Glutenin is involved in the gluten-driven mucosal T cell response

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Gluten ingestion causes coeliac disease in susceptible individuals. Gluten is a heterogeneous mixture of glutenin and gliadin, the latter of which is considered responsible for disease induction. By combining high-performance liquid chromatography purification steps of gluten with a T cell bioassay and mass spectral analyses, we have identified a glutenin peptide (glt04 707–742) that activates T cells from the small intestine of a coeliac disease patient and results in the secretion of large amounts of IFN-γ. The minimal T cell stimulatory core of the peptide (residues 724–734) is repetitively present in glutenin molecules. Moreover, it was observed that a large number of naturally occurring variants of this peptide are recognized by the T cells. These data suggest that the large heterogeneity of glutenin proteins dramatically increases the number of available T cell epitopes. Together, the results provide new insight into the nature of the gluten antigens that lead to coeliac disease and suggest that glutenin, next to gliadin-derived antigens, may be involved in the disease process.

Key words: Coeliac disease / Small intestine / HLA-DQ8

1 Introduction

Coeliac disease (CD) results from the ingestion of gluten in sensitive individuals (for a review see [1]). The identity of the peptide antigens that lead to the typical flat mucosa in the proximal small intestine of CD patients, however, remains largely unclear [2]. This is in part due to the complex composition of gluten antigens, which has hampered the analysis of individual components. The major protein fractions of gluten are the Q- and P-rich glutenin and gliadin molecules, and early investigations have suggested that the alcohol-soluble gliadin fraction contains the factor that is toxic for coeliac patients [3]. In support of this, both in vivo [4–6] and in vitro [7–9] studies using relatively pure gliadin fractions or synthetic gliadin peptides have shown that gliadin can indeed induce morphological changes in the gut. Since no such studies have been performed using glutenin-derived peptides, its role in the disease process is unclear. The presence of high titers of anti-glutenin antibodies in sera of a subset of CD patients [10–12] is indicative of the presence of glutenin-specific T helper cells, and suggests that glutenin may, like gliadin, be involved in the aberrant immune response against gluten. [I 18981]

Abbreviations: CD: Coeliac disease tTG: Tissue transglutaminase TCC: T cell clone

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from the same patient. Briefly, HPLC purification steps of a gluten preparation were combined with a T cell bioassay (Fig. 1). Several peptide fragments in the most active T cell stimulatory fraction were individually subjected to tandem mass spectral analyses to obtain amino acid sequence information. The identity of several peptide peaks in the active fraction was elucidated by combining partial amino acid sequence information with database searching (not shown). Synthetic versions of the identified peptide sequences were tested in a T cell proliferation assay which demonstrated that the stimulatory activity resided in a glutenin fragment (glt04 residues 707–742, Swiss Prot. Accession Number P08489, sequence SGQGQRPGWLPQQGQQYYPSTSPQQ- SGQGQQLGQ) (not shown).

Next, we determined the core peptide sufficient to induce optimal proliferation of TCC S12. First, overlapping 18-mer peptides spanning the glutenin 707–742 fragment were tested (Fig. 2A). Further N- and C-terminal truncations of the 18-mer glutenin peptide 719–736 demonstrated that the minimal epitope required for optimal T cell stimulation for TCC S12 comprises residues 724–734 (QGYYPTSPQSQ, referred to as “glt peptide”) (Fig. 2B). Based on the published peptide binding motifs for DQ8 [23, 24], Q724 is to be expected at relative position p1 and residue Q732 at p9. This was supported by molecular modelling (not shown).

2.2 Effect of deamidation of glt-specific recognition

Recently, it was found that the enzyme tissue transglutaminase (tTG) enhances gluten-specific T cell reactivity due to selective deamidation (the conversion of a Q to E) of gliadin epitopes [25, 26]. This appears to be a common phenomenon since tTG was found to enhance T cell reactivity of T cell clones from several individuals [25, 26]. In addition, the reactivity of polyclonal gluten-
responsive T cell lines from patients is increased by a factor of 3 to 10 due to the treatment of gluten with tTG (not shown). In the T cell line from which the glutenin-specific TCC S12 was isolated, tTG treatment resulted in the increase of T cell reactivity with a factor of 4. We therefore wished to determine the effect of tTG on recognition of the glutenin epitope. The glt peptide was treated with tTG for 2 h and subsequently tested with TCC S12. In contrast to what was found for two gliadin-specific TCC and several additional gluten-specific TCC with unknown peptide specificity [25, 26], a marked decrease in T cell proliferation was found upon tTG treatment of the glutenin epitope (Fig. 3). We also evaluated whether tTG had an effect on the T cell stimulatory capacity of the original glutenin sequence identified (residues 707–742). The results indicate that tTG does not influence the recognition of this peptide (Fig. 3). Mass spectrometric analysis indicated that in the short version of the peptide both the p1 and p9 Q residues were partly converted to E (not shown). In contrast, no Q residues were modified in the longer version of the peptide (not shown), indicating that the activity of tTG is not only dependent on the primary sequence of the peptide. To further evaluate the effects of Q to E substitutions, analogs of the glt peptide in which individual Q residues were replaced by an E were tested for their T cell stimulatory capacity. A slight increase in antigenicity was found for the p1 Q724 to E analog. However, the p0 Q723 and the p10 Q733 to E analogs were less efficiently recognized compared to the wild-type peptide, whereas the introduction of an E at p9 (Q730) of the peptide almost completely abolished proliferation of TCC S12 (Fig. 4). In conclusion, Q > E substitutions in the glt peptide decrease rather than increase its antigenicity. This likely explains the observation that tTG treatment of the (short version of the) glt peptide results in a decrease in T cell stimulatory capacity.

2.3 T cell responses of TCC S12 towards a set of natural glt homologs

Database similarity searches with the glt peptide sequence resulted in the identification of homologs within 32 glutenin variants, differing at either one or two positions from the glt peptide. Such peptide sequences are repeated up to 16 times within one glutenin protein. To evaluate potential T cell cross-reactivity against the naturally occurring sequences, the homologous glutenin sequences were tested for their T cell stimulatory capacity. Nineteen of the 32 glutenin sequences induced proliferation of TCC S12 (Table 1). Natural variants carrying amino acids other than the glt peptide at positions 725, 729, 730 and 731 did not or only marginally stimulate TCC S12. These results are in line with those obtained after single amino acid substitution analysis, where the residues G725, Y729, P730 and P731 appeared to be indispensable for T cell recognition (not shown). The critical role of the P residues at position 730 and 731 likely reflects a function of this amino acid in the maintenance of the correct conformation of the peptide in the binding groove. The stimulation of TCC S12 with the glutenin peptide and its analogs not only resulted in the induction

![Figure 3](image1.png)

**Figure 3.** Effect of tTG treatment of the glt peptide on T cell recognition. T cells (TCC S12; 10⁴ cells) were tested against a concentration range of two length variants of the glutenin peptide (short version: QQGYYPTEPQDQG, long version: SGQGQRPGQWLOPGQGQGQGQYYPTEPQDQGQGQQLGQ) that were either untreated with tTG (−tTG) or treated with tTG (+tTG) in the presence of 10⁸ irradiated (3000 rad) PBMC from an HLA-DQ2/DQ8-positive donor as APC.

![Figure 4](image2.png)

**Figure 4.** Q to E substitution analysis of the glt peptide. T cells (TCC S12; 10⁴ cells) were stimulated with 10⁵ PBMC (3000 rad) from an HLA-DQ8-positive donor in the presence of several concentrations of the indicated peptide sequences. Amino acids are shown as single-letter codes. Values show mean cpm (× 10⁻³) of triplicate cultures. SD is < 10 %. Residues at position 724 and 732 are the predicted p1 and p9 anchor residues.
Table 1. T cell responses of TCC S12 towards a set of naturally occurring glutenin 722–736 homologs. T cells (10^4) were stimulated with 10^5 PBMC (3000 rad) from an HLA-DQ8-positive donor in the presence of 13.4 μg/ml of the indicated peptides. Amino acids are shown as single-letter codes. Amino acids displayed in bold differ from the residues of the original glt sequence (GQQGYYPQTPQSQSQ). Stimulation with the latter peptide resulted in 42,500 counts. Values show mean cpm of triplicate cultures. SD is < 10%. Values displayed in bold are considered positive.

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of proliferation but also in the production of relatively large amounts of IFN-γ (up to 600 ng/ml), a cytokine that has been implicated in the process of tissue destruction in the gut [27, 28]. The levels of IFN-γ production correlated well with the levels of T cell proliferation induced by the glutenin peptide and its analogs (not shown).

3 Discussion

CD or gluten-sensitive enteropathy is characterized by small intestinal mucosal injury and malabsorption [1]. In support of a role of T cells in the pathogenesis, Lundin et al. [18] have first demonstrated that gluten-specific T cells can be isolated from small intestinal biopsy specimens of patients. So far, two intestinal gluten-derived T cell epitopes have been identified, both of which represent gliadin fragments [21, 22]. We now report on the identification of a glutenin-derived epitope that is recognized by DQ8-restricted T cells from the small intestine of a CD patient.

The gluten epitope is located in domain B of the high molecular weight glutenin subunit glt04 (residues 707–742). The minimal epitope comprises residues 723–735 and contains the repetitive QQQYPTS motif. Based on the minimal determinant, 32 highly homologous versions were identified in naturally occurring glutenin variants, 19 of which were found to be recognized by TCC S12. The promiscuous recognition of the homologous variants together with the presence of multiple copies of these epitopes within one glutenin molecule (up to 16 repeats) indicate that the antigenic sequences are available at a relatively high concentration in wheat products.

To investigate whether the glutenin peptide is also recognized by T cell lines from other CD patients, we have tested T cell lines from seven additional patients, two from adult patients and five from recently diagnosed children. These cell lines were tested against the glutenin peptide and its Q→E analogs. No T cell reactivity was found in the T cell lines derived from the children. In contrast, one of the cell lines from the adult patients responded to the glutenin peptide but not the Q→E analogs (stimulation index to glutenin peptide: 3.5; to gluten: 10; background cpm: 1500). These results indicate that the glutenin peptide could be a more common T cell epitope. The lack of reactivity in the T cell lines from the children, however, may indicate that this peptide may be a secondary target for mucosal T cells. This will be the subject of further investigations.

The association of CD to DQ2 [13, 14] and DQ8 [15–17] and the dominant restriction of mucosal gluten-specific T cells by these DQ alleles [18–20] strongly suggest that the HLA association reflects a preferential binding and presentation capacity of the disease-associated molecules with respect to gluten-derived peptides. As discussed previously [22, 29], both DQ2 [30–32] and DQ8 [23, 24] prefer the presence of negatively charged residues at several positions in the bound peptide. This appears to be an important characteristic of these DQ molecules with respect to gluten sensitivity: since gluten proteins consist of large amounts of Q residues, deamidation (the conversion of a Q to an E) potentially results in the presence of relatively large amounts of negatively charged (E) residues in gluten-derived peptides.
Recently, we and others [25, 26] have shown that tTG is capable of selective deamidation of Q residues in gluten-derived peptides. Interestingly, tTG treatment of two defined gliadin epitopes was found to strongly enhance both DQ2- and DQ8-restricted T cell reactivity, presumably by increasing the affinity of the antigens for the DQ molecules. Since tTG represents the target for the anti-endomysial antibodies that are characteristic for CD [33], these results suggest a link between the gluten-specific cellular and the tTG-directed humoral response in CD. In contrast to the gliadin epitopes tTG treatment of the glt peptide was found to decrease T cell reactivity, which correlates with the finding that most of the Q to E substitutions in the glt peptide are negatively involved in the T cell response. Only at p1 (Q724), the Q to E substitution led to a modest increase in antigenicity, which is likely due to the preference for negatively charged or polar residues at p9 of DQ8-bound peptides [31, 32]. It should be noted, however, that the DQ8 peptide binding motif also favors negatively charged residues at p9 by virtue of a supposed interaction with the positively charged R at position 79 of the DQα chain. Therefore, also the glt P9 (Q723) Q to E analog is predicted to bind with high affinity to DQ8. As only marginal T cell activation was found for the p9 Q to E analog, the results suggest that the glt p9 Q to E substitution directly affects T cell recognition, as is sometimes observed for anchor residues [34–36]. Thus, for the present glt epitope, we observed that deamidation decreases rather than increases the glutenin-specific T cell response. Additional epitopes need to be determined to reveal whether the destruction of epitopes due to tTG-induced deamidation only accounts for glutenin-, and not for gliadin-derived peptides.

In conclusion, this study for the first time demonstrates that, next to gliadin-derived antigens, glutenin-derived peptides are involved in gluten-specific T cell responses. These glutenin-specific T cells may, like gliadin-specific T cells, contribute to the disease pathogenesis by the secretion of large amounts of IFN-γ. Our findings indicate that the role of glutenin in the disease process may have to be re-evaluated. Further research should aim to establish whether the glutenin epitope can induce intestinal morphological changes that are characteristic of CD.

4 Materials and methods

4.1 Antigens and peptides

A pepsin/trypsin digest of gluten was prepared as described [22]. Peptides were synthesized by standard 9-fluorenylmethoxycarbonyl chemistry on a multiple peptide synthesizer (Abimed AMS 422). tTG treatment was performed by incubating the glutenin 723–735 peptide with tTG (Sigma; T-5398) at a concentration of 500 and 100 μg/ml, respectively, at 37 °C for 2 h in PBS with 1 mM CaCl₂.

4.2 Isolation of gluten-specific T cell lines

TCC S12 (CD3⁺, CD4⁺, TCRα/β⁺) was isolated from a small intestinal biopsy specimens of the HLA-DR3/4, DQ2/8-positive, Dutch CD patient S as described [22]. In proliferation assays in which matched and mismatched APC were used it was found that the TCC responded to stimulation with a trypsin/pepsin-treated gluten preparation in the presence of HLA-DQ8-positive APC only. Moreover, the response could be blocked with DQ-specific antibodies. Polyclonal gluten-specific T cell lines from seven additional CD patients (all DQ2 positive, one DQ2/8 positive) were generated by co-culture of small intestinal biopsy specimens with the trypsin/pepsin-treated gluten preparation. After one round of restimulation with the gluten preparation in the presence of autologous PBMC the cells were expanded with IL-2, tested for specificity and frozen until further use. All patients gave informed consent to the study, which was approved by the hospital ethics committee.

4.3 T cell proliferation assays

Proliferation assay were performed in duplicate in 150 μl culture medium [RPMI1640 (Gibco), containing 100 % human serum] in 96-well-flat-bottom plates (Falcon) using 10⁴ T cells stimulated with 10⁵ irradiated PBMC (3000 rad) in the absence or presence of antigen at the indicated concentrations. After 48 h, cultures were pulsed with 0.5 μCi [³H]thymidine and harvested 18 h later.

4.4 IFN-γ production

IFN-γ production was measured by ELISA in supernatants of antigen-stimulated T cells. T cells (0.25 × 10⁶), were stimulated with 2 × 10⁵ irradiated PBMC (3000 rad) in the absence or presence of peptide at a concentration of 10 μg/ml. Assays were performed in 150 μl culture medium [RPMI1640, containing 10 % FCS (Gibco)] in 96-well round-bottom plates (Falcon).

4.5 HPLC purification of the pepsin/trypsin digest of gluten

Approximately 1 mg of an enzymatic digest of gluten was fractionated via micro-reversed-phase HPLC (SMART system, column C2/C18, sc 2.1/10, Pharmacia) using an acetonitrile gradient from 0 to 70 % (flow rate 100 μl/min, 2 % increase acetonitrile/min, containing 0.1 % trifluoroacetic acid).
4.6 Mass spectrometry

Electrospray ionization mass spectrometry was performed on the most abundant peaks present in the bioactive HPLC fraction using a Quadrupole Time of Flight (Q-TOF) hybrid mass spectrometer (Micromass, Manchester, GB) as described [22]. Briefly, precursors were selected with the quadrupole and fragments were collected with high efficiency with the orthogonal time of flight mass spectrometer. The collision gas applied was argon (pressure 4 × 10⁻⁵ mbar) and the collision voltage approximately 30 V.

4.7 Database searching

The program PeptideSearch was used for sequence elucidation. This program has been developed to identify sequences in databases using mass spectral information [37]. Database similarity searches were done on the basis of a selected subset of wheat proteins from the Swiss Prot database.

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