Serologic Assay Based on Gliadin-Related Nonapeptides as a Highly Sensitive and Specific Diagnostic Aid in Celiac Disease

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Background: Celiac disease (CD) is induced by wheat gliadins and related cereal proteins. Anti-gliadin antibodies (AGAs) are present in the serum of CD patients, but these antibodies have lower diagnostic specificity and sensitivity than autoantibodies [anti-endomysium antibodies (AEmAs) and anti-tissue transglutaminase antibodies (AtTGAs)]. Recently, AGAs from CD patients were found to recognize deamidated gliadin peptides, probably formed by the action of tissue transglutaminase.

Methods: We synthesized several gliadin peptides and their glutamine-glutamic acid-substituted counterparts on cellulose membranes and tested their recognition by IgA in sera of 52 AEmA-positive CD patients and 76 AEmA-negative controls in a luminescence assay. For comparison, we assayed IgA concentrations of AGAs, AtTGAs, and AEmAs. For measurement of AtTGAs, we used the human recombinant antigen.

Results: We identified several nonapeptides that were detected with high specificity by IgA in CD patients. Diagnostic accuracy of the peptide antibody assay was highest when peptide PLQPEQPFP was used in combination with peptide PEQLPQFEE within one assay. AGAs were above the cutoff in 14 of the controls, but only 5 of the controls were positive for peptide antibodies. For comparison, 82% and 94% of samples were correctly classified by AGAs and the combination nonapeptide assay, respectively (P = 0.007), and the AtTGAs correctly classified 98%.

Conclusion: The peptide antibody assay has higher diagnostic accuracy than AGAs for distinguishing patients with CD from controls, and has diagnostic accuracy similar to that of AtTGAs.

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Patients with celiac disease (CD)7 are sensitive to gliadins from wheat and to related proteins (prolamins) from other cereals (rye, barley, and possibly oats) in their diet (1). After ingestion, these food proteins damage the mucosa of the small intestine (2), leading to malabsorption. The symptoms, however, are variable and, often, atypical. Patients must adhere strictly to a gluten-free diet to show clinical and histologic improvement. CD is a complex inflammatory disorder with strong (auto)immune features (3–5). Tissue transglutaminase (tTG) has been identified as an autoantigen (6). CD is characterized by the presence of antibodies against gliadin (AGAs) and of IgA autoantibodies against tTG (AtTGAs) (7). Autoreactivity against tTG is reflected mainly by typical immunofluorescence staining of extracellular connective tissue components by endomysium antibodies (AEmAs) (8). Both AGAs and autoantibodies represent important diagnostic markers for the disease. Whereas AGAs are also found in patients with other disorders, AEmAs and their ELISA correlate, AtTGAs, are considered highly specific and sensitive for CD (9).

The autoantigen tTG is a calcium-dependent enzyme that catalyzes an acyl transfer between the γ-carboxamide

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7 Nonstandard abbreviations: CD, celiac disease; tTG, tissue transglutaminase; AGA, anti-gliadin antibody; AtTGA, anti-tissue transglutaminase antibody; AEmA, anti-endomysium antibody; and TBS-T, Tris-buffered saline containing Tween 20.

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group of a polypeptide-bound glutamine and the ε-amino group of a polypeptide-bound lysine residue to form an isopeptide bond (10, 11). Glutamine-rich gliadins have been shown to be good substrates for the enzyme, which can be cross-linked to tTG itself (12). In the absence of primary amines, tTG can also deamidate glutamine residues, leading to the formation of glutamic acid residues in gliadin (13–16). This deamidation reaction improves binding of gliadin peptides to MHC class II molecules and is important for subsequent stimulation of gliadin-specific T cells (17, 18). Furthermore, deamidation of gliadin increases binding of AGAs in the serum of CD patients, but not control individuals, to gliadin (19, 20). The tripeptide PEQ was discovered to be the core epitope of gliadin autoantibodies.

Here we describe an assay for antibodies to nonapeptides that has a diagnostic accuracy considerable higher than that of AGAs and close to that of autoantibodies.

**Patients and Methods**

**HUMAN SERA**

We tested 52 sera from biopsy-confirmed celiac patients ([30 children (age range, 1.5–16 years), including 16 females and 14 males; and 22 adults (age range, 22–66 years), including 17 females and 5 males], all positive for IgA-AEmAs (titer, 1:5–1:5120), with enhanced IgA antibody concentrations of AtTGAs [79.1–1431 kilounits/L (mean, 769 kilounits/L), with three sera below the cutoff value of 186 kilounits/L] and with increased IgA-AGAs (10.4–836 kilounits/L; mean, 193 kilounits/L) for reactivity with peptides. For controls, we tested 76 AEmA-negative sera [16 children (1.5–14 years), including 7 females and 9 males; and 60 adults (19–61 years), including 18 females, 42 males] with AtTGA concentrations below the cutoff value of 186 kilounits/L (5.6–176 kilounits/L; mean, 53.0 kilounits/L). Controls comprised 42 blood donors, 23 patients with inflammatory bowel disease, 1 patient with diabetes mellitus, 1 patient with fructose malabsorption, 1 patient receiving antirheumatic medication, 7 patients in whom CD was excluded biotically, and 1 CD patient on a strict gluten-free diet. Control sera were selected for high AGA titers (6.8–665 kilounits/L; mean, 52.2 kilounits/L). AGAs in 14 of the controls were above the cutoff (see below). Patient and control sera were collected between 1994 and 2004 in the University Hospital and the Department of Paediatrics of St. George’s Hospital of Leipzig, Children’s Hospital of the University of Tübingen, the Institute of Immunology of the Technical University of Dresden, and in the Department of Paediatrics of the University of Münster, Germany. Every individual was included only once in the study.

**ASSAY OF ANTIBODIES**

IgA-AGAs were determined by enzyme immunoassay in microwells coated with gliadin as described previously (21). A titer >50 kilounits/L was considered as positive. IgA class AEmAs were assayed by immunofluorescence on monkey esophagus sections (Virimmun) (22). IgA AtTGAs were assayed by ELISA using human recombinant tTG as antigen, as described previously (23). Antibodies detecting synthetic peptides were estimated as described below in samples with known concentrations of AEmAs, AtTGAs, and AGAs. The different antibody species were assayed nonblind in the same serum sample.

**PEPTIDES**

The peptides on cellulose membranes were prepared by automated spot synthesis (24), as described in detail previously (25). The peptides were covalently bound to a cellulose membrane (Abimed) through their COOH termini. The peptides were N-terminally acetylated. The one-letter code for amino acids was used. All 51 nonapeptides from α/β-gliadin precursor (accession number C22364) (26), γ-gliadin precursor (accession number P21292) (27), and w-secalin (28) carrying PQQ as a central tripeptide core and their 51 PEQ-containing counterparts were synthesized. Additional nona- and dodecapeptides and one octadecapeptide as fragments of the above mentioned prolamins and differently Q→E-substituted variants were synthesized as described below.

**BINDING ASSAY**

After being washed in methanol and Tris-buffered saline with Tween (TBS-T; 137 mmol/L NaCl, 2.7 mmol/L KCl, 50.4 mmol/L Tris, 0.5 mL/L Tween 20, pH 8.0), the membranes were blocked (TBS-T containing 50 g/L sucrose and 25 g/L skim milk powder), washed in TBS-T, and incubated in human serum in blocking solution (1:200). After further washing steps, membranes were incubated with anti-human IgA conjugated with peroxidase (code P216, diluted 1:1000 in blocking solution; Dako). After washing, luminescence was measured with the Supersignal CL-HPR reagent set, according to the manufacturer’s instructions (code 34080; Pierce) in a ChemilImager (Alpha Innotech Corp.). The binding score was assessed quantitatively by means of Alpha Ease software (Alpha Innotech). A luminescence score of 170 units was used as the cutoff value. This cutoff was chosen to obtain similar sensitivity and specificity values for the assay.

When two different peptides were used in combination to test for reactivity with human IgA, both peptides were spotted individually at different sites on the membrane, and the test was considered positive if there was a signal above the cutoff value for one or for both of the peptides.
STATISTICS
For calculation of results, all data were considered and no outliers were removed. The χ² test was applied to compare the proportions of observations in different categories in a contingency table. The proportions were considered significantly different if P was < 0.05. The z-test was applied to calculate 95% confidence intervals for differences in proportions.

Results
We compared binding of human IgA antibodies from 41 sera of EmA-positive CD patients to 51 nonapeptides bearing a central PQQ motif with binding to their 51 PEQ-containing counterparts. The number of sera that bound was increased when PQQ was substituted by PEQ in all except one case. The difference between binding to the substituted and the native peptide was statistically significant for 22 of the peptides (peptide group 1). The remaining 29 peptides for which no difference was found formed group 2 (Table 1). A common feature of the PEQ-containing peptides of group 1 was a C-terminal PFP formed group 2 (Table 1). A common feature of the remaining 29 peptides for which no difference was found was the substituted and the native peptide was statistically significant in all except one case. The difference between binding to PEQ-containing counterparts. The number of sera that reacted with the above-mentioned nonapeptides. These seven sera were from two blood donors, four patients with chronic inflammatory bowel disease, and one patient in whom CD was excluded bioptically. All seven were negative for AEmAs and ATTGAs, but three of them had increased AGAs.

To increase the sensitivity for CD antibodies, we searched for other peptides. The PEQ motif occurs in the native unmodified sequence of gliadin. We investigated the peptides WQPEQSQRC, PQRPEQQFP, and AQQPEQ-QIS, but these peptides were recognized by only a small fraction of the sera from CD patients. The CD sera that were not reactive with the QPEQPP-containing peptides did not bind these additional peptides.

To test whether the reactivity of antibodies can be enhanced by increasing the length of the peptides, we investigated Q→E-substituted octadecapeptides from γ-type gliadin (LPFPQOPQFOPQFPQ; positions 86–103, which contains three PQQ motifs; Table 3). In the singly substituted forms, only the introduction of glutamic acid at positions 8 and 13 significantly increased binding of AEmA-positive patient IgA. Substitution in position 13 gives rise to the PEP sequence. The triple-substituted peptide was recognized by the highest number of patient sera. Only a small number of control sera bound to the amidated 18mer and its differently substituted variants. Estimation of antibodies with the triple-substituted 18mer had a diagnostic sensitivity of 85.0% and a diagnostic specificity of 95.0%.

Table 1. Amino acids predominating in different positions of two groups of PEQ-containing nonapeptides. a

<table>
<thead>
<tr>
<th>Peptide group</th>
<th>Position in nonapeptide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>50% P a</td>
</tr>
<tr>
<td>2</td>
<td>41% Q a</td>
</tr>
</tbody>
</table>

a Only amino acids with frequency >30% at the respective positions are shown.

b The 22 peptides in group 1 were detected by significantly more sera than their PQQ counterparts, whereas this difference is not significant for the 29 peptides in group 2. Forty-one sera from CD patients were tested.

c The frequencies of the amino acids indicated are different between the two peptide groups (P < 0.05).

Table 2. Reactivity of IgA in AEmA-positive CD sera and in sera from controls with different nonapeptides.

<table>
<thead>
<tr>
<th>Antibodies against</th>
<th>CD patients (n = 52)</th>
<th>Controls (n = 76)</th>
<th>Diagnostic sensitivity, %</th>
<th>Diagnostic specificity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSQPEQPPFP</td>
<td>47</td>
<td>6</td>
<td>90</td>
<td>92</td>
</tr>
<tr>
<td>SQQPEQPPFP</td>
<td>43</td>
<td>7</td>
<td>83</td>
<td>93</td>
</tr>
<tr>
<td>LPQPEQPPFP</td>
<td>45</td>
<td>4</td>
<td>83</td>
<td>96</td>
</tr>
<tr>
<td>LPQPEQPPFP</td>
<td>47</td>
<td>5</td>
<td>90</td>
<td>93</td>
</tr>
<tr>
<td>TQQPEQPPFP</td>
<td>45</td>
<td>4</td>
<td>86</td>
<td>95</td>
</tr>
</tbody>
</table>

Table 3. Reactivity of human IgA with an octadecapeptide from γ-type gliadin and different Q→E-substituted forms.

<table>
<thead>
<tr>
<th>Antibodies against a</th>
<th>40 CD patients</th>
<th>44 controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPFPQOPQFOPQFPQ</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>LPFPQOPQFOPQFPQ</td>
<td>27</td>
<td>2</td>
</tr>
<tr>
<td>LPFPQOPQFOPQFPQ</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>LPFPQOPQFOPQFPQ</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>LPFPQOPQFOPQFPQ</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>LPFPQOPQFOPQFPQ</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>LPFPQOPQFOPQFPQ</td>
<td>23</td>
<td>1</td>
</tr>
<tr>
<td>LPFPQOPQFOPQFPQ</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>LPFPQOPQFOPQFPQ</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>LPFPQOPQFOPQFPQ</td>
<td>33</td>
<td>2</td>
</tr>
<tr>
<td>LPFPQOPQFOPQFPQ</td>
<td>28</td>
<td>2</td>
</tr>
<tr>
<td>LPFPQOPQFOPQFPQ</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>LPFPQOPQFOPQFPQ</td>
<td>34</td>
<td>2</td>
</tr>
</tbody>
</table>

a Bold "E" indicates a Q→E substitution.

b P < 0.05 compared with native peptide.
The high reactivity of CD sera with the PEP sequence in the 18mer led us to test dodecapeptides from $\alpha$- and $\gamma$-type gliadin and $\omega$-secalin containing PQP and the Q$\rightarrow$E-substituted forms, but fewer than one-half of the CD sera were reactive. The CD sera unreactive with the QPEQPFP-containing nonapeptides did not bind.

According to our previous findings (19), the sequences QQQPFP, WQIPEQ, and PQQLPQ may also be targets of human AGAs. The hexapeptide QQQPFP occurs twice in $\alpha$-type gliadin as the sequences QWPQQQPFP and FQGQQQPFP. We examined single, double, and triple Q$\rightarrow$E substitutions within the QQ tripeptide, but none of the substituted forms appeared to be a good epitope for CD antibodies. We investigated the WQIEQ motif as part of the nonapeptide QLWQIEQ. This peptide was recognized by a large fraction of CD sera, which may be attributable to the fact that it contains primarily the PEQ stretch. There was an increase (although statistically not significant) in reactive sera when the Q$\rightarrow$E-substituted peptide QLWEIEQ was used. We observed a large and statistically significant enhancement of binding of IgA from celiac patients when we changed the PQQLPQ sequence (as part the peptides PQQQLPQF and FQQQLLPQF) to PEQLPQ but not when we changed it to PQELPQ.

Two of the five sera that were not reactive with the six QPEQPFP-containing nonapeptides (Table 2) gave a positive signal with PEQLPQFEE. None of the 76 control sera were positive with this peptide. Thus, use of peptide PLQPEQPFP in combination with PEQLPQFEE gave a diagnostic accuracy of 93.8%. When we used PEQLPQFEE in combination with another one of the QPEQPFP-containing nonapeptides, the diagnostic accuracy was lower (Table 4). For comparison, also shown in Table 4 are data on AEmAs, ATGAs, and AGAs in CD patients and controls. In five of the six cases, the diagnostic accuracy of the peptide assay was significantly higher than that of AGAs and came close to that of autoantibodies.

The reactivities of the individual sera with peptides PLQPEQPFP and PEQLPQFEE are shown in Fig. 1.

**Discussion**

Antibodies against gliadin are usually considered as much less specific and much less sensitive for CD than autoantibodies (AEmAs and ATGAs) (29). Increased AGA titers can be found in a variety of other conditions not related to CD, e.g., inflammatory bowel disease (30), IgA nephropathy (31), HIV infections (32), neurologic disorders (33, 34), and rheumatoid arthritis (35) as well as in some apparently healthy individuals. With the discovery of tTG as an autoantigen of CD and its availability as a recombinant human protein for ELISA, the role of AGAs in diagnosis of CD has been decreased. However, the specificity of AGAs in CD differs from that for other diseases. In CD, gliadin entering the intestinal mucosa is modified (deamidated) by tTG. The modified antigens stimulate B lymphocytes to produce antibodies against the deamidated peptides (19). We wanted to examine whether deamidated short gliadin-homologous peptides are more specifically detected by CD antibodies than native whole gliadin and whether such peptides may be suited for diagnosis of CD. The tripeptide motif PEQ has been shown to be an important structural requirement for binding of antibodies in CD patients (19) and was the starting point of our investigations.

The finding that the majority of CD patients can be detected with only a few short peptides suggests that the epitope repertoire is restricted in gliadin. This seems to be mainly attributable to the monotone amino acid composition and to the repetitive nature of the prolams and is favorable for diagnostic purposes. In the sequences of the three prolams tested ($\alpha$- and $\gamma$-type gliadin and $\omega$-secalin...
Fig. 1. Reactivity of human IgA with peptides PLQPEQPFP and PEQLPQFEE.

Shown on the right is the reactivity of sera from 52 AEmA-positive CD patients with peptide PLQPEQPFP (⊙). Five sera were unreactive with PLQPEQPFP; three of them are shown on the x axis. The other two sera were reactive with peptide PEQLPQFEE (∆). Shown on the left is the reactivity of 76 control sera with peptides PLQPEQPFP and PEQLPQFEE. The large gray circle on the x axis indicates that 67 sera were reactive with none of the peptides. Five of the controls were positive with peptide PLQPEQPFP. Dotted horizontal line, cutoff.

lin), 37% of amino acids are represented by Q and 22% by P. Consequently, tripeptide motifs consisting of two Q residues and one P residue (PQQ, QPQ, and QQP) are the most numerous, and each occurs more than 50 times in the three species of prolamins. Of these, the sequence PQQ is most abundant (66 times). If the sequence PQQ is extended one position in the direction to the NH2 terminus and three positions in the direction to the COOH terminus, then the most often occurring heptapeptide is QPQQPFP (13 times). This building block is contained in γ-type gliadin and ω-secalin but not in α-type gliadin. As is known from investigations on the substrate specificity of tTG (15), this enzyme prefers a Q residue in gliadin for deamidation if it is followed two positions in the direction to the COOH terminus by a P and then by an F. However, a P immediately following the Q residue was found to inhibit deamidation. The sequence QXPF occurs 29 times in the three prolamins investigated, 25 times as QQPF. Thus, after deamidation, the EQPF motif should be formed. This pattern exactly matches the core of the QPEQPF heptapeptide detected by the antibodies from the majority of CD patients. Thus, the epitope specificities of AGAs in CD patients reflect the substrate specificity of tTG.

Interestingly, the celiac AGAs specifically detect only PEQ peptides from γ-type gliadin and ω-secalin but not from α-type gliadin. In α-type gliadin, five nonapeptides contain PEQ: in three cases followed by PYP, and in one of the three preceded by Q. Nevertheless, reactivity with the antibodies is very weak. This suggests that substitution of F by Y in the PFP motif decreases the deamidating activity of tTG.

Of the 51 Q→E-substituted nonapeptides, FSQPEQPFP, SQPPEQPFP, PQPPEQPFP, PLQPEQPFP, PIQPEQPFP, and TQPPEQPFP were recognized by the highest number of celiac sera. We examined whether longer peptides containing the QPEQPFP sequence might be better binders of celiac antibodies, but none of the Q→E-substituted forms of the octadecapeptide LPFQQPQQPFPQPQQPQ from γ-type gliadin was a better epitope. This may suggest that the number of amino acids forming the antibody binding site is restricted to a small number, possibly even less than nine.

Searching for peptides that might complement our assay, we found PEQLPQFEE. This peptide was recognized by only approximately one-half of the CD sera, but it detected two of the five patients not detected when the QPEQPF-containing peptides were used alone in the test. Thus, for diagnostic purposes, PEQLPQFEE should be used in combination with PLQPEQPFP within one assay, yielding a diagnostic accuracy of 93.8%, which is substantially higher than the diagnostic accuracy obtained with AGAs.

It should be kept in mind that a control group was selected that also included 14 patients with high AGA titers. Only 3 of the 14 AGA-positive controls were positive for peptide antibodies, which underscores the specificity of the test. The validity of the peptide antibody assay is lower but very close to that of autoantibodies. To date, no serologic test for diagnosis of CD has demonstrated 100% sensitivity and 100% specificity (29, 36, 37). The diagnostic accuracy of AEmAs was 97.6%, which is very similar to that reported recently for the same recombinant antigen (23). Concerning the high accuracy of the AEmA assay, it should be kept in mind that we have selected patients with biopsy-confirmed CD who were positive for AEmAs. Thus, the sensitivity of AEmAs was set in our work to 100% per definition.

In children younger than 2 years with CD, autoantibodies have been reported to frequently be absent (38, 39). Because the new peptide assay is not based on estimation of autoantibodies but on modified gliadin peptides, it might also be suitable for diagnosis of CD in small children.

Further variation of the amino acid sequence of peptides could improve the sensitivity and specificity of the peptide antibody test. For routine use, however, the technique must be simplified and transferred from a luminescence assay performed on cellulose membranes to a microplate format.

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References


