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In vivo antigen challenge in celiac disease identifies a single transglutaminase-modified peptide as the dominant A-gliadin T-cell epitope

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Celiac disease (CD) is an increasingly diagnosed enteropathy (prevalence, 1:200–1:300)¹ that is induced by dietary exposure to wheat gliadins² (as well as related proteins in rye and barley) and is strongly associated with HLA-DQ2 ($\alpha 1^*0501$, $\beta 1^*0201$), which is present in over 90% of CD patients³. Because a variety of gliadin peptides have been identified as epitopes for gliadin-specific T-cell clones^{4–6} and as bioactive sequences in feeding studies and in *ex vivo* CD intestinal biopsy challenge^{7–9}, it has been unclear whether a 'dominant' T-cell epitope is associated with CD. Here, we used fresh peripheral blood lymphocytes from individual subjects undergoing short-term antigen challenge and tissue transglutaminase-treated, overlapping synthetic peptides spanning A-gliadin to demonstrate a transient, disease-specific, DQ2-restricted, CD4 T-cell response to a single dominant epitope. Optimal gamma interferon release in an ELISPOT assay was elicited by a 17-amino-acid peptide corresponding to the partially deamidated peptide of A-gliadin amino acids 57–73 (Q65E). Consistent with earlier reports indicating that host tissue transglutaminase modification of gliadin enhances gliadin-specific CD T-cell responses¹⁰, tissue transglutaminase specifically deamidated Q65 in the peptide of A-gliadin amino acids 56–75. Discovery of this dominant epitope may allow development of antigen-specific immunotherapy for CD.

Because α -gliadins induce intestinal inflammation in celiac disease (CD)⁶, attempts to define 'toxic' peptides have focused on peptides from the first fully sequenced α -gliadin, A-gliadin (SwissProt accession number, Q41545)¹¹. *In vivo* and *ex vivo* peptide challenge experiments have been unable to identify dominant 'toxic' peptides because of the impracticality of directly comparing all potential T-cell epitopes *in vivo* or using the limited material obtainable at biopsy, whereas clone-based strategies may be susceptible to artifacts (Fig. 1). Although peripheral blood lymphocyte responses to gliadin are more easily studied, epitope mapping studies in CD have used intestinal T-cell clones because of concern that local and systemic immune responses to gliadin are qualitatively different¹⁰. However, recent studies using T-cell receptor transgenic animals indicate that enteral exposure to antigen alters antigen-specific systemic T-cell dynamics¹².

In contrast to previous studies of CD peripheral blood T-cell responses that used the uncharacterized mixture of peptides contained in peptidase-digested gliadin, we used a set of 51 synthetic 15-amino-acid peptides spanning the complete se-

quence of A-gliadin and assessed peripheral blood mononuclear cell (PBMC) secretion of the T-helper-cell type 1-associated cytokine gamma interferon (IFN- γ) and the anti-inflammatory cytokine interleukin (IL)-10 in ELISPOT assays. Tissue transglutaminase (tTG) converts glutamine (Q) residues in peptides to glutamate (E), or if amine donors such as lysine alone or in peptides or proteins are present, glutamyl-lysine (E-E-K) conjugates are formed¹³. Thus, we assessed immune responses to A-gliadin peptides that were unmodified, tTG-treated or tTG-treated in the presence of lysine.

Initial studies of healthy normal subjects (HLA-DQ2 and other DQ genotypes) showed no consistent IFN- γ responses when PBMCs were stimulated with gliadin peptides. Similarly, we measured no responses in patients with celiac disease either before they started a gluten-free diet or while they were maintained on a gluten-free diet (data not shown). The T-cell response to gliadin in CD might be analogous to that described in experimental models of autoimmunity and chronic infection with an initial response to a limited number of dominant T-cell epitopes, followed by epitope 'spreading'¹⁴ and 'exhaus-

Table 1 Clinical details of subjects studied

	Age Sex	Celiac diagnosis	DQ2	Bread challenge	Symptoms with bread
1	64 F	14 yr	++	3 d	P, L, U, D
2 ^a	57 M	1 yr	+	10 d	L, N
3	35 F	7 yr	+	3 d	N
4 ^a	36 M	6 wk	++	3 d	P, U, D
5	26 M	19 yr	+	3 d	None
6	58 M	37 yr	+	3 d	None
7	55 M	1 yr	+	3 d	D
8	48 F	15 yr	++	3 d	P, D
9 ^a	37 M	3 mo	+	3 d	None
10 ^a	25 F	3 mo	+	3 d	None
11	38 F	14 yr	+	3 d	None
12 ^b	62 F	11 yr	+	— d	N, V, P
13	52 F	No	+	3 d	P
14	28 F	No	+	3 d	None
15	33 M	No	+	3 d	None
16	30 M	No	+	3 d	None

Patients with CD (1–12) had followed gluten-free diets since diagnosis of their CD, and control subjects (13–16), for 4 weeks. ^aSubjects had no antibody against endomysium before bread challenge except for subjects 4, 9 and 10; this remained unchanged except for subject 2, who had bread for 10 d. ^bSubject 12 became unwell 3 h after first bread-containing meal and had no further bread. HLA-DQ2 status: +, heterozygous; ++, homozygous. yr, years; mo, months; wk, weeks. P, abdominal pain; L, lethargy; U, mouth ulcers; D, diarrhea; N, nausea; V, vomiting.

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VRVPVQLQLP QNPSSQQQPOE QVPLVQQQF PGQQQQQFPQ QPYPQPQFP SQQPYL **QLQP** FPQPQLPYPQ
 1 11 21 31 41 51 61
PQSFPQQPY PQPQPYQSP QQPISQQQAQ QQQQQQQQQQ QQQILQQILQ QQLIPCMDV LQQHNIHAR
 71 81 91 101 111 121 131
 SQVLQOSTYQ LLQELCCQHL WQIEPQSQCQ AIHNVVHAIL HQQQKQQQQ PSSQVSFQQP LQQYP LGQGS
 141 151 161 171 181 191 201
 FRPSQQNPQA QGSVQPQQLP QFEEIRNLAL QTLPAMCNVY IAPYCTIAPF GIFGTN
 211 221 231 241 251 261

Fig. 1 Amino-acid sequence of A-gliadin A-gliadin 57-73 Q65E is the dominant epitope (bold); sequences identified as bioactive in other studies are underlined.

tion¹⁵. Thus, we tested immune responses in response to 'gluten challenge'.

For this study, 'gluten challenge' (or 'bread challenge') consisted of the consumption of gluten-containing white bread (four slices, or 200 g, daily) for one-half day ($n = 1$), 3 days ($n = 10$) or 10 days ($n = 1$). We compared the immune responses induced by this gluten challenge in CD subjects, initially in remission maintained by a gluten-free diet, with those in healthy HLA-DQ2 subjects who had followed a gluten-free diet for 4 weeks and were then challenged with gluten-containing bread

for 3 days (Table 1). Because T-cell responses to gliadin peptides in peripheral blood were transient, we studied CD subjects in groups of two to four to allow a 'stepwise' analysis, with screening of peptides followed by synthesis of further peptides, which produced an increasingly detailed description of the dominant epitope.

Gluten challenge induced a transient IFN- γ response to one of the ten pools of A-gliadin peptides that had been treated with tTG (with or without lysine) (pool 3) and included peptides spanning A-gliadin amino acids 51–85, at a concentration of 10 $\mu\text{g}/\text{ml}$ (Fig. 2a). Induced responses to tTG-treated pool 3 were maximal on days 6–8 after subjects began eating bread and were present in 11 of 12 CD subjects, compared with 0 of 4 control subjects (Fig. 2b). PBMC IFN- γ responses to 50 $\mu\text{g}/\text{ml}$ tTG-treated chymotrypsin-digested gliadin were also induced in CD subjects and were of similar magnitude to those induced by tTG-treated pool 3 (Fig. 2b).

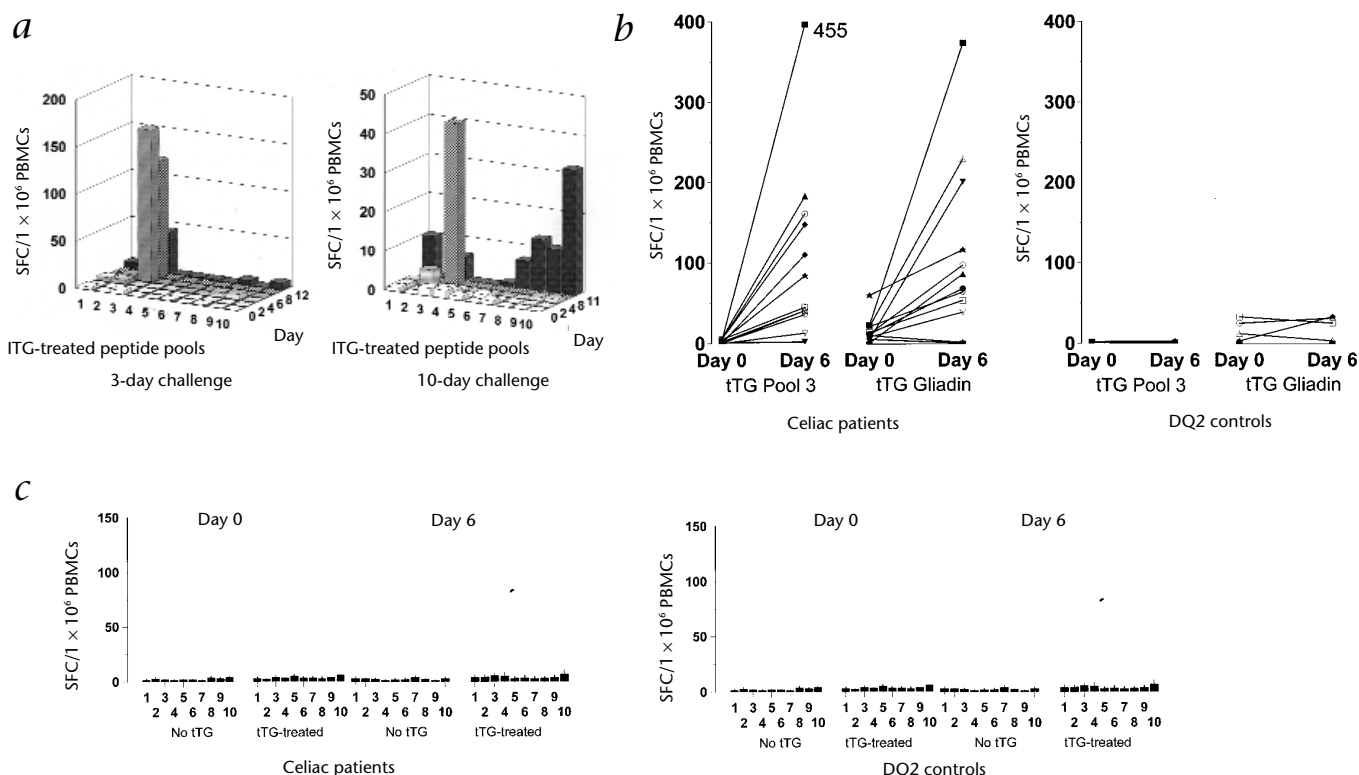


Fig. 2 Ex vivo IFN- γ responses of PBMCs to A-gliadin peptides. **a**, IFN- γ responses, by ELISPOT assay, of freshly isolated PBMCs to pools (1–10) of five overlapping 15-amino-acid A-gliadin peptides (10 $\mu\text{g}/\text{ml}$) treated with tTG, up to 12 d after bread challenge began, in CD patients who consumed bread for 3 d (left; representative of five CD patients) or 10 d (right). **b**, IFN- γ responses to tTG-treated pool 3 were induced by 6 d after bread challenge began in 11 of 12 CD and 0 of 4 DQ2 control subjects (all previously following a gluten-free diet for up to 4 weeks). Results, by ELISPOT assay, are in SFC/ 1×10^6 PBMCs (mean \pm s.e.m.). On day 0: pool 3 plus tTG: CD, 3.9 ± 1.1 (range, 0–14) and control, 3.8 ± 1.4 (range, 2–8); buffer plus tTG: CD, 2.1 ± 0.7 (range, 0–7) and control, 2.1 ± 1.8 (range, 0–7.3). On day 6: pool 3 plus tTG: CD, 110.6 ± 35.9 (range, 2.3–455) and

control, 5.2 ± 1.8 (range, 2–10); buffer plus tTG: CD, 3.5 ± 0.8 (range, 0–10) and control, 5.2 ± 2.3 (range, 2–12). IFN- γ responses to tTG-treated chymotrypsin-digested gliadin (50 $\mu\text{g}/\text{ml}$) were similar in CD and control subjects before bread challenge, but by day 6, mean responses increased 660% in CD subjects compared with 37% in control subjects, by ELISPOT assay. SFC/ 1×10^6 PBMCs (mean \pm s.e.m.) on day 0: CD, 16.9 ± 4.5 (range, 1.5–61) and control, 19.3 ± 6.7 (range, 4–34); on day 6: CD, 112.4 ± 31.5 (range, 1.1–377) and control, 24 ± 6.9 (range, 4–34). **c**, Induction of IFN- γ responses with bread challenge, as shown by ELISPOT assay, was specific to pool 3 and only occurred in CD subjects (left; $n = 12$), not in DQ controls (right; $n = 4$); responses to tTG-treated pool 3 were on average 630% greater than pool 3 without tTG treatment.

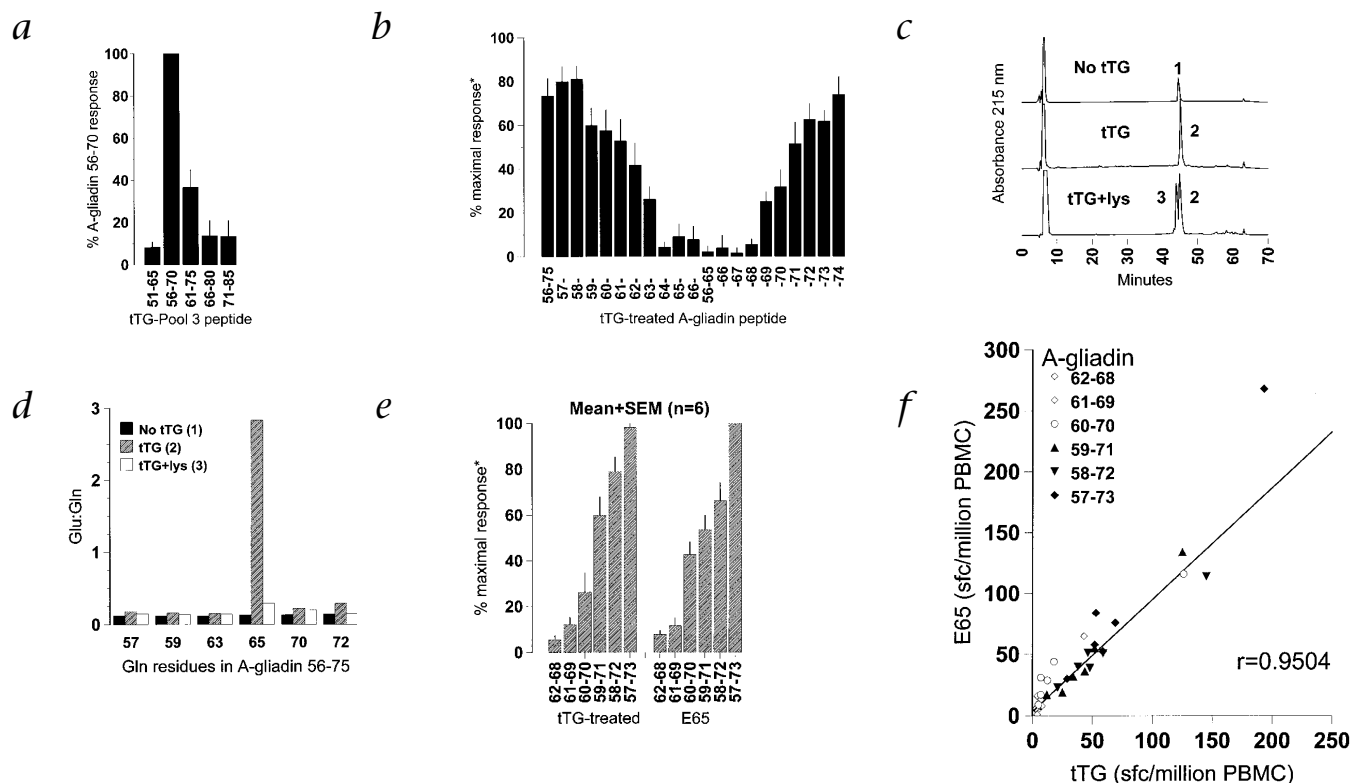


Fig. 3. Definition of the minimal epitope in tTG-treated pool 3. **a** and **b**, IFN- γ responses to tTG-treated peptides, by ELISPOT assay. **a**, 15-amino-acid peptides were included in pool 3. In individual CD subjects, A-gliadin 56-70 elicited $29\text{--}424\text{ SFC}/1 \times 10^6\text{ PBMCs}$, and buffer with or without tTG elicited $1\text{--}8\text{ SFC}/10^6$. **b**, Truncations of A-gliadin 56-75 (LQLQPFQPPQ LPYPQPQSFP) were assessed. IFN- γ results are expressed as % of maximal response elicited by a single peptide in individual experiments. **c**, HPLC profiles of A-gliadin 56-75 after treatment with tTG with or without lysine. tTG plus lysine generated two products (peaks 2 and 3), with peak 2 having the same retention time as the product of tTG treatment alone. **d**, Amino-acid analysis of peaks 1 (A-gliadin 56-75; no tTG), 2 (tTG) and 3 (tTG plus lysine), showing approximately 65% deamidation of the glutamine at position 65 with tTG treatment alone. A new 'moiety' not eluting with

glutamate or glutamine, possibly Glu-Lys, was apparent at position 65 after sequencing of the product of treatment with tTG plus lysine (data not shown). **e**, IFN- γ responses, by ELISPOT assay, to tTG-treated and Q65E-substituted A-gliadin peptides (amino acids, horizontal axis), indicating that the 17-amino-acid peptide has optimal bioactivity. Maximal responses ($\text{SFC}/1 \times 10^6\text{ PBMCs}$) are in the range of $29\text{--}268$; buffer with or without tTG, $1\text{--}8$. **f**, IFN- γ responses, by ELISPOT assay, to tTG-treated and Q65E-substituted versions of A-gliadin peptides. Data are consistent with the conversion Q65E accounting for the bioactivity of tTG-treated A-gliadin peptides: $r = 0.9504$; $y = ax + b$: $a = 1.08$ (95% confidence interval (CI), $0.957\text{--}1.205$); $b = 2.18$ (95% CI, $-5.07\text{--}9.53$); $n = 6$ CD subjects. PBMCs used for IFN- γ ELISPOT assays were drawn from CD subjects 6–8 d after bread challenge began.

None of the subjects had responses to any A-gliadin peptide pools before gluten challenge, and only pool 3 elicited responses on days 6–8 (Fig. 2c). Epitope spreading to other A-gliadin peptide pools was not apparent up to day 12 with 3-day bread challenge ($n = 5$), but in the CD subject who consumed bread for 10 days, IFN- γ responses to tTG-treated A-gliadin peptide pools 1, 7, 8, 9 and 10 were apparent on day 11 (Fig. 2a).

Symptoms associated with gluten challenge were generally mild (Table 1) with the exception of one subject who vomited 3 hours after the first two slices of bread, and two others who developed mouth ulcers after 3 days. The one CD subject who did not show induction of responses to tTG-treated pool 3 had no symptoms associated with gluten challenge. Serum antibody against endomysium was induced with bread challenge for 10 days but not 3 days.

Two overlapping tTG-treated peptides corresponding to A-gliadin amino acids 56–70 and 61–75 (that is, A-gliadin 56-70 and A-gliadin 61-75) accounted for the bioactivity associated with tTG-treated pool 3 (Fig. 3a), and only tTG-treated truncations of A-gliadin 56-75 that included residues 64–68 (PQLPY)

elicited IFN- γ responses (Fig. 3b). High-performance liquid chromatography (HPLC) confirmed the purity of the synthetic A-gliadin 56-75 (greater than 95%) (peak 1) and identified a single product resulting from tTG treatment of A-gliadin 56-75 (peak 2) (Fig. 3c). Amino-acid analysis of tTG-treated A-gliadin 56-75 demonstrated that deamidation due to tTG was restricted to glutamine residue 65 (Q65E) (Fig. 3d). Comparison of IFN- γ responses induced with tTG-treated peptides beginning with the 7-amino-acid peptide spanning the core antigenic region of A-gliadin indicated that the 17-amino-acid peptide A-gliadin 57-73 was optimal (Fig. 3e). IFN- γ responses to synthetic peptides with the substitution Q65E were equivalent to the responses to homologous tTG-treated A-gliadin peptides, by ELISPOT assay (Fig. 3f). The substitutions Q57E and/or Q72E in addition to Q65E did not enhance the bioactivity of A-gliadin Q65E; IFN- γ responses, with a range of $37\text{--}111\text{ spot-forming cells (SFC)}/1 \times 10^6\text{ PBMCs}$ ($n = 6$ CD subjects), were: Q57E and Q65E, $94 \pm 2\%$; Q65E and Q72E, $84 \pm 7\%$; Q57E, Q65E and Q72E, $88 \pm 10\%$ (% of A-gliadin E65 response, mean \pm s.e.m.).

Although treatment of A-gliadin 56-75 with tTG plus lysine

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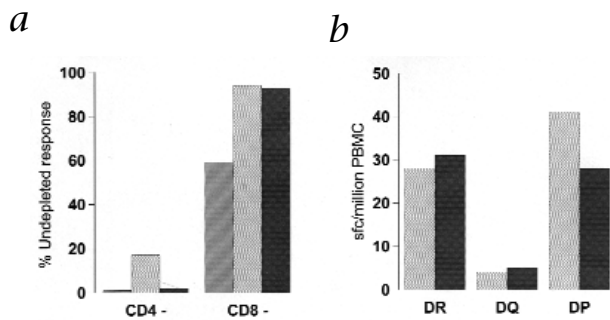


Fig 4 Analysis of dominant A-gliadin epitope. **a**, IFN- γ responses to A-gliadin 57-73 Q65E, by ELISPOT assay, are substantially reduced by depletion of PBMCs using magnetic beads coated with antibody against CD. $n = 3$ CD patients. **b**, Preincubation of PBMCs from two CD subjects homozygous for HLA-DQ $\alpha 1^*0501$, $\beta 1^*0201$ with antibody against DQ substantially reduces responses to tTG-treated A-gliadin 56-75, but antibodies against DR and DP do not. PBMCs used for IFN- γ ELISPOT assays were drawn from CD subjects 6–8 days after bread challenge began.

generated one new moiety (peak 3) and one co-eluting with A-gliadin 56-75 treated with tTG alone (peak 2) (Fig. 3c), IFN- γ responses to peptide pools treated with tTG plus lysine and tTG alone were similar in all CD subjects, by ELISPOT assay. We synthesized to more than 99% purity the peptide predicted to result from cross-linking of A-gliadin 57-73 to lysine or pep-

tide/protein-bound lysine (A-gliadin 57-73 Q65E- ϵ -K). IFN- γ responses to A-gliadin 57-73 Q65E- ϵ -K were 17% of those elicited by A-gliadin 57-73 Q65E on day 6 with 3-day gluten challenge, by ELISPOT assay (range, 4–35%; $n = 3$ CD subjects), indicating that the dominant epitope is not the result of A-gliadin forming a hapten with tTG or other proteins by covalent cross-linking through Q65, as the isopeptide bond E- ϵ -K is relatively resistant to peptidase attack¹³.

Depletion of CD4 T cells from PBMC populations substantially reduced IFN- γ responses to A-gliadin 57-73 Q65E (CD4 depletion: mean, 6% (range, 0.3–17%); CD8 depletion: mean, 72% (range 59–94%); undepleted responses, 24–115 SFC/ 1×10^6 PBMCs; $n = 3$ CD subjects on day 8) (Fig. 4a). In two CD subjects homozygous for HLA-DQ $\alpha 1^*0501$, $\beta 1^*0201$, antibody against DQ (L2) blocked IFN- γ responses to tTG-treated A-gliadin 56-75 (Fig. 4b).

A-gliadin 56-70 or A-gliadin 60-75 alone treated with tTG with or without lysine, plus pool 3, did not induce significantly more responses than buffer plus tTG with or without lysine in IL-10 ELISPOT assays (SFC/ 1×10^6 PBMCs (mean \pm s.e.m.): 1,177 \pm 576, 455 \pm 226 and 226 \pm 109, respectively; buffer plus tTG, 1,024 \pm 483; differences not significant by paired t -test; $n = 6$ CD subjects on day 6). But consistent with a recent report¹⁶, chymotrypsin-digested gliadin with or without tTG treatment was a potent inducer of IL-10 before and during bread challenge (tTG-treated gliadin on day 6: 8,129 \pm 3,570 SFC/ 1×10^6 PBMCs (mean \pm s.e.m.); $P < 0.001$, compared with buffer plus tTG, by paired t -test).

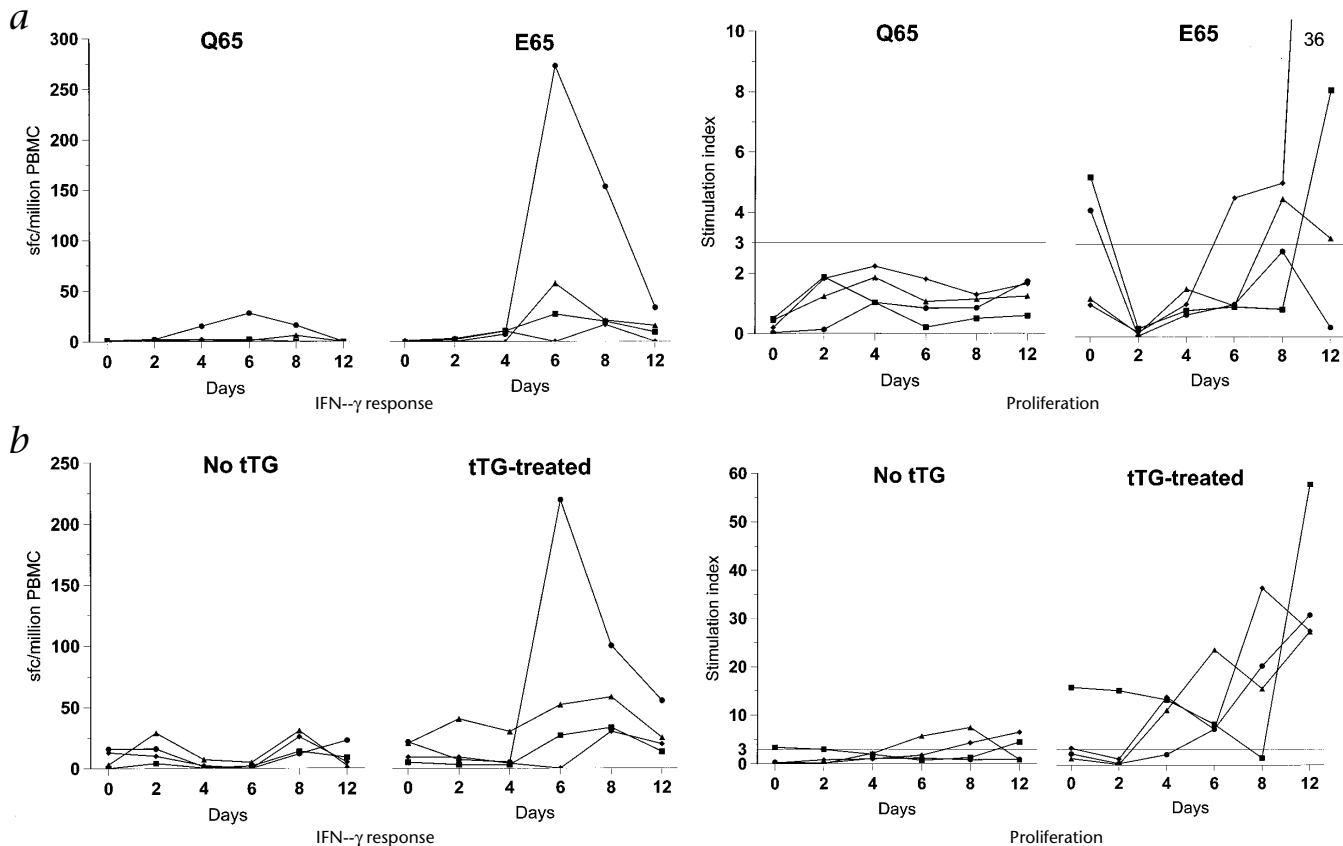


Fig 5 Dynamics of PBMC IFN- γ responses by ELISPOT assay and proliferative responses. Responses were measured in four patients with CD, challenged with bread starting on day 1. One patient (■) tolerated only one breakfast (100 g) of bread; the other three patients

completed 3 days of bread challenge. **a**, Responses to 12 μ g/ml A-gliadin 57-73 with glutamine (Q65) or glutamate (E65) at position 65. **b**, Responses to 50 μ g/ml chymotrypsin-digested gliadin with or without tTG treatment.

Proliferation assays done in parallel with IFN- γ ELISPOT assays during bread challenge demonstrated stimulation indices of more than three induced by A-gliadin 57-73 Q65E in three of four subjects by day 12 but none of four with A-gliadin 57-73 Q65. Similarly tTG-treated chymotrypsin-digested gliadin induced substantially greater proliferative responses than untreated chymotrypsin-digested gliadin (Fig. 5). However, maximum proliferative responses were delayed to at least day 12, in contrast to IFN- γ responses in PBMCs, which were maximum on day 6 or 8.

Short-term antigen challenge elicited a transient population of anti-gliadin T cells in peripheral blood that allowed identification of the dominant HLA-DQ2-restricted, CD-specific epitope in A-gliadin 57-73, QLQPFQPELPYPQPS. This epitope resulted from tTG-catalyzed deamidation of one glutamine residue at position 65. The binding motif for HLA-DQ2 indicates a preference for negatively charged amino acids at three of five anchor sites¹⁷, indicating that binding of this A-gliadin peptide to HLA-DQ2 is likely to be enhanced by the tTG catalyzed deamidation. Although we studied only one subject during a 10-day gluten challenge, epitope spreading occurred subsequent to an initial response to the dominant epitope. Epitope spreading may account for the variety of unrelated A-gliadin peptides previously identified as bioactive.

The core 7-amino-acid sequence in A-gliadin 57-73 (PQPQLPY) is also present in several other gliadins (SwissProt and Trembl accession numbers, P02863, Q41528, Q41531, Q41533, Q9ZP09, P04722, P04724, P18573). The two peptides most commonly recognized by gliadin-specific T-cell clones derived from CD intestinal biopsies are the tTG-modified gliadin peptides QLQPFQPELPY and PQPELPYPQPELPY (L. Sollid, personal communication). T cells responsive to the same A-gliadin peptide are readily induced in CD despite many years of following a gluten-free diet, indicating persistence of memory T cells that may be due to continuing exposure to trace amounts of gluten. In fact, labeling of food as 'gluten-free' indicates gluten content is less than 0.02% (ref. 18), and it is not uncommon for CD patients to have occasional accidental exposures to gluten.

Our studies here have identified an immunodominant CD-specific epitope and an immune response induced by bread challenge, thereby providing a defined target for immunomodulation. These findings indicate several possible alternatives for the treatment of celiac disease. For example, 'non-toxic' wheat might be created by the removal or modification of the antigenic sequence in gliadin proteins, or therapy with an 'altered peptide ligand antagonist' could be developed.

Methods

Subjects and antigen challenge: The study was approved by the Oxfordshire Regional Ethics Committee. Patients attending the Coeliac Clinic at the John Radcliffe Hospital (Oxford, UK) were invited to participate in the study. CD was diagnosed on the basis of small intestinal histology, and by normalization of symptoms and histology with gluten-free diet. All CD subjects had followed a gluten-free diet since the diagnosis of their CD, and antibody against endomysium (detected by indirect immunofluorescence using patient serum diluted 1:5 with normal saline, followed by fluorescein isothiocyanate-conjugated goat antibody against human IgA) was absent before bread challenge except for subjects 4, 9 and 10, who had begun to exclude gluten from their diets within the previous 3 months. Healthy DQ2 control subjects (all negative for antibody against endomysium), were staff members of the Coeliac Clinic; they were educated about the gluten-free diet by a dietitian. The HLA-DQA and DQB genotype of all subjects was determined using peripheral blood

DNA and PCR with sequence-specific primer mixes¹⁹⁻²¹.

For the gluten challenge, in addition to their usual gluten-free diet, subjects consumed two slices of gluten-containing bread (50g/slice, Sainsbury's "standard white sandwich bread") at breakfast and lunch for 3 d (or in one case each, 1 or 10 d, the former because of onset of vomiting 3 h after the first gluten meal). The presence or absence of antibody against endomysium was assessed the week before and up to two months after the bread challenge was begun.

Peptides. Synthetic peptides were purchased from Research Genetics (Huntsville, Alabama). Mass-spectroscopy and HPLC were used to confirm the peptides' authenticity and more-than-70% purity. Gliadin (100 mg/ml; G-3375; Sigma) was digested with α -chymotrypsin (200:1 (weight:weight); C-3142; Sigma) at room temperature in 0.1 M NH_4HCO_3 with 2 M urea; this was stopped after 24 h by heating samples to 98 °C for 10 min. After centrifugation at 13,000g for 10 minutes, the chymotrypsin-digested gliadin supernatant was filter-sterilized (pore size, 0.2 μm). Digestion of gliadin was verified by SDS-PAGE, and protein concentrations were assessed. Gliadin (640 $\mu\text{g}/\text{ml}$) and synthetic gliadin peptides (15-amino-acid peptides: 160 $\mu\text{g}/\text{ml}$, other peptides: 0.1 mM) digested with α -chymotrypsin were individually treated for 2 h at 37°C with 50 $\mu\text{g}/\text{ml}$ tTG (T-5398; Sigma) in PBS plus 1 mM CaCl_2 . Peptides and peptide pools were separated into aliquots in sterile 96-well plates and stored frozen at -20°C.

IFN- γ and IL-10 enzyme-linked immunospot (ELISPOT) assays. PBMCs were prepared from 50–100 ml venous blood by Ficoll-Hypaque density centrifugation. After being washed three times, PBMCs were resuspended in complete RPMI containing 10% heat-inactivated human AB serum. ELISPOT assays for single-cell secretion of IFN- γ and IL-10 used commercial kits (Mabtech, Stockholm, Sweden) with 96-well plates (MAIP-S-45; Millipore, Bedford, Massachusetts) according to the manufacturers' instructions, as described²², with 2×10^5 – 5×10^5 (for IFN- γ) or 0.4×10^5 – 1×10^5 (for IL-10) PBMCs in each well. Peptides were assessed in duplicate wells, and 20 $\mu\text{g}/\text{ml}$ *Mycobacterium tuberculosis* purified protein derivative (PPD RT49; Serum Institute, Copenhagen, Denmark) was included as a positive control in all assays. Intra-assay variability was determined in one experiment using 24 replicates (mean, 42 SFC/ 0.5×10^6 ; s.d., 6.3 SFC/ 0.5×10^6), indicating that the percent coefficient of variation of SFC/ 1×10^6 PBMCs based on duplicate measures using 0.5×10^6 cells was 10.7%.

Proliferation assay. PBMCs in complete RPMI plus 10% heat-inactivated human AB serum were prepared as described above, and were added to round-bottomed, 96-well plates, with 1×10^5 cells per well, together with 12 $\mu\text{g}/\text{ml}$ peptide, 50 $\mu\text{g}/\text{ml}$ chymotrypsin-digested gliadin, or phosphate buffered saline, with or without tTG, in a total volume of 200 μl . These were then incubated at 37 °C in humidified air with 5% CO_2 for 5 d. ^3H -thymidine (1 $\mu\text{Ci}/\text{well}$; Amersham-Pharmacia, Amersham, UK) was added, and after further incubation for 18 h, ^3H incorporation into DNA was measured using a cell harvester (Packard FilterMate™) and radioactivity counter (TopCount NXT Microplate Counter; both from Packard, Meriden, Connecticut). Results were expressed as counts per minute (c.p.m.) of uptake in test sample /c.p.m. in appropriate control.

Peptide analysis. All samples were separated on a Jupiter C4 reverse-phase column (250 mm in length, 2 mm in diameter; Phenomenex, Macclesfield, UK) equilibrated in 0.1% trifluoroacetic acid and 2% acetonitrile. A linear gradient of 2–50% acetonitrile was applied over 50 min and then a further linear gradient of 50–90% acetonitrile was applied over 18 min. The flow rate was 150 $\mu\text{l}/\text{min}$ and the peptides were detected at a wavelength of 215 nm. Peaks of interest were sequenced on an Applied Biosystems 494A Procise protein sequencer (PE Biosystems, Warrington, UK).

CD4/CD8 and HLA class II restriction. Magnetic beads coated with antibody against CD4 or CD8 (Dyna, Oslo, Norway) were washed four times with RPMI, then incubated with PBMCs in complete RPMI containing 10% heat-inactivated human AB serum (5×10^6 cells/ml) for 30 min on ice. Beads were removed using a magnet, and the cells remaining were counted. *In vivo* HLA class II restriction of the immune response to tTG-

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treated A-gliadin 56-75 was established by incubating PBMCs (5×10^6 cells/ml) with 10 µg/ml monoclonal antibodies against HLA-DR (L243), HLA-DQ (L2) and HLA-DP (B7.21) at room temperature for 1 h before the addition of peptide.

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