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Antigen Presentation to Celiac Lesion-Derived T Cells of a 33-Mer Gliadin Peptide Naturally Formed by Gastrointestinal Digestion

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Celiac disease is a chronic inflammatory disease of the small intestine caused by the ingestion of proline- and glutamine-rich wheat gluten (consisting of the gliadin and glutenin subcomponents) or related proteins from rye and barley. The lesion is characterized by villous atrophy, crypt hyperplasia, and increased number of infiltrating lymphocytes in both epithelium and lamina propria. Celiac disease has a strong HLA association; ~90% of the patients are HLA-DQ2, and the majority of the remaining patients are HLA-DQ8 (1). Gluten-reactive CD4+ T cells can be readily isolated from the small intestine of celiac disease patients, but not of controls, and such gluten-reactive T cells are exclusively restricted by the disease-associated DQ2 or DQ8 HLA molecules. The activation of gluten-reactive T cells probably represents a key event in disease development. Gluten-reactive T cells from celiac lesions predominantly recognize gluten peptides in which glutamine residues at certain positions have been converted to glutamic acid by tissue transglutaminase (TG2)-mediated deamidation (1). The negative charges introduced by TG2 generally increase the binding affinity of the gluten peptides to DQ2 (2, 3).

Several different celiac T cell epitopes derived from gliadin proteins have been identified during the last few years (2, 4–7), and these epitopes cluster in the proline-rich regions of the proteins (5). The epitopes identified in α-gliadins appear to be particularly important because they are recognized by intestinal T cells of the majority of adult celiac patients (2). By mimicking the enzymatic gastrointestinal digestion of a representative α-gliadin (α2-gliadin), Shan and coworkers (8) found a 33-mer (LQLQQPFQQLYPQQLYPQQLYPQPF; α2-gliadin 56–88) to be particularly interesting. This fragment was resistant to further breakdown by luminal proteases and intestinal brush-border enzymes due to its high proline content (13 of 33 residues are proline). Moreover, it showed strong type II polyproline helical conformation in solution; the same conformation that is adopted by peptides bound to MHC class II molecules (9). The 33-mer contains six partly overlapping copies of three DQ2-restricted T cell α epitopes [Fig. 1], and the deamidated 33-mer was found to be an extremely potent T cell stimulator, several-fold more potent than any other known gluten peptide (8). Given the fact that this highly immunostimulatory peptide is an end product of the gastrointestinal proteolytic digestion and given its central role in the pathogenesis of celiac disease, we aimed to characterize its binding to the disease-associated DQ2 molecule and the mechanism of its presentation to T cells.

Materials and Methods

Peptide synthesis, gliadin protein Ag, and GST-TG2 production

The γ1 peptides YQQLPPOEQPQSQFPEGRPF (γ-gliadin Y134–153) and qPEQQQSF were posttranslationally converted from Gln residues.

4 Abbreviations used in this paper: TG2, tissue transglutaminase; B-LCL, EBV-transformed B lymphoblastoid cell line; DC, dendritic cell; HMWF-PT-gliadin, high m.w. fraction of PT-gliadin; Hsp, heat shock protein; MB, Mycobacterium bovis; PT-gliadin, peptic trypsin-treated gliadin; TCC, T cell clone.
FIGURE 1. The 33-mer and its epitopes. The 33-mer (α2–gliadin 56–88) contains, in total, six overlapping copies of three DQ2-restricted T cell epitopes. Three glutenine residues (underlined), which we predict to be primarily selected for deamidation by TG2 (10, 11), were converted to glutamic acid in the synthetic deamidated 33-mer.

**Results**

The 33-mer has a relatively high binding affinity to DQ2

Binding of the 33-mer to DQ2 was assessed in a competitive inhibition assay at pH 4.9 using an 125I-labeled MB 65 kDa Hsp DR3/DQ2 homozygous individual (CD114) was cultured in RPMI 1640 with 10% FCS (both from Invitrogen Life Technologies, Carlsbad, CA).

The DC were prepared from PBMC of DR3/DQ2-positive blood donors.

Monocytes were positively selected from the PBMC with anti-CD14 mAb-coated MicroBeads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) and then cultured in RPMI 1640 with 10% FCS containing 1000 U/ml GM-CSF and 500 U/ml IL-4 (both from R&D Systems, Abingdon, U.K.). On day 6, some DC were matured with 150 ng/ml LPS (Sigma-Aldrich) for 48 h, and the rest were kept immature in the GM-CSF/IL-4 medium. Both immature and mature DC were used as APC on day 8, and both cell sets were >95% CD11c+, as ascertained by flow cytometry. The mature DC expressed CD83 and showed up-regulated expression of CD86 and HLA class II molecules compared with the immature DC.

**T cell assays**

For use in T cell assays, HMWF–PT gliadin and the 33-mer were preincubated with 200 μg/ml GST-TG2 in PBS with 2 mM CaCl2 for 2 h at 37°C. In glutaraldehyde fixation of the APC, B–LCL or DC were washed with serum-free RPMI 1640 and incubated with 0.05% glutaraldehyde in RPMI 1640 for 90 s at 22°C before 0.2 M glycine were added for an additional 60 s. The cells were then thoroughly washed. Live B–LCL were irradiated (75 Gy). Fifty thousand fixed or live B–LCL or 20,000 fixed or live DC were coincubated overnight at 37°C in U-bottom, 96-well plates with various concentrations of Ags in RPMI 1640/10% FCS. After the incubation, the APC were washed twice with cold RPMI 1640 to remove unbound Ags before 50,000 gliadin-specific T cells were added to each well. The final part of the T cell assay was performed in RPMI 1640 with 15% heat-inactivated, pooled human serum. The following T cell clones (TCC) were used: TCC387.E9, TCC430.1.142 and TCC423.1.3.8. TCC387.E9 and TCC430.1.142 both recognize the DQ2–epitope (9 mer core region PPQFPPELPY), and the closely related α-III epitope (9-mer core region PYPPQP). TCC423.1.3.8 recognizes the DQ2–epitope (9-mer core region PQQSFPQPE). All these TCC are derived from intestinal biopsies of celiac disease patients and are DQ2 restricted (17). T cell proliferation was measured by the uptake of 1'H[thymidine (1 Ci/well) (0.037 MBq/well); Hartmann Analytic, Braunschweig, Germany), which was added 24 h before harvesting. Cells were harvested after 72 h onto glass-fiber filter paper with an automated harvester (Matsch TEC, Hamden, CT), and [1'H]thymidine incorporation was measured by liquid scintillation counting (Wallac MicroBeta TriLux 1450; PerkinElmer, Wellesley, MA). Each Ag concentration was studied in triplicate. At least two independent experiments were performed for each assay.

**Detergent-solubilized DQ2 molecules and peptide binding assays**

Detergent-solubilized DQ2 molecules were purified from DQ2 homozygous, EBV-transformed, B lymphoblastoid cell lysates as described previously (14). The Mycobacterium bovis (MB) 65-kDa heat shock protein (Hsp)-derived peptide (KPLIIAQDVEDVEY; MB 65 kDa Hsp 243–255Y), the synthetic deamidated 33-mer, and the 33-mer deamidated by TG2 were 125I-labeled by the chloramine-T method (15). Labeled peptides (30,000 cpm; 1–5 nM) were incubated with DQ2 (1 μM for both deamidated 33-mer variants or 300 nM for MB 65 kDa Hsp 243–255Y) overnight at 37°C in the presence of a mixture of protease inhibitors (14). In the competitive inhibition assays, various concentrations of unlabeled peptides were added to inhibit binding of the isolate-labeled MB 65 kDa Hsp 243–255Y peptide. DQ2 molecules and peptides were separated on Sephadex G-50 Superfine (Pharmacia Biotech) mini spin columns as described previously (16). The radioactivity was counted, and the concentrations of the competing peptides required to give 50% inhibition of binding of the indicator peptide (IC50) were calculated.

**APCs**

An EBV-transformed B lymphoblastoid cell line (B–LCL) and monocyte-derived dendritic cells (DC) were used as APC. The B–LCL of an HLA-DQ2 molecules and peptide binding assays

**Results**

The 33-mer has a relatively high binding affinity to DQ2

Binding of the 33-mer to DQ2 was assessed in a competitive inhibition assay at pH 4.9 using an 125I-labeled MB 65 kDa Hsp...
Processing dependence of the 33-mer and other gliadin T cell epitopes

To address whether presentation of the 33-mer requires further processing, we first used live and glutaraldehyde-fixed B-LCL as APC. To benchmark the assay, we used DQ2-ι-like epitope, which has previously been described to be processing dependent when part of partially proteolysed and dialyzed gliadin (18). By using pepsin- and trypsin-digested gliadin that was dialyzed against a membrane with an exclusion size of 12–14 kDa (HMWF-PT-gliadin) and the DQ2-ι-specific T cell clone TCC423.1.3.8, we reproduced this finding. A 21-mer deamidated peptide containing the DQ2-ι epitope (underlined; YQQLPQPEQPOQSFPEQERPF) was presented by both live and fixed B-LCL (Fig. 3A). In contrast, HMWF-PT-gliadin was presented to TCC423.1.3.8 by live DQ2-ι fixed B-LCL, but not by same cells fixed by glutaraldehyde (Fig. 3B).

Using the same live and fixed B-LCL, a 12-mer peptide containing the DQ2-αI epitope (underlined; QLQPFPQPELPY) was efficiently recognized by the DQ2-αI-specific T cell clone TCC430.1.142 (Fig. 4A). The TG2-treated 33-mer, which contains three copies of DQ2-αI and -αIII epitopes, was also presented to the same T cell clone equally well by live and fixed B-LCL. The deamidated 33-mer was much more potent than the αI peptide, and this increased relative stimulatory potency was unchanged by using fixed B-LCL. Also, HMWF-PT-gliadin was presented by B-LCL (Fig. 4B), suggesting that even among the higher m.w. material retained by the dialysis membrane there were fragments that did not require further processing. Similar results were obtained with another DQ2-αI/III-specific T cell clone (TCC387.E9) as the readout (data not shown). These results indicate that the presentation of the 33-mer and larger fragments containing the DQ2-αI or DQ2-αIII epitopes does not require processing by B-LCL for effective T cell presentation.

We next repeated some of these experiments with the T cell clone TCC430.1.142 using live and fixed mature and immature DC as APC. The results are shown for the immature DC (Fig. 4, C and D), but the results were comparable with those for the mature DC. The 12-mer DQ2-αI peptide was equally efficiently presented by fixed and live DC. The TG2-treated 33-mer was also effectively presented by fixed DC, but not as efficiently as live DC (Fig. 4C). Fixed DC also presented the HMWF-PT-gliadin, but as with the 33-mer, the live cells were more effective than the fixed cells (Fig. 4D). The increased potency of the 33-mer compared with the 12-mer was also observed with DC as APC (Fig. 4C).

To further assess the molecular sizes of PT-gliadin fragments that are presented by fixed APC, PT-gliadin was subjected to fractionation by gel filtration. Fractions 7 and 8, containing larger molecules than the 33-mer (which elutes in fraction 10 under the same chromatographic conditions; Fig. 5A), were tested in T cell assays using both live and fixed B-LCL. Similar to results obtained with dialyzed PT-gliadin, the DQ2-αI/III-specific T cell clone TCC430.1.142, but not the DQ2-ι-specific T cell clone TCC423.1.3.8, recognized the high m.w. fractions 7 and 8 when presented by fixed B-LCL (Fig. 5B). Using live B-LCL, both T cell clones recognized the same fractions, showing that both the DQ2-αI/III and DQ2-ι epitopes were present in fractions 7 and 8 (Fig. 5B).

The 33-mer binds DQ2 efficiently at neutral pH in peptide binding assays and T cell assays with fixed B-LCL

We next analyzed the pH dependence of binding of the 33-mer to DQ2. The binding to DQ2 molecules purified from B-LCL was first tested, then in another set of experiments binding to fixed APC was tested using T cell proliferation as the readout. In the experiments with purified DQ2 molecules, the synthetic deamidated 33-mer, the 33-mer deamidated by TG2, and MB 65 kDa Hsp 243–255Y were labeled with 125I and incubated overnight with detergent-solubilized DQ2 at different pH, and binding was assessed in a gel filtration assay. Peptide MB 65 kDa Hsp 243–255Y bound to DQ2 with a maximum of approximately pH 5.5, in accordance with earlier reports (14), whereas the binding of both 33-mer variants was maximal at pH 6.3 (Fig. 6).

In the T cell assay either TG2-treated 33-mer or synthetic 33-mer peptide with three glutamine residues substituted with glutamic acid were coincubated overnight with fixed B-LCL at three different pH values. The APC were then thoroughly washed to remove unbound peptides and pH-adjusting buffers. Thereafter, the DQ2-αI/III-reactive T cell clone TCC430.1.142 was added to cell culture medium at pH 7.3 and cultured in a standard 3-day T cell proliferation assay. The T cell responses to both deamidated 33-mer variants were higher when APC and 33-mer coinubation was performed at pH 6.3 compared with those at pH 4.9 and pH 7.2 (Fig. 7).
Discussion

The 33-mer (LQLQPFPQPQLPYPQPQLPYPQPQPF, α2-gliadin 56–88) is produced by normal gastrointestinal digestion of recombinant α2-gliadin. It is extremely resistant to proteolytic degradation, contains six partly overlapping copies of three different DQ2-restricted T cell epitopes, and is superstimulatory for T cells (8). Understanding how this peptide binds to DQ2 and how it is handled by APC for presentation to T cells is thus particularly instructive for the learning about the disease mechanisms of celiac disease. In this study we confirm the initial observations on the stimulatory potency of the 33-mer with live APC and show that the 33-mer is presented efficiently by fixed APC. Notably, this increased stimulatory potency is also retained after fixation of the APC.

Extracellular Ags can be presented by APC in either of two ways. The classical Ag processing and presentation pathway includes Ag uptake into the endosomal compartment, proteolytic fragmentation in the lysosomal compartments, and binding of the resultant peptide fragments to MHC class II molecules. Most proteins are processing dependent and presented via this pathway. The alternative pathway for Ag binding is the direct binding of HLA-DQ2 most efficiently at approximately pH 6.3 in cell-free peptide binding assay. The binding efficiency of 125I-labeled synthetic deamidated 33-mer, TG2-treated 33-mer, and the high affinity binder MB 65 kDa Hsp 243–255Y to purified detergent-solubilized DQ2 molecules were assessed in a direct peptide binding assay at various pH. For each peptide, the binding is presented as a percentage of the maximum binding of this peptide. Maximum binding was 14.3% for the synthetic deamidated 33-mer, 7.2% for the TG2-treated 33-mer, and 10.7% for MB 65 kDa Hsp 243–255Y. Error bars indicate the observed range.
polypeptides to surface-expressed MHC class II molecules, without being internalized and processed. Shorter peptides are usually able to use this presentation pathway, but even some longer peptides of 30–39 aa (19, 20), intact proteins (i.e., fibrinogen) (21), and denatured proteins (i.e., bovine albumin, hen egg lysozyme, OVA, and human transferrin) (22) have been shown to bind to the appropriate MHC class II molecules in the absence of processing. Our observations that the 33-mer α-gliadin peptide can bind directly to DQ2 and that both 33-mer and pepsin, trypsin-generated fragments >10 kDa, can be presented by fixed APC, suggest that the 33-mer and larger fragments containing this peptide are not dependent on further processing by APC for T cell presentation. The observation that live DC present the TG2-treated 33-mer better than fixed DC is interesting, particularly because no such difference is observed for the shorter TG2-treated 12-mer T cell epitope. DC may thus be particularly suited to present the 33-mer. The lack of difference in presentation of the TG2-treated 33-mer between live and fixed B-LCL and the data showing that the deamidated 33-mer is a good DQ2 binder by itself suggest that it is impaired Ag uptake or HLA loading, rather than processing, that is affected by fixation of the DC. In this respect it is interesting that TG2 associated with surface integrins may be involved in the uptake of gluten peptides in celiac disease (1).

Our data provide some insight into why the 33-mer is a more potent T cell stimulator than the shorter peptides containing single epitopes within the 33-mer. The relative stimulatory potency of the 33-mer cannot be solely explained by the increased number of epitope copies contained, because the 33-mer has only three copies of the DQ2-αI and DQ2-αIII epitopes. The 33-mer has increased binding affinity to detergent-solubilized DQ, and the increased potency is seen also with fixed APC that are metabolically inactive. This suggests that at least part of the effect is related directly to HLA binding and does not involve signal transduction in the APC, which has been shown to be important for the superstimulatory activity of artificially multimerized T cell epitopes (23). Increasing antigenicity as a function of peptide length has also been reported for a cytochrome c epitope (24). T cell recognition of this epitope was independent of processing, and the antigenicity was continuously improved by extension of the core peptide region. A 23-mer peptide was >30 times more potent than the 12-mer core peptide. Moreover, a hybrid peptide of 51 residues containing the cytochrome c epitope and a T cell epitope derived from tetanus toxoid not only required no processing, but was 1 log more efficient than the cytochrome c of less than half its size. This length-dependent antigenicity of the cytochrome c peptides was also observed using lipid membrane-associated I-Ek, suggesting that this phenomenon is related to MHC binding per se. Our results with the 33-mer, including the binding data, are analogous to these observations. They raise the possibility that long gluten protein fragments surviving gastrointestinal digestion have the potential to be particularly immunogenic as a function of their length or because they can act multivalently for binding to DQ2. The identification of additional gluten T cell epitopes in celiac disease should take this into account.

Among the peptides eluted from DQ2 molecules of B-LCL, CLIP peptides are remarkably abundant (25–28). This may relate to binding of an unusual register of CLIP peptides in addition to the conventional register (25–26) as well as a potentially low efficiency of HLA-DM to unload CLIP peptides from DQ2. At any rate, cell surface DQ2 with abundant CLIP peptides available for exchange reactions should be particularly fit for cell surface binding of peptides. This may be one of the reasons why DQ2 predisposes to celiac disease. Not only can DQ2 bind the register-constraining, proline-rich gluten peptides with high affinities by accommodating glutamate residues in the P4, P6, or P7 pocket (29), but it can also directly bind gluten peptides that are produced by the gastrointestinal enzymatic digestion. The 33-mer is particularly proline rich and adopts type II polyproline helical conformation in solution. The fact that the free 33-mer has already adopted the required conformation for MHC class II binding may promote its binding to cell surface DQ2 by exchange with CLIP or other prebound peptides without the need for HLA-DM.

A limited set of peptides with high affinity binding to DQ2 has been found to exhibit a pH binding optimum at ~5 (14). Interestingly, the 33-mer has a pH optimum closer to what is found in the extracellular milieu, which is relevant for binding to DQ2 at the cell surface. At this point, we do not know the factors involved in this peptide-dependent pH effect on binding. It could relate to variable protonation of the peptides. The fact that the 33-mer adopts a type II polyproline helical conformation in solution may also be relevant.

The requirement for processing of food Ags by APC in the gut may be a special case, because these Ags are preprocessed by gastrointestinal digestion. Although most dietary proteins are broken down to amino acids or di- or tripeptides by the consecutive proteolytic actions of gastric, pancreatic, and small intestine brush-border membrane enzymes (30), significant amounts of the 33-mer remain intact after the digestion of α5-gliadin by all these enzymes (8). Interestingly, a corresponding 12-mer peptide, QLQPFPQQLPY (α5-gliadin 57–68), containing the native sequence of the αI epitope, is produced by similar proteolytic digestion of α5-gliadin (2). T cells that become activated in intestinal biopsies by gluten stimulation in vitro are mainly CD4+ T cells located in the lamina propria (1, 13). The gluten peptides will thus have to access DQ2-positive APCs in this subepithelial compartment. Although
enterocytes have been suggested to play a role in gliadin uptake and presentation in celiac disease (31), these cells generally lack expression of HLA-DQ molecules (32), and it therefore seems unlikely that DQ2-binding gliadin peptides should associate with HLA molecules of epithelial cells. Recently, intestinal transport and degradation of the 33-mer and other gliadin peptides were studied by mounting duodenal biopsies in Ussing chambers (33). After intestinal transport, gliadin peptides were found to be fully digested by enterocytes in controls and celiac patients in remission and to a somewhat smaller degree in patients with active celiac disease. Of note, however, selective uptake of luminal peptides by DC would not be measured in this system. Interestingly, DC express tight junction proteins and penetrate gut epithelial monolayers; they can thereby sample Ags directly from the intestinal lumen (34). DC also express vast amounts of TG2 (35). Our observation speaks to the possibility that luminal 33-mer and other gluten peptides produced by the gastrointestinal digestion can be deamidated by TG2 expressed in the enterocyte brush border or by DC and then be picked up by DC, partly by direct binding to DQ2, for later presentation to T cells in the lamina propria.

In summary, we demonstrate that a 33-mer of α-gliadin that is the end product of normal gastrointestinal proteolysis can be presented to T cells without further processing by APCs. The binding of the 33-mer does not show the pH 5 optimum found for other DQ2 ligands (14), but, rather, has an optimum around pH 6, closer to the pH at the cell surface. Direct binding of this peptide to cell surface DQ2 molecules may be an important route for Ag presentation in vivo. These results are interesting in relation to blocking of peptide presentation by competing compounds, a possible therapeutic approach for treatment of celiac disease.

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References