varieties may be identified with a reduced or even absent toxicity profile for CD patients. This may contribute to an improved and safe wheat-containing diet for CD patients.

Table 1. Amino Acid Sequences of Previously Identified T-Cell Stimulatory Epitopes of Gluten Used for Sequence Alignments With the Gluten Proteins Present in the Uniprot Database

HLA restriction	Protein	Epitope	Reference
HLA-DQ8	α-gliadin	QGSFQPSQQ	10
	HMW-glutenin	QGYYPTSPQ	17
HLA-DQ2	α-gliadin	PFPQPQLPY (α-9)	27
		PQPQLPYPQ (α-2)	27
		FRPQQPYPQ (α-20)	16
	γ-gliadin	PQQSFPQQQ (γ-1)	6
		FPQQPQQPF (γ-2)	16
		IQPQQPAQL (γ-30)	16
	LMW-glutenin	FSQQQQSPF (glt- 156)	16
		FSQQQQQPL (glt-17)	16
		QXPQQPQQF (glu-5)	16

Materials and Methods

Database Searches

A wheat gliadin and glutenin subset was extracted from the Uniprot database using the SRS program (www.ebi.ac.uk). All epitope sequences listed in Table 1 were searched for full similarity against this subset using the stand-alone Macintosh (Apple, Cupertino, CA) version of the program PeptideSearch (http://www.mann.embl-heidelberg.de/GroupPages/PageLink/peptidesearchpage.html).

Description of the Different Wheat Accessions

The wheat accessions used in this study were obtained from the small-grain cereal collection maintained by the Centre for Genetic Resources (Wageningen, The Netherlands). The wheat accessions used in this study were selected on the basis of the species known to have played a role in the evolution of bread wheat, and they include all 3 genomes of the cereal crop with the highest production worldwide of all cereal crops. Diploid, tetraploid, and hexaploid accessions were used (Table 2). In all figures the numbers of the wheat accessions shown refer to the numbers of the wheat accessions described in Table 2.

The accessions were selected from a small-grain cereal collection that contains well over 5000 wheat accessions. The selection criteria were a difference in the following: (1) genetic background, (2) ploidy level, (3) geographic distribution, and (4) growth season (Table 2).

Preparation of Gluten-Containing Samples From Different Wheat Varieties

Samples of different wheat varieties were ground and a trypsin/pepsin digest was prepared as follows: 2 g of wheat

Table 2. List of Different Diploid, Tetraploid, and Hexaploid Wheat Accessions Used for Screening of the Presence of T-Cell Stimulatory Epitopes Involved in CD

Species (number in Figure)	Genome	Accession number	Country of origin	Winter/ spring type
Triticum monococcum (1)	AA	CGN 06602		
T monococcum (2)	AA	CGN 10542		Spring
T speltoides (3)	SS, later mutated to BB	CGN 10682	Israel	Spring
T speltoides (4)	SS, later mutated to BB	CGN 10684	Turkey	Spring
T squarrosum (5)	DD	CGN 10719	Israel	Spring
T squarrosum subsp. strangulata (6)	DD	CGN 10757	Iran	Spring
T turgidum variety dicoccoides (7)	AABB	Rec. 965418 (1975)	Israel	Winter
T turgidum variety dicoccoides (8)	AABB	Rec. 965521 (1975)	Israel	Winter
T turgidum group carthlicum (9)	AABB	CGN 04221	Iran	Spring
T turgidum group carthlicum (10)	AABB	CGN 08360	Russia	Spring
T aestivum group aestivum (11)	AABBDD	CGN variety Arcade (1989)	Belgium	Spring
T aestivum group aestivum (12)	AABBDD	CGN variety Minaret (1983)	The Netherlands	Spring
T aestivum group compactum (13)	AABBDD	CGN 04210	Italy	Spring
T aestivum group compactum (14)	AABBDD	CGN 08315	Switzerland	Spring
T aestivum (15)	AABBDD	CGN 08510		
T aestivum (16)	AABBDD	CGN 12393		

NOTE. Only Arcade and Minaret have been on the market as commercially available varieties.

flour was solubilized in 20 mL 1 mol/L acetic acid and boiled for 10 minutes. After cooling, 10 mg pepsin A (Sigma P-7012; Sigma Chemical Co, St. Louis, MO) was added and the mixture was incubated for 4 hours at 37°C. Subsequently, the pH level was adjusted to 7.8 with NaOH, followed by addition of 20 mg trypsin (Sigma T-4665). After overnight incubation at 37°C, trypsin inhibitor type II (Sigma) was added and the sample was dialyzed against water for 48 hours. The dialysate was centrifuged and the supernatant was fractionated over a 30-kilodalton membrane (Centriprep YM-30, Amicon Inc., Beverly, MA). For the subsequent experiments the fraction smaller than 30 kilodaltons was used. A control sample was prepared from a commercial gliadin preparation (no. 48960; Fluka Chemie, Zwijndrecht, The Netherlands) using the same protocol. For the T-cell assay the pepsin/trypsin digests were treated with tissue transglutaminase as described.[10]

T-Cell Proliferation Assays

To test for the presence of T-cell–stimulatory epitopes in different wheat varieties, 6 different T-cell clones each recognizing a different epitope were used: a Glia-α2/9–specific T-cell clone (recognizing both the Glia-α2 and the Glia-α9 T-cell epitope), a Glia-γ1–specific T-cell clone, a Glia-γ30–specific T-cell clone, a LMW-Glt-156–specific T-cell clone, a LMW-Glt-17–specific T-cell clone, and a HMW-glutenin–specific T-cell clone. The clones originated from gluten-specific T-cell lines generated from small intestinal biopsy specimens of different CD patients.[7,8,16,17] With the exception of the HLA-DQ8 restricted, HMW-glutenin–specific T-cell clone, all T-cell clones are HLA-DQ2 restricted. For the HLA-DQ2–restricted T-cell clones the pepsin/trypsin digests of the different wheat varieties were treated with tissue transglutaminase to facilitate T-cell recognition of the T-cell–stimulatory gluten peptides.[3,8]

Proliferation assays were performed in triplicate in 150 μL Iscove's modified Dulbecco's medium (Bio Whittaker, Verviers, Belgium) with 10% pooled normal human serum in 96-well flat-bottom plates using 10^4 gluten-specific T cells stimulated with 10^5 irradiated HLA-DQ2– or HLA-DQ8–matched (HMW glutenin) allogeneic peripheral blood mononuclear cells (3000 rad) in the presence or absence of antigen (1–10 μg/mL). After 2 days, ^{3}H-thymidine (.5 μCi/well) was added to the cultures, and 18–20 hours thereafter cells were harvested. ^{3}H-thymidine incorporation in the T-cell DNA was counted on a liquid scintillation counter (1205 Betaplate Liquid Scintillation Counter, LKB Instruments, Gaithersburg, MD). For the detection of interferon-γ (IFN-γ) production, 50 μL supernatant was collected from the cultures before the addition of ^{3}H-thymidine. IFN-γ production was measured using the Cytometric Bead Array (Becton Dickinson, San Diego, CA). The experiment was repeated twice for each T-cell clone.

Synthetic Peptides

Peptides were synthesized by standard fluorenylmethoxycarbonyl (Fmoc) chemistry on a SyroII (MultiSyntech, Germany) peptide synthesizer. The integrity of the peptides was checked by reversed phase (rp) high-performance liquid chromatography and mass spectrometry. The S-acetyl-mercaptoacetic acid group was introduced in the resin-bound peptides by the coupling of a 6-fold equimolar mixture of S-acetyl mercaptoacetic acid N-hydroxysuccinimide ester and 1-hydroxybenzotriazole in N-methylpyrrolidon (NMP) for 2 hours. Biotin was introduced in the resin-bound peptides by a 2-hour coupling with a 6-fold equimolar preactivating mixture of biotin and benzotriazol-1-yloxypyrrolidino phosphonium hexafluorophosphate (PyBob).

Monoclonal Antibodies Against T-Cell Stimulatory Epitopes of LMW and HMW Glutenin Proteins

For generation of monoclonal antibodies (mAbs) specific for the LMW-glutenin–derived T-cell–stimulatory epitope Glt-

Table 3. Database Analysis of Gluten Proteins for the Presence of T-Cell Stimulatory Sequences Involved in CD

	Number of T-cell stimulatory gluten sequences found in a single database entry							Genes without T-cell–stimulatory sequences
	0	1	2	3	4	5	≥6	
α-gliadins	10	7	7	2	1	0	2	10/29 (34%)
γ-gliadins	0	1	1	10	3	0	2	0/17 (0%)
LMW-glutenin	54	3	0	0	0	0	0	54/57 (95%)
HMW-glutenin	1	5	5	4	3	3	3	1/22 (4.5%)

156, mice were immunized with peptides chemically cross-linked to tetanus toxoid (TTd-DDDXPPFSQQQQSPFS-amide). For generation of mAbs specific for the HMW-glutenin–derived T-cell–stimulatory epitope presented by HLA-DQ8 molecules, peptides chemically cross-linked to tetanus toxoid (TTd-DDDXPGQGQ(Q/P)GYYPTS(L/Q)QQP-amide and TTd-DDDXQGQQGYYPTSPQQ(P/S)-amide were used for immunization.

Fusion and screening of the hybridomas was performed as described.[24]

Competition Assays for the Quantitative Detection of T-Cell Stimulatory Epitopes of Gluten Proteins

Competition assays were performed as described earlier.[24] For the competition assay detecting LMW-glutenin, a biotinylated peptide encoding the LMW-Glt-156 T-cell epitope was used as the indicator peptide (Bio-XKAKAKAX-PPFSQQQQSPFS-amide). For the competition assay detecting HMW-glutenin, a biotinylated peptide encoding the HMW-glutenin epitope was used as the indicator peptide (Bio-XKAKAKAKAXQGQQGYYPTSPQQP-amide). For quantification of the gliadin and LMW-glutenin assays a standard curve was made by the European gliadin reference IRMM-480[25] in a concentration range of 100 μg/mL to 3 ng/mL. For the HMW-glutenin assay a standard curve was made using a trypsin/chemotropism digest of recombinant HMW-glutenin proteins (kindly provided by P. Shewry, Rothamsted Research, Hampenden, United Kingdom) in a concentration range from 10 μg/mL to 10 ng/mL. The assays were repeated twice.

Protein Analysis by 1-Dimensional Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Western Blotting

For protein analysis wheat grains were ground and the wheat flour was resuspended in 8 mol/L urea, 10 mmol/L Tris HCl, at a pH of 8.0. After 1 hour of head-over-heel rotation the wheat flour suspensions were spun down and the soluble fraction was used for 1D sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12.5% acrylamide gel) was performed under standard conditions. After separation, proteins either were stained with Coomassie Brilliant Blue or transferred to nitrocellulose. On the blots the proteins were visualized with mAbs specific for stimulatory T-cell epitopes from α- and γ-gliadin[24] and LMW-glutenin (this study).

Results

Matching of Gliadin- and Glutenin-Derived T-Cell Epitopes With Gluten Proteins

It is unknown if all gluten genes are equally toxic for CD patients. The sequences of hundreds of gluten proteins are available through databases. We have aligned the sequence of 11 T-cell–stimulatory sequences from α-gliadin, γ-gliadin, LMW-glutenin, and HMW-glutenin with the gliadin and glutenin proteins present in the Uniprot database (Table 1). Strikingly, although all γ-gliadins contained 1 or more T-cell–stimulatory sequences, the large majority of the LMW-glutenin and one third of the α-gliadin proteins did not contain the T-cell–stimulatory epitopes for which we searched (Table 3). Also, some of the HMW-glutenin proteins contained no or a few T-cell–stimulatory sequences. The results also indicate that although some gluten proteins lack T-cell–stimulatory sequences, others may contain up to 6. Thus, considerable variability in the toxicity of individual gluten proteins is likely to exist.

Detection of T-Cell Stimulatory Epitopes by T-Cell Based Assays

We previously have described T cells specific for T-cell–stimulatory epitopes in α-gliadin, γ-gliadin, and LMW-glutenin.[7,8,16] We have used these T cells to screen wheat accessions for the presence of these epitopes. For this, 16 wheat accessions were selected (Table 2). Pepsin/trypsin digests of grinded seeds of these *Triticum* accessions were prepared, treated with tissue transglutaminase, and tested with T-cell clones specific for the Glia-α2/9, Glia-γ1, Glia-γ30, LMW-Glt-17, and LMW-Glt-156 peptides. The results show striking differences in the T-cell responses to the individual wheat preparations. Representative results are shown in Figure 1*A* and *D* and a summary of all results is shown in Table 4. Wheat varieties either induced low, medium, or high T-cell responses, independent of the ploidy level or genome

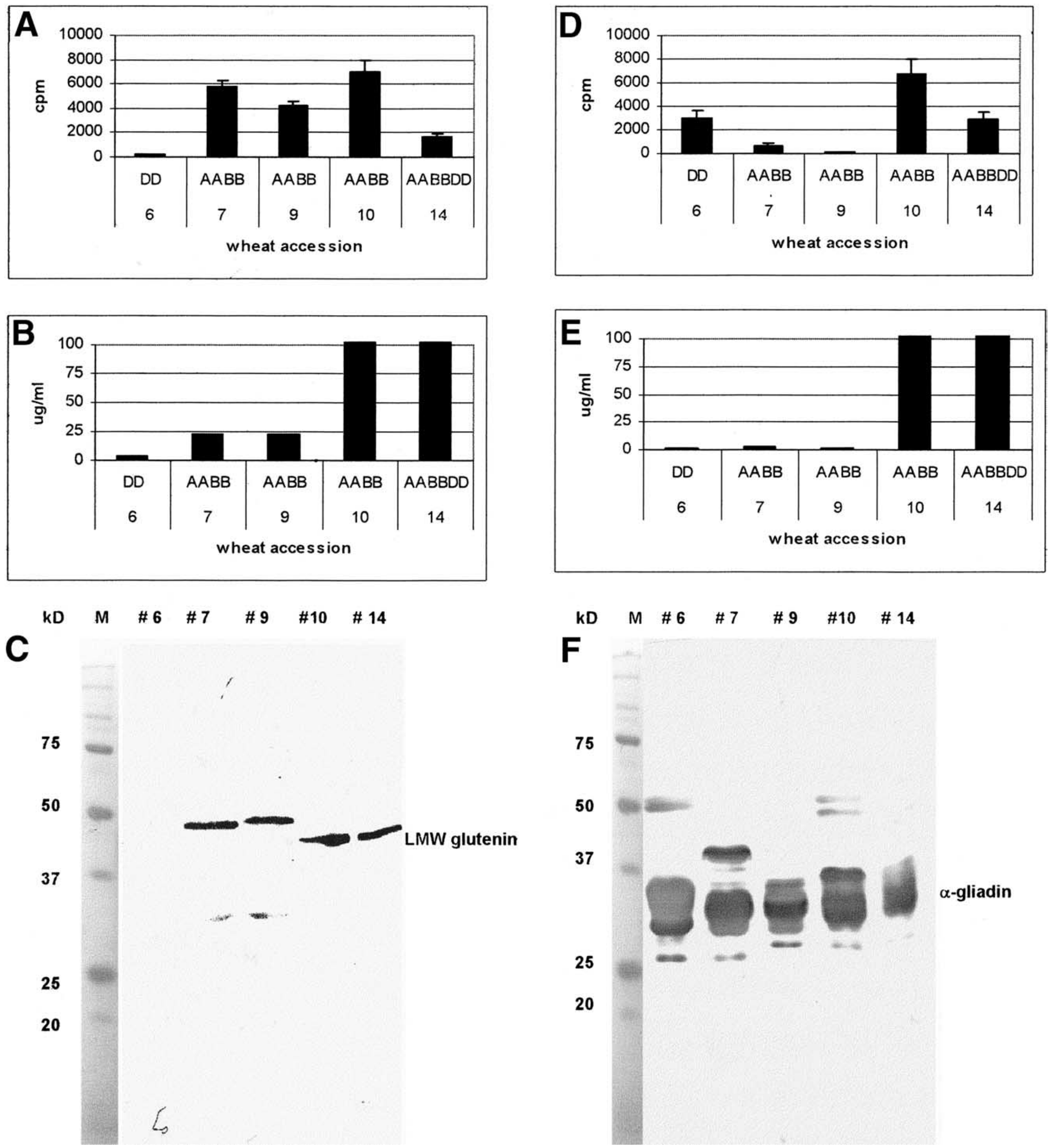

Figure 1. Analysis of gluten proteins of *Triticum* accessions with different levels of T-cell–stimulatory epitopes in the (*A*, *D*) T-cell assay and (*B*, *D*) mAb-based competition assay. (*A*) Stimulation of the LMW-Glt-156–specific T-cell clone, (*B*) mAb-based competition assay specific for the LMW-Glt-156 T-cell epitope, (*C*) Western blot stained with the anti-LMW-Glt-156 T-cell epitope–specific MAb, (*D*) stimulation of the Glia-α2/9–specific T-cell clone, (*E*) mAb-based competition assay specific for the Glia-α9 T-cell epitope, and (*F*) Western blot stained with the anti-Glia-α9 T-cell epitope–specific mAb. The experiments shown in *A* and *D* were performed in triplicate and the mean ± SD of each measurement is shown.

background of the accessions (Table 4; Figure 1*A* and *D*). Very low responses were found for the diploid accession 4 and the hexaploid accessions 13 and 16 (Table 4). Next we determined if the observed differences in proliferative T-cell responses correlated with similar differences in IFN-γ production. An α-gliadin and an LMW-glutenin T-cell clone were tested against protein extracts from wheat accessions 4, 9, 10, 13, 14, and 16; T-cell proliferation and IFN-γ production were measured simultaneously. In all cases a clear

Table 4. Overview of the Results of Screening of the Different Wheat Accessions for the Presence of T-Cell–Stimulatory Epitopes Involved In CD

		T-cell assay						mAb assay			
	Genome	Glia-α-2/9	Glia-γ-30	Glia-γ1	LMW-Glt-156	LMW-Glt-17	HMW-glt	Glia-α-9	Glia-γ-1	LMW-Glt-156	HMW-glt
1	AA	++	+++	+++++	++	+/−	+	+++	+++	++++	+/−
2	AA	+/−	+	+/−	+++++	+/−	+/−	+	+++	++++	+/−
3	SS/ BB	+/−	+/−	+++	++	++++	+	+	+/−	+	+/−
4	SS/ BB	+/−	+/−	+	+/−	+	+/−	+/−	+/−	+/−	+/−
5	DD	++++	+++	+++	+	+++	+++	++	+++	+/−	+/−
6	DD	+++	+/−	+	+/−	++	++	+/−	++	+/−	+/−
7	AABB	+	++	++	++++	++++	+++	+/−	++	++	+
8	AABB	+	+++++	+++	+++	++++	++++	++	+++	++	+++
9	AABB	+/−	+	+/−	+++	+++	+++	+/−	++	++	+++
10	AABB	+++++	++++	++	+++++	+++++	++++	++++	++++	++++	++++
11	AABBDD	+	+	+	+++	+++	+	+/−	+	++	+/−
12	AABBDD	+	+/−	++	+++	+++	++	+++	+++	+++	++
13	AABBDD	+	+/−	+/−	+	+	+/−	−	+	+	+/−
14	AABBDD	+++	+/−	++	++	+++	++	++++	++++	++++	++
15	AABBDD	++++	++++	++	+++	+++	+++++	++	+++	++	++++
16	AABBDD	+/−	+/−	+	++	+	+++	+/−	+	+++	++

NOTE. Pepsin/trypsin digests of the different accessions were tested by the T-cell based and mAb-based assays. For the T-cell assay, the responses are represented relative to the maximum response given by the T-cell clone indicated by +++++. Therefore, +/− corresponds with <5%; + corresponds with 5%–20%; ++ corresponds with 20%–35%; +++ corresponds with 35%–65%; ++++ corresponds with >65%; +++++ corresponds with 100% of the maximum response given by the T-cell clone. For the mAb assay the amount of antigen detected is represented relative to the maximum amount (μg/mL) detected in a given accession indicated by ++++. Therefore, +/− corresponds with <5%; + corresponds with 5%–10%; ++ corresponds with 10%–30%; +++ corresponds with 30%–60%; ++++ corresponds to 60%–100% of the maximum amount of antigen measured in the mAb-based competition assay.

correlation was found between T-cell proliferation (Figure 2*A* and *C*) and IFN-γ production (Figure 2*B* and *D*). Together these results indicate that wheat varieties differ significantly in the level of T-cell–stimulatory epitopes present.

Detection of T-Cell Stimulatory Epitopes by mAb-Based Competition Assays

Subsequently, pepsin/trypsin digests of ground seeds of the *Triticum* accessions were tested with mAbs specific for the T-cell epitopes Glia-α9, Glia-γ1,[24] and LMW-Glt-156 (this study) in a competition assay as described.[24] In agreement with the results of the T-cell assay, we observed large differences in the levels of the 3 gluten epitopes in the wheat accessions tested (Table 4; Figure 1*B* and *E*). Also, with this assay we observed that varieties 4, 13, and 16 contain low levels of the gluten peptides (Table 4). Thus, these results confirm the results of the T-cell–based assay and indicate that not all vari-

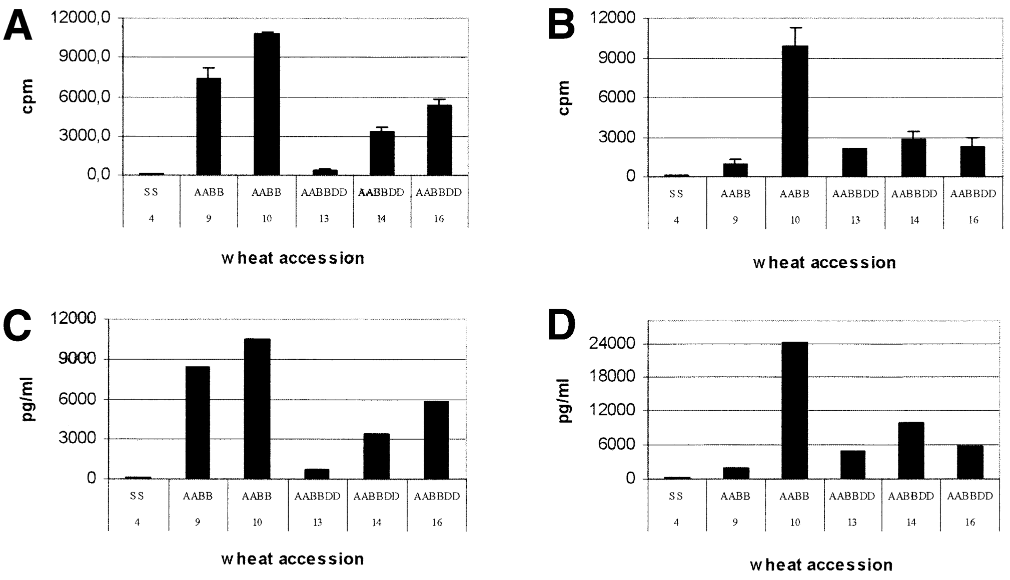

Figure 2. Correlation between T-cell proliferation and IFN-γ production after stimulation of T-cell clones with protein extracts of 6 wheat accessions. Two T-cell clones, specific for the gluten peptides (*A*, *B*) LMW-Glt-156 and (C, D) Glia-α2/9 were tested against protein extracts of 6 wheat accessions at a concentration of 2 μg/mL protein. T-cell stimulation was determined by measurement of (*A*, *C*) proliferation and (*B*, *D*) IFN-γ production. The experiments shown in *A* and *C* were performed in triplicate and the mean ± SD of each measurement is shown.

eties contain similar amounts of T-cell–stimulatory gluten sequences.

Western Blot Analysis of Gluten Proteins in Wheat Accessions

The α-gliadin and LMW-glutenin–specific MAbs also are suitable for the specific detection of α-gliadins and LMW-glutenins on Western blots. We therefore have used these mAbs to stain gluten proteins expressed in the wheat accessions. Protein extracts were prepared from 5 wheat accessions that were found to contain different amounts of the T-cell–stimulatory epitopes in the T-cell and mAb-based competition assays: varieties 6 (diploid, DD genome), 7, 9, 10 (tetraploid, AABB), and 14 (hexaploid, AABBDD). Next, the proteins were separated by 1-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis and, after blotting onto nitrocellulose membrane, were stained with the mAb used in the competition assays (Figure 1*C* and *F*). With the LMW-glutenin mAb staining was observed for all but the diploid (DD) accession 6 (Figure 1*C*). In this accession relatively low levels of the LMW-glutenin–derived T-cell–stimulatory epitope were detected with both the T-cell– and the mAb-based assay (Figure 1*A* and *B*). Moreover, in accessions 7 and 9 the antibody stained a protein that was distinct from that in accessions 14 and 10. This correlates with the presence of an intermediate level of the T-cell–stimulatory glutenin epitopes in accessions 7 and 9, as compared with a high level of the T-cell–stimulatory epitopes in accessions 10 and 14 (Figure 1*B* and *C*). With the α-gliadin–specific MAb staining was found for all accessions. However, a distinct staining pattern was obtained for each variety, indicating the presence of different gliadin proteins in the accessions (Figure 1*F*). Together these results indicate that the observed differences in the levels of T-cell–stimulatory epitopes found in the wheat varieties are caused by differences in protein composition between the wheat accessions.

Detection of a T-Cell Stimulatory HMW-Glutenin Peptide in Wheat Accessions

The baking quality of wheat-derived flours to a large extent depends on the presence of HMW-glutenins. These HMW-glutenins long have been considered not to be involved in CD but recent evidence has indicated that the HMW-glutenins contain T-cell–stimulatory peptides,[17,18] although only 1 such peptide has been identified at present.[17] We therefore also tested the pepsin/trypsin digest of the wheat accessions with a T-cell clone specific for this HMW-glutenin peptide[17] and a mAb raised against this peptide (this study). In this case the digests were not treated with tissue transglutaminase because both the T-cell clone and the mAb preferentially respond to native HMW-glutenin (data not shown).[17] The results (Figure 3, Table 4) indicate that large differences exist in the presence of the HMW-glutenin peptide. The lowest values are found in the AA and SS diploid accessions and in some of the tetraploid accessions.

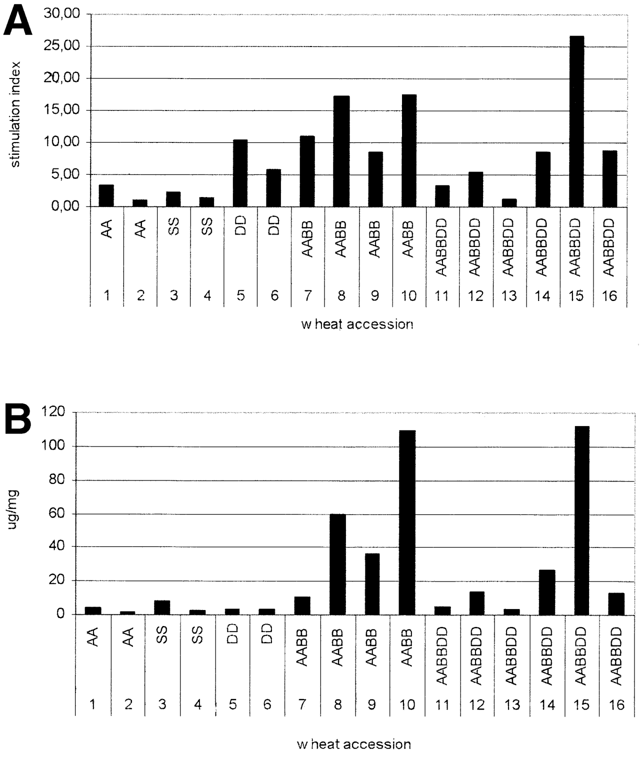

Figure 3. Detection of HMW-glutenin T-cell epitope in different wheat accessions in a (*A*) T-cell assay and a (*B*) mAb-based competition assay. The T-cell responses were defined as a stimulation index (*[T + APC + antigen]/[T + APC]*).

Thus, in addition to the gliadins and LMW-glutenins, considerable variability exists with regard to the presence of the HMW-glutenin peptide in the wheat accessions.

Discussion

CD is the most common food intolerance in the Western hemisphere: approximately .5% to 1% of the population is now known to have the disease, although the symptoms vary widely among patients. Classic symptoms include diarrhea, stomachache, and failure to thrive. These are the result of a lesion in the small intestine characterized by villous atrophy. T cells isolated from small intestinal biopsy specimens of patients have been shown to respond specifically to gluten-derived peptides bound to the disease predisposing HLA-DQ2 and/or HLA-DQ8 molecules.[9,10] These inflammatory T-cell responses are thought to be a key player in disease development. T-cell–stimulatory gluten peptides are found in the α- and γ-gliadins and in the LMW- and HMW-glutenins.[6,10,15–18] Because different combinations of gluten genes are present in individual wheat accessions and varieties, this indicates that such wheat accessions also may have a distinct toxicity profile. This prompted us to analyze a selection of diploid, tetraploid, and hexaploid accessions for the presence of a number of T-cell–stimulatory sequences. For this purpose we used T-cell clones isolated from small intestinal biopsy specimens of patients[7,8,16,17] and mAbs specific for T-cell–stimulatory sequences present in α-gliadin, γ-gliadin, LMW-glutenin, and HMW-glutenin.

The results indicate that large differences exist with regard to the presence of T-cell–stimulatory gluten peptides in the wheat accessions investigated, independent of the ploidy level. To substantiate these observations a large number of gluten genes present in public databases were screened for the presence of 11 T-cell–stimulatory sequences. In agreement with the results of our T-cell and mAb screening, a surprisingly large number of gluten genes were found to lack the T-cell–stimulatory sequences for which we searched. About one third of the α-gliadin genes encode proteins that lacked T-cell–stimulatory sequences and a large proportion of the LMW-glutenins also were found to lack the T-cell–stimulatory sequences. It should be noted that this does not implicate that such gluten proteins may not have any T-cell–stimulatory properties. They may contain a variant of the T-cell–stimulatory sequence for which we searched, and some of such variants may have T-cell–stimulatory properties. In contrast to the α-gliadins and LMW-glutenins the majority of HMW-glutenins and all of the γ-gliadins were found to contain T-cell–stimulatory sequences. Together, these results indicate that individual gluten proteins are likely to have a distinct toxicity profile.

Several studies have suggested that the α-gliadins encode the immunodominant T-cell response inducing gluten peptides, although the γ-gliadins and glutenins have received much less attention.[1,26] Our database searches, however, indicate that the γ-gliadins appear to contain T-cell–stimulatory sequences most frequently. Thus, it may prove to be easier to select wheat varieties that lack harmful α-gliadins as γ-gliadins. This raises the question of whether the composition of the γ-gliadin gene clusters is sufficiently diverse to allow the identification of clusters that are completely free of toxic peptides. In this respect it is worth noting that varieties 4, 13, and 16 appear to contain low levels of all peptides for which we tested, including the γ-gliadin peptides. Apparently, depending on which γ-gliadin genes actually are expressed, considerable differences in protein composition are present between the varieties and this may be exploited to select for varieties that contain low levels of T-cell–stimulatory γ-gliadin peptides. Alternatively, silencing of the entire γ-gliadin gene locus may be used to remove γ-gliadin–related toxicity from wheat accessions. Such an approach would be less favored for the LMW- and the HMW-glutenins because they are important for the baking quality of wheat-based doughs.

It is known that early exposure to gluten and a double HLA-DQ2 gene dose both promote CD development. In Sweden the addition of gluten to infant food led to a 5-fold increase in the occurrence of CD in the 1980s,[27] and HLA-DQ2 homozygous individuals have a 5-fold increased risk for developing CD compared with HLA-DQ2 heterozygous individuals.[28] A large repertoire of abundant immunogenic gluten peptides in the diet, together with a high copy number of HLA-DQ2,[29] thus favors the breaking of oral tolerance.[30] In present-day practice, gluten is introduced into the diet of infants at 6–7 months of age. Because there is no restriction in the amount of gluten given, gluten intake at age 12 months is between 6 and 9 g/day,[30] whereas gluten-specific T cells of CD patients are known to respond to microgram amounts. The sudden introduction of grams of gluten thus may play an important role in the breaking of oral tolerance. As we have suggested previously, the current understanding of the development of the disease may call for a more gradual and/or reduced intake of gluten in infants.[29] The breeding of wheat varieties with a lower amount of T-cell–stimulatory gluten peptides potentially could aid in reaching that goal.

In general, the results of our study are in agreement with the results published recently by the Norwegian group[31] with 1 exception: we also observed a low to absent presence of the α-gliadin peptide in the AA and BB/SS genome. Similarly, we observed a variability in the presence of γ-gliadin–derived peptides. In addition to the analysis of these ancient haplotypes and AABB-containing varieties, as reported by the Norwegian group,[31] we also analyzed 6 more modern wheat varieties that typically are used for bread making and that contain 3 genomes: AABBDD. Moreover, we also analyzed the presence of LMW- and HMW-glutenin–derived peptides and showed considerable heterogeneity in the levels of these peptides in the wheat accessions as well. We believe that this is relevant particularly because the HMW-glutenins are a major factor determining the baking quality of wheat flour and we reported previously that T-cell responses to LMW-glutenins frequently are observed in children.[16]

In conclusion, our data indicate that with the use of gluten-specific T cells and mAbs directed to T-cell–stimulatory gluten peptides, wheat accessions can be selected that contain minimal to absent quantities of particular harmful gluten sequences. This is promising for future breeding programs aimed at generating new varieties that will be acceptable for the manufacturing of food products for CD patients. Moreover, such varieties may prove to be valuable tools for disease prevention in individuals at risk.

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