Fine specificity of monoclonal antibodies against celiac disease–inducing peptides in the gluteome

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ABSTRACT

Background: In celiac disease patients, peptides derived from dietary gluten are recognized by HLA-DQ2-restricted CD4$^+$ T cells, which results in inflammation. Such immune-stimulatory peptides are found in both gliadins and glutenins. Monoclonal antibodies (mAbs) against these peptides can be used to screen food for the presence of such peptides.

Objective: We aimed to determine the specificity of 5 mAbs raised against T cell stimulatory peptides found in α- and γ-gliadins and low and high-molecular-weight glutenins and to compare it with the specificity of patient-derived T cells.

Design: The reactivity of the mAbs with gluten peptides, enzymatic gluten digests, and intact gluten proteins was determined and compared with that of gluten-specific T cells by using a combination of immunologic and biochemical techniques. Furthermore, the reactivity of the mAbs with gluten homologues in barley, rye, and oat was determined.

Results: The specificity of the mAbs largely overlaps with that of gluten-specific T cells. Moreover, mAbs detect several homologous peptides present in gluten proteins. All except the LMW-specific mAbs also detect storage proteins present in barley and rye, whereas the γ-gliadin-specific mAbs also recognize oat proteins.

Conclusion: The mAbs raised against T cell stimulatory peptides in gliadins and glutenins allow a comprehensive screen for the presence of harmful gluten and gluten-like proteins and peptides in food products. They can thus be used to guarantee the safety of food for celiac disease patients. Am J Clin Nutr 2008:88:1057–66.

INTRODUCTION

Celiac disease (CD) is caused by an intolerance to gluten, storage proteins found in wheat. Typical symptoms are chronic diarrhea or constipation, malnutrition, anemia, fatigue, growth retardation, and migraine. These symptoms are the result of an inflammatory process that causes (sub) total villous atrophy in the small intestine, which decreases the normal uptake of nutrients from food. With an incidence of 0.5–1.0%, CD is the most common immune-mediated food intolerance condition in the Western world.

During digestion, the gluten proteins are enzymatically broken down in the gastrointestinal tract. However, because of the high proline content of gluten, the degradation is not efficient and relatively large gluten peptides persist (1, 2). In CD patients, such peptides trigger a CD4$^+$ T cell–mediated inflammatory immune response (3–5). These T cell stimulatory gluten peptides originate from the α- and γ-gliadins and from the low-molecular-weight (LMW) and high-molecular-weight (HMW) glutenins (6–8). Next to wheat, barley, rye, and oat are known to contain storage proteins that can trigger the immune system of CD patients (9). The collection of all gluten and gluten-like proteins is referred to as the gluteome (10).

A strict lifelong gluten-free diet is currently the only available treatment for CD. According to the guidelines of the Codex Alimentarius gluten-free food should meet strict criteria (ftp.fao.org/codex/alinorm07/al30_26e.pdf). To determine whether foods intended for the gluten-free market meet these requirements, sensitive assays for gluten detection are required. The detection of gluten in food is complicated because gluten is a large family of proteins that is divided into 2 biochemical distinct subfamilies, the glutenins and gliadins, that can both be further subdivided (11). Because both intact gluten proteins and gluten hydrolysates are used in the food industry, gluten detection assays should be able to detect both intact gluten molecules and fragments thereof. Moreover, because the disease-related T cell stimulatory peptides of gluten have been identified, it would be a major advantage if such gluten detection assays would specifically detect these peptides rather than indicate a level of overall gluten content.

To develop a robust assay that would meet these requirements, we developed mAbs against T cell stimulatory peptides present in α-gliadin, γ-gliadin, LMW glutenin, and HMW glutenin. Some of these antibodies have already been used to develop highly sensitive competition assays for the detection of proteins and peptides that harbor these sequences (12–14). In the present

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study, an extensive characterization of the specificity of these mAbs was carried out to determine their usefulness for the detection of various gluten and gluten-like peptides present in cereals that are implicated in CD.

**MATERIALS AND METHODS**

**Generation of monoclonal antibodies**

For the generation of mAbs, Balb/c mice were immunized with synthetic peptides corresponding to known T cell stimulatory epitopes that were coupled to tetanus toxoid (Table 1). Fusion, purification, and screening of the hybridomas were performed as described previously (12–14).

**Database searches**

Searches were performed in the UniProt kB database for variants of the Glia-α9, Glia-α20, Glia-γ1, and LMW- and HMW-glutenin gluten epitopes by using FASTA alignment as described previously (15). The following sequences were used in the searches: GLQFPQPQQLPYE for Glia-α9, PFRPQFPQYPQPO for Glia-α20, PQQSFPQQRPFFQPSL for Glia-γ1, and PPFSQOQPQSPFS for LMW. Previously described results were used for the variants of the HMW epitope (7). Variants of the Glia-α2, Glia-α9, Glia-α20, and Glia-γ1 epitopes present in gluten-like proteins from barley, rye, and oat were identified previously (15).

**Synthetic peptides**

Peptides were synthesized by standard Fmoc chemistry on a SyroII peptide synthesizer as described previously. The integrity of the peptides was checked by reversed-phase HPLC and mass spectrometry. When required, biotin was introduced in the resin-bound peptides by a 2-h coupling with a 6-fold excess of streptavidin-HRP (Sigma Aldrich). The color reaction was stopped by the addition of 100 μL/well of 2 mol H2SO4/L. Finally, absorbance was read on a multispan plate reader (Wallac, Turku, Finland).

**Direct binding assay**

Direct binding assays were, in general, performed as was the peptide binding assay. In brief, ELISA plates (Nunc Maxisorb immunoplate) were coated overnight at room temperature with the various peptides in a concentration range between 10 and 1.25 μg/mL in PBS (100 μL/well). The plates were washed and the residual binding sites were blocked. After a washing step, the plates were incubated for 1 h with the various mAbs at a concentration of 1.5 μg/mL in PBS/0.2% Tween20/0.2% skim milk. After this step, the plates were washed and incubated for 30 min with an excess of rat-anti-mouse HRP conjugated polyclonal antibodies (Sigma Aldrich). After a washing step, the assay was stained and absorbance was read as described above.

**Immune precipitation assay**

For immune precipitation, a pepsin/trypsin digest of wheat flour was incubated with the various monoclonal antibodies covalently coupled to sepharose beads (2–3 mg/mL). After being gently mixed for 120 min at 4 °C, the beads were washed sequentially with 120 mM NaCl and 20 mM Tris HCl (pH 8.0), 1 mol NaCl/L and 20 mM Tris HCl (pH 8.0), 20 mM Tris HCl (pH 8.0), and 10 mM Tris HCl (pH 8.0). Bound peptides were eluted with 10 mL 10% (by vol) acetic acid and analyzed by mass spectrometry followed by database searches as described previously (10).

**T cell proliferation assays**

Proliferation assays were performed in triplicate in 150 μL RPMI-1640 (Gibco; Lonza, Verviers, Belgium) supplemented with 10% human serum in 96-well flat-bottom plates (Falcon; Corning Inc, New York, NY) by using 2 × 104 gluten-specific T cells stimulated with 105 irradiated HLA-DQ2-matched allogenic peripheral blood mononuclear cell (3000 RAD) in the presence of 1 μg peptides. All the peptides were deamidated by tissue transglutaminase (N-Zyme BioTech, Darmstadt, Germany) in the presence of calcium chloride overnight at 37 °C. After 48 h at 37 °C, the cultures were pulsed with 0.5 μCi [3H]thymidine and harvested 18 h later; [3H]thymidine incorporation was quantified with a liquid scintillation counter.

**Protein analysis by 1D sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12.5% acrylamide gel) was performed under standard conditions and then the separated proteins were either stained with Imperial Protein Stain (Pierce, Rockford, IL) or transferred to polyvinylidene difluoride membranes (Bio-Rad,
Hercules, CA). For the Western blot analysis, the proteins were visualized with mAbs specific for stimulatory T cell epitopes from α- and γ-gliadin and LMW and HMW glutenin (12–14).

In-gel digestion of proteins

The desired gel bands, isolated from a Coomassie stained gel, were digested with chymotrypsin by using the Proteineer DP digestion robot (Bruker, Bremen, Germany). The protocol supplied by the manufacturer was followed. Digested proteins were analyzed by mass spectrometry as described previously (10).

RESULTS

Generation of monoclonal antibodies specific for immune-stimulatory gluten sequences

For the development of reagents that detect immune-stimulatory gluten peptides, mAbs were raised against synthetic peptides corresponding to T cell stimulatory sequences present in gliadin (Glia-α9, Glia-α20, and Glia-γ1), LMW glutenin (glt-156), and HMW glutenin (Table 1). The minimal amino acid sequences recognized by the mAb specific for the Glia-α9 and the Glia-γ1 epitopes were determined previously (13). With the use of a set of partially overlapping synthetic peptides, the minimal amino acid sequences recognized by the mAb specific for the Glia-α20 and LMW and HMW glutenin peptides were determined. Representative results are shown in Figure 1, whereas an overview of all results is given in Table 2. The results indicate that the sequence recognized by the mAb specific for the Glia-α9, Glia-α20, and HMW-glutenin peptides largely overlap with the T cell stimulatory sequences (Table 2). In contrast, the mAb specific for the Glia-γ1 and LMW glutenin epitopes recognize sequences that (partially) overlap with either the C- or N-terminus of the T cell epitopes (Table 2).

![Graph A](image1.png)

![Graph B](image2.png)

**FIGURE 1.** Minimal epitope sequences recognized by anti-Glia-α20 monoclonal antibody (mAb; A) and anti-HMW-glt mAb (B) raised against T cell stimulatory epitopes of gliadin and glutenin. The minimal epitopes recognized by the mAbs was determined in a peptide recognition assay by testing their reactivity against a set of overlapping peptides. OD, optical density.
Because many gluten proteins share a high degree of homology, we determined whether the mAbs reacted specifically with only the peptide used for immunization or whether they also detected the other T cell stimulatory sequences. The results (Figure 2, A, B, D, and E) indicated that the mAb specific for the Glia-α9, Glia-γ1, and LMW and HMW glutenin peptides are highly specific for the peptide used for immunization. In contrast, the Glia-α20–specific mAb also reacted with the Glia-α9 and Glia-γ1 peptides (Figure 2C). Whereas the reactivity of this mAb with the Glia-α9 peptide is likely based on the shared sequence PQXPY between the Glia-α20 and Glia-α9 peptides, the basis for the reactivity with the Glia-γ1 peptide is less clear.

**TABLE 2**
Comparison of minimal amino acid sequences recognized by gluten-specific monoclonal antibodies (mAbs) and known T cell stimulatory gluten sequences

<table>
<thead>
<tr>
<th>Epitope</th>
<th>T cell</th>
<th>mAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glia-α9</td>
<td>QLQPFPQPQLPY</td>
<td>QLQPFPQPQLPY</td>
</tr>
<tr>
<td>Glia-α20</td>
<td>PPQPQYPQPQPQ</td>
<td>PPQPQYPQPQPQ</td>
</tr>
<tr>
<td>Glia-γ1</td>
<td>QPQPQPQPQPQPQPQPQPQPQ</td>
<td>QPQPQPQPQPQPQPQPQPQPQ</td>
</tr>
<tr>
<td>LMW-glt</td>
<td>QPQPQPQPQPQPQPQPQPQPQ</td>
<td>QPQPQPQPQPQPQPQPQPQPQ</td>
</tr>
<tr>
<td>HMW-glt</td>
<td>QGQQGYPTSQSQS</td>
<td>QGQQGYPTSQSQS</td>
</tr>
</tbody>
</table>

The minimal amino acid sequence recognized by human T cells is underlined, and the mAb epitope is in boldface. Glia, gliadin; glt, glutenin; HMW, high molecular weight; LMW, low molecular weight.

**FIGURE 2.** Limited cross-reactivity of the various monoclonal antibodies (mAbs) for the T cell stimulatory epitopes. Cross-reactivity of the mAbs was determined in a direct binding experiment. BSA-coupled peptides encoding the T cell stimulatory epitopes were coated on an enzyme-linked immunosorbent assay plate, and the binding of the following mAbs to the indicated T cell epitopes was determined: anti-Glia-α9 (A), anti-Glia-γ1 (B), anti-Glia-α20 (C), anti-LMW-glt (D), and anti-HMW-glt mAb (E). Glia, gliadin; glt, glutenin; HMW, high molecular weight; OD, optical density.
Monoclonal antibodies and gluten-specific T cells recognize an overlapping set of natural gluten-derived homologues of the T cell stimulatory epitopes

In previous studies it was shown that human gluten-specific T cells can react to several homologous gluten peptides if they share a certain degree of sequence similarity (16). To compare the specificity of the human T cells with that of the gluten-specific mAb, natural variants of the Glia-α9, Glia-α20, Glia-γ1, and LMW and HMW glutenin epitopes were identified by database searches. Subsequently, the corresponding peptides were synthesized, and the reactivity of T cells and mAbs to these peptides was determined.

Eleven variants of the Glia-α9 T cell epitope (QLQPFPQPQLPYQPQP, the minimal 9 amino acid T cell epitope is underlined) that contain single or multiple substitutions were identified (17). In general, substitutions in the region flanking the minimal epitope affected T cell and mAb recognition in the same way (Figure 3A). Substitutions within the 9 amino acid core sequence at positions 3, 4, and 5 significantly reduced or even abolished both T cell and mAb recognition, whereas a substitution at position 8 affected T cell recognition only. The latter was due to the fact that position 8 is outside the antibody epitope (Table 2).

In total, 4 of the 12 peptides tested were detected by both T cells and mAbs, 3 by T cells only, 2 by mAbs only, and the remaining 3 by neither (Figure 3, A and B). Thus, the specificity of the T cells and the antibody partially overlap.

A similar analysis was performed for the other antibodies, a graphical representation of the results obtained is shown in Figure 3B. Of 21 variants of the Glia-α20 T cell epitope, 6 were recognized by T cells and mAb, 10 by T cells only, and 1 by mAb only (Figure 3B). Also, for the 8 variants of the LMW glutenin epitope and the 33 homologues of the HMW glutenin epitope, it was observed that a subset was detected by both T cells and mAbs (Figure 3B). In contrast, of the 6 variants of the Glia-γ1 epitope, only 1 was recognized by the T cells, whereas 2 others were recognized by mAbs only. Thus, with the exception of the Glia-γ1 mAb, all other mAbs reacted with multiple variants of the T cell epitopes.

Gluten-specific monoclonal antibodies recognize their epitopes in pepsin/trypsin digests of wheat flour

Next, we determined the reactivity of the mAb against a pepsin/trypsin digest of wheat flour. For this purpose the mAbs were covalently coupled to sepharose beads and incubated with a pepsin/trypsin digest of gluten. Subsequently, unbound material was removed by extensive washing, and the bound material was eluted with acid and analyzed by mass spectrometry. This approach was successful with the Glia-α9, Glia-α20, and LMW and HMW glutenin mAbs. In all cases the mass spectrometric analysis identified peptides that contained the minimal amino acid sequence for which these mAbs are specific. The 5 most abundant peptides identified for each mAb are shown in Table 3. These mAbs thus react with gluten peptides that are naturally formed during digestion in the gastrointestinal tract resulting from the activity of pepsin and trypsin.

Gluten-specific antibodies show the presence of T cell stimulatory epitopes in various gluten proteins

To study the suitability of the mAbs for the detection of gluten proteins, the mAbs were extracted with isopropanol and dithiothreitol from a wheat variety and separated on SDS-PAGE.
TABLE 3
Protein fragments precipitated by gluten-specific monoclonal antibodies (mAbs)

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Peptides precipitated</th>
<th>Antibody</th>
<th>Peptides precipitated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Glia-α9</td>
<td>QVQWPQQQQPSGVPQQPPQQPF PSQGVQVQPPQQPPQQPF QVQWPQQPPQQPF QVQWPQQPPQQPF</td>
<td>Anti-Glia-α20</td>
<td>RPQQPYPQQPQQY SQPQPFRQQYPQQPQY SQPQPFRQQYPQQPQY SQPQPFRQQYPQQPQY</td>
</tr>
<tr>
<td>Anti-HMW-glt</td>
<td>PISPQPPQGQSGQPQGYPTSL QISPQPPQGQSGQPQGYPT PSQGPQGQSGQPQGYPT</td>
<td>Anti-LMW-glt (1)</td>
<td>SQQPFRWWQQPFPSSQQQQPL SQQPFRWWQQPFPSSQQQQPL SQQPFRWWQQPFPSSQQQQPL SQQPFRWWQQPFPSSQQQQPL</td>
</tr>
</tbody>
</table>

\*The amino acid sequences of gluten peptides were detected by the gluten-specific mAbs in a pepsin/trypsin digest of gluten. The 5 most abundant peptides are shown. mAb epitopes are in boldface; variants of the epitopes are in italics. Glia, gliadin; glt, glutenin; HMW, high molecular weight; LMW, low molecular weight.

Subsequently, the proteins were either stained directly with Coomassie blue or, after transfer to a membrane, by the gluten-specific mAb in a Western blot analysis (Figure 4). To identify the proteins stained by the various mAbs in the Western blot analysis, gel slices were excised from the Coomassie blue-stained gel at positions corresponding to the mAb-stained bands. Subsequently, the proteins in these gel slices were isolated, digested with chymotrypsin, and analyzed by mass spectrometry. All chymotrypsin fragments isolated from the gel slice corresponding to the proteins stained by the HMW-specific mAb (Figure 4; lane 2, proteins 1 and 2) were found to be derived from HMW glutenin proteins. These fragments contained multiple copies of the minimal amino acid sequences recognized by both the mAb and T cells specific for HMW glutenin (data not shown). Similarly, both of the proteins recognized by the anti-LMW-1 mAb (Figure 4; lane 3, proteins 3 and 4) were identified as LMW glutenin proteins and contained the minimal amino acid sequences recognized by the mAb and the T cell (data not shown). The gel slices corresponding to the regions stained by the Glia-α9, Glia-α20, and Glia-γ1-specific mAb (Figure 4; lanes 4, 5, and 6; proteins 5-8) were found to contain α/β-gliadin proteins that contained both the Glia-α9 and Glia-α20 T cell and mAb epitopes (Figure 4; lanes 4, 5, and 6; proteins 5, 6, 7, and 8). In addition, a γ-gliadin protein (Figure 4; lanes 4, 5, and 6; protein 8) and an LMW glutenin protein that contained the LMW-1 mAb epitope (1) (Figure 4; lane 4, 5, and 6; proteins 5, 6, 7, and 8) were identified. Thus, the mAbs are useful for the detection of gluten proteins that harbor harmful peptides involved in CD.

Gluten-specific antibodies show the presence of gluten homologs in barley, rye triticale, and oat

Previously, we noted that barley, rye, and oat contain sequences that are highly similar to the T cell stimulatory sequences in gluten (Table 4) and that some of these sequences are recognized by gluten-specific T cells (15). We determined that gluten-specific mAbs likewise react with the homologous peptides from barley, rye, and oat. The Glia-α9 and Glia-α20 mAbs were found to primarily react with secalin- and hordein-derived peptides but not with those from avenins (Figure 5, A and B). In contrast, the Glia-γ1 mAb strongly reacted with avenin peptides and not, or hardly, with the hordein and secalin peptides (Figure 5C).

To demonstrate that the mAbs were capable of detecting hordeins, secalins, and avenins, protein extracts of 5 cereals (wheat, barley, oat, rye, and triticale) were prepared and separated by SDS-PAGE. Subsequently, the proteins were stained with the

![FIGURE 4. Detection of gluten proteins by Western blot analysis. Gluten proteins were extracted from flour of a wheat variety and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were either stained directly (lane 1) or after being blotted to a polyvinylidene difluoride membrane and visualized with the monoclonal antibody (mAb) specific for HMW-glt (lane 2), LMW-glt 1 (lane 3), Glia-γ1 (lane 4), Glia-α9 (lane 5), and Glia-α20 (lane 6). Proteins recognized by the antibodies (numbered and indicated by arrows) were excised from the directly stained gel and digested with chymotrypsin. After extraction, the identification of the isolated proteins was determined by a combination of tandem mass spectrometry/mass spectrometry and bioinformatics. Glia, gliadin; glt, glutenin; HMW, high molecular weight; LMW, low molecular weight; M, mass marker.](image-url)
TABLE 4
Gluten epitopes and the homologous peptide sequences found in hordein, secalin, and avenin

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gliadin α2/α9</td>
<td>PYLQLQQFPQPLPYPQLPYQFPQ</td>
</tr>
<tr>
<td>Secalin α2</td>
<td>QQPFPQPPQFPQPQ</td>
</tr>
<tr>
<td>Hordein α2</td>
<td>QQPFPQPPQFPQPQ</td>
</tr>
<tr>
<td>Gliadin α2/α9</td>
<td>PYLQLQQFPQPLPYPQLPYQFPQ</td>
</tr>
<tr>
<td>Secalin α9</td>
<td>PQQPFPQPPQFPQPQ</td>
</tr>
<tr>
<td>Hordein α9</td>
<td>PQQPFPQPPQFPQPQ</td>
</tr>
<tr>
<td>Avenin α9I</td>
<td>QYQPEPQEQFPVQ</td>
</tr>
<tr>
<td>Avenin α9II</td>
<td>QYQPEPQEQFPVQ</td>
</tr>
<tr>
<td>Gliadin α20</td>
<td>PQPFPQPPQFPQPQ</td>
</tr>
<tr>
<td>Hordein α20</td>
<td>QQFPFPQPPQFPQPQ</td>
</tr>
<tr>
<td>Gliadin γ1</td>
<td>PIPQFPQQSPQPQRPQPSLQ</td>
</tr>
<tr>
<td>Secalin γ1</td>
<td>QIPQFPQQSPQPQRPQPSLQ</td>
</tr>
<tr>
<td>Hordein γ2</td>
<td>QIPQFPQQSPQPQRPQPSLQ</td>
</tr>
<tr>
<td>Avenin γ2I</td>
<td>QIPQFPQQSPQPQRPQPSLQ</td>
</tr>
<tr>
<td>Avenin γ2II</td>
<td>QIPQFPQQSPQPQRPQPSLQ</td>
</tr>
</tbody>
</table>

1 Cereal storage protein sequences were obtained by database searches using the protein family as the keyword (15; see Table 1). T cell stimulatory sequences are underlined. Sequences recognized by monoclonal antibodies are shown in boldface.

mAb in a Western blot analysis (Figure 6, Table 5). A commercial gluten preparation was used as a positive control. As expected, the staining pattern of the gluten preparation and the wheat protein extract were very similar. In contrast, distinct staining patterns were observed for oat, rye, and barley, whereas the staining pattern of triticale—a hybrid containing both the wheat and the rye genome—closely matches the sum of the staining patterns of wheat and rye. The results (Figure 6, Table 5) indicate that the Glia-α9 mAb reacts with all cereals except oat. The HMW mAb only reacts with wheat and rye, and the LMW mAb reacts only with wheat. The reactivity of the HMW mAb with rye is presumably based on the presence of proteins in rye that have a high degree of homology with both the x- and y type of HMW glutenins of wheat (18). The staining patterns obtained with the Glia-α20 and Glia-γ1 mAbs are more complex, the latter having a high affinity for the oat proteins. The results also indicate that the mAb-staining patterns obtained are distinct for each cereal, which may be useful for the identification of the type of (contaminating) cereals present in (gluten-free) food products.

Gluten-specific antibodies do not react with milk proteins

Because the gluten-specific antibodies are used in assays in which milk is used as a blocking agent, we used a Western blot analysis to test whether the antibodies cross-react with proteins in milk as an additional control for the assay. Milk was diluted in water, and the proteins were precipitated with acetone. Subsequently, the proteins were separated on SDS-PAGE, transferred to blots, and stained with the antibodies against Glia-α9, Glia-α20, Glia-γ1, and LMW and HMW glutenin gluten epitopes. None of the antibodies reacted with proteins in milk, whereas gluten proteins were clearly detected (Figure 7).

Comparison of Ridascreen Gliadin kit and the homemade ELISA for the Glia-α9 epitope

A commercial test kit based on the R5 mAb specific for the sequence QQFPQ (Ridascreen Gliadin kit; R-Biopharm AG, Darmstadt, Germany) is now available. Because this sequence partially overlaps with that recognized by the mAb specific for the α-9 T cell epitope (QQFPQ), we compared the reactivity of the α-9-specific mAb with that of the R5 antibody. For this purpose we tested a number of samples with both our mAb against the α-9 peptide and with the commercially available R5-based test kit (Table 6). The gluten-free samples tested were negative in both assays (data not shown). Gluten-containing samples could easily be detected by both assays, although the actual values obtained varied. In general, the values obtained

FIGURE 5. Recognition of hordein (Hor), secalin (Sec), and avenin (Av) peptides by the following gliadin (Glia)-specific monoclonal antibodies (mAbs): anti-Glia-α9 mAb (A), anti-Glia-α20 mAb (B), and anti-Glia-γ1 mAb (C). Gluten peptides and homologous peptide sequences in Hor, Sec, and Av identified by database screening (15) were tested for recognition in a direct binding assay by the gluten-specific mAbs. OD, optical density.
molecules in disease development. Recent work has shown the basis for this association: HLA-DQ2 and HLA-DQ8 can bind gluten-derived peptides and present these to inflammatory T cells that are present in the small intestine of CD patients, which leads to disease (19–21). Consequently, the gluten-derived peptides that can bind to HLA-DQ2 or HLA-DQ8 and stimulate T cells are the true culprits and should not be present in food intended for consumption by CD patients.

Importantly, such T cell stimulatory gluten peptides have now been identified in α- and γ-gliadin and LMW and HMW glutenin. Both gliadins and glutenins are multiprotein families with a high degree of sequence homology between different members. Therefore, variants of the identified T cell stimulatory peptides are known that can also elicit T cell responses (7). Moreover, peptides with similar T cell stimulatory properties have been identified in barley, rye, and oat as well (15). To guarantee the safety of food products intended for the gluten-free market, it would therefore be highly desirable to use tools that detect as many of the potentially harmful gluten and gluten-like fragments as possible.

With this in mind, mAbs were raised against non-deamidated forms of known T cell stimulatory peptides present in gluten. This will allow detection of native gluten peptides that on deamidation by tissue transglutaminase in the small intestine can trigger an immune response. We aimed at detecting both the α- and γ-gliadins and HMW and LMW glutenins. With these antibodies we can thus monitor the presence of 4 different classes of gluten proteins, which is a large improvement compared with the existing assays based on the R5 mAb that only recognizes gliadin proteins.

**TABLE 5**
Recognition of various cereals by gluten-specific monoclonal antibodies (mAbs)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glia-α9</th>
<th>Glia-α20</th>
<th>Glia-γ1</th>
<th>HMW-glt</th>
<th>LMW-glt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Rye</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Triticale</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oat</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Barley</td>
<td>+</td>
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<tr>
<td>Gluten preparation</td>
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<td>+</td>
</tr>
</tbody>
</table>

1 Reactivity of the mAbs against gluten and gluten-like proteins in wheat, barley, rye, triticale, and oat. Gluten preparation was used as a control. Glia, gliadin; glt, glutenin; LMW, low molecular weight; HMW, high molecular weight.
In the present study we provide a detailed analysis of the reactivity of these antibodies against gluten proteins, gluten peptides, and gluten-like proteins present in barley, rye, and oat. The minimal amino acid sequences detected by these mAb were determined and found to partly overlap with T cell stimulatory gluten sequences. Moreover, like gluten-specific T cells (13), the mAbs were found to react not only with the peptide against which they were raised but also with homologous gluten proteins and peptides present in wheat, barley, rye, and oat. In addition, the proteins recognized by the mAb did contain epitopes involved in CD. By Western blot analysis, these antibodies were found to give characteristic staining patterns depending of the cereal being analyzed, a property that may be useful for the identification of the type of cereal present in and/or contaminating particular food products.

In addition to its reactivity with gluten and gluten-like proteins and peptides, the mAb against the Glia-γ1 epitope also reacted strongly with oat—a finding that may be explained by the homology between the N-terminal sequence of the Glia-γ1 T cell epitope (ie, QQRPFI) and an amino acid sequence that is frequently present in the α- and γ-avenins (ie, QQQPFV). Similarly, oat protein can be recognized by Glia-γ1–specific T cells (13), although it should be indicated that the specificity of the mAbs and T cells overlap only minimally (Table 2).

In separate studies it was tested whether the antibodies react with proteins from teff, rice, and maize, cereals that are nontoxic for CD patients. In all instances we observed reactivity with wheat, rye, and/or barley only (22).

The Codex Alimentarius defines gluten-free foods as wheat starch–containing food products with a gluten content <200 ppm and naturally gluten-free food products with a gluten content <20 ppm. In a new draft for the standards used for gluten-free food products, the level of 100 ppm for food rendered gluten-free is recommended (ftp://ftp.fao.org/codex/alinorm07/al30_26e.pdf). The best known commercially available assays for the detection of gluten are sandwich ELISAs based on the R5 antibody, which is specific for a common sequence present in α-, β-, and γ gliadins (23) and on an antibody directed against a sequence in ω-gliadin (24). However, because these sequences do not match T cell stimulatory gluten peptides, these assays do not measure the presence of harmful fragments for CD patients. Moreover, these assays fail to detect the HMW and LMW glutenins, whereas there is mounting evidence that these proteins are harmful for CD patients as well (7, 8, 17, 25). With the availability of antibodies specific for α- and γ-gliadins as well as for LMW and HMW glutenins, assays can now be developed that overcome these problems. Moreover, because these antibodies do not only react with gluten proteins but with peptides as well, they are suitable for use in competition assays in which not only intact gluten proteins can be measured but also gluten peptides of sizes recognized by T cells. This is of particular interest because gluten hydrolysates are often used in the food industry and cannot be measured in sandwich ELISAs.

In conclusion, the antibodies described in this study allow a comprehensive screen for the presence of harmful gluten and gluten-like peptides and proteins in foods intended for consumption by CD patients with a level of detail that is as yet unprecedented. This will likely contribute to food safety and thereby improve the quality of life of CD patients.

TABLE 6
Comparison of the Ridascreen Gliadin kit with the Glia-α9 specific assay 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ridascreen gliadin</th>
<th>Glia-α9 specific assay ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>ppm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toast</td>
<td>1.52</td>
<td>8.08</td>
</tr>
<tr>
<td>Butter biscuits</td>
<td>20.67</td>
<td>27.19</td>
</tr>
<tr>
<td>Cake bakery</td>
<td>3.15</td>
<td>18.66</td>
</tr>
<tr>
<td>Twello’s quinoa flour</td>
<td>2.81</td>
<td>11.23</td>
</tr>
<tr>
<td>Couscous</td>
<td>—</td>
<td>349.9</td>
</tr>
</tbody>
</table>

1 Gluten-containing samples were tested with both the Ridascreen gliadin kit (R-Biopharm AG, Darmstadt, Germany), based on the R5 antibody, and the assay specific for the Glia-α9 epitope developed in our laboratory. Glia, gliadin.

2 LePoole, Twello, Netherlands.

3 De Wijn, Kaag ald Zaan, Netherlands.

4 Nestle, Amsterdam, Netherlands.

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The authors’ responsibilities were as follows—CM: performed experiments with the monoclonal antibodies; YK-W: tested the hybridoma-producing antibodies; PrV and AdR: performed the mass spectrometry measurements and analyzed the results; JWD: involved in the synthesis of the synthetic peptides; LD: obtained and tested the monoclonal antibodies and developed the monoclonal antibody–based assays; and CM, FK, and

FIGURE 7. No cross-reactivity of gluten-specific antibodies with milk proteins was observed. Two different amounts of a milk-water solution were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were either stained directly with Coomassie blue or after transfer to a polyvinylidene difluoride membrane with the mAb specific for HMW-glt, LMW-glt 1, Glia-γ1, Glia-α9, and Glia-α20. C, gluten preparation used as positive control; M, mass marker.
REFERENCES