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Coeliac disease

Endocytotic segregation of gliadin peptide 31–49 in enterocytes
Klaus-Peter Zimmer,1 Ina Fischer,2 Thomas Mothes,3 Gabriele Weissen-Plenz,4 Martina Schmitz,5 Herbert Wieser,6 Jürgen Büning,7 Markus M Lerch,8 Paul C Ciclitira,9 Peter Weber,10 Hassan Y Naim2

ABSTRACT
Objective Coeliac disease (CD) is a multisystemic autoimmune inflammation of the intestinal tract induced by wheat gluten and related cereals in human leucocyte antigen (HLA)-DQ2/8-positive individuals. The molecular mechanisms relevant to oral tolerance induction towards toxic cereals such as gliadin remain poorly understood. Enterocytes, which express predominantly HLA-DR proteins, are capable of processing, transcytosing and presenting food antigens from the intestinal lumen to T lymphocytes of the lamina propria.

Methods Epitope-specific monoclonal antigliadin antibodies are utilised to unravel the intraepithelial transport processes of gliadins in peptides in human duodenal biopsy specimens from patients with CD and reconstitute the transepithelial and endocytic pathways of gliadin in intestinal epithelial HT29 cells.

Results The gliadin peptide AA 31–49 is segregated from the peptides AA 56–68 and AA 229–246 along the endosomal pathway. Thus, AA 31–49 bypasses HLA-DR-positive late endosomes in intestinal cells and in biopsy specimens of patients with untreated CD. Further, it is localised in early endosomes and consequently escapes antigen presentation at the basolateral membrane, unlike peptides AA 56–68 and AA 229–246 that reach HLA-DR-positive late endosomes. Strikingly, forms of gliadin peptide AA 31–49 conjugated to cholera toxin B are sorted into late endosomes of HT29 cells.

Conclusions Endocytic segregation of gliadin peptide AA 31–49 seems to be a constitutive process. It explains why this peptide cannot stimulate gluten-sensitive T cells. Presentation of gliadin peptides by HLA-DR proteins via late endosomes within enterocytes might induce a tolerogenic effect and constitutes a potentially promising therapeutic approach for induction of tolerance towards gliadin.

INTRODUCTION
Coeliac disease (CD) is a multisystemic inflammatory of the intestinal tract induced by wheat gluten and related cereals in human leucocyte antigen (HLA)-DQ2/8-positive individuals. The disease affects up to 1% of the population and is elicited by a failure of oral tolerance towards gliadin, the major component of several dietary cereals.1 Several lines of evidence have implicated HLA class DQ2/8 as mediators of antigen presentation of toxic cereal peptides by dendritic cells of the lamina propria to gluten-sensitive mucosal T cells.2

Gluten proteins can be divided into gliadins, which are alcohol-soluble storage proteins, and relatively insoluble glutenins. Tissue transglutaminase, the autoantigen of CD, is able to increase the antigenicity of gliadin by deamidation of its glutamine residues.4 Gliadin peptide AA 51–49 as well as AA 56–68 exacerbate CD in vitro and in vivo.5–7 The non-immunodominant gliadin peptide 31–45 rapidly induced the expression of interleukin-15 (IL-15), cyclo-oxygenase-2, CD83 and CD25 of macrophages and/or dendritic cells of the lamina propria, underlining its ability to activate innate immunity.8 Peptide 51–49 upregulates not only IL-15 but also epithelial MICA (major histocompatibility complex class I chain-related gene A), the target of the natural killer (NK) cell receptor NK2D, which mediates the cytoxicity of intraepithelial lymphocytes (IELs) in active CD.9 In contrast to the immunodominant gliadin peptide AA 56–68, however, gliadin peptide AA 51–49 does not stimulate small intestinal gluten-sensitive T lymphocytes of patients with CD,8 10 11 strongly suggesting the existence of discriminatory processing mechanisms of gliadin peptides that either alter their antigenic properties or hinder their presentation to major histocompatibility class (MHC) antigens.

Current concepts have established that the enterocytes possess a high capacity for transcytotic processing and transcellular passage of food antigens to the paracellular space.12 13 The binding of gliadin to the brush border membrane in intestinal cell lines and intestinal tissue could be substantially upregulated by interferon γ (IFNγ),14 15 suggesting a modulatory role for this cytokine and possibly other associated components in the uptake and further processing of gliadin peptides. An interesting feature with respect to gliadin uptake is the intracellular accumulation of gliadin peptides in intracellular compartments of CD enterocytes as compared with control enterocytes.16 17

The molecular mechanisms relevant to antigen-presenting cells and antigen presentation in the onset of CD remain poorly understood. It is also unclear how HLA class DQ2/8-positive individuals develop tolerance towards cereal proteins in spite of their genetic predisposition. CD8+ IELs may be involved in this tolerance mechanism.18 Enterocytes, which express predominantly HLA-DR proteins, are capable of processing, transcytosing and presenting food antigens from the intestinal lumen to T lymphocytes of the subjacent lamina propria.19 It has been demonstrated that exogenous antigens associate with MHC class II molecules within late endosomes before they are recycled back to the

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surface for presentation to CD4+ T lymphocytes. Targeting of antigens to late endosomes, which contain lysosome-associated membrane proteins (LAMPS) and MHC class II proteins, enhances presentation to CD4+ T lymphocytes. We have previously reported that the food antigen ovalbumin (OVA) was targeted to late endosomes of enterocytes and delivered by exosomes into the paracellular space of naïve BALB/c mice, which were able to induce oral tolerance. In contrast, this translocation process does not occur in SCID (severe combined immune deficient) mice that do not develop tolerance mechanisms.

The central issue in the current investigation was to unravel the intraepithelial transport of gliadin peptides in human duodenal biopsy specimens from patients with CD and to reconstitute these pathways in epithelial cell lines. Our findings emphasise the key role of endosomal discrimination between specific gliadin peptides in the pathogenesis of CD which is relevant to antigen presentation, T cell stimulation and tolerance induction. Strikingly, gliadin peptide AA 31−49 could be rerouted in intestinal cells from early to late endosomes via conjugation with the B subunit of cholera toxin (CTB) and thus offers a promising therapeutic approach that could elicit tolerance towards gliadin peptides in the enterocytes.

**MATERIALS AND METHODS**

**Patients**

We studied duodenal biopsy specimens from children with CD diagnosed according to the ESPGHAN criteria. Seven specimens were obtained from patients with untreated CD (between 1 and 12 years old). Three biopsy specimens were taken from adult patients with CD who had received a gluten-free diet for at least 6 months. Duodenal biopsy specimens from patients subsequently diagnosed with functional dyspepsia (two patients, 5 and 4 years old) and postenteritis syndrome (two patients, 2 and 3 years old) served as controls. The adult patients with CD were in remission (under a gluten-free diet) and were challenged by intraduodenal infusion of FF III (Frazer et al., 2010; In situ hybridisation of HLA-DR).

**Antibodies**

The primary antibodies used in this study are summarised in table 1. Binding sites were visualised at the electron microscope level by gold-conjugated goat antirabbit serum (diameter of 6 and 12 nm (Dianova, Hamburg, Germany)), dilution of 1:50) and gold-conjugated goat antimouse (rat) serum (diameter of 6 and 12 nm (Dianova), dilution of 1:10), which were affinity-purified against human immunoglobulins (Igs). For light microscopy, a Texas Red-conjugated goat antirabbit antibody (1:50) and a fluorescein-conjugated goat antimouse antibody (1:10) (both from Cappel, Eppelheim, Germany) were used. Large streptavidin–gold particles (12 nm) were purchased from Dianova. Goat antirabbit- and goat antimouse-conjugated Alexa Fluor dyes 488 and 568 (Invitrogen, Karlsruhe, Germany), dilution of 1:1000 were used to label HT29 cells.

**Epitope mapping of WB8 and R5 antibodies**

We investigated the binding of WB8 and R5 to the following gliadinSb peptides: VPVPQLOPQPSQIQPEQVPLVQQQFPGQQQPFPQOPQ YPQPQOFPSQQY PYL, the corresponding sequence of A-gliadin VPVPQLOPQPSQIQPEOE QVLV QQQFLQGQQQFPQPQPQ YPQPQOFPSQQPYL, the 35mer gliadin peptide LQLQFPQFPP YLPFPQGPLYQPQPLYPQFPQPF and a repetitive motif occurring in γ-gliadins: FQPQFPQFPQPFQPF. Peptide synthesis on cellulose membranes and luminescence binding assays were performed as described. Mean luminescence scores (MLS, on a scale from 0 to 3) are indicated as the means of 3–6 experiments.

**Immunofluorescence**

Semithin frozen sections (0.5 μm) of the duodenal biopsy specimens were prepared with a Leica EM Ultrakut RFCs cryoultramicrotome (Leica, Bensheim, Germany) at −60 to −70°C as described in detail elsewhere. The specimens were photographed in a Zeiss Axioskop fluorescence microscope. HT29 cells were incubated with synthetic peptides at a concentration of 0.5 mg/ml in cell culture medium, fixed with 4% paraformaldehyde and prepared for indirect immunofluorescence. The primary antibodies were polyclonal or monoclonal antibodies against gliadin or endosomal protein markers (LAMP-2, cathepsin D and early endosome antigen 1 (EEA-1)). The secondary antibodies were conjugated to Alexa Fluo 568 (for gliadin detection) or Alexa Fluo 488 (for endosomal markers). Confocal images were acquired utilising a Leica TCS SP II microscope (Leica Microsystems, Wetzlar, Germany).

**In situ hybridisation of HLA-DR**

RNA was isolated from B lymphocytes (RNAeasy MiniKit, Qiagen, Hilden, Germany) and reverse transcribed (cDNA synthesis Kit, Fermentas, St Leon-Roth, Germany). The reverse transcribed products were used for PCRs with the following upstream and downstream PCR primers for HLA-DR (NM_019111, NCBI): CGGGGTACCATGAGCGCTCAGGAAT CGGGAAGCTTCCTCCATGTGCCTTACAGAGG with a HindIII site (sequence location 133–153) and CGGAAGCTTCTCCATGTGCCTTACAGAGG with a HindIII site (sequence location 838–860).

HLA-DR cDNA was subcloned into a pGem-4Z vector and an in vitro transcription was performed according to the manufacturer’s protocol (Fermentas) with digoxigenin-labelled UTP (Roche, Penzberg, Germany).

In situ hybridisation of frozen sections obtained from duodenal biopsy specimens was performed according to the procedure described previously. It was performed either with 0.2 mg digoxigenin-labelled cRNA probe/ml (antisense or sense strand of HLA-DR) or with hybridisation solution only at 52°C in a humidified chamber. The digoxigenin-labelled probes were detected using antidigoxigenin–alkaline phosphatase according to the manufacturer’s instructions (Boehringer, Mannheim, Germany).
**Coeliac disease**

### Table 1  Primary antibodies

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<th>Antibody</th>
<th>Host/clone/ isotype</th>
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<td>rabbit</td>
<td>α-Gliadin</td>
<td>1:100</td>
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<td>24 25</td>
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<td>Human LAMP-1</td>
<td>1:10</td>
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<td>Human LAMP-2</td>
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<tr>
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<td>Human LAMP-1 and -2</td>
<td>1:10</td>
<td>M Fukuda, San Diego, California, USA</td>
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<tr>
<td>Cathepsin D</td>
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<td>β-Chain</td>
<td>1:5</td>
<td>J Cordell, Oxford, UK</td>
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<td>α-Chain</td>
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<td>PJ Peters, Amsterdam, The Netherlands</td>
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</table>

*Biotinylated.

WB8 was raised against the gliadin peptide B3144, which comprises the amino acid sequence 3–56 of A-gliadin. For immunofluorescence experiments the primary antibodies were used at up to 10 times higher dilutions as indicated.

HLA, human leucocyte antigen; IgG, immunoglobulin G; LAMP, lysosomal-associated membrane protein; m, mouse.

### Biosynthetic labelling and peptide internalisation of HT29 cells

Gliadin peptides were synthesised by Fmoc chemistry with a solid-phase synthesiser 431A (Applied Biosystems, Darmstadt, Germany) and accordingly they were free of lipopolysaccharide. The crude peptides were purified by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) on a C18 silica gel Nucleosil 100-5 (Macherey-Nagel, Düren, Germany). Analytical RP-HPLC showed that the peptides were chromatographically pure (>98%), and electrospray mass spectrometry (LCQ, Finnigan Mat, Bremen, Germany) confirmed their correct amino acid sequences. The following gliadin peptides were synthesised: peptide 31–59, LGQQQFPFPQYFPQFPF; peptide 56–68, LQLQPFPQPQLPY; and the control peptide 229–246, LPQFEIRNLAQTLIAM.

HT29 and T84 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) high glucose (PAA, Pasching, Austria) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. For HLA class II analysis, cells were stimulated with 200 U/ml IFNγ (kindly provided by Professor B Otto, Hannover, Germany). The cells were biosynthetically labelled with 35S methionine and the cells were solubilised with 0.5% Nonidet P40 (NP40, Fluka, Deisenhofen, Germany) in 50 mM Tris–HCl, pH 7.4, 300 mM NaCl. Proteins were immunoprecipitated with the relevant antibody as described elsewhere and the immunoprecipitates were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli.27 The gels were visualised after electrophoresis with a Phosphorimager (Bio-Rad, Munich, Germany).

The conjugate with CTB (SBL Vaccin, Stockholm, Sweden) was incubated at a concentration of 50 μg/ml.

### Conjugation of peptide 31–49 with CTB

Peptides were coupled to CTB (SBL Vaccin) using the heterobifunctional cross-linker m-maleimidobenzoyl-N-hydroxysuccinimide (MBS; Pierce, Bonn, Germany). The amino groups of the carrier proteins were first activated by 50 min incubation at room temperature with a 25-fold molar excess of MBS. Excess MBS was removed by a desalting column (PD-10 column, Amersham Bioscience, Freiburg, Germany). In a second step, peptides were added at a molar ratio of 20:1 (peptide/carrier protein). Cross-linking at the cysteine residues was performed for 3 h at room temperature. Unbound peptides were removed by dialysis against phosphate-buffered saline (PBS). The concentration was determined with a BCA (bicinchoninic acid) protein assay (Pierce). The successful conjugation was tested in dot blot analysis using antigliadin antibodies. HT29 cells were incubated with this conjugate at a concentration of 50 μg/ml and prepared for immunofluorescence as described above.

### Western blot analysis

Western blot analysis was performed using NP40 detergent extracts of HT29 cells and the antibody CR3/43 directed against HLA class II. The secondary antibody employed sheep anti-mouse IgG linked to peroxidase (Amersham Biosciences). The detection was performed with a Super Signal ELISA Femto kit (Pierce, Pernio, Bonn, Germany).

### RESULTS

#### Gliadin epitopes of monoclonal antibodies WB8 and R5

Detection of gliadin and its peptides was performed with the epitope-specific monoclonal antibodies R5 and WB8 or with commercially available polyclonal antibodies that possess a wide range of binding capacity towards toxic as well as non-toxic epitopes. The core epitope of the R5 antibody comprises only five amino acids with the sequences QQQFP, QQQFP, QLPFP and QLPFP. In comparison, WB8 requires a larger peptide, QQQQFPFPFPQ, for optimal binding. Truncation of the nonapeptide at the N-terminal end yields a pentapeptide QQPFP that is no longer capable of binding WB8, but could be recognised by the R5 antibody. Furthermore, the antibody WB8 does not tolerate the substitutions Q2 to P, Q3 to L, Q4 to E, P5 to Q, or Q9 to E in the nonapeptide QQQQFPFPQ. Obviously, the epitope specificity of WB8 is more restricted than that of R5. This explains why both antibodies react with the peptides comprising amino acids 3–56 of A-gliadin and to a typical repetitive sequence of γ-gliadin (figure 1). The pentapeptide epitopes of R5 are widely distributed over the different species of gliadins (α, γ, and ω type) but nevertheless occur most often in γ- and ω-type gliadins. The reactivity of WB8, on the other hand, is almost restricted to α-type gliadins and only a few epitopes in γ-type gliadins. As already shown, substitution of various Q residues by E decreases the reactivity of R5 with the corresponding deamidated peptides and could be tolerated by WB8.

#### Divergent transcytosis of gliadin in enterocytes of biopsy specimens

Polyclonal antibodies against gliadin label HLA-DR-positive endosomes of enterocytes from untreated patients with CD.16
Co-labelling enterocytes of patients with CD with R5 and WB8 antibodies revealed a positive labelling of the Golgi complex (figure 2a), vesicles and the apical and basolateral membranes. In comparison with R5, the labelling densities of WB8 were stronger in the Golgi apparatus of untreated patients with CD. The polyclonal antibodies against gliadin label the apical membrane including endocytic invaginations (figure 2b) more intensely than the monoclonal antibodies, while the opposite applies to the labelling of Golgi complexes. Incubation of duodenal biopsy samples from patients with CD with FF III for 5–10 min resulted in a rapid internalisation of gliadin (figure 2b) into endocytotic vesicles (figure 2c) and, after 10 min, to labelling of the Golgi complex. Most binding sites of gliadin antibodies were situated close to the apical and the basolateral (not shown) membrane as well as to the outer and inner membranes of vesicles.

To unravel the potential role of HLA-DR molecules in the induction of immune tolerance towards gliadin peptides in the enterocytes we first sought to assess their cellular localisation and compare it with that of gliadin peptides. Enterocytes expressing HLA-DR molecules revealed many binding sites inside the Golgi complexes (figure 2d), vacuoles and basolateral membranes. It should be noted that some HLA-DR molecules were also detected at the apical membrane of those enterocytes in which HLA-DR was intensively revealed in the Golgi (figure 2e). This default transport of HLA-DR molecules to the apical membrane is probably due to oversaturation of the sorting machinery in the trans-Golgi network and subsequent failure to recognise the FXXL sorting signal within the cytosolic tail of the β-chain. In contrast to HLA-DR, HLA-DQ was detected in the enterocytes of only one of the biopsy specimens, which at the same time also showed a stronger HLA-DR labelling.

Immunofluorescence (figure 3a,c) and immunoelectron microscopic (figure 3b–f) experiments were performed to define co-localisation sites of gliadin within the endocytotic compartment. Vacuolar HLA-DR co-localised with LAMP-positive vacuoles of CD enterocytes (figure 3a). As assessed by polyclonal antibodies, gliadin is not only localised in the late endosomes, but also within HLA-DR-positive vacuoles of enterocytes (figure 3b). Interestingly, LAMP- and cathepsin D-positive vacuoles of CD enterocytes were not labelled with WB8 (figure 3c,d) while R5 revealed only a weak labelling signal in two of eight patients with CD in late endosomes (data not shown). In contrast, polyclonal antibodies against gliadin clearly labelled LAMP-positive endosomes of CD enterocytes (figure 3e), lending strong support to the data mentioned above. The lack of labelling with WB8 and R5 within HLA-DR-containing vacuoles was also confirmed using a biotinylated anti-HLA-DR monoclonal antibody (figure 3f) as well as with a polyclonal antibody against MHC class II proteins (data not shown). There was no labelling of the monoclonal and polyclonal antibodies against gliadin in patients with CD on a gluten-free diet.

Expression of HLA-DR antigens by enterocytes

The predominant expression of HLA-DR antigens was previously shown in tissue culture of intestinal epithelial cells. We confirmed HLA-DR expression by in situ hybridisation of biopsy specimens from patients with CD. Staining for HLA-DR in biopsy specimens from healthy controls as well as patients with CD in remission was detected only in single cells within the lamina propria (figure 4a). Control experiments with the corresponding sense probe were completely negative (figure 4b). The strongest labelling of HLA-DR mRNA was found in biopsy specimens from patients with CD on a gluten-free diet.
The holotoxin enters the body by binding to LAMP-2, in sucrose density gradients. Other studies assigned a substantial role for intestinal epithelial cells as non-conventional antigen-presenting cells with a major suppressive function that maintains homeostasis and induces tolerance reactions. The current study supports the concept that HLA-DR-expressing enterocytes are able to generate a tolerogenic effect in contrast to the toxic reaction mediated by HLA-DQ-positive dendritic cells of the lamina propria.

We further analysed the expression of HLA class II antigens at the protein level in intestinal HT29 cells. Interestingly, the expression of the α- and β-chains of HLA class II antigens increased substantially after 72 h stimulation of HT29 cells with IFNγ (figure 4d). In contrast, unstimulated HT29 cells did not reveal HLA class II proteins in comparison with HLA-DR3-expressing B lymphocytes (figure 4e).

As already shown in duodenal biopsy specimens, HLA class II antigens are localised in late endosomes (figure 5a). HLA class II proteins in HT29 cells were also detected in late endosomal vesicles (figure 5a) as there is co-localisation of both LAMP-2 and cathepsin D (figure 5b). HT29 cells incubated with gliadin peptide AA 31–49 show co-localisation with EEA-1 (figure 5c). To rule out any overlap between EEA-1- and LAMP-2-positive vacuoles, cell fractionation was performed in HT29 cells. Figure 5d clearly demonstrates that early endosomes, represented by EEA-1, could be separated from late endosomes, represented by LAMP-2, in sucrose density gradients.

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In vitro transcytosis of specific gliadin peptides in HT29 cells

While gliadin peptides AA 31–49 and AA 56–68 exacerbate CD in vitro and in vivo, only the latter stimulates small intestinal gluten-sensitive T lymphocytes of patients with CD. In addition to these peptides we used the C-terminal sequence comprising AA 229–246 of A-gliadin as a non-toxic control peptide lacking the characteristic repetition of proline and glutamine residues.

Following internalisation of each of the peptides in IFNγ-stimulated intestinal HT29 cells, gliadin was detected with indirect immunofluorescence. We found no difference in endocytic uptake and targeting between IFNγ-stimulated and unstimulated cells. All the peptides as well as gliadin from FF III were found to be localised in early endosomes, co-localising with EEA-1 (data not shown). The control sequence (figure 6a) and the peptide AA 56–68 (figure 6b) were both transported to late endosomal compartments, co-localising with LAMP and cathepsin D, respectively. The peptide AA 31–49, in contrast, reached neither late endosomes, since it did not co-localise with LAMP-2 (figure 6c), nor HLA class II-positive vacuoles (figure 6d). This transport pattern was confirmed in T84 cells where peptide AA 31–49 did not show any co-localisation with LAMP-2 and cathepsin D, in contrast to peptide AA 56–68 and AA 229–246.

Targeting of peptide AA 31–49 to late endosomes in HT29 cells

In view of the localisation of AA 31–49 in early endosomes and its relevance to the onset of CD we designed a procedure by which this peptide can be redirected to late endosomes with the ultimate goal of generating oral tolerance towards gluten. For this, the intracellular routing of the chimeric form of AA 31–49 fused to CTB was investigated in HT29 cells. In this experiment HT29 cells were not stimulated by IFNγ to examine the endocytic targeting of the CTB-conjugated peptide AA 31–49 independently of upregulation of HLA class II. CTB is well known as an efficient mucosal carrier for oral immunisation with antigen–CTB complexes. The holotoxin enters the body by binding via the B subunit to GM-1 molecules at the surface of enterocytes and further transport to the lysosomes. Strikingly, the results of our experiments show that the conjugated forms of gliadin peptide AA 31–49 with CTB were co-localised with both marker proteins for late endosomes, LAMP-2 (figure 7a) and cathepsin D (figure 7b), indicating that this peptide construct could reach a compartment that expresses HLA-DR antigens in HT29 cells.

DISCUSSION

The translocation of luminal antigens to late endosomes of enterocytes of the mouse small intestine has been proposed to constitute a crucial mechanism for the induction of oral tolerance. Other studies assigned a substantial role for intestinal epithelial cells as non-conventional antigen-presenting cells with a major suppressive function that maintains homeostasis and induces tolerance reactions. The current study supports the concept that HLA-DR-expressing enterocytes are able to generate a tolerogenic effect in contrast to the toxic reaction mediated by HLA-DQ-positive dendritic cells of the lamina propria.

In fact, mapping the specificity of a panel of monoclonal and polyclonal antibodies towards different gliadin peptides in relation to their intracellular localisation in intestinal cells could...
unequivocally reveal an essential and discriminatory role for the endosomal system in sorting of gliadin peptides. Hence, polyclonal antibodies against gliadin label gliadin peptides in late endosomes of duodenal enterocytes,\(^\text{16}\) while the monoclonal antibodies R5 and WB8 (WB8 more strongly than R5) label predominantly early endosomal compartments. It is important to note that the R5 antibody recognises several gliadin peptides including AA 31\(^\text{e}\) 49 and AA 56\(^\text{e}\) 68 with a decreased reactivity to deamidated peptides, while WB8 binds to larger peptides including AA 31\(^\text{e}\) 49, with or without deamidation, but not to AA 56\(^\text{e}\) 68. These in situ analyses could be corroborated by the endocytosis experiments in unstimulated HT29 cells using the three gliadin peptides AA 31\(^\text{e}\) 49, AA 56\(^\text{e}\) 68 and AA 229\(^\text{e}\) 246. According to these experiments, the following transport pattern of gliadin peptides could be identified within enterocytes: (1) gliadin peptides AA 56\(^\text{e}\) 68 and AA 229\(^\text{e}\) 246 are transported to HLA-DR-positive late endosomes; and (2) gliadin peptide AA 31\(^\text{e}\) 49 can be detected in HLA-DR-negative early endosomes and bypasses HLA-DR-positive late endosomes.

These results are supported by data in which mucosal to serosal peptide fluxes and peptide processing during transcytosis of intestinal epithelium were measured in duodenal biopsy explants.\(^\text{37}\) Following 3 h of contact with the mucosal side, 50% of the peptide corresponding to AA 31\(^\text{e}\) 49 of A-gliadin arrived intact in the serosal compartment in enterocytes from patients with active CD in contrast to 12% of the peptide corresponding to AA 57\(^\text{e}\) 68 of this protein. The processing defect of AA 31\(^\text{e}\) 49 was interpreted to be non-constitutive because controls showed only “slight abnormalities”.\(^\text{37}\) In contrast to this study, our results support an intrinsic property of AA 31\(^\text{e}\) 49 because endocytotic segregation of this peptide can be demonstrated in HT29 and T84 cells.

The failure of peptide AA 31\(^\text{e}\) 49 to reach an HLA class II-positive late endosomal compartment for antigen presentation strongly suggests that intestinal gluten-sensitive T lymphocytes of patients with CD cannot be stimulated with this peptide despite its ability to exacerbate CD in vitro and in vivo.\(^\text{7}\) The in situ and in vitro expression of HLA class II presented herein further support the notion that luminal antigens are transported to late endosomes of enterocytes independently of MHC class II protein expression.\(^\text{38}\)

Association of exogenous antigens with HLA class II molecules occurs in late endosomes and represents a necessary event in the induction of oral tolerance that requires CD4\(^+\) T cells as well as HLA class II molecules.\(^\text{39}\) The targeting of gliadin peptides (such as gliadin peptide AA 31\(^\text{e}\) 49) to early endosomes and bypassing HLA-DR-positive late endosomes in the enterocytes hampers the presentation of luminal antigens and also the

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**Figure 3** Segregation of gliadin peptides from late endosomes. Subcellular localisation of human leucocyte antigen (HLA)-DR antigens (a, b, f), lysosomal-associated membrane protein (LAMP) (a, c, e), cathepsin D (d) and gliadin (b–f) within enterocytes of duodenal biopsy specimens obtained from patients with active coeliac disease. The biopsies were treated as described in figure 2. Binding sites were visualised by immunofluorescence (a, c) and at the immuno-electron microscopic (b, d–f) level. HLA-DR antigens and α-gliadin were detected in late endosomes (a, b, e) in contrast to WB8 (c, d, f). Arrows refer to 6 nm large gold particles labelling α-gliadin (e). V, vacuole; AM, apical membrane; M, mitochondrion; N, nucleus. Bars, 0.1 μm.
Coeliac disease

Figure 4  Expression of human leucocyte antigen (HLA) class II antigens by enterocytes in vivo. In situ hybridisation experiments performed with the antisense probe of HLA-DR (a, c) and the sense probe of HLA-DR (b) in duodenal biopsy specimens of healthy control (a, b), and untreated patients with coeliac disease (CD) (c). HLA-DR expression was restricted to lamina propria cells in healthy patients (a), but was additionally detected in enterocytes of patients with CD (c). HT29 cells were stimulated with 200 U of interferon γ (IFNγ) or not stimulated (w/o). HLA class I and class II antigens were immunoprecipitated from detergent extracts of the biosynthetically labelled cells using anti-HLA-ABC (W6/32) and anti-HLA-DQ-DP-DR (clone CR3/43), respectively (d). As a control for HLA class II expression, a B lymphocyte cell line was used in western blot analysis (e). Here, the cell lysates were treated at 95°C (+) or incubated at room temperature (w/o) to preserve the dimer before loading for sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS—PAGE) (e).

stimulation of CD4 and α/β TCR (T cell receptor)-positive T lymphocytes with suppressor function. Antigen presentation of intestinal epithelial cells seems to depend on HLA-DR molecules, whose expression level in enterocytes is higher than that of HLA-DQ molecules. This mechanism of tolerance has to be distinguished from the CD immune reaction mediated by HLA-DQ molecules and dendritic cells of the lamina propria. Recent studies have shown that intestinal epithelial cells stimulate T cells through an HLA-DR-mediated event. It is possible that gliadin peptides such as AA 56–68 associate with HLA-DQ in enterocytes, contributing to the toxic reaction of CD, and that unbound peptide AA 56–68 directly reaches dendritic cells of the lamina propria. Moreover, the tolerance reaction may also be disturbed if a single or few gliadin peptides such as AA 31–49 escape presentation by HLA-DR antigens (figure 8 online).

While a tolerogenic (adaptive) T cell response still needs to be demonstrated in individuals without CD, our results support the concept that innate immunity is activated by AA 31–49 through enterocytes in active CD, leading to induction of IL-15 and epithelial expression of MICA.
demonstrated that the WB8 antibody co-localises with α/β TCR and γ/δ TCR (but not CD4, leucocyte common antigen (LCA) and CD3) within enterocytes and proposed a cleavage and uptake mechanism for these innate TCRs at contact sites between T lymphocytes and the basolateral membrane of enterocytes. Using the biopsy specimens and HT29 cells of the present study we were able to show transport of AA 31-49 together with HLA class I proteins through early endosomes and a proteasome-mediated process including MHC class I proteins as well as CD1d in enterocytes of active CD (Zimmer et al manuscript unpublished results). The transferrin receptor and secretory IgA may play a role in the transport of gliadin peptides through the early endosomal compartment. It will be interesting to examine whether MICA is involved in the fast targeting of gliadin peptide 31-49 through early endosomes of enterocytes.

It is well known that CTB functions as a carrier for immunisation when coupled to an antigen. In the present study we could show that an antigen—CTB complex—that is, the peptide corresponding to AA 31-49 of A-gliadin bound to CTB—could be redirected into late endosomes instead of early endosomes in HT29 enterocytes. In several studies the application of such conjugates via a nasal or an oral route resulted in a suppressive immune response against the coupled antigen with the generation of Foxp3+CD25+CD4+ regulatory T lymphocytes. The mucosa and the associated lymphoid tissues deal with the coupled antigen in a different way compared with the antigen alone. This could be due to different transport, processing and possibly different presentation of antigens by epithelial cells.

Taken together, we have shown that enterocytes internalise gliadin from FF III and synthetic peptides, which can be detected in early endosomes. Transport of the control peptide as well as the peptide AA 56-68 to the late endosomes can be reconstituted in HT29 cells. The peptide AA 31-49, on the other hand, did not reach HLA class II-positive late endosomes in the HT29 and T84

**Figure 5** Intracellular co-localisation of human leucocyte antigen (HLA) class II complexes and gliadin peptide 31-49 in HT29 cells. Indirect immunofluorescence was performed in HT29 cells with the dyes Alexa Fluor 568 (HLA class II, lysosomal-associated membrane protein-2 (LAMP-2) and polyclonal gliadin antibody) and 488 (cathepsin D and early endosome antigen 1 (EEA-1)). HLA class II antigens co-localise in late endosomes with cathepsin D (a). Double labelling of LAMP-2 and cathepsin D as useful markers for the late endosomal compartment (b). Gliadin peptide 31-49 is present in EEA-1-positive vacuoles after its incubation with HT29 cells (c). HT29 homogenates were separated on a sucrose gradient followed by western blot analysis using antibodies against EEA-1 (early endosomes) and LAMP-2 (late endosomes) (d). Bars, 5 μm.
cell line or in enterocytes from human duodenal biopsy specimens, which seems to be a constitutive defect related to the peptide structure. Furthermore, the intracellular transport of peptide AA 31–49 conjugated with CTB can be redirected to LAMP-/cathepsin D-positive late endosomes in the HT29 cell line. It will be interesting to examine whether physiological ligands such as antigliadin IgA are able to redirect peptide AA 31–49 to a late endosomal compartment.

We conclude that the antigenic structure determines the sorting of gliadin peptides to either the early or late endosomes in enterocytes with subsequent implications on the association with MHC II antigens of enterocytes. Endocytic segregation of gliadin peptide AA 31–49 explains why this peptide cannot stimulate gluten-sensitive T cells. Our experiments indicate that presentation of gliadin peptides by HLA-DR proteins of enterocytes might induce a tolerogenic effect in contrast to the toxic reaction of gliadin peptides mediated by HLA-DQ proteins in dendritic cells. The sorting of a conjugated form of gliadin peptide AA 31–49 to late endosomes presents a potentially promising therapeutic approach for induction of primary, or possibly secondary, tolerance towards gliadin.

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Figure 7  The AA 31—49 peptide of α-gliadin was chemically cross-linked to the cholera toxin B subunit (CTB) and HT29 cells were incubated with the conjugate for 2.5 h at 37°C. Indirect immunofluorescence was performed with the dyes Alexa Fluor 568 (peptide) and 488 (lysosomal-associated membrane protein-2 (LAMP-2) and cathepsin D) (a, b). Together with CTB the AA 31—49 peptide co-localises in the late endosomes with LAMP-2 (a) and cathepsin D (b). Arrows indicate co-localisation sites. Bars, 5 μm.

Competing interests None.

Ethics approval This study was conducted with the approval of the University of Muenster.

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REFERENCES

Endoscopic view of the lesion from the stomach.

Editor’s quiz: GI snapshot

Troublesome belching with fetor odour

CLINICAL PRESENTATION
A 57-year-old lady presented with a 5-month history of upper abdominal pain, belching with odour which was occasionally feculent. She also noticed intermittent postprandial epigastric fullness associated with excessive burping. The patient denied any heart burn, weight loss or fever. On examination, she was minimally tender in the epigastric region. Routine bloods were normal. Ultrasound of the abdomen did not show any pathology. Upper gastrointestinal endoscopy revealed an unusual appearance of a tight orifice leading to a small pocket in gastric fundus (figure 1) containing the food debris. A computerised tomography scan was performed and a selected image is shown below confirming the endoscopic findings (figure 2).

QUESTION
What is the lesion shown and what is the appropriate management?

See page 324 for answer

Figure 1 Endoscopic view of the lesion from the stomach.

Figure 2 Pre operative upper abdominal computerised tomography.

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