IgA antibodies of coeliac disease patients recognise a dominant T cell epitope of A-gliadin

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Background: In coeliac disease (CD) patients, the dominant DQ2-A1-gliadin peptide recognised by CD4 T cells is contained within peptide sequence 57–73 (p57-73) of A-gliadin. This peptide sequence is also located within a 33-mer protease resistant gliadin fragment and therefore is likely to play an important role in the pathogenesis of CD.

Aims: Our aim was to determine whether a B cell epitope was present within the immunodominant T cell epitope of A-gliadin and, if so, to elucidate its sequence and determine the importance of deamidation and/or modification of the amino acid at position 65 for IgA binding.

Patients and methods: A cohort of CD patients, disease controls, and healthy individuals were examined. Serum IgA antibodies to the native and modified p57-73 fragment of A-gliadin were analysed using enzyme linked immunosorbent assays. Peptide scanning experiments were further used to elucidate the B cell epitope.

Results and conclusion: IgA antibodies to p57-73 were found in 29/72 (40.2%) endomysial antibody positive patients, all of whom had CD. The peptide antibody appeared to be present when patients were on a diet containing gluten and declined on a gluten free diet. The p57-73 antibody was very specific for CD (98%) and had a sensitivity of 56%. The amino acid at position 65 was not important for IgA binding but was crucial for T cell recognition of p57-73. Pentapeptide PXPQP emerges as a potentially strong candidate for the IgA binding motif in this region of A-gliadin. This study shows that a significant proportion of newly diagnosed CD patients have an antibody response to the immunodominant T cell epitope.

METHODS

Patient samples

A total of 192 serum samples were investigated for the presence of anti-p57-73 gliadin antibodies. Subjects studied were divided as follows: 72 CD (41 untreated CD, 25 patients on a successful gluten free diet (GFD), and six CD of unknown dietary status), disease controls (n = 70), assay controls (10 patients with rheumatoid factor, eight patients with IgA paraproteins, and eight patients with a raised polyclonal IgA greater than 5 g/l), and 24 healthy laboratory volunteers. Ethics approval for use of stored serum samples was obtained from the Central Oxfordshire Ethics Committee. All patients had previously been typed for HLA DQ antigens and were either HLA DQ2 or DQ8 positive.

Assay of EMA and gliadin antibodies

IgA endomysium antibodies (EMA) were determined after incubation of serum with primate oesophagus tissue (The Binding Site, UK) by indirect immunofluorescence. Serum IgA was measured using rate nephelometry (Beckman Coulter, UK). Samples with an IgA value of less than 0.1 g/l were excluded from the study. Total IgG and IgA gliadin antibodies were measured by enzyme linked immunosorbent assay (ELISA; Aesku Lab, Germany).

IgA anti-p57-73 A-gliadin ELISA

Maxisorp microtitre plates (Life Technologies, UK) were coated with 100 μl of peptide 57-73 per well at 5 μg/ml

Abbreviations: CD, coeliac disease; p57-73, A-gliadin peptide sequence 57-73; GFD, gluten free diet; EMA, endomysial antibody; RF, rheumatoid factor positive; PP, paraprotein; CV, coefficient of variation; ELISA, enzyme linked immunosorbent assay; TBS, Tris buffered saline; OD, optical density
(diluted in Tris buffered saline (TBS), pH 7.5). The 57-73 peptide, used in this study, is a common sequence found in various alpha-gliadins and is identical to the A-gliadin protein sequence (DNA accession No Q41545) described by Kasarda and colleagues16 and differing from gene derived sequences by substitution of S73 for Leu or Pro. The coated plates were incubated at 37°C for two hours before being left overnight for 16 hours at 4°C. The plates were washed four times with TBS (pH 8.0) with 0.01 mol/l EDTA plus 0.1% Tween 20 (TTBS) using a Dynex plate washer (Thermo Life Sciences, Basingstoke, UK) before being blocked with 100 l/well of 10% fetal calf serum in TTBS for one hour at room temperature. After a further wash step, serum samples were diluted 1/100 in TTBS and 100 l/well incubated at room temperature for one hour. Substrate (100 l of 1 mg/ml 3,3′-diaminobenzidine [DAB] in 0.1 mol/l sodium citrate buffer) was then added to the plates in duplicate for one hour at room temperature. After four washes, serum samples were diluted 1:100 in TTBS and 100 l/well incubated at 4°C for 16 hours. The assay was then carried out as described previously, except the antihuman IgA peroxidase conjugate could be used at a lower concentration of 1:4000 as the biotinylated peptide bound tightly to the plates. A mixture of peptide positive and negative samples were tested in duplicate on these plates. Each plate was repeated twice and mean results used.

**Statistics**

One way measures ANOVA and Spearman correlation were performed using GraphPad Prism version 3.0 for Windows (GraphPad Software, San Diego California, USA). Positive and negative predictive values of this assay for CD were calculated using 41 untreated newly diagnosed CD patients that were EMA positive and 103 EMA negative non-CD samples.

### RESULTS

**Proportion of IgA anti-p57-73 A-gliadin peptide antibodies from EMA positive and negative subjects**

Four different sera (two anti-p57-73 positive and two p57-73 negative) examined over 10 different ELISA assays were used to determine inter- and intra-assay coefficients of variation (CV) for the ELISA. Interassay CV varied from 9.3% to 15.9%, and intra-assay CV varied from 3.2% to 14.7%. To validate p57-73 specificity in this ELISA, direct inhibition studies using A-gliadin were carried out. Overnight incubation of p57-73 positive sera with three different concentrations of A-gliadin resulted in maximal 82% inhibition of p57-73 antibody binding in the ELISA (data not shown) suggesting good specificity for gliadin in this assay.

IgA anti-p57-73 gliadin peptide antibodies were found in 29 of 72 EMA positive samples and in two EMA negative samples (fig 1). The latter samples had an IgA paraprotein. The proportion of patients with untreated CD who had IgA anti-p57-73 gliadin peptides antibodies was 57% whereas...
only 14% of patients on a GFD had anti-p57-72 gliadin peptide antibodies. All patients on a GFD diet who were gliadin peptide positive had endomysium antibodies. IgA peptide 57-73 antibody levels and IgA gliadin antibody tities were found to correlate with a high degree of significance \( r = 0.70, p < 0.0001, n = 71 \), Spearman correlation, data not shown).

The sensitivity and specificity of the anti-p57-73 gliadin peptide antibody for the diagnosis of untreated CD were 56% and 98%, respectively. The positive predictive value was 92% and the negative predictive value was 86%.

**Time course of the disappearance of anti-p57-73 antibodies on a GFD**

For one patient, sera were available over a 26 month period from the start and maintenance on a GFD. Five samples spanning this period were tested for EMA and anti-p57-73 IgA antibodies. Patient A was strongly peptide and EMA positive at the point of diagnosis (table 1). Two months later, the patient had biopsy proven coeliac disease and was on a GFD. Successive samples monitoring the progress of the GFD showed that the anti-p57-73 antibody waned markedly from as early as two months post GFD and became almost negative. EMA took longer to become negative.

**Effect of a truncated peptide on anti-p57-73 gliadin antibody binding**

A shorter peptide (62-68), from the centre of peptide 57-73, was used in the ELISA to see whether this shortened sequence bound IgA antibodies. IgA antibodies \( n = 47 \) from the same subject binding to sequences p57-73 and p62-68 of A-gliadin gave equivalent OD values suggesting that the binding motif of 57-73 incorporates the peptide 62-68 (data not shown).

**Binding in the p57-73 A-gliadin ELISA with alternative amino acids at position 65**

The sequence of peptide 57-73 of A-gliadin recognised by CD4 T cells is deamidated at position 65, with a glutamine altered to glutamic acid (3,6). To ascertain whether the amino acid at position 65 is also important for IgA antibody binding, experiments with four different amino acids (proline, lysine, arginine, and glutamic acid) at position 65 (p65) of peptide 57-73 were carried out. Figure 2 shows ODs of 18 sera samples, all anti-p57-73 antibody positive, against the five peptides with different p65 amino acids. No significant difference between the p65 amino acid substituted peptides could be demonstrated \( p = 0.266, n = 18 \), one way ANOVA.)
Scanning the p57-73 region of α-gliadin to determine specific B cell epitopes

To identify the antibody binding region of the peptide 57-73, nine octapeptides, spanning the whole 57-73 sequence, were examined in the ELISA (fig 3). Any significant changes in binding should elucidate specific binding sequences and thus the B cell epitopes. Peptides were biotinylated to eliminate ELISA plate coating variability. An initial experiment ascertained that biotinylating peptides along 57-73 did not alter binding of anti-p57-73 IgA antibodies. The antibodies of sample binding and the cut off values remained the same for both un-biotinylated and biotinylated peptide data (not shown). Thirty nine samples (29 EMA positives and 10 EMA negatives) with a range of anti-peptide 57-73 antibody levels were tested against these nine peptides in ELISAs. Figure 4 shows binding to the nine biotinylated peptides for a range of representative samples with ODs less than 2. Figure 4 demonstrates that, in general, binding to different peptides appears uniform across the whole sequence, inferring a short common motif or a polyclonal response to the region.

DISCUSSION

In this study, we found that 57% of untreated patients with CD had IgA gliadin peptide antibodies against the immunodominant T cell epitope 57-73 (DQ2-A1-gliadin). IgA antibodies to this epitope are highly specific for untreated CD, unlike IgA antibodies to whole gliadin and to different epitopes of A-gliadin.16 The ELISA showed good positive (92%) and negative (86%) predictive values and was highly specific for CD (98%). The limited sensitivity of the anti-p57-73 gliadin peptide antibodies implies that it will not be useful as a diagnostic screening tool for CD. Serial serum samples obtained from a single patient showed that the p57-73 IgA antibody disappeared on a GFD. Further analysis is required to establish whether this assay has a role in monitoring compliance with a GFD.

Although T cells require deamidation of glutamine at position 65 to recognise the A2-gliadin 57-73 peptide sequence, it is unclear whether deamidation plays an important role in antibody recognition. Our data showing that deamidation of gliadin at position 65 does not influence IgA binding is in agreement with the findings of Aleanzi and colleagues15 who however showed that deamidation of glutamine within this immunodominant epitope did enhance IgG binding to this peptide in some patients. A number of groups have reported that deamidation of glutamine residues in y-gliadin promotes IgG and IgA gliadin peptide binding,15 suggesting that the role of this process in antibody recognition is complex and may vary for different gliadins and different antibody isotypes.

Substitutions of glutamine at position 65 by either positively or negatively charged amino acids did not have a statistically significant effect on IgA binding to the 57-73 gliadin peptide sequence, supporting the view that IgA binding is not markedly influenced by the presence of glutamine deamidation at position 65.

Peptide scanning experiments identified the hexapeptide QXPQP (X being P, Q, or L) shown previously15 to be a significant IgA and IgG epitope, as one potential epitope for IgA binding within p57-73 (fig 3). QXPQP occurs only at the beginning of p57-73 (fig 3) and the uniform binding observed across the whole p57-73 sequence infers another common motif or a polyclonal antibody response (fig 4) to this region of A-gliadin.

Both the 33-mer peptide10 and the shorter p57-73 peptide studied here exhibit highly repetitive sequences. The longer 33-mer has three repetitive amino acid motifs PQPQP (with X being either phenylalanine or tyrosine) repeated four times, LPYPQP repeated three times, and PQPQL repeated three times along its length. Of these, PQPQP is repeated twice within p57-73 whereas LPYPQP is present only once and PQPQL is not present (as the deamidated form of p57-73 was used in the scanning experiments). Figure 3 shows that 9/9 peptides had at least three of the five amino acids of PQPQP, 8/9 had four, and 6/9 had the complete motif, making PQPQP a potentially strong candidate as the IgA binding motif in this region of A-gliadin. It is possible that due to its repetitive nature within p57-73, this sequence was not elucidated by the scanning experiment as the motif fragments in peptides 5–7 (fig 3) may have been enough to sustain antibody binding at the maximal level.

Identification of T cell epitopes and the primary initiator peptide14 opens up the possibility of peptide immunotherapy.15–17 Whole A-gliadin has been administered intranasally to successfully inhibit gliadin specific T cell responses in a murine model of CD.14 The finding that a peptide, which contains an immunodominant T cell epitope, can elicit an antibody response raises the possibility that peptide immunotherapy could give rise to inappropriate immune responses, such as those described in recent trials involving therapeutic vaccination in patients with Alzheimer’s disease.17, 18 Our data show for the first time that antibody responses in patients with untreated CD are directed against the gliadin peptide fragment that contains the immunodominant T cell epitope. Finally, this work adds to the evidence that antibody responses against immunodominant T cell epitopes do occur in human autoimmune disease.19–21 The finding that this gliadin peptide fragment contains both B and T cell epitopes suggests that it plays an important role in the pathogenesis of coeliac disease as B and T cell interactions play a vital role in induction of immune responses.

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REFERENCES

EDITOR’S QUIZ: GI SNAPSHOT

An unusual cause of postoperative dyspnoea

Clinical presentation
A 79 year old gentleman underwent exploration of a dislocated hip arthroplasty. During surgery, he regurgitated bile stained fluid into his laryngeal mask airway. His chest remained clear, saturation did not fall, and chest radiography immediately postoperatively was unremarkable.

Twelve days postoperatively he developed respiratory distress associated with right sided pleuritic chest pain. Air entry was symmetrical and he was tachypnoeic (respiratory rate 26), hypotensive, and tachycardic (heart rate 140 beats/min). Electrocardiography revealed a right heart strain pattern. Oxygen saturation initially was 80%. Arterial blood gases on initial oxygen therapy and their subsequent improvement are given in table 1.

At medical review, chest radiography was interpreted as right lower zone shadowing and a preliminary diagnosis of postoperative pulmonary embolus was postulated, treatment with oxygen continued, and low molecular weight heparin was commenced.

An urgent computed tomography (CT) scan of the thorax was arranged and the scanogram revealed the true diagnosis.

Question
What does the CT scanogram show and what is the underlying cause?
See page 1286 for answer
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