Activation of Celiac Disease Immune System by Specific α-Gliadin Peptides

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ABSTRACT

Cereal Chem. 72(5):475–479

Two different gliadin molecules (designated α-gliadin and α/β-gliadin) were synthesized as 52 and 58 ten amino acid (aa) long overlapping peptides for the determination of their B-cell epitopes. Monoclonal antibodies and human serum pools revealed two epitopes common for both gliadins (peptide 14 aa:s 66–75 and peptides 34α aa:s 166–175, 36α/β aa:s 176–185) and two unique epitopes (α-gliadin peptides 48 aa:s 236–245 and α/β-gliadin peptide 52 aa:s 256–275). In addition, peptide 9 (QPYPQPQPFP) aa:s 41–50 and peptide 42 (LGQGSFRPSQ) aa:s 206–215 were detectable by monoclonal antibodies and serum pools from patients with untreated celiac disease but not by serum pools from disease control patients who had antigliadin antibodies. Patients

with celiac disease were also studied for their human leukocyte antigen (HLA) class II status (the presence of genetically determined proteins on antigen-presenting cells that are important for immunological recognition). Antigliadin antibody response to peptide QPYPQPQPFP was restricted by celiac disease (and HLA class II) because relative amounts of the antipeptide antibodies were significantly ($P < 0.05$) increased in celiac disease patients. The HLA alleles DQA1*0501 and DQB1*0201 are strongly associated with celiac disease. The difference between patients with celiac disease and healthy control subjects with regard to peptide QPYPQPQPFP suggest that this region in the gliadin molecule is of pathogenetic importance in celiac disease.

Celiac disease, or gluten-sensitive enteropathy, is provoked in genetically susceptible individuals by dietary exposure to wheat gluten and similar proteins in other closely related cereals (Trier 1991, Marsh 1992). The typical lesion in small intestinal epithelium is villous atrophy with crypt hyperplasia leading to malabsorption of most nutrients. Common symptoms that result from the malabsorption include diarrhea and wasting away in adults or a failure to thrive in children. Although symptoms are wide ranging, monosymptomatic or symptomless manifestations are also often present (Visakorpi and Mäki 1994). Removal of wheat, rye, barley, and oats from the diet results in histological and clinical recovery.

Most work has focused on the gliadin proteins and the peptides derived from them by digestion with pepsin and trypsin as being primarily responsible for initiating changes in susceptible individuals that ultimately lead to epithelial damage (Shewry et al 1992). All the different types of gliadins appear to be active (Howdle et al 1984). The mechanisms by which the gliadin-triggered immunological changes in the epithelium are initiated and lead to damage are not understood in any significant detail. At present, the most favored hypothesis involves interactions between human leucocyte antigen (HLA) class II peptide complex on the antigen-presenting cells and cell surface receptors of T-cell lymphocytes and other cells of the immune system (Marsh 1992). The most susceptible HLA class II DQ α/β heterodimer (DQ2) on antigen-presenting cells in celiac disease is encoded by the DQA1*0501 and DQB1*0201 alleles.

The presence of high levels of circulating antibodies to gliadins is a common (but not specific) feature of active celiac disease (Savilahti et al 1983). Whether or not there is a connection between the gliadin peptide sequences that stimulate production of circulating antibodies and the peptide sequences that initiate

the process leading to intestinal damage is not known. The most immunogenic stretches of amino acid sequences in gliadins are also not well characterized.

A few specific α-gliadin peptides have been studied both in vivo and in vitro (Karagiannis et al 1987, Mantzaris et al 1990, Sturgess et al 1994), but there have not been any systematic investigations published in which a series of peptides scanning through complete gliadin protein sequences have been investigated for the ability either to react with circulating antibodies or to produce cell-mediated responses that might lead to intestinal damage. In this investigation, we have evaluated the reactivity of linear B-cell epitopes (immunogenic peptide sequences) of an α-gliadin and an α/β-gliadin (sequence based on a cDNA clone that might correspond to either an α- or β-gliadin because they are structurally similar) by analyzing the reactivity with several mouse monoclonal antibodies (MAbs) and with serum pools from celiac patients of a series of 10-residue peptides that span the complete sequences in five-residue increments. We found several highly active peptides that react with circulating antibodies in human sera.

MATERIALS AND METHODS

Study Population

During 1990–1992, 98 children attending the Department of Pediatrics at the Tampere University Hospital for suspected celiac disease underwent small bowel biopsy. Venous blood samples were taken on admission at the time of their initial visit. Seventeen children showed severe jejunal mucosal damage (subtotal villous atrophy) with crypt hyperplasia consistent with celiac disease. They were treated with gluten-free diet and they fulfilled the ESPGAN criteria for celiac disease (Mäki et al 1989, Walker-Smith et al 1990). The other children who were suspected for celiac disease but had normal jejunal mucosal morphology were thus excluded for celiac disease (disease controls).

Serum Pool Study Groups

For the epitope scanning, patients were grouped by serum pools consisting of four individuals each. The first study group comprised three pools derived from 12 patients with untreated celiac disease. One of these pools consisted of patients with known HLA class II antigen (DQA1*0501 and DQB1*0201). The

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celiac disease patients in the other two pools of this study group were not tested for the DQA and DQB alleles. All these patients were positive for antigliadin IgG antibodies. The second study group comprised three pools derived from 12 patients who had antigliadin IgG antibodies but were excluded for celiac disease. Two of these pools consisted individuals negative for HLA DQA1*0501 and DQB1*0201. Individuals in one pool were not tested. The third study group was made up of one pool with four disease control patients who were negative for HLA DQA1*0501 and HLA DQB1*0201 and who had no antigliadin antibodies. An additional pool of three MAbs was also used in epitope scanning. MAb clone numbers 15E10-1, 16C4-6, and 2C11-12 were raised in mice against commercial gliadin (a mixture of α-, β-, γ-, and ω-gliadins) polypeptides (30–40 kDa) obtained by preparative gel electrophoresis (Vainio and Varjonen 1995). They were of IgG1 isotype except for 2C11-12 (not determined for isotype).

Individual Serum Samples

The antipeptide antibody levels were further determined for 17 patients with celiac disease (mean age 8.2 years, range 1.3–16.3 years) and 21 patients suspected for celiac disease but with normal jejunal morphology (disease controls) (mean age 7.6 years, range 0.4–13.8 years). The sera were from the diagnostic phase, and all the celiac disease patients and 19 disease control patients were positive for antigliadin antibodies (mean IgG level 82 and 84 EIU, respectively). The celiac disease patients were positive for HLA DQA1*0501 and DQB1*0201, and the disease

Fig. 1. Epitope scanning of α-gliadin (**A**) and α/β-gliadin (**B**) with monoclonal antibodies. Histogram of antibody reactivity (absorbance at 450 nm) to individual ten amino acid long peptides. ↓ = Reactive regions.

control patients were negative for HLA DQA1*0501 and DQB1*0201.

Determination of B-Cell Epitopes

Amino acid sequences of α- and α/β-gliadins were identified by Genetic Computer Group (GCG) sequence analysis software (University of Wisconsin, Madison, WI) on the basis of their open reading frames (ORF) available in the European Molecular Biology Laboratory (EMBL) gene bank. The α- and α/β-gliadins (clone A42) (Kasarda et al 1984, Okita et al 1985) containing 266 and 299 amino acids, respectively, were synthetized as 52 and 58 overlapping peptides by an epitope scanning kit (Cambridge Research Biochemicals Ltd, CRB, UK), which is described in detail elsewhere (Geysen et al 1987, Lehtinen et al 1990). Briefly, synthesis of 10 amino acid long peptides on polyethylene rods (≈20 ug of peptide per rod) was conducted by Fmoc-chemistry stepping five amino acids from peptide to peptide (Geysen et al 1987, Lehtinen et al 1990). The rods were assembled in a microtiter plate format to be used in enzyme-linked immunosorbent assay (ELISA) for the determination of B-cell epitopes in the gliadins.

For ELISA, the rods were saturated with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS, pH 7.2) for 1 hr at +37°C, after which different serum pools were added. Eight pools were used: 1) MAb; 2) disease control patients with no antigliadin antibodies; 3–5) disease control patients with antigliadin antibodies; and 6–8) celiac disease patients with antigliadin antibodies. The pooled sera were diluted 1:200 (for MAb, 1:100) in a buffer containing 1% BSA, 1% ovalbumin, 0.1% Tween 20 in PBS. They were incubated overnight at +4°C. Next, the plates were washed four times with PBS + 0.5% Tween 20 and once with distilled water. Antihuman (antimouse in the MAb analyses) IgG peroxidase conjugate (Dakopatts a/s, Glostrup, Denmark) was diluted 1:2,500 and allowed to react for 1 hr at +37°C. After a washing step, tetramethyl benzidine substrate (Kirkegaard & Perry Laboratories, Gaithesburg, MD) was applied; the reaction was stopped with 1N HCl after 30 min. Absorbance was measured at 450 nm (Multiscan photometer, Labsystems, Helsinki, Finland). The rods were tested and reused after a standardized ultrasonic disruption procedure with 0.1% mercaptoethanol in SDS at +60°C.

Computer Analyses

Search for amino acid homology was done by the FASTA program of GCG.

ELISA for Antipeptide Antibodies

Based on the epitope scanning, peptides 9 (QPYPQPQPFP SQQ-GGC) and 42 (LGQGSFRPSQQN-GGC) were chosen for further ELISA analyses. The peptides containing an extra –GGC spacer were made with a Zinsser Analytic SMPS 350 peptide synthetizer (Zinsser Analytics, Frankfurt, Germany) as described in Lähdeaho et al (1993b) and Paavonen et al (1994). Fmoc-protected amino acids were activated by HOBt (1-hydroxybenzotriazole, Sigma, St. Louis, MO) and N'N'-di-isopropylcarboimide (DIC, Sigma). Deprotection was performed with 25% piperidine (Applied Biosystems Api, Kelvin Close, Warrington, UK) in dimethylformamide (Api). The peptides were cleaved by a solid phase method according to King et al (1990) and checked for purity with HPLC analysis (System-Gold, Beckmann Instruments, Fullerton, CA). The IgG class antipeptide antibodies were determined by ELISA. Briefly, Nunc Macrosorb F-plates (Nunc InterMed a/s Århus, Denmark) were coated with 50 mg/ml of antigen in carbonate buffer (pH 9.5). After an overnight incubation, the plates were saturated for 1 hr at room temperature with 10% FCS in PBS. The serum samples and IgG peroxidase conjugate (Dakopatts a/s) were diluted 1:20 and 1:1000, respectively, in a dilution buffer containing 10% FCS + 0.05%
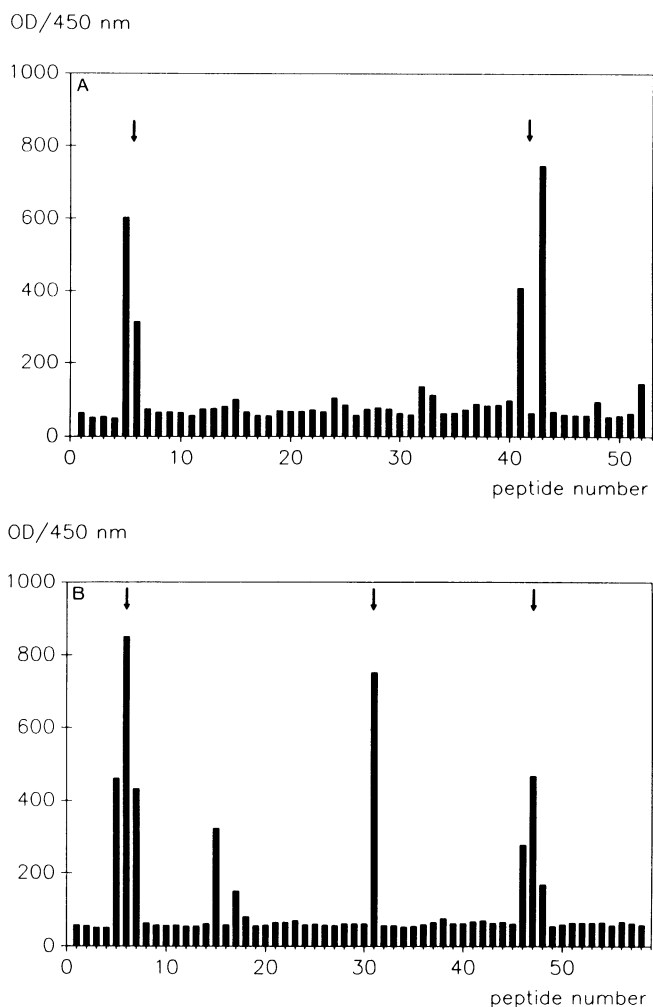
Tween 20 in PBS. Otherwise the ELISA was conducted as described previously (Lähdeaho et al 1993b).

Antigliadin Antibodies

The analyses of antigliadin antibodies against crude gliadin were conducted by ELISA as described (Vainio et al 1983, Lähdeaho et al 1993b) using a 1:20 serum dilution.

Statistics

The Mann-Whitney U-test was used for statistical comparisons.

RESULTS

Identification of Antigenic B-Cell Epitopes

Epitope scanning revealed two major reactive sites (peptides 5–6 and 41–43) in the α-gliadin and three reactive sites in the α/β-gliadin (peptides 5–7, 31, and 46–48) when the four MAbs were applied (Fig. 1).

The α-gliadin derived peptides (one letter code) 5–6 (^{21}QVPLVQQQQF^{30}, ^{26}QQQQFLGQQQ^{35}) and the α/β-gliadin derived peptides 5–6 (^{21}QVPLVQQQQF^{30}, ^{26}QQQQFPGQQQ^{35}) are equal except for one amino acid. On the other hand, the α-gliadin peptides 41–43 (^{201}LQQYPLGQGS^{210}, ^{206}LGQGSFRPSQ^{215}, ^{211}FRPSQQNPQA^{220}) and the corresponding α/β-gliadin peptides 46–48 (^{226}VSFQQPQQQY^{235}, ^{231}PQQQYPSSQV^{240}, ^{236}PSSQVSFQPS^{245}) showed no more than 50–60% identity, even when the frame shift was considered. Antigenic peptide 31 (^{151}QQSTYQLLQQ^{160}) was found only in the α/β-gliadin.

### Identification of Immunogenic B-Cell Epitopes

When the three different serum pools from patients with newly diagnosed, untreated celiac disease were applied for the epitope scanning, both the gliadins showed consistently similar, albeit wide, reactivity (Fig. 2). The reactive peptides 9, 14, 34, 42, and 48 in the α-gliadin, and corresponding peptides 9, 14, 36, and 52–54 in the α/β-gliadin were identified as the major B-cell epitopes based on parallell repeated analyses (Fig. 2). Peptides 9 (^{41}QPYPQPQPFP^{50}), 14a (^{66}LPYSQPQPFR^{75}) / 14 α/β (^{66}PFPPQL PYPQ^{75}), 34a (^{166}QSQCQAIHNV^{175}) / 36 α/β (^{176}QAIHNVAH AI^{185}), and 48a (^{236}RNLALQTLPA^{245}) / 52–54 α/β (^{256}QPQ QLPQFAEIRNLALQ TLP^{275}) were similar, or almost similar, in their reactivity and amino acid content, whereas the α-gliadin peptide 42 (^{206}LGQGSFRPSQ^{215}) did not have an immunogenic counterpart in the α/β-gliadin. No specific reactivity was found with antigliadin antibody negative human sera (data not shown). When antibody reactivity in the disease controls with antigliadin antibodies was considered, peptides 14, 35/36, 48, and 52–54 were again readily detectable. However, peptide 9 and the α-gliadin peptide 42 remained consistently undetectable (Fig. 2 [A2 and B2]).

Free peptides 9 (QPYPQPQPFPSQQ-GGC) and 42 (LGQG SFRPSQQN-GGC), and crude gliadin were attached to ELISA plates for analysis of individual antipeptide antibody responses in HLA-typed patients with celiac disease and disease controls. There was no correlation between the serum antipeptide antibody levels and the antigliadin antibody levels (data not shown). However, the relative proportions of IgG antibody levels to
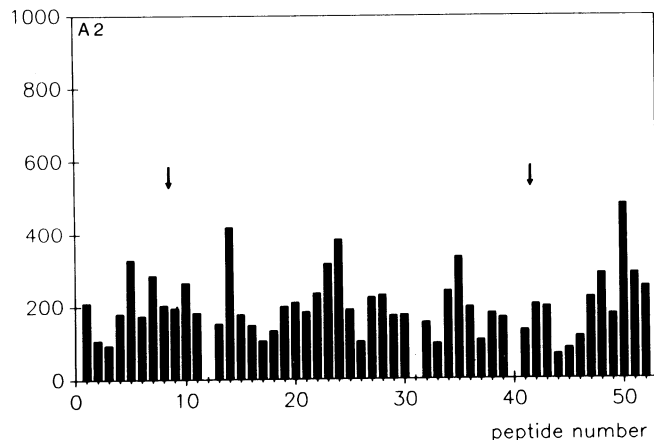
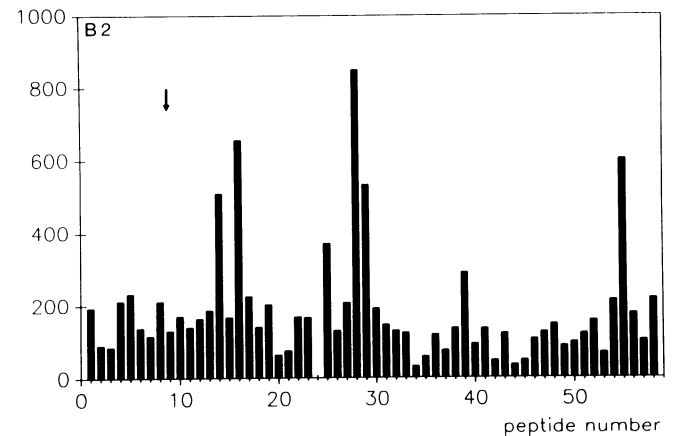OD/450 nm
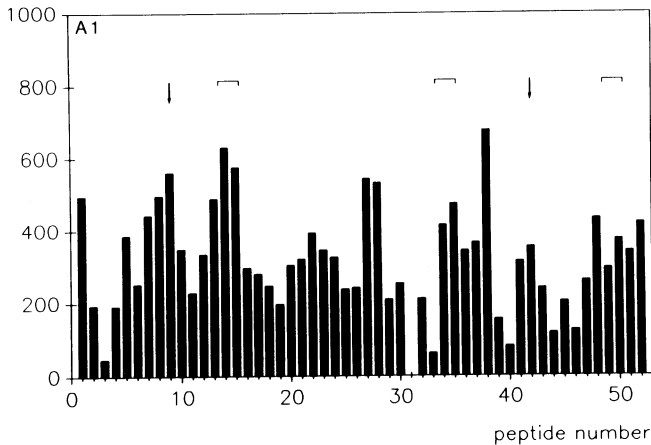
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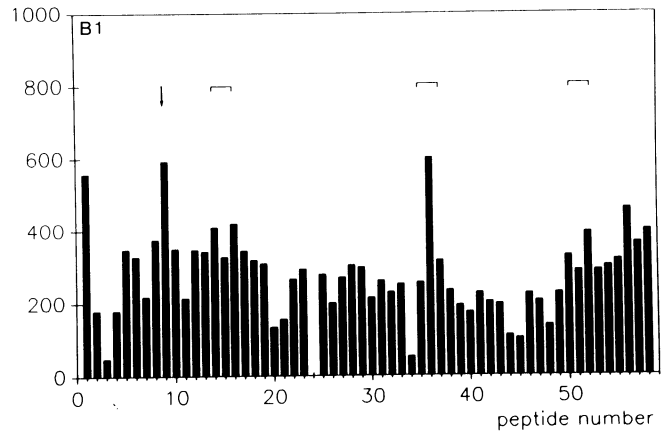
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Fig. 2. Epitope scanning of α-gliadin (**A1** and **A2**) and α/β-gliadin (**B1** and **B2**) with human serum antibodies. Histogram of antibody reactivity (absorbance at 450 nm) to individual ten amino acid long peptides. Antigliadin antibodies from patients with celiac disease (**A1** and **B1** [↓ and ⌐ = reactive regions]) and antigliadin antibodies from disease controls (**A2** and **B2** [↓ = missing epitopes]). Representative histograms of parallel analyses are shown.

peptide 9 (antipeptide antibody levels divided by antigliadin antibody levels) were significantly ($P < 0.05$, Mann-Whitney U-test) higher among the 17 celiac disease patients (mean 0.37) than among the 19 antigliadin antibody positive disease controls (mean 0.33) not carrying the HLA-DQA1*0501 or DQB1*0201 alleles (Fig. 3). No significant differences between the cases and controls were found for peptide 42.

B-Cell Epitopes Specific to Celiac Disease

To further evaluate the nature of the celiac disease associated peptides 9 and 42, the aa-homology, secondary structure, and associated T-cell epitopes were outlined. The GCG FASTA program revealed considerable homology between peptides 9 and 42, and two adenovirus proteins: the 100 kDa late protein and the E1b protein, respectively (Table I). Human annexin also showed 50% homology with peptide 9, but no obvious amino acid homology with other mammalian proteins was identified.

According to the hydrophilicity index of Kyte and Doolittle (1982), peptides 9 and 42 are both hydrophilic (data not shown), suggesting antigenicity. A previously defined T-helper cell epitope lies in the vicinity of the peptide 9 (Gjertsen et al 1993, Lundin et al 1993). Peptide 42 is also overlapped by a T-helper cell epitope.

DISCUSSION

Celiac disease patients differ from healthy controls with regard to their levels of antigliadin antibodies (Savilahti et al 1983, Friis et al 1986), but so far only a few epitope-specific differences between the celiac disease patients and controls have been reported (Devery et al 1989, Devery et al 1991, Lähdeaho et al 1993b). We identified two major, celiac disease-associated B-cell epitopes: peptide 9 ([41]QPYPQPQPFP[50]) in α- and α/β-gliadins, and peptide 42 ([206]LGQGSFRPSQ[215]) only in α-gliadin. Both the peptides were reactive with previously described gliadin-specific MAbs and pooled sera of celiac disease patients but showed only low reactivity with serum pools of disease control patients with equal antigliadin antibody levels.

Epitope scanning has recently been successfully used for the analysis of defined epitopes in wheat glutenin (Andrews and Skerritt 1994). Stepping five amino acids at a time reveals more than half of existing B-cell epitopes (M. Lehtinen et al, *unpublished*). In our hands, the use of human serum pools instead of individual sera precipitated this analysis of major B-cell epitopes. Unfortunately, the procedure recognizes only linear epitopes, and discovery of possible celiac disease-specific

differences in conformational epitopes has to await improved techniques.

The presence or absence of antigliadin antibodies is independent of HLA genotype, i.e., whatever DR specificity may present gliadin peptides to T-helper cells (Mäki et al 1991, Pettersson 1993). Thus, our study also had individuals with normal jejunal mucosal morphology who had serum antigliadin antibody levels as high as that detected in untreated celiac disease patients. In the present study, individuals with untreated celiac disease who carry the celiac disease-specific HLA DQA1*0501 and DQB1*0201 alleles (Sollid et al 1989) showed distinguishable IgG antibody responses to a specific epitope spanning over peptide 9 in both α- and α/β-gliadins. These IgG antibody reactive epitopes may have a pathogenetic role in celiac disease because IgG is known to activate complement and thus may induce damage of the jejunal epithelium (Halstensen et al 1992). The relative proportions of individual IgG antibody levels to peptides 9 and 42 appeared to be similarly distributed, but the difference between cases and controls did not reach statistical significance for peptide 42.

Peptide 9 is located in the domain I of α- gliadins and α/β-gliadins, and has not been described previously. Significantly lower relative proportions of antipeptide antibodies in the disease control patients, who possess antigliadin antibodies but are devoid of appropiate HLA, suggest that augmentation of the antibody response to peptide 9 in celiac disease may be HLA-restricted. The presence of a celiac disease-specific T-helper cell epitope (Gjertsen et al 1993, Lundin et al 1993) in this region is in line with this assumption. Further proof of the HLA restriction could be achieved by studying individuals positive for antigliadin antibodies who carry the celiac disease-specific DQA/DQB alleles but have normal jejunal mucosal morphology. It is also of interest that a toxic sequence of α-gliadin (PSQQ) lies in the vicinity of peptide 9 (de Ritis et al 1988). In a recent study, a synthetic peptide that overlaps peptide 9 also damaged tissue in vivo (Sturgess et al 1994).

We have previously shown that patients with celiac disease have significantly higher antipeptide antibody levels to a B-cell epitope spanning peptide 42 (Lähdeaho et al 1993a,b) than do healthy controls who are age- and sex-matched but not antigliadin antibody matched. The discrepancy between this study and previous studies may be due to the differences in matching (Carter et al 1989, Devery et al 1989, Howdle et al 1989). Furthermore, abnormal T-cell responses to peptide 42 in patients with celiac disease have been described (Karagiannis et al 1987). Thus, the human antibody response to the α-gliadin peptide 42, but not to
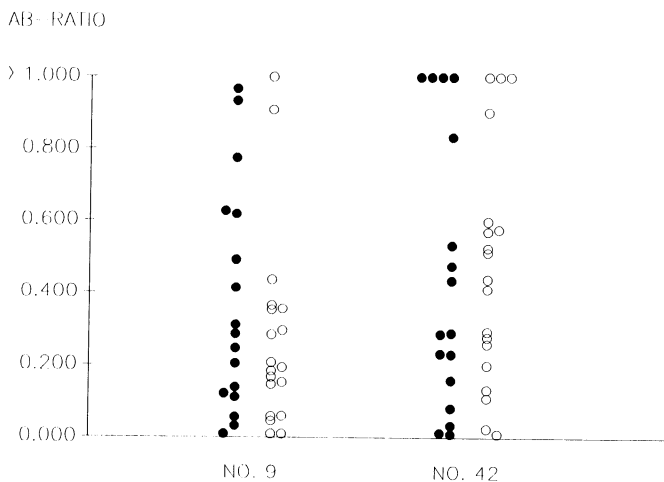
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Fig. 3. Relative proportions of antipeptide antibodies to α-gliadin peptides 9 and 42 in 17 genetically susceptible patients with celiac disease (●) and 19 disease controls (O).

TABLE I
Amino Acid Homology of α- and α/β-Gliadin Peptides 9 and 42 Aligned with *Saccharomyces cerevisiae*, Drosophila melanogaster, Rat, Human Annexin, and γ -Crystal and Adenovirus Types 5 and 12

Peptide 9	[41]QPYPQPQPFP[50]
α- and α/β-gliadin	PFPPQQPYPQPQPFPSQQPY
S. cerevisiae	PFDPQINITKKMPFPYYEDQ
Rat (creatine kinase)	LFPPHADYPDLRKHNNCMAE
Human (annexin)	SFPPSGQYPYPSGFPPMGGGA
Adenovirus type 5 (100 kDa late protein)	IPPPPQPYQQPRALASQDGTQ
Peptide 42	[206]LGQGSFRPSQ[215]
α-gliadin	LQQYPLGQGSFRPSQQNPQA
S. cerevisiae	LQYYGLTEASFYFVLFGVAR
Drosophila	IQLAPLGQGSFGMVYEGILK
Rat (β-crystal)	GYQYLLEPGDFRHWNEWGAF
Human (γ-crystal)	GQQYLLRRGEYPDYQQWMGL
Adenovirus type 12 (E1b protein)	TIHLGLRRGMFRPSQCNFSH

corresponding α/β-gliadin peptide 48, needs to be discussed. The α/β-gliadin peptide 48 does not contain or overlap similar T-helper cell epitopes as does the α-gliadin peptide 42. Lack of human serum antibodies to peptide 48 might, again, be due to lack of T-cell help. Peptide 42 is located in domain V and also overlaps a toxic sequence (PSQQ) that is not present in peptide 48. Thus it is plausible that this particular region of α/β-gliadin, besides being non-toxic in vitro, will not provoke harmful immune response in vivo.

Our studies suggest that specific regions of gliadin molecules may be involved in triggering the production of gliadin antibodies in patients with celiac disease. Whether or not all naturally occurring gliadin molecules are involved in the development of the disease warrants further investigation.

ACKNOWLEDGMENTS

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