Identification of common epitopes on gliadin, enterocytes, and calreticulin recognised by antigliadin antibodies of patients with coeliac disease


Abstract
Background—Sera of patients with coeliac disease, containing IgA and IgG antigliadin antibodies (AGA) and various IgA autoantibodies, react with isolated enterocytes. AGA cross react with enterocyte antigens, one of which has been identified as calreticulin.

Aims—To characterise the antigenic structures of gliadin, enterocytes, and calreticulin recognised by AGA from patients with active coeliac disease.

Methods—AGA were isolated from sera of nine patients by affinity chromatography and tested by competitive ELISA using 40 α-gliadin synthetic dodecapeptides (A1–F6).

Results—Reactivity of gliadin with all purified AGA tested was inhibited by peptide A4 at the N-terminal region; by C2, C3, and D4 at the central region; and by F3 and F4 at the C-terminal region of the gliadin molecule. AGA cross reactivity with enterocytes was inhibited by peptides A4, D1–D4, and F6 and with calreticulin by peptides A4, D3, and D4. As dominant epitopes AGA of coeliac patients recognise similar structures corresponding to peptides A4, D3, D4, and F6 present on gliadin, enterocytes, and calreticulin. Substitution of glutamine in the A4 peptide by glutamic acid caused loss of inhibitory capacity. Shortening of peptide A4 at the N-terminal by three amino acids increased its inhibitory effect.

Conclusions—AGA of patients with coeliac disease react with similar structures on gliadin and potential autoantigens on enterocytes.

(Keywords: coeliac disease; antigliadin antibodies; enterocyte autoantigens; cross reactivity)

Coeliac disease is a chronic inflammatory disease of the gut occurring in genetically susceptible individuals after ingestion of gluten. It is characterised by a flattened mucosa, villous atrophy, and crypt hyperplasia in the small intestine; dietary avoidance of gluten leads to normalisation of morphological abnormalities.1-3

The presence of autoantibodies in sera of patients with coeliac disease, a strong genetic

major histocompatibility complex (MHC) class II (HLA-DQ2 and HLA-DQ8) linked susceptibility, association with other autoimmune diseases, and a local inflammatory response, suggest that coeliac disease has many similarities with autoimmune diseases.4-8 Sera of patients with coeliac disease contain high concentrations of antibodies to gliadin (ethanol soluble fraction of gluten) and IgA autoantibodies against reticulin, endomysium, and jejunum, which are used for disease screening.9-13 The molecular nature of the target structures of these autoantigens is being intensively studied. Tissue transglutaminase has recently been suggested to be an endomysial autoantigen.14-17

We have found that sera and media from cultured biopsy specimens of patients with coeliac disease also contain antibodies against isolated rat and human enterocytes.18 Furthermore, it has been observed that some monoclonal antibodies to gliadin, as well as isolated patients’ antigliadin antibodies (AGA), cross react with similar epitopes on rat and human enterocytes.19 Both types of AGA, monoclonal antibodies, and antibodies isolated from patients with coeliac disease recognise three main proteins in the lysate of rat or human enterocytes. One of these cross reacting molecules has been identified as calreticulin. The fact that the concentration of antibodies to enterocytes and calreticulin is significantly higher in sera of patients with active coeliac disease than in sera of patients on a gluten-free diet or in healthy controls suggests a possible role of these cross reactive antibodies in the pathogenesis of coeliac disease.20 21

The structural similarity of epitopes on calreticulin and gliadin was supported by previous results of inhibition experiments showing that two gliadin peptides inhibit the reactivity of anticalreticulin antibodies with calreticulin.20

The aims of this study were: (1) to analyse the structures on α-gliadin recognised by isolated AGA of patients with active coeliac disease; (2) to characterise the cross reactive epitopes on gliadin, isolated enterocytes, and human calreticulin by competitive enzyme linked immunosorbent assay (ELISA) using synthetic peptides of α-gliadin; and (3) to use

Abbreviations used in this paper: AGA, antigliadin antibodies; MHC, major histocompatibility complex; EMA, endomysial antibody.
modified and truncated peptides to analyse the binding specificity.

Material and methods
PATIENTS AND HEALTHY CONTROLS
Sera were obtained from nine patients with active coeliac disease (eight adults and one child) diagnosed on the basis of jejunal biopsy findings (according to ESPGAN criteria). Patients’ sera were routinely tested for endomysial antibody and found to be positive. The local ethics committee approved the study protocol.

SYNTHETIC PEPTIDES OF α-GLIADIN
Based on the amino acid sequence of α-gliadin, 40 synthetic dodecapeptides (A1–F5) which overlapped by six amino acids, and one decapetide (F6) were synthesised using the Fmoc/tBu protection strategy on a miniaturised synthesiser. All peptides were purified by gel filtration on Sephadex G15 (purity ranged from 62 to 95% according to HPLC). Peptides chosen for further screening were purified using HPLC and characterised by amino acid analysis and mass spectrometry. Positive ion MALDI (matrix assisted laser desorption ionisation) mass spectra were measured on a Bruker BIFLEX reflectron time of flight mass spectrometer (Bruker-Franzen, Bremen, Germany) equipped with a multiprobe inlet and a gridless delayed extraction ion source.

PURIFICATION OF ANTIBODIES FROM PATIENT SERA
Sera from nine coeliac patients, taken for diagnostic purposes, were used for AGA isolation by affinity chromatography using CNBr activated Sepharose 2B (Pharmacia, Sweden) coupled with α-gliadin (gift of Prof. Hekkens) solubilised in 0.3 M borate buffer, pH 8.8. In brief, patients’ sera were loaded onto an immunoadsorbent column and adsorbed molecules were subsequently eluted with 0.3 M borate buffer, pH 8.0; 0.036 M glycine-HCl buffer, pH 3.4 (fraction of antibodies with lower affinity—eluate 1); and 0.022 M glycine-HCl buffer, pH 2.4 (fraction of antibodies with higher affinity, firmly bound to immobilised gliadin—eluate 2). Eluted proteins were concentrated by ultrafiltration using Diaflo membranes (Amicon, Massachusetts, USA). Protein concentration was measured using an ultraviolet spectrophotometer (Pye, Unicam).

A similar approach was applied for isolation of anti-SSB/La (Sjögren Syndrome B/La) antibodies from sera of patients with systemic lupus erythematosus using SSB/La antigen purified from rabbit thymus.

ISOLATION OF RAT ENTEROCYTES
Enterocytes were isolated from 2–3 month old female Wistar rats essentially according to Raul et al. and Kolinská et al. Intestinal cells were isolated in sequential populations at 37°C with gentle shaking of inverted rat jejunum in phosphate buffered saline (PBS) with addition of 1.5 mM Na₂EDTA and 0.5 mM Dl-dithiothreitol; Ca²⁺ and Mg²⁺ were omitted.

ELISA
Screening of patients’ sera for AGA was performed by ELISA using crude gliadin (Sigma). The binding of isolated AGA to α-gliadin, rat enterocytes, and human calreticulin or isolated anti-SSB/La antibodies to purified SSB/La antigen was determined as follows: in brief, α-gliadin, human calreticulin, or SSB/La antigen or SSB/La antibodies were used at a final concentration of 5 µg/ml; isolated rat enterocytes were diluted in PBS to a final concentration of 5x10⁵ cells/ml. The antigens were applied to microtitre plates (Gama, a.s., České Budějovice), incubated overnight at 4°C, and fixed with glutaraldehyde. After blocking with 1% bovine serum albumin (BSA; SEVAC, Prague) and repeated washings (with PBS and PBS-0.1% Tween), 50 µl of isolated AGA (or anti-SSB/La antibodies) diluted in PBS containing 2% BSA (PBS-BSA) was added and incubated for one hour at room temperature. After further washings, plates were incubated

Figure 1 Typical elution profile of antibodies isolated from the sera of patients with coeliac disease by affinity chromatography on an α-gliadin-Sepharose 2B column. Elution was performed gradually with (a) 0.3 M borate buffer, pH 8.0; (b) 0.036 M glycine-HCl, 0.15 M NaCl buffer, pH 3.4 (eluate 1); and (c) 0.022 M glycine-HCl buffer, pH 2.4 (eluate 2).

Figure 2 Amino acid sequences of synthetic dodecapeptides, overlapping by six amino acids, derived from α-gliadin.

| A1 | VRFVPYPQLPQNP | D1 | VLGQSTYGQLLOE |
| A2 | GLQDNPSQGQP | D2 | YQLQLGELCQGQL |
| A3 | PSSQPQEOEVPL | D3 | LCCQLWQLIPEQ |
| A4 | QEYVPLQYQQQ | D4 | WQPEOSQCQAI |
| A5 | VQQDFLQGQQP | D5 | SQCAIHNYYVHA |
| A6 | LGQDFPFPQPQ | D6 | HNVVHAILHOD |
| A7 | FPPQOPYPPQPO | D7 | IILHQQOQKQOD |
| B1 | YPPDFPISPQ | E1 | KQDGQOPSSQS |
| B2 | FPPQPYLQLQP | E2 | PSSQSVFAQPQ |
| B3 | YLQLQFFPQPOL | E3 | FQDPQQDYPFG |
| B4 | FPQPLPYSPQO | E4 | QYPQGSSFRPS |
| B5 | PYSQPFFRPQO | E5 | GSFPRQSGQNPQ |
| B6 | PFPQDPPYPQO | E6 | QNOQPAGGQSVQ |
| B7 | YPPQPSQPOSQP | E7 | GQSVPQQLOPQ |
| C1 | PYOSQPQISO | F1 | QGLPQFEFIRNL |
| C2 | GQPISOQGIQO | F2 | EIERNLALOLTP |
| C3 | QILQQILQQLI | F3 | ALGTLPAACNYY |
| C4 | LGQNFRCMDVV | F4 | AMCNYYIFPYCT |
| C5 | FCMDVLPQGOHNI | F5 | 1PPYCTIAPFPG |
| C6 | LOQHNIAHGRSD | F6 | IAPFGIGFNT |
| C7 | AHPGRSQLQOGS |
for two hours with 50 µl of peroxidase labelled swine antihuman IgA or IgG (Px-SWAHu/IgA or Px-SWAHu/IgG, SEVAC, Prague) diluted 1/1000 in PBS-BSA. The enzyme reaction was developed by adding a solution containing H2O2 and 1,2-o-phenylenediamine and stopped by 2 M H2SO4. The optical density was read on a spectrophotometer at 492 nm (Titer-trek Multiscan MCC/340 Flow Laboratories, Irwine, Scotland, UK).

**PEPTIDE COMPETITIVE ASSAY**

For inhibition experiments, 50 µl of isolated AGA (or anti-SSB/La antibodies) at a concentration giving 50% of the maximal optical density (from 0.4 to 0.8) in a titration curve was mixed with 60 µl of gliadin peptide (1 mg/ml in PBS) determined from titration curves. After overnight incubation at 4°C the mixture was centrifuged and the supernatants were transferred into microtitration plates precoated with α-gliadin, calreticulin, enterocytes, or SSB/La antigen. The level of binding of isolated AGA was measured by ELISA run in duplicate or triplicate, as described above. Percentage of inhibition (I) was calculated by the formula:

\[ I = 100 - 100 \times \frac{O}{O} \]

where o corresponds to the optical density of the sample (incubated with peptide) and O to the optical density of the positive control (without peptide).

Evaluation of two independent experiments using two different antibodies by paired t test showed a highly significant degree of reproducibility of competitive ELISA (t=0.210, t=0.167; coefficient of variance (CV)=3–6%).

**STATISTICAL ANALYSIS**

Data of competitive ELISA were evaluated by cluster analysis and analysis of variance (BMDP statistical software, University of California). The reproducibility of independent experiments was tested by paired t test and the CV was determined.

**Results**

**ANALYSIS OF α-GLIADIN EPITOPES RECOGNISED BY ANTIBODIES FROM PATIENTS WITH COELIAC DISEASE**

To identify the epitopes recognised by AGA from patients with coeliac disease on the α-gliadin molecule, antibodies were isolated from the sera of nine patients with active disease (A–I) by affinity chromatography; fig 1 shows an example elution curve. The set of 40 synthetic dodecapeptides A1–F5 and one decapeptide F6 covering the amino acid sequence of α-gliadin (except for the poly-Q region, amino acids 97–112) were synthesised (see fig 2).

Initially, synthetic peptides derived from gliadin were used in competitive ELISA to inhibit the reaction of AGA isolated from patients with coeliac disease, with α-gliadin.

![Figure 3](http://example.com/fig3.jpg)

**Figure 3** Inhibitory effect of 40 α-gliadin derived synthetic dodecapeptides and one decapeptide on the binding of antigliadin antibodies isolated from sera of one patient (E/2) with α-gliadin (A) and isolated enterocytes (B). Results are expressed as mean values of two or three measurements.

![Figure 4](http://example.com/fig4.jpg)

**Figure 4** Inhibition of binding of antigliadin antibodies isolated from patients with coeliac disease, to gliadin by gliadin peptides estimated by competitive ELISA (%). Antibodies (A–I) with lower (1) or higher (2) binding affinity were isolated from nine patients with coeliac disease. Gliadin peptides (A1–F6) are numbered from the N-terminus of the molecule.
For this reason antibodies were diluted according to titration curves to 50% of maximum binding. A representative experiment (fig 3A) shows the inhibition effect of the whole set of synthetic peptides on the binding of gliadin with AGA.

Figure 4 summarises results obtained by the analysis of six different antibodies isolated from patients with coeliac disease. The binding of all patient antibodies tested was inhibited by A4, C2, C3, D4, F3, and F4 dodecapeptides. Figure 5 shows the location of these inhibitory peptides on the α-gliadin molecule. The competitive effect of other peptides such as A5, B1, C4, C7, D2, D3, D5, D6, F2, F5, or F6 was determined only in the case of some patients’ AGA.

**Identification of cross reactive epitopes on gliadin, enterocytes, and calreticulin**

The same set of 40 synthetic dodecapeptides of gliadin (fig 2) was used to determine the cross reactive epitopes of autoantigens on enterocytes. The capacity of peptides to inhibit the reaction of patients’ AGA with isolated enterocytes is shown in fig 3B and summarised in fig 6. The dodecapeptides A4, D1–D4, and F6 competed with the binding of patients’ AGA with enterocytes.

Cluster analysis divided the peptides into two clusters. One cluster included peptides A4, C2, C3, D1–D4, F2, and F6; the second cluster covered all other peptides. The analysis of variance performed for each of 10 antibodies showed highly significant differences (p<0.001) between the two clusters of peptides; the only exception was when antibody E/2 was analysed.

The synthetic peptides, efficient at inhibiting antibody reactivity with gliadin and enterocytes, were also tested in competitive assays using calreticulin as the target antigen (fig 7). The reactivity of AGA tested with calreticulin was diminished mainly by A4, D3, and D4.

The specificity of inhibition by gliadin peptides A3, A4, C2, C3, and D2–D4 was confirmed in an unrelated system (binding of isolated anti-SSB/La antibodies and purified SSB/La antigen). Neither of the peptides...
enterocytes were used as target structures, specific epitopes. In experiments in which isolated AGA and may correspond to individually specified in most patients with coeliac disease. We have shown by competitive ELISA that AGA react with monoclonal anti-gliadin antibodies isolated from three patients with coeliac disease. The results of one representative experiment (antibody E2) are shown. Plates coated with gliadin (full columns) and enterocytes (empty columns) were used in a competitive ELISA. The data are expressed as mean values of assays run in duplicate.

exhibited any inhibitory activity (in contrast to 70% inhibition by SSB/La antigen, data not shown).

ANALYSIS OF BINDING SPECIFICITY
The significance of the amino acid glutamine (Q) in the A4 peptide was studied by replacing Q with glutamic acid (E). No inhibitory effect was detected when this modified peptide was tested in competitive ELISA in reactions of isolated AGA, with either gliadin or enterocytes. Absence of inhibition was also observed when a poly-E dodecapeptide was used. Conversely, the reactivity was notably reduced when poly-Q octapeptide was tested (fig 8).

To identify the amino acid residues critical for binding, shorter peptides were synthesised by reducing the length of the A4 peptide from the N-terminus of the molecule. The most efficient in inhibition assays with the selected three AGAs tested was the nine amino acid peptide, VPLVQQQOF (fig 8).

Discussion
In the present study we attempted to map the immunogenic regions of α-gliadin and to identify similar structures on enterocytes and calreticulin recognised by AGA isolated from patients with coeliac disease. We have shown by competitive ELISA that AGA react with peptide A4 localised at the N-terminal region, C2, C3 and D4 in the central region, and F3 and F4 at the C-terminal region of the gliadin molecule. These peptides may represent common gliadin epitopes recognised by antibodies present in most patients with coeliac disease. Peptides A5, B1, C4, C7, D2, D3, D5, D6, F2, F5, or F6 inhibited the binding of only some AGA and may correspond to individually specific epitopes. In experiments in which isolated enterocytes were used as target structures, peptides A4, D1–D4, and F6 significantly inhibited the cross-reactivity of all patients’ AGA. The inhibitory effect of peptides in all competitive assays was stronger and the spectrum of inhibitory peptides was broader when higher affinity antibodies were tested. From the set of peptides efficient in inhibition assays performed with gliadin and enterocytes only A4, D3, and D4 dodecapeptides significantly reduced the binding of AGA with isolated calreticulin. The peptides seem to correspond to similar epitopes recognised by AGA on gliadin as well as on isolated enterocytes and calreticulin. The presence of similar structures on gliadin and calreticulin is also supported by the fact that gliadin dodecapeptides A4 and D2 inhibit the interaction of calreticulin with rabbit anticalreticulin antibodies. Moreover, the A4 peptide revealed the amino acid sequence similarity with calreticulin.

Interestingly, we have found, using the Multiple Alignment Construction and Analysis Workbench (MACAW) program, sequence similarities between inhibitory gliadin peptides and the calcium binding, proline rich P domain of human calreticulin (peptides A4, D3, D4, and F6 correspond to respective amino acid sequences 262–270, 317–322, 236–242, and 310–316) (unpublished data).

The peptides from the N-terminal region of α-gliadin have been reported to affect mucosal structure in biopsy samples cultured in vitro. Furthermore, in vivo challenge with gliadin peptides led to morphometric variables of the jejunal mucosa and to changes in CD3+ intraepithelial lymphocyte numbers.

From analysis of T cell specificity to gliadin in coeliac patients, it has been shown that T cells from peripheral blood and intestinal mucosa recognise a peptide from the N-terminal region of α-gliadin presented by HLA-DQ2 or DR7 molecules.

Despite intensive efforts, the peptide specificity of T cells from patients with coeliac disease has not yet been completely determined. Interestingly, the A4 and A5 overlapping dodecapeptides efficient in our inhibition studies are nearly identical with the N-terminal nonadecapeptide 21–40 of α-gliadin, which was found to bind with the highest affinity to DQ2 molecules in both functional and biochemical assays.

These findings focused our attention on the A4 peptide from the N-terminal region of α-gliadin, which could play a pathogenic role in coeliac disease. To characterise more precisely the binding site of patients’ AGA we prepared N-terminal truncated modified A4 peptides. The most efficient inhibitor was a peptide three amino acids shorter than the original A4 peptide, consisting of the following nine amino acids: VPLVQQQOF. When the last amino acid V was removed, the ability to inhibit the binding of isolated AGA with both gliadin and enterocytes was dramatically reduced. The importance of amino acid Q in the A4 peptide was shown by the loss of the inhibitory capacity caused by substitution of Q with E, associated with a change in the peptide charge, and by the significant inhibitory effect of poly-Q
octapeptide when compared with poly-E dopecaptepe, which was completely ineffective.

In conclusion, we have identified similar immunogenic regions in an inducing external antigen, gliadin, and potential autoantigens. The presence of similar epitopes on gliadin, enterocytes, and calreticulin suggests that molecular mimicry may participate in the pathogenesis of coeliac disease. Moreover, we can speculate that the potential effect of autoantibodies can be blocked by most efficient inhibitory peptides.

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