COELIAC DISEASE

Antagonists and non-toxic variants of the dominant wheat gliadin T cell epitope in coeliac disease

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Background: Coeliac disease (CD) is due to an inappropriate T cell mediated response to specific gluten peptides. Measured by interferon γ (IFN- γ) ELISPOT, about half of the gliadin specific T cells induced with in vivo wheat gluten exposure in HLA-DQ2+ CD are specific for an α/β -gliadin peptide (p57-73 QE65; QLQPFQPELPYPQPQS) that includes two overlapping T cell epitopes (PFPQPELPY and PQPELPYPQ).

Aim: To define minimally substituted variants of p57-73 QE65 universally devoid of IFN- γ stimulatory capacity but capable of antagonising IFN- γ secretion from polyclonal T cells specific for p57-73 QE65.

Methods: Peripheral blood mononuclear cells collected from 75 HLA-DQ2+ CD patients after in vivo gluten challenge were used in overnight ELISPOT assays to screen 218 single or double substituted variants of p57-73 QE65 for cytokine stimulatory and antagonist activity.

Results: The region p60-71 (PFPQPELPYPQP) and especially p64-67 (PELP) was sensitive to substitution. Twelve substitutions in p64-67 stimulated no IFN- γ ELISPOT response. Among 131 partial agonists identified, 45 produced statistically significant inhibition of IFN- γ ELISPOT responses when cocultured in fivefold excess with p57-73 QE65 ($n=10$). Four substituted variants of p57-73 QE65 were inactive by IFN- γ ELISPOT but consistently antagonised IFN- γ ELISPOT responses to p57-73 QE65, and also retained interleukin 10 stimulatory capacity similar to p57-73 QE65.

Conclusions: Altered peptide ligands of p57-73 QE65, identified using polyclonal T cells from multiple HLA-DQ2+ CD donors, have properties in vitro that suggest that a single substitution to certain α/β -gliadins could abolish their capacity to stimulate IFN- γ from CD4 T cells and also have anti-inflammatory or protective effects in HLA-DQ2+ CD.

Understanding the immunological toxicity of gluten in coeliac disease leads to the possibility of designing non-toxic wheat, rye, or barley, or altered gluten peptides that may inhibit pathogenic T cell responses.

Because individual wheat, rye, and barley cultivars express many different gluten proteins¹ that are likely to include many different sequences that activate diverse T cell populations, the most practical strategy for production of non-toxic gluten is design of modified gluten proteins that are expressed in a crop without being toxic to individuals with coeliac disease (CD). This approach requires knowledge of the bioactive sequences within a single gluten protein, and discovery of modifications to such sequences that are universally non-toxic but do not adversely affect gluten cooking properties. The goal of modified crops expressing non-toxic gluten would be to provide low cost, high quality gluten suitable for individuals with CD, but this would not protect against the possibility of inadvertent gluten exposure from other sources.

A second possibility is to identify modified versions of gluten T cell epitopes that are not toxic and also inhibit the toxicity of gluten. In animal models, substitution of T cell epitopes at critical positions converts peptides into highly specific antagonists (altered peptide ligand (APL) antagonists).^{2,3} Translating the success of APLs in controlling autoimmunity in animal models⁴ to human T cell mediated diseases has been problematic^{5,6} because pathogenic T cell epitopes are not well defined or are inconsistent between individuals with the same disease.⁷ Most importantly, the frequency of disease relevant T cells in peripheral blood is often close to or below the levels of detection for T cell assays without in vitro expansion with mitogenic factors that may alter T cell phenotype (for example, see Bielekova and colleagues⁸). Hence interindividual heterogeneity and

intraindividual diversity of pathogenic T cells, as well as inability to access "unmanipulated" epitope specific T cells in sufficient numbers, have been major obstacles to systematic design of APLs for human disease.

In CD, a deamidated α/β -gliadin peptide (p57-73 QE65; QLQPFQPELPYPQPQS) has been identified as the dominant epitope in A-gliadin for T cells present in peripheral blood after in vivo gluten challenge in HLA-DQ2+ CD.⁹ Intestinal T cell clones are relatively specific for one of two overlapping nine amino acid sequences (DQ2- α -I: PFPQPELPY; and DQ2- α -II: PQPELPYPQ) within p57-73 QE65.^{10,11} One other HLA-DQ2 restricted T cell epitope has been identified in α/β -gliadin (GLIA-20E PFRPEQPYPQPQY)¹² but its contribution to the gluten induced T cell response in HLA-DQ2+ CD measured by interferon γ (IFN- γ) ELISPOT is rarely more than 10% of that to p57-73 QE65 (manuscript submitted).

In this study, T cells specific for p57-73 QE65 present in blood after in vivo gluten challenge of HLA-DQ2+ patients with CD were used in overnight ELISPOT assays to define amino acid modifications that abolish IFN- γ induction, retain interleukin (IL)-10 stimulatory capacity, and act as antagonists of p57-73 QE65.

MATERIALS AND METHODS

Subjects and gluten challenge

Seventy five subjects (Oxford 41, Melbourne 34; median age 47 years (range 19-70); 53 females) were included in this study (Oxford subjects also participated in a concurrent study).¹³ The Oxfordshire Regional Ethics Committee and Melbourne Health Human Ethics Committee approved the

Abbreviations: APL, altered peptide ligand; CD, coeliac disease; IFN- γ , interferon γ ; IL, interleukin; PBMC, peripheral blood mononuclear cells; SFU, spot forming units; TCR, T cell receptor

study. CD was diagnosed on the basis of small intestinal histology and all subjects were following a long term gluten free diet and had *HLA-DQB1*02* (31 homozygotes), as determined by polymerase chain reaction with sequence specific primer mixes of peripheral blood DNA.^{14–16} Wheat gluten challenge consisted of 200 g of commercial white bread (four slices) daily for three days. Blood was obtained six and seven days after commencing the gluten challenge. Six subjects had repeat challenges.

Antigens and cytokine ELISPOT

Synthetic peptides were purchased from Research Genetics (Huntsville, Alabama, USA). Mass spectroscopy and high performance liquid chromatography verified peptide authenticity and purity (>70%). Peptides were prepared and gliadin (endotoxin free; generous gift from A Tatham) deamidated with transglutaminase, as previously described.⁸ ELISPOT assays for IFN- γ , IL-4, IL-5, and IL-10 were performed using kits (Mabtech, Nacka Strand, Sweden) according to the manufacturer's instructions with peripheral blood mononuclear cells (PBMC) isolated by Ficoll-Hypaque density centrifugation from heparinised blood collected six days after commencing gluten challenge.⁹ Spot forming units (SFU) in individual wells were counted using an automated ELISPOT reader (AID ELISPOT Reader System; AID Autoimmun Diagnostika, Germany). Each peptide was incubated in duplicate wells. As our goal was to identify antagonists acting on the T cell receptor rather than directly competing for binding to HLA-DQ2, antagonism assays were performed by incubating PBMC with p57–73 QE65 (10 μ g/ml) at 37°C for 30 minutes followed by addition of the candidate antagonist peptides (50 μ g/ml) that were then incubated overnight in standard IFN- γ ELISPOT assay format.¹⁷ In each assay of altered peptides for either agonist or antagonist activity, at least 20 wells were used to assess p57–73 QE65 (50 μ g/ml, agonist assays; or 10 μ g/ml, antagonist assays). In 10 subjects, mean per cent intra-assay coefficient of variation of 16 duplicate IFN- γ ELISPOT assays of p57–73 QE65 (10 μ g/ml) was 16%, and mean IFN- γ ELISPOT response in three duplicate assays of medium alone was equal to 5 (1%) (mean (SEM)) of the response to p57–73 QE65 (10 μ g/ml).

Statistics

ELISPOT responses were compared following normalisation (%) against an individual's mean response to p57–73 QE65 (in agonist assays, 50 μ g/ml; in antagonist assays, 10 μ g/ml) after subtraction of SFU present with phosphate buffered saline (25 μ l) in medium (100 μ l). In experiments comparing peptide potency by the IFN- γ ELISPOT assay, data from 16/41 subjects were not included for analysis because p57–73 QE65 stimulated fewer than 30 SFU greater than medium alone in duplicate wells. ELISPOT responses to peptides indexed

against p57–73 QE65 were compared by the Student's *t* test using groups of at least eight individuals (SPSS software package).

RESULTS

Fine mapping p57–73 QE65

To identify the residues in p57–73 QE65 that affect its recognition by T cells, alanine and lysine substitution scans were performed (fig 1). Substitutions of residues 60–71 (PFPQPELPYPQP) (that is, positions 4–15) reduced responses in the IFN- γ ELISPOT using PBMC induced by wheat gluten challenge in eight subjects. Substitution with lysine, and to a lesser extent alanine, revealed the bioactivity of p57–73 QE65 to be critically dependent on a core sequence of four amino acids (PELP, p64–67). In the "core" sequence PELP (p64–67), alanine substitution reduced bioactivity by greater than 50% while lysine substituted for any position in p64–67 reduced mean bioactivity to less than 5%.

Variants of p57–73 QE65 were synthesised with each naturally occurring amino acid except cysteine at positions 61–69 and a selection of 10 representative amino acids at positions 59, 60, and 70. IFN- γ ELISPOT responses to each of these 201 single amino acid substituted variants of p57–73 QE65 were compared with the parent peptide at a concentration shown to stimulate maximal responses (50 μ g/ml) in eight subjects. Superagonists, partial agonists, and non-agonists were identified (fig 2).

In general, bioactivity of substituted peptides was consistent between subjects. By far the majority of substitutions of p57–73 QE65 created partial agonists, and only 12/201 substitutions, all in the core region p64–67, resulted in IFN- γ ELISPOT responses less than 5% of p57–73 QE65 (not significantly different from medium alone) (table 1). Half of the inactive peptides were created by substitutions with polar amino acids (Lys or Arg in five, Glu in one). Substitutions with polar residues, especially cationic amino acids, also resulted in the weakest agonists outside p64–67. Although 64P, 67P, and 69P were sensitive to substitution, the best tolerated substitutions were with serine or threonine. Interestingly, proline is often found substituted for serine or threonine in wheat gliadin sequences. Substitution of Leu 66 for glutamine to create a naturally occurring sequence in gliadin (xxxPFPQPEQFPQxxx) reduced bioactivity to 28 (6)%.

Antagonists of A-gliadin 57–73 QE65

Antagonism assays were performed in which PBMC collected six days after commencing gluten challenge were incubated in a standard ELISPOT assay, except that PBMC were preincubated with a concentration (10 μ g/ml) of p57–73 QE65 shown to produce a near optimal response (81 (5)% of maximum; $n=8$)¹³ in the IFN- γ ELISPOT for 30 minutes

Figure 1 Interferon γ (IFN- γ) ELISPOT responses to p57–73 QE65 (50 μ g/ml) substituted with alanine (A) or lysine (B) at positions 1–17 using peripheral blood mononuclear cells collected six days after commencing gluten challenge, expressed as per cent of a subject's response to p57–73 QE65. Values are mean (SEM); $n=8$.

Figure 2 Interferon γ ELISPOT responses to p57–73 QE65 (50 μ g/ml) with single amino acid substitutions, as indicated, from positions 3 (59Q) to 14 (70Q) using peripheral blood mononuclear cells collected six days after commencing gluten challenge, expressed as per cent of a subject's response to p57–73 QE65. Values are mean (SEM); $n=8$. Median spot forming units induced by p57–73 QE65 in duplicate wells was 55 (range 30–824), and by medium alone, 5 (range 1–9). *Indicates response to unmodified p57–73 QE65 (100%).

prior to addition of the test peptides (50 μ g/ml). Antagonism was measured as mean per cent reduction in responses compared with incubation of PBMC with p57–73 QE65 (10 μ g/ml) alone in 10 subjects.

Initially we assumed weak agonists would make more attractive antagonists and assessed 67 single amino acid substituted variants with less than 30% of the IFN- γ ELISPOT

response to p57–73 QE65, but antagonism was not inversely associated with agonism (fig 3) and so a total of 131 peptides with less than 60% of the bioactivity of p57–73 QE65 were eventually assessed for antagonism. With an enlarged number of peptides with greater agonist activity, a weak but significant inverse relationship was found between mean agonist and mean antagonist activity for individual peptides ($n=131$, $r=0.26$, $p<0.0023$).

At fivefold excess, 45 single amino acid substituted peptides inhibited IFN- γ ELISPOT responses to p57–73 QE65 at a significance level of $p<0.05$ (>6% inhibition, one tailed t test), and 18 at a significance level of $p<0.01$ (>11% inhibition) (table 2). The three most potent antagonists, Thr for Pro64 (64P→T), Thr for Glu65 (65E→T) 28 (5)%, and Val for Pro67 (67P→V), inhibited p57–73 QE65 responses by between 24% and 28% and remained statistically significant ($p<0.02$) after Bonferroni correction for multiple comparisons. Agonist activity of these three potent antagonists varied between 3 (5)% (65E→T) and 47 (6)% (64P→T). A peptide unrelated to p57–73 QE65 that is known to bind to HLA-DQ2 (TPO; Y00406 632–645Y) was inactive in the IFN- γ ELISPOT with CD PBMC but did significantly inhibit IFN- γ

Table 1 Inactive* variants of p57–73 QE65 with a single amino acid substitution

Substitution	Agonist (%)	Substitution	Agonist (%)
65E→P	−0.05 (3.5)	64P→Q	3.2 (5.21)
65E→Y	0.47 (1.23)	67P→Y	3.21 (2.63)
66L→R	1.29 (2.05)	65E→T	3.36 (4.53)
64P→K	1.58 (1.79)	66L→E	3.53 (4.18)
65E→K	2.01 (1.77)	66L→K	4.36 (3.12)
67P→K	2.96 (2.11)	64P→M	4.56 (5.36)

* Interferon γ ELISPOT response not significantly different from medium alone (Student's t test); $n=8$. Values are mean (SEM).

Figure 3 Agonist and antagonist activity against altered peptide ligand (ALP, single amino acid substituted variants) of p57-73 QE65. "Agonism" (x axis) of variant peptides is the mean interferon γ ELISPOT response, expressed as per cent of response to p57-73 QE65 (peptide concentration 50 μ g/ml) ($n=8$ subjects). "Antagonism" (y axis) is the mean interferon γ ELISPOT response of peripheral blood mononuclear cells incubated with variant peptide (50 μ g/ml) and p57-73 QE65 (10 μ g/ml), expressed as per cent of response to p57-73 QE65 (10 μ g/ml) ($n=10$ subjects).

ELISPOT responses to p57-73 QE65 (22 (7)%; $p<0.0003$). A second HLA-DQ2 binding peptide, HLA1 α (P30433 46-60; 50 μ g/ml) did not inhibit p57-73 QE65 IFN- γ ELISPOT activity.

Substitutions creating antagonists in p57-73 QE65 were only found in positions 62-69, and were especially abundant in three positions: 64, 65, and 67 (27/45, $p<0.05$; 14/17 $p<0.01$). The overlapping T cell epitopes (DQ2- α -I and DQ2- α -II) in p57-73 QE65 span p60-70. The sequence, p62-68, is common to DQ2- α -I and DQ2- α -II, but only 65E and 68Y are predicted to be HLA-DQ2 anchors for both epitopes, and only 64P is predicted to be a T cell receptor (TCR) contact for both epitopes.^{11, 18} Other residues in the sequence p62-68 are a TCR contact for one epitope and an HLA-DQ2 anchor for the other epitope. Hence variants of p57-73 QE65 with inhibitory activity were substituted at positions predicted to be purely anchors for HLA-DQ2 (65E), purely a TCR contact (64P), or a

TCR contact for DQ2- α -I and anchor HLA-DQ2 for DQ- α -II (67P).

As no single substitution of p57-73 QE65 created a potent antagonist measured by IFN- γ ELISPOT, the possibility of combining antagonists in a mixture or creating single peptides with two substitutions that individually created antagonists was explored. Single amino acid substituted variants with weak agonist activity ($<30\%$ p57-73 QE65 bioactivity) and antagonist activity were added together (each 50 μ g/ml) to PBMC preincubated with p57-73 QE65 (10 μ g/ml) and compared with individual single substituted peptides (50 μ g/ml) or peptides synthesised to incorporate two "equivalent" substitutions (50 μ g/ml). PBMC from 10 CD subjects not previously used in antagonism assays were studied.

Four single substituted peptides previously identified as antagonists were again effective antagonists using PBMC from 10 further subjects (table 2). Three variants of p57-73 QE65 with two compound substitutions retained antagonist activity but were non-agonists: 64P $\rightarrow$ W+67P $\rightarrow$ I, 64P $\rightarrow$ W+65E $\rightarrow$ T, and 64P $\rightarrow$ W+65E $\rightarrow$ I (table 3). However, in only 3/16 cases were peptides with two substitutions as potent as mixtures of two peptides each incorporating one of the amino acid substitutions, and inhibition of p57-73 QE65 IFN- γ ELISPOT responses was greater for cocktails of two antagonists than either antagonist alone for 15/16 pairs of single amino acid substituted peptides (see table 3). However, combinations of two antagonist single substituted peptides invariably possessed greater agonist activity than a single peptide encompassing two equivalent substitutions. The most potent inhibitory combinations were 64P $\rightarrow$ W with 65E $\rightarrow$ W (32 (8)%) and 64P $\rightarrow$ Y with 65E $\rightarrow$ W (32 (4)%), while alone 64P $\rightarrow$ W inhibited by 16 (5)%, 64P $\rightarrow$ Y by 21 (5)%, and WE $\rightarrow$ 65 alone was not inhibitory.

Hence a restricted set of substitutions in the core region of p57-73 QE65 produce peptides that are pure antagonists of p57-73 QE65 induced IFN- γ secretion from polyclonal T cells. The observation that antagonists result from substitutions in p57-73 QE65 at positions predicted to be both anchors for HLA-DQ2 and/or TCR contact residues, and that the antagonists are often without agonist activity suggests that substituted peptides are simply competing with p57-73 QE65 for binding to HLA-DQ2. Alternatively, if antagonist peptides are true APLs, it might be that they alter T cell activation and induce a TH2 cytokine response.

Table 2 Amino acid substitutions resulting in antagonists ($p<0.02$)

Substitution	Antagonism (%) [p value]	Agonism (%)	Substitution	Antagonism (%) [p value]	Agonism (%)
63Q $\rightarrow$ W	14 (3) [0.001]	38 (7)	67P $\rightarrow$ A	14 (5) [0.01]	29 (5)
64P $\rightarrow$ F	14 (4) [0.006]	8 (4)	67P $\rightarrow$ D	18 (4) [0.001]	25 (10)
64P $\rightarrow$ G	15 (5) [0.01]	18 (4)	67P $\rightarrow$ G	12 (3) [0.002]	13 (3)
64P $\rightarrow$ R	18 (5) [0.002]	18 (14)	67P $\rightarrow$ H	14 (5) [0.01]	15 (6)
64P $\rightarrow$ S	16 (3) [0.0004]	32 (7)	67P $\rightarrow$ I $\ddagger$	24 (6) [0.001]	24 (7)
64P $\rightarrow$ T*	28 (4) [0.0001]	47 (6)	67P $\rightarrow$ K	14 (6) [0.02]	3 (2)
64P $\rightarrow$ W $\ddagger$	21 (6) [0.004]	10 (6)	67P $\rightarrow$ V*	24 (4) [0.0001]	30 (7)
64P $\rightarrow$ Y $\ddagger$	17 (7) [0.01]	29 (11)	68Y $\rightarrow$ E	15 (6) [0.02]	33 (5)
65E $\rightarrow$ H $\ddagger$	18 (6) [0.01]	40 (6)	68Y $\rightarrow$ L	12 (4) [0.004]	43 (5)
65E $\rightarrow$ I $\ddagger$	20 (5) [0.001]	19 (5)	68Y $\rightarrow$ S	16 (5) [0.004]	58 (9)
65E $\rightarrow$ Q	13 (5) [0.02]	12 (2)	69P $\rightarrow$ G	13 (5) [0.01]	50 (6)
65E $\rightarrow$ R $\ddagger$	16 (6) [0.02]	7 (6)	69P $\rightarrow$ I $\ddagger$	10 (4) [0.01]	57 (22)
65E $\rightarrow$ S	15 (3) [0.001]	48 (5)	69P $\rightarrow$ L	17 (7) [0.02]	31 (3)
65E $\rightarrow$ T $\ddagger$	28 (5) [0.0001]	3 (5)	69P $\rightarrow$ N	14 (6) [0.02]	46 (10)
65E $\rightarrow$ W	17 (6) [0.01]	8 (4)	69P $\rightarrow$ Q	12 (4) [0.01]	35 (5)
66L $\rightarrow$ G	11 (4) [0.01]	6 (4)			

Values are mean (SEM).

*Significant at $p<0.02$ after Bonferroni correction for multiple comparisons (131).

$\ddagger$ Antagonism significant ($p\leq 0.005$) when assessed in a second sample of 10 CD subjects with coeliac disease (CD).

$\ddagger$ Antagonism not significant ($p>0.05$) when assessed in a second sample of 10 CD subjects.

Table 3 Antagonist and agonist activity of double substituted or mixtures of single substituted variants of p57-73 QE65

Double substitution	Antagonism (%) [p value]	Agonism (%)	Single substitution combination	Antagonism (%) [p value]	*Agonism (%)
64P→W,65E→H	20 (8) [0.02]	1 (4)	64P→W+65E→H	28 (5) [0.00008]	25 (11)
64P→W,65E→I	28 (6) [0.0003]	2 (5)	64P→W+65E→I	28 (5) [0.0003]	26 (10)
64P→W,65E→R	13 (9) NS	-1 (2)	64P→W+65E→R	26 (9) [0.01]	3 (3)
64P→W,65E→T	28 (5) [0.0004]	-1 (3)	64P→W+65E→T	30 (4) [0.00003]	20 (7)
64P→W,65E→W	12 (6) [0.04]	1 (4)	64P→W+65E→W	32 (8) [0.001]	16 (6)
64P→W,67P→I	29 (5) [0.0002]	5 (8)	64P→W+67P→I	22 (7) [0.006]	25 (6)
64P→Y,65E→H	7 (7) NS	7 (10)	64P→Y+65E→H	17 (6) [0.008]	22 (7)
64P→Y,65E→I	9 (5) [0.049]	2 (5)	64P→Y+65E→I	25 (5) [0.0003]	25 (4)
64P→Y,65E→R	4 (7) NS	2 (5)	64P→Y+65E→R	26 (5) [0.0003]	7 (2)
64P→Y,65E→T	18 (5) [0.003]	12 (15)	64P→Y+65E→T	13 (5) [0.01]	17 (7)
64P→Y,65E→W	10 (4) NS	3 (6)	64P→Y+65E→W	32 (4) [0.00001]	7 (1)
64P→Y,67P→I	11 (5) [0.04]	2 (5)	64P→Y+67P→I	15 (8) [0.05]	22 (5)
65E→H,67P→I	-2 (5) NS	3 (6)	65E→H+67P→I	12 (5) [0.02]	31 (5)
65E→I,67P→I	-10 (4) NS	4 (7)	65E→I+67P→I	11 (5) [0.02]	17 (6)
65E→R,67P→I	-3 (5) NS	2 (5)	65E→R+67P→I	12 (6) [0.04]	17 (3)
65E→T,67P→I	-1 (5) NS	3 (6)	65E→T+67P→I	6 (6) NS	16 (5)
65E→W,67P→I	-7 (3) NS	1 (4)	65E→W+67P→I	18 (5) [0.002]	11 (6)

NS, not significant by paired Student's *t* test.

Antagonism is per cent reduction in interferon γ (IFN- γ) ELISPOT responses to p57-73 QE65 (10 μ g/ml) alone compared with coincubation with substituted peptide (50 μ g/ml).

Agonism is IFN- γ ELISPOT response to double-substituted peptide (50 μ g/ml) or two single substituted peptides (each 50 μ g/ml) compared with p57-73 QE65 (50 μ g/ml). (*Agonism of single substituted peptide combinations was assessed in eight subjects separately from other assays.)

Values are mean (SEM); *n* = 10 subjects.

To test whether TH2 associated cytokines were preferentially stimulated by antagonist peptides, IL-4, IL-5, IL-10, and IFN- γ ELISPOT assays were performed in parallel using PBMC collected from 25 HLA-DQ2+ CD subjects six days after commencing gluten challenge.

Deamidated gliadin stimulated IL-4, IL-5, and IL-10 from PBMC, similar in magnitude to tetanus toxoid (fig 4). Deamidation enhanced IL-4, IL-5, and IL-10 ELISPOT responses to gliadin as we have previously shown for IFN- γ ELISPOT responses in PBMC after gluten challenge. IL-10 ELISPOT responses to deamidated gliadin (100 μ g/ml) were present (>3 times medium alone) in PBMC from 19/25 subjects (median 178 (range 13–1039) in the 19 responders) while IL-4 ELISPOT responses were present in only 9/25

subjects (8–88 SFU/million) and IL-5 responses in 13/25 subjects (7–91 SFU/million). IL-10 responses were consistently greater than IL-4 and IL-5 responses. ELISPOT detected IL-10 responses to p57-73 QE65 in PBMC from 11/25 subjects (median 15 SFU/million (range 0–48)). Relative to the contribution made by p57-73 QE65 to the IFN- γ ELISPOT response (median 64% (range 0–270)) to deamidated gliadin, the IL-10 response to p57-73 QE65 was modest (median IL-10 ELISPOT response to p57-73 QE65 was 11% of the response to deamidated gliadin (range 0–112); *n* = 15).

Altered peptide ligands of p57-73 QE65 with antagonist activity but lacking IFN- γ stimulatory activity often retained IL-10 stimulatory activity only slightly weaker than p57-73 QE65. IL-4 and IL-5 ELISPOT responses to substituted

Figure 4 Cytokine ELISPOT responses (spot forming units/ 10^6 peripheral blood mononuclear cells) to gliadin (100 μ g/ml) (Gli) alone, gliadin after deamidation by transglutaminase (Gli+ITG), transglutaminase (ITG) (10 μ g/ml) alone, p57-73 QE65 (50 μ g/ml), or tetanus toxoid (TT, 10 light forming units/ml) (A–D), or to substituted variants of p57-73 QE65 (50 μ g/ml) that antagonise p57-73 QE65 stimulated interferon γ ELISPOT responses (64P→T[64T], 64W, 64Y, 65T, 67V) and have minimal interferon γ stimulatory activity (64W65I, 64W65T, and 64W67I), or do not have interferon γ stimulatory activity (65P and 65Y) (E–H) using peripheral blood mononuclear cells collected six days after commencing gluten challenge (each point is an individual subject; *n* = 20 for interferon γ ELISPOT; all others *n* = 25).

peptides were uncommon and weak (<10 SFU/million), similar to those induced by p57–73 QE65.

DISCUSSION

Molecular modification of T cell epitopes in gluten to make useful therapies and guide design of non-toxic gluten requires detailed understanding of the polyclonal T cell response to gluten. The diversity of TCR V α and V β genes of gliadin specific T cell clones¹⁹ suggests that studies based on individual T cell clones are unlikely to be informative or enabling in design of therapeutic or non-toxic peptides. In addition, the dominant stimulatory peptide in A-gliadin, p57–73 QE65, includes two distinct overlapping epitopes ensuring that at least two populations of T cells respond to this peptide.¹¹ Despite these challenges, gliadin specific T cells, whether cloned from intestinal biopsies¹¹ or present in peripheral blood after gluten challenge,¹³ are nearly all HLA-DQ2 restricted in HLA-DQ2+DQ8– individuals with CD, implying common requirements for amino acids that are HLA-DQ2 anchors in positions 1, 4, 6, 7, and 9.²⁰

Fine molecular mapping of p57–73 QE65 indicates four residues, p64–67 (PELP), corresponding to anchors for HLA-DQ2 and/or TCR contacts for the two overlapping epitopes, DQ2- α -I (PFPQPELPY) and DQ2- α -II (PQPELPYPQ), are particularly sensitive to substitution. Other positions within p60–70 (PFPQPELPYPQ), but not 57Q, 58L, 72Q, or 73S, are sensitive to particular substitutions, consistent with p60–70 activating T cells with specificities corresponding to either DQ2- α -I or DQ2- α -II. Residues flanking DQ2- α -I and DQ2- α -II that lie outside the HLA-DQ2 binding groove (59Q at position –1 for DQ2- α -I, and 71P at position +10 for DQ2- α -II) were also modestly sensitive to certain substitutions (Glu and Ala, respectively).

The crystal structure of HLA-DQ2 bound to DQ2- α -I, the less active of the two epitopes in p57–73 QE65, has been reported.¹⁸ The positive electrostatic effect of Lys β 71 in the DQB1*02 chain of the HLA-DQ2 heterodimer affects binding with positions 4 (63Q), 6 (65E), and 7 (66L) in DQ2- α -I and explains the loss of bioactivity associated with Arg or Lys substitutions of 63Q, 65E, 66L, 67P, and 68Y (corresponding to positions 4, 6, and 7 in DQ2- α -I and DQ2- α -II). While the crystal structure of DQ2- α -I and HLA-DQ2 indicates that the side chain of the amino acid at position 9 in DQ- α -I (68Y) does not interact with the binding groove, our finding that 70Q (position 9 for DQ2- α -II) substituted with bulky hydrophobic amino acids (Trp [W] or Tyr [Y]) is more bioactive than Gln and nearly 50% more bioactive than Pro suggests that the interactions of HLA-DQ2 with DQ2- α -I and II may be subtly different.

It is of interest that a traditional approach to design of APL antagonists using alanine substitutions generated only one antagonist sequence for p57–73 QE65 (67P→A) using PBMC collected after gluten challenge, yet a series of alanine substitutions have been reported to abolish IFN- γ production and create antagonists for a DQ2- α -II specific intestinal T cell clone.²¹ In a separate study of three DQ- α -I specific intestinal T cell clones,²² proliferative responses to the 64P→Q substituted variant (QLQFPQQLPY) of p57–68 QE65 varied from none to the same as the unmodified epitope. In contrast, 64P→Q elicited 3% of the IFN- γ ELISPOT response induced by p57–73 QE65 and was not significantly different from medium alone in PBMC from eight subjects. Clearly, the polyclonal p57–73 QE65 specific T cell population present in PBMC after gluten challenge in a range of individuals is a more complex system than a homogeneous T cell clone, yet only in vivo challenge studies can establish the non-toxicity of peptides and these are impractical for more than a few peptides.

For PBMC, substitutions of p57–73 QE65 that created antagonists were unpredictable, except that they tended to be weak agonists and none created potent antagonists for IFN- γ production in vitro. Four peptides with single (65E→T) or double substitutions were non-agonists for IFN- γ but retained the capacity to stimulate IL-10 and reduced p57–73 QE65 stimulated IFN- γ secretion. Substitutions of HLA-DQ2 anchors (for example, 65E→T) that produce antagonists without stimulating IFN- γ are potentially of great interest as they may have general application to HLA-DQ2 restricted gluten epitopes with glutamate at anchor positions 4 and 6.

Rather than design non-toxic grains by deleting substantial segments of gliadin proteins containing sequences resembling or corresponding to T cell epitopes, the cooking properties of gluten proteins are more likely to be retained by minimal modification to only a small number of key residues. Substitution with cationic amino acids, arginine, or lysine, predictably abolishes the bioactivity of p57–73 QE65 more effectively than other functional groups of amino acids, yet these amino acids are unusual in glutamine, proline-rich regions of gliadins and it may be that the functionality of gliadin proteins is adversely affected by such modifications. Alternatively, threonine substitution for glutamine at positions predicted to be deamidated by transglutaminase (Q followed by P at position +2 and/or hydrophobic amino acids at position +3)²³ may be less disruptive to gliadin functionality as serine and threonine are found as naturally occurring substitutions for proline in glutamine, proline-rich regions of gliadins and may produce immunologically inactive or even inhibitory peptides. T cell epitopes in A-gliadin have already been mapped⁹ and only one sequence, p57–73 QE65, predictably stimulates IFN- γ using PBMC after gluten challenge in CD subjects and hence A-gliadin may be a useful protein for initial efforts to design “non-toxic” gluten proteins.

It is intriguing that deamidated gliadin is a potent stimulus for IL-10, yet the dominant epitope, p57–73 QE65, is a rather weak stimulus for IL-10, raising the possibility that certain gliadin epitopes may stimulate T cell secretion of cytokines skewed towards IL-10 rather than IFN- γ . It may be that mapping T cell epitopes using the IL-10 ELISPOT could identify a hierarchy of peptides different to those defined using the IFN- γ ELISPOT.

This study describes a systematic approach to the discovery of non-toxic and antagonist peptides derived from the dominant HLA-DQ2 restricted gliadin T cell epitope in CD. Such peptides may be useful in modifying grains and producing functional non-toxic gluten, and offer a general principle for design of APL antagonists in CD.

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Conflict of interest: declared (the declaration can be viewed on the Gut website at <http://www.gutjnl.com/> supplemental).

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EDITOR'S QUIZ: GI SNAPSHOT

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Answer

From question on page 462

Computed tomography of the abdomen showed diffuse calcifications of the aorta (A in fig 2) and other major arterial branches, with limited progression of the oral contrast to the proximal jejunum only. Far more impressive pathologies however were the dilated loops of small bowel with absence of intravenous contrast enhancement but diffuse pneumatosis intestinalis (air in the bowel wall, arrowheads in right panel) as well as the widespread air in the portal vein system (arrowheads in left panel).

These findings were consistent with massive intestinal ischaemia with diffuse bowel necrosis and venous translocation of gas, in its extent not compatible with life.

The fully alert patient and her family had to be informed about the shortly anticipated death. To rule out a less than fatal degree of intestinal ischaemia, a small diagnostic laparotomy was performed which confirmed the incurable extent of the necrosis. The patient was extubated, obtained best palliative care, and expired after several hours.

Figure 2 Mesenteric ischaemia announcing death (see text). A, aorta.