Mapping of Gluten T-Cell Epitopes in the Bread Wheat Ancestors: Implications for Celiac Disease

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Background & Aims: Celiac disease is a prevalent disorder characterized by a chronic intestinal inflammation driven by HLA-DQ2 or -DQ8-restricted T cells specific for ingested wheat gluten peptides. The dominant T-cell responses are to epitopes that cluster within a stable 33mer fragment formed by physiologic digestion of distinct α-gliadins. Celiac disease is treated by excluding all gluten proteins from the diet. Conceivably, a diet based on baking-quality gluten from a wheat species that expresses no or few T-cell stimulatory gluten peptides should be equally well tolerated by the celiac patients and, importantly, also be beneficial for disease prevention.

Methods: To identify baking quality, harmless wheat, we followed the evolution of the wheat back to the species that most likely have contributed the AA, BB, and DD genomes to the bread wheat. Gluten were extracted from a large collection of these ancient wheat species and screened for T-cell stimulatory gluten peptides.

Results: Distinct differences in the intestinal T-cell responses to the diploid species were identified. Interestingly, we found that the fragments identical or equivalent to the immunodominant 33mer fragment are encoded by α-gliadin genes on the wheat chromosome 6D and thus absent from gluten of diploid einkorn (AA) and even certain cultivars of the tetraploid (AABB) pasta wheat. Conclusions: These findings have implications for celiac disease because they raise the prospect of identifying or producing by breeding wheat species with low or absent levels of harmful gluten proteins.

Celiac disease is a multifactorial disorder that affects children and adults with high prevalence. It is characterized by an inflammatory response to ingested wheat gluten proteins, which is genetically determined and which leads to atrophy of the intestinal villi and malabsorption. Celiac disease in children often presents with diarrhea and malnutrition, whereas, in adults, extraintestinal symptoms such as fatigue, osteoporosis, and anemia dominate. The disease is unique in that critical etiologic factors have been identified: the ingested gluten proteins as obligate environmental factors and the genes that encode the HLA variants DQ2 (DQA1*05/DQB1*02) or DQ8 (DQA1*03/DQB1*0302) as primary genetic factors. These HLA-DQ variants most likely confer disease susceptibility by their ability to bind in vivo deamidated gluten peptides and form complexes that are recognized by interferon (IFN)-γ producing CD4+ T cells within the celiac lesions. Both classes of gluten proteins, gliadins and glutenins, contain peptides that can bind DQ2 or DQ8 and be recognized by intestinal T cells, but their relative importance differs. Peptides derived from α-gliadins are recognized by T cells from almost all celiac patients, whereas T-cell responses to γ-gliadins and glutenins are much less frequent. This hierarchy probably reflects that certain α-gliadin (αG) proteins contain a stable 33mer fragment that contains a cluster of epitopes. This αG-33mer fragment is naturally formed by digestion with gastric and pancreatic enzymes, it binds well to DQ2 after deamination by tissue transglutaminase (TG2), and it is recognized much more effectively by intestinal T-cell lines than shorter peptides covering the DQ2-α-I, -α-II, and α-III epitopes.

Celiac disease goes in remission when the patients are put on a gluten-exclusion diet, and patients relapse when gluten is reintroduced into the diet. Complying with a gluten-free diet is difficult and affects the patients’ quality of life, but a strict diet is critical to reduce morbidity and mortality. New treatment strategies are thus actively pursued. Most of these treatments aim to put the celiac patients on a normal diet and add a drug designed to abolish the T-cell stimulatory capacity of the gluten. An alternative possibility, explored here, is to

Abbreviations used in this paper: αG, α-gliadin; CT, chymotrypsin; TG2, tissue transglutaminase.
improve the celiac diet with food items made from baking-quality wheat that do not contain harmful gluten proteins. Previous studies have failed to identify such harmless wheat, but they were done long before molecular-level knowledge on T-cell stimulatory gluten sequences was available.

The hexaploid bread wheat contains 11 complex gluten gene loci, 3 of which encode α-gliadin proteins. How frequent T-cell stimulatory sequences occur in the more than 100 distinct α-gliadins encoded by these loci is not clear, but it is interesting that only 2 of 29 α-gliadin entries in the Entrez database (www.ncbi.nlm.nih.gov/entrez) contain the complete αG-33mer fragment (4/29 and 9/29, respectively, contain the α-II and α-I epitopes). If correct, these low frequencies argue that wheat without the most important T-cell epitopes could exist. To resolve this issue, one could, in theory, perform complete mapping of all the genes or proteins expressed. These methods would, however, not be suitable for screening purposes. For this reason, we favor an alternative strategy, which is used here: to test the wheat with a panel of celiac lesion T-cell clones specific for defined epitopes.

To reduce the complexity, but keep focus on a bread-quality gene pool, we decided to follow the evolution of the wheat and screen the Aegilops and Triticum species that most likely have contributed the AA, BB, and DD genome to the bread wheat (Figure 1). Only very few gluten gene sequences from these ancient wheat species exist, but protein expression pattern analyses indicate that there are highly polymorphic genes, which vary widely between cultivars and which include allelic variants not found in bread wheat. All these factors argue that T-cell assays, rather than genetic approaches, are the more rational and efficient method to screen accessions of these species. Interestingly, we find distinct differences in the intestinal T-cell responses to the 3 diploid species. The gluten digest from the selected cultivars of Triticum tauschii, the DD genome donor, contains all the T-cell epitopes identified to date. Gluten from the AA, and most likely also BB genome species, in contrast, lacks sequences equivalent to the αG-33 fragment. Experiments with mutant wheat lines suggest that these sequences are exclusively encoded by α-gliadin genes on chromosome 6D. This explains why some durum wheat (AABB) cultivars are not recognized by T cells specific for epitopes within the αG-33mer fragment. These findings have implications for programs aiming to produce wheat species with no or low contents of gluten proteins harmful to celiac disease patients.

**Materials and Methods**

**Collection of the Grain Samples**

Accessions of Triticum urartu (n = 40), Triticum monococcum (n = 15), Triticum tauschii (n = 9), Ae speltoides (n = 3), Triticum dicoccites (n = 4), Triticum dicoccon (n = 3), and Triticum durrum (n = 10) were collected. Some T urartu accessions had low grain numbers and were thus pooled. (Data on the individual accessions can be provided on request.)

**Deletion Mutants of the Chinese Spring Bread Wheat Cultivar**

Three ditelosomal lines that lack the short arm of chromosomes 6A (Dt 6 AL), 6B (Dt 6 BL), and 6D (Dt 6 DL) and 5 deletion mutants (6AS-1, 6BS-1, 6DS-2, 6DS-4, and 6DS-6) were a gift from Dr. B. Gill at the Wheat Genetics Resource Centre, Kansas State University, KS.
Table 1. Overview of the Celiac Lesion-Derived T-Cell Clones Included in This Study

<table>
<thead>
<tr>
<th>Intestinal T-cell clone</th>
<th>Epitope</th>
<th>Sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCC 380.E2</td>
<td>DQ2-αI</td>
<td>LQPFPQGLPY</td>
</tr>
<tr>
<td>TCC 387.E9</td>
<td>DQ2-αI</td>
<td>PQPQLPPQQL</td>
</tr>
<tr>
<td>TCC 430.1.142</td>
<td>DQ2-αI</td>
<td>LQPFPQGLPY</td>
</tr>
<tr>
<td>TCC 412.5.16</td>
<td>DQ2-αII</td>
<td>QPQPQLPPQQ</td>
</tr>
<tr>
<td>TCC 430.1.135</td>
<td>DQ2-αII</td>
<td>QPQPQLPPQQ</td>
</tr>
<tr>
<td>TCC 436.5.4</td>
<td>DQ2-αII</td>
<td>QPQPQLPPQQ</td>
</tr>
<tr>
<td>TCC 450.2.2.6</td>
<td>DQ2-αII</td>
<td>QPQPQLPPQQ</td>
</tr>
<tr>
<td>TCC 370.E3.19</td>
<td>DQ2-αIII</td>
<td>QLPYPQQLPPQ</td>
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<tr>
<td>TCC 370.2.25</td>
<td>DQ2-αIII</td>
<td>QLPYPQQLPPQ</td>
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<tr>
<td>TCC 423.1.3.8</td>
<td>DQ2-γI</td>
<td>QPQPQLPPQQP</td>
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<td>TCC 430.1.41</td>
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<td>TCC 437.1.3.11</td>
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<td>FQPQPQLPPQQ</td>
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<td>TCC 430.1.112</td>
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<td>FSQPQPQLPPQQ</td>
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<tr>
<td>TCC 387.3</td>
<td>DQ2-γVI</td>
<td>PQQPQLPPQQ</td>
</tr>
</tbody>
</table>

*The T-cell clones do not recognize the native peptide but rather peptides in which certain glutamines (underlined) are deamidated by TG2.

Extraction and Preparation of Gluten From the Wheat Samples

Hulled species were dehulled by hand, and the grains were milled to whole-meal flours. The small, shrunken kernels of the *Ae speltoides* were crushed by a mortar. Gluten was extracted and digested with chymotrypsin (C-4129; Sigma Chemical Co., St. Louis, MO) as described. The gluten digests were treated with human recombinant TG2 in 2 mmol/L Ca²⁺/PBS as described.

Generation of Gluten-Specific T-Cell Lines From Celiac Biopsy Samples

Polyclonal T-cell lines were established from intestinal biopsy samples of celiac patients as described. Briefly, single cell suspensions were isolated from biopsies incubated with 100 μg/mL chymotrypsin digested bread wheat gluten (CT-gluten) in complete medium (CM; 15% human serum in RPMI 1640) for 20 hours at 37°C, cultured with autologous, irradiated PBMC, IL-2 (R&D Systems, Abingdon, United Kingdom), and interleukin (IL)-15 (R&D) and screened for gluten-specificity on day 15. T-cell clones were generated from the intestinal T-cell lines as described.

Establishing a Panel of Intestinal T-Cell Lines and Clones

Biopsy sample-derived T-cell lines from DQ2⁺ celiac patients were selected for this study based on 2 criteria: effective recognition of tissue transglutaminase (TG2)-treated digests of chymotrypsin (CT)-gluten and significant responses to at least 2 different gluten epitopes, including the γ-M2, γ-M7, and γ-M12 of γ-gliadin M-36999. The intestinal T-cell clones used are described in Table 1. All the clones, except TCC 387.3, which recognizes an epitope defined as γ-V1 contained within residues 62–72 (PQPQPQPQPQQ) of recombinant γ-5 gliadin, have been described elsewhere.

Testing of Gluten Proteins and Peptides in T-Cell Assays

Antigen-presenting cells (APC; DQ2 homozygous, Epstein-Barr virus (EBV) transformed B-lymphoblast cell lines (B-LCL)) were irradiated 75 gray, seeded at 7.5 × 10⁴ cells per well, incubated overnight with gluten antigens in 96-well, U-bottom plates in a volume of 100 μL complete medium (CM) before T cells (2.5 to 5 × 10⁴ cells per well) were added. T-cell proliferation was measured by ³H thymidine incorporation after 48–72 hours. Cytokines in the culture supernatants were measured after 24, 48, and 72 hours with the Human Th1/Th2 Cytokine Bead Array (BD Pharmingen, San Diego, CA), according to the manufacturer’s instructions. Briefly, 25 μL of the supernatants were incubated with 10 μL capture beads (IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ) for 90 minutes before addition of PE detection reagent and sample analysis on a FACSCalibur instrument (BD Pharmingen).

Isolation and Identification of T-Cell Stimulatory Peptides in *T urartu* No. 2

The CT-gluten of *T urartu* No. 2 (in 0.01 mol/L NH₄HCO₃) was separated by gel filtration (FPLC, Superdex peptide HR 10/30, Amersham Biosciences, Little Chalfont, United Kingdom), and each fraction was split: One part was TG2-treated and tested with an α-I-specific T-cell clone, and the other part was run using reverse-phase (RP) high-performance liquid chromatography (HPLC) (μRPC, C2/C18, Amersham) and split again. RP-HPLC fractions recognized by T cells after TG2 treatment were analyzed by matrix-assisted, laser desorption ionization, time of flight mass spectrometry (Ultraflex; Bruker-Daltonik, Bremen, Germany). Tandem mass spectra of selected peptide ions were recorded by collision-induced dissociation (collision gas argon) on a MALDI-quadrupole time-of-flight mass spectrometer (Micromass, Manchester, United Kingdom) using the orthogonal TOF mass analyzer.

Results

Screening the AA, BB, and DD Genome Wheat Species With Intestinal T-Cell Clones

Gluten from selected samples of the AA donor (*T urartu* No. 2), the potential BB donor (*Ae speltoides* No. 1), and the DD donor (*T tauschii* No. 1) were treated with TG2 and tested for recognition by 14 different celiac lesion-derived T-cell clones specific for 3 α-gliadin epitopes and 5 γ-gliadin epitopes (Table 1 and Figure 2, upper panel).

The DD Genome Donor Expresses all the Gluten T-Cell Epitopes

The gluten digests of all the nine *T tauschii* accessions were efficiently recognized by all the α- and γ-gliadin specific T-cell clones (data not shown), suggesting that the DD genome encodes most of the hitherto identified harmful gluten proteins.
Gluten T-Cell Epitopes in AA Genome Species

A surprising finding in the initial screening was that *T. urartu* No. 2 was not recognized by celiac lesion-derived α-II- or α-III-specific T-cell clones. The same sample was, however, efficiently recognized by T-cell clones specific for the α-I epitope, which is part of αG-33mer fragment in the α-2 gliadin (Figure 2, upper panel). Broad titrations of *T. urartu* No. 2 and another AA genome accession (*T. monococcum* No. 5) with α-I- and α-II-specific T-cell clones demonstrated that these differences were qualitative and not due to low expression of α-gliadins in the einkorn samples; no responses were detected with the α-II-specific T-cell clones, whereas the α-I-specific clones displayed dose-dependent responses (Figure 3).

To clarify whether AA genome species in general lack the α-II and α-III epitopes, we screened gluten digests from all the einkorn samples against the whole panel of T-cell clones (Figure 2, lower panel). Strikingly, none of the 33 samples were recognized by the α-II- and α-III-specific T-cell clones, whereas the α-I-specific T-cell clones responded to all of them. Thus, it appears that all the 30–40 distinct α-gliadins expressed by each of these diploid AA accessions lack peptide sequences that are recognized by α-II- and α-III-specific T-cell clones, or, in other words, their gluten does not contain peptide sequences that are identical or equivalent to the αG-33mer fragment.

Interestingly, the various accessions of *T. monococcum* were differentially recognized by the γ-gliadin-specific intestinal T-cell clones. This suggests that large intercultivar difference in the γ-gliadin genes of the domesticated einkorn may exist.

Fine Specificity Analysis of the *T. urartu* Peptide Recognized by the α-I-Specific T Cells

Because α-I-specific T-cell clones can cross-react with deamidated γ-gliadin peptides, it was important to identify the *T. urartu* fragments recognized by the α-I-specific T-cell clones. A CT digest of *T. urartu* No. 2 was thus separated, first by size and then by RP-HPLC. Fractions stimulatory after TG2-treatment were analyzed by mass spectrometry. Peaks that corresponded exactly to the mass of the synthetic α-I peptide 59–68 and its shorter fragments were identified. MS/MS sequencing confirmed that the peptide in the *T. urartu* sample indeed had the sequence QLQPFPQPQLPY (Figure 4).
T-Cell Responses to the Potential BB Genome Donor

In the initial screening, *Ae speltoideus* No. 1 was not recognized by α-I-, α-II-, or α-III-specific T-cell clones (Figure 2, upper panel). Similar results were later also obtained with the 2 additional accessions of *Ae speltoideus* (Figure 3). Taken together, these data demonstrate that the potential BB wheat genome donor *Ae speltoideus* lacks the whole α/H9251 G-33mer fragment.

Differential T-Cell Recognition of AABB Genome Species

Because *T Turgidum* arose from 2 diploid species that lack the α-II and -III epitopes, one could predict it to lack sequences equivalent to the αG-33mer fragment. Although oversimplified, because *T turgidum* is not a uniform species, but ranges from the wild, hulled emmer to the cultivated pasta wheat, support for this prediction was indeed found. Eighteen different accessions of *T. turgidum* were screened; all were, as expected, recognized by T-cell clones specific for the α-I and the γ-epitopes. With the α-II- and α-III-specific clones, a different pattern was found; 2 of the 4 cultivated emmer accessions and 3 of the 10 durum wheat cultivars were not recognized by these T-cell clones (data not shown).

Differential Responses to the Ancient Species by Celiac Lesion-Derived T-Cell Lines

All the T-cell clones in this study stem from polyclonal T-cell lines derived from intestinal biopsy specimens of adult celiac disease patients. From previous analyses of such polyclonal T-cell lines, we know that all celiac patients tested to date respond to the αG-33mer fragment and that the other identified gluten epitopes are much less frequently recognized. To determine the overall intestinal T-cell reactivity to primitive wheat, we tested selected diploid and tetraploid accessions with a panel of these polyclonal T-cell lines. Interestingly, we found that the polyclonal T-cell lines that predominantly recognize the αG-33mer fragment displayed only marginal proliferative responses and IFN-γ secretion to samples not recognized by α-II- and α-III-specific T-cell clones (Figure 5). In fact, it appeared that the overall response of each T-cell line to the selected accessions mirrored its responses to the γ-gliadin epitopes (Figure 5A).

Intestinal T-Cell Responses to Bread Wheat Deletion Mutants

Collectively, the data above indicated that sequences equivalent to the αG-33mer sequence are not present in the α-gliadin loci of the A or B chromosomes (*Gli*-2A or *Gli*-2B) but only in *Gli*-2D on the short arm of chromosome 6D. To test this directly, gluten was extracted from ditelosomic lines that lack the short arm...
of 6A (Dt 6 AL), 6B (Dt 6 BL), and 6D (Dt 6 DL). All these lines are derived from Chinese Spring, a bread wheat cultivar that contains an αG-33mer fragment encoding gene.21 The Dt 6 DL line was recognized by T-cell clones specific for the α-H9251-I–, α-H9253-III–, and α-H9253-VI– but not α-H9251-II– and α-H9251-III– specific clones (Figure 6A). To map further the location of the α-H9251 G-33mer fragment containing loci is positioned between the 6-DS4 and 6-DS6 deletion (Figure 6B).

**Discussion**

Here, we describe how insights into the evolution of the modern bread wheat can be combined with knowledge on celiac disease pathogenesis in an integrated approach to identify wheat strains that are devoid of or have low contents of T-cell stimulatory gluten sequences. We have extracted the gluten fraction from selected samples of the
diploid wheat species that are the origin of the bread wheat and tested for recognition by a panel of celiac lesion-derived, gluten-specific T cells. Our main findings are as follows: (1) The relative contribution to the overall immune reactivity of gluten differs widely between the AA, BB, and DD genome-encoded gliadin proteins; (2) the sequences equivalent to the immunodominant and highly immunostimulatory α-G-33mer fragment appear to be encoded by genes located in the Gli-2 locus on chromosome 6D; and (3) pronounced differences in the expression of the various γ-gliadin T-cell epitopes are noted between individual AA genome cultivars.

Intestinal T-cell clones with defined specificities are sensitive and accurate monitors of individual peptides in complex protein digests. They are hence well suited for assessing the content of harmful proteins in ancient wheat. A potential pitfall is that the clones cover only a limited number of specificities and thus may not fully represent the repertoire of gluten-reactive T cells within the celiac lesions. To minimize this problem, we have used polyclonal, gluten-reactive T-cell lines from a number of patients for complementary testing. We believe that these assays, when combined, should detect most of the gluten peptides in any unknown gluten sample.

Figure 6. Sequences equivalent to α-G-33 are only present on chromosome 6D. (A) Reactivity of α-I-specific T-cell clones to TG2-treated gluten digests extracted from ditelosomic lines of the hexaploid bread wheat Chinese Spring. The lines lack the short arm of chromosome 6A (Dt 6 AL), 6B (Dt 6 BL), and 6D (Dt 6 DL). (B) Responses to TG2-treated gluten of Chinese Spring deletion mutants that lack parts of the short arm of chromosome 6D. The numbers opposite the chromosome drawing indicate the relative distance (i.e., the FL value) from the centromere at which the 3 deletions are localized.

Given the heterogeneity of T-cell epitopes in wheat gluten, the important question is whether it will be feasible to establish wheat cultivars that are devoid of all T-cell stimulatory sequences. Our results do not give a definite answer to this but indicate that it should at least be possible to identify cultivars devoid of the strongly immunostimulatory α-gliadin-derived sequences. This could be done by first identifying AA genome cultivars that do not express the α-I epitope, either, as done here, by screening with α-I-specific T cells or, alternatively, by T-cell epitope specific antibodies or DNA markers. Next, the α-I-negative AA cultivars should be screened for expression of the various γ-gliadin T-cell epitopes. Our finding of differences in the expression of α-gliadin T-cell epitopes between individual AA genome cultivars (Figure 3) indicates that wheat cultivars with few or no epitope-encoding γ-gliadin genes should exist. There is also evidence that low molecular weight (LMW) and high molecular weight (HMW) glutenins harbor sequences that infrequently are recognized by celiac lesion T cells, and the selection algorithm will at some point need to take this into account. Even if it should prove impossible to generate a wheat cultivar completely devoid of harmful proteins, a cultivar low in T-cell stimulatory sequences can possibly be tolerated by most celiac disease patients. The experience from oats consumption by celiac disease patients is relevant in this respect. Oats avenin contain some few gliadin-like sequences that can
be deamidated by TG2 and be recognized by DQ2-restricted T cells of certain celiac disease patients.24 Nevertheless, oats seem to be tolerated by most celiac disease patients.25

Celiac disease prevention with a diet based on wheat cultivars with few or no harmful sequences has perhaps a greater potential than the treatment of subjects who have already contracted the disease. There is evidence in the literature that the amount of gluten exposure is related to population incidence,26 and theoretic considerations support this notion.27 Widespread usage of harmless cultivars will require competitive production yields and baking qualities. Because good bread-making performance traits map to D genome gluten genes, the latter is a potential concern, even though the production of high-quality einkorn bread has been reported.15,28 Another factor that limits the use of ancient wheat species is their adherent glumes. Breeding of free-threshing ancient wheat cultivars are, however, in progress.29 This breeding can be extended to include for selection of traits as described in this paper.

The production of transgenic crops is another approach for the production of wheat harmless to celiac disease patients. The public resistance to gene-modified food is however substantial, and transgenic wheat have so far not been put on the market. Our results suggest that screening of primitive wheat cultivars followed by breeding and selection based on the absence of certain gluten protein sequences has the potential to produce wheat cultivars that are devoid or low in sequences harmful to patients with celiac disease. The motivation to embark on this task should be fueled by the reports that celiac disease, with a prevalence of 1:100 in many populations, is one of the most common chronic inflammatory diseases of our societies.1,2

References


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