Gliadin intake alters the small intestinal mucosa in indomethacin-treated HLA-DQ8 transgenic mice

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1Department of Food Sciences, National Research Council, Avellino, Italy; 2Department of Protein Biochemistry, National Research Council, Naples, Italy; 3European Laboratory for Investigation of Food Induced Diseases and Department of Pediatrics, University “Federico II” of Naples, Naples, Italy; and 4Department of Immunology, Mayo Clinic College of Medicine, Rochester, Minnesota

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Gliadin intake alters the small intestinal mucosa in indomethacin-treated HLA-DQ8 transgenic mice. Am J Physiol Gastrointest Liver Physiol 307: G302–G312, 2014. First published June 12, 2014; doi:10.1152/ajpgi.00002.2014.—Celiac disease (CD) is an enteropathy caused by the ingestion of wheat gluten in genetically susceptible individuals. A complete understanding of the pathogenic mechanisms in CD has been hindered because of the lack of adequate in vivo models. In the present study, we explored the events after the intragastric administration of gliadin and of the albumin/globulin fraction from wheat in human leukocyte antigen-DQ8 transgenic mice (DQ8 mice) treated with indomethacin, an inhibitor of cyclooxygenases (COXs). After 10 days of treatment, mice showed a significant reduction of villus height, increased crypt depth, increased number of lamina propria-activated macrophages, and high basal interferon-γ secretion in mesenteric lymph nodes, all of which were specifically related to gliadin intake, whereas the albumin/globulin fraction of wheat was unable to induce similar changes. Cotreatment with NS-398, a specific inhibitor of COX-2, also induced the intestinal lesion. Enteropathy onset was further characterized by high levels of oxidative stress markers, similar to CD. Biochemical assessment of the small intestine revealed the specific activation of matrix metalloproteinases 2 and 9, high caspase-3 activity, and a significant increase of tissue transglutaminase protein levels associated with the intestinal lesion. Notably, after 30 days of treatment, enteropathic mice developed serum antibodies toward gliadin (IgA) and tissue transglutaminase (IgG). We concluded that gliadin intake in combination with COX inhibition caused a basal inflammatory status and an oxidative stress condition in the small intestine of DQ8 mice, thus triggering the mucosal lesion and, subsequently, an antigen-specific immunity.

Celiac disease (CD) is defined as a chronic small intestinal immune-mediated enteropathy precipitated by exposure to dietary gluten in genetically predisposed individuals (27). The central role of adaptive immunity in CD pathogenesis is supported by the identification of gluten-specific intestinal CD4+ T cells in celiac patients and the strong association of disease with human leukocyte antigen (HLA)-DQ2 and HLA-DQ8 genes (45). Accordingly, the intestinal lesion is believed to be induced by the secretion of interferon (IFN)-γ from these gluten-specific T cells (38). In line with this hypothesis, CD4+ T cell immunity to gluten leads to a loss of oral gluten tolerance and small intestinal pathology in lymphopenic mice (19). On the other hand, gluten cytotoxicity and activation of innate immunity in CD have been reported (24). In particular, a study (31) using tissue culture models showed that gliadin can induce IL-15 secretion, which results in the upregulation of natural killer group 2, member D (NKG2D), on intraepithelial lymphocytes (IELs) and the NKG2D ligand MICA on epithelial cells, causing target cell killing. Recently, other cereal components (β-amylase/trypsin inhibitors) have been defined as novel contributors to innate immunity activation (25).

To date, the lack of adequate in vivo models has hampered the chronological assessment of the pathogenic mechanisms occurring in CD. The main difficulty resides in the ability to develop a strategy to interrupt oral tolerance and induce a HLA-restricted cell-mediated enteropathy. The models developed so far only partially address this issue. Indeed, an association of gluten sensitivity with expression of the DQ8 heterodimer is indicated by the greater immune response to gluten induced in HLA-DQ8 transgenic mice (DQ8 mice) compared with HLA-DQ6 transgenic mice (5) as well as the occurrence of nonimmune effects in the former, such as intestinal permeability changes (33). Intriguingly, gliadin alone decreases intestinal antioxidant and detoxifying defenses in these mice, but it is unable to induce any enteropathy (2). After intragastric immunization of DQ8 mice, we identified two immunodominant peptides that induce IFN-γ secretion in vitro, as expected in CD (44). However, we still observed the absence of enteropathy in this model. Exacerbation of the intestinal gliadin-specific immune response by coadministration of Lactobacillus casei also did not cause any mucosal damage (10). On the other hand, gliadin-sensitized NOD-DQ8 mice develop moderate enteropathy, intraepithelial lymphocytosis, and barrier dysfunction (20). The existence of a complex mechanism of oral tolerance to counterbalance the hyperactivation status toward luminal antigens could explain the absence of mucosal damage in mice transgenic for HLA-DQ2.5 and a gluten-specific T cell receptor upon gluten feeding (12). Interestingly, in mice expressing the T cell receptor for peptide 46–61 of the dietary hen egg white lysozyme, the simultaneous administration of peptide and indomethacin (Indo), a nonsteroidal anti-inflammatory drug that inhibits cyclooxygenases (COXs), causes crypt expansion and villus blunting (35). COXs are enzymes involved in the synthesis of prostaglandins from arachidonic acid. In particular, PGE2 enhances the production of anti-inflammatory cytokines and/or inhibits the production of pro-
inflammatory cytokines in in vitro models of T lymphocyte stimulation (3, 13, 47). The downregulatory effects of PGE2 are normally operative in the intestinal system but not in the systemic immune system, as evidenced by the high levels of PGE2 produced by mononuclear cells in the lamina propria (LP) (35). Recently, in vivo suppression of oral tolerance by inhibition of COX-2 has been associated with enhanced differentiation of IL-4-producing T cells and reduced Foxp3+ Tr cell differentiation in mesenteric lymph nodes (MLNs) (6). We have found that intestinal immunization toward gliadin, followed by COX inhibition, decreases villus height in DQ8 mice (11). In the present study, the effects of oral gliadin challenge with Indo were explored in this model in the absence of a mucosal immunization. Our results indicate that the intrinsic toxicity of gliadin in the absence of COX-2 activity was sufficient to trigger several typical pathological signs of CD.

MATERIALS AND METHODS

Protein preparation. Flour from wheat cultivar Sagittario (1 g) was defatted twice with petroleum ether (5 ml) for 30 min. Albumin and globulin (Alb/Glob) were extracted by resuspending the defatted flour in 0.4 M NaCl in 0.067 NaH2PO4 buffer at pH 7.6 (4 ml). The suspension was shaken for 10 min and centrifuged at 15,000 g for 15 min. The supernatant containing Alb/Glob was freeze dried and stored at −80°C. Gliadin was extracted from the pellet by adding a water-ethanol mixture (5 ml) to a final concentration of 70% (vol/vol) ethanol. The suspension was shaken for 45 min and centrifuged at 15,000 g for 10 min. The supernatant was freeze dried and stored at −80°C. For in vivo experiments, wheat proteins were suspended at 1 mg/ml in 200 mM acetic acid.

Mice. DQ8 mice, which expressed the HLA-DQ8 molecule in the absence of endogenous mouse class II genes and were nontransgenic for human CD4 (7), were maintained in pathogen-free conditions at our animal facility (Accreditation No. DM.161/99). Animals were reared on a gluten-free diet for several generations and used at the age of 6–12 wk. These experiments were approved by the National Institutional Review Committee and performed in accordance with European regulations (EU Directive 2010/63/EU).

In vivo treatments. DQ8 mice were intragastrically administered 25 mg/kg doses of wheat proteins (gliadin or Alb/Glob) on days 0, 3, 5, and 7. A subenteropathic dose of Indo (1.5 mg/100 ml or 0.75 mg/100 ml, Sigma-Aldrich, Milan, Italy) (11) was given in the drinking water and changed every 3–4 days. Mice were euthanized on day 10 or 30. In some experiments, mice were challenged with wheat proteins for 7
days and injected intraperitoneally with the selective COX-2 inhibitor NS-398 (1 mg/kg, Sigma-Aldrich) at 8-h intervals, as previously described by Newberry et al. (35). Fragments of the small intestine were collected for biochemical and immunomorphometric measurements. MLN cells were also isolated for the in vitro assessment of cytokine patterns.

**Morphometry and immunohistochemistry.** Tissue fragments were placed on black filter paper and covered with normal saline, and their mucosal surfaces were immediately examined and photographed under a stereomicroscope. Tissue was then embedded in OCT compound, snap frozen in liquid nitrogen, and prepared for histological and immunohistochemical evaluation. Cryostat sections (5 μm) were air dried at room temperature, fixed in acetone, and tested with rat anti-mouse CD3 or Ly-6B.2 (neutrophil) antibody (Serotec, DBA, Milan, Italy). Sections were then incubated with rabbit anti-rat serum (Dako, Copenhagen, Denmark) and finally with rat peroxidase anti-peroxidase complex (Rockland Immunochemicals, Gilbertsville, PA). Slides were developed with 2-amino-9-ethyl-carbazole (Sigma-Aldrich). Nonspecific binding was monitored by omitting the primary antibody. Sections were finally stained with Mayer’s hematoxylin and mounted. Villus height and crypt depth were measured with an ocular micrometer. Twenty to thirty individual measurements were made on each slide, and average crypt depths and villus heights were calculated. The density of intraepithelial cells expressing CD3 and of LP neutrophils was determined by counting the number of stained cells per millimeter of epithelium and per millimeter squared of LP, respectively. Immunofluorescence confocal microscopy was applied to detect macrophage F4/80 (F4/80) or expression of the costimulatory molecule B7-2 (CD86). Acetone-fixed sections (6 μm) were incubated with rabbit anti-mouse F4/80 or rat anti-mouse CD86 antibodies (Biolegend, San Diego, CA) for 1 h. Sections were then incubated with rabbit FITC anti-rat antibody (Life Technologies, Italia, Monza MB, Italy). Finally, all sections were counterstained with ToPro-3 (Life Technologies), mounted in PBS-glycerol (1:1), and imaged with a Leica SP confocal microscope (Leica TCS-SP, Heidelberg, Germany). The density of cells expressing F4/80 and CD86 was evaluated within a total area of 1 mm² of LP.

**Cytokine protein assay.** MLN cells were passed through a stainless steel wire mesh to dissociate cells. MLN cells were cultured at 2.5 × 10⁶ cells/ml in the presence or absence of gliadin (100 μg/ml) after 72 h, supernatants were collected and analyzed for IFN-γ, IL-4, and IL-10 protein levels by ELISA.

**Analysis of mRNAs.** RNA was extracted from small intestinal samples using TRIzol reagent (Invitrogen, Milan, Italy). cDNA was prepared by reverse transcription. Real-time PCR was performed on an iCycler iQ (Bio-Rad Laboratories, Hercules, CA). The reaction conditions for 39 cycles were 95°C for 30 s, 54.6°C (L-32 and COX-2), 58.0°C (E-cadherin), or 60.0°C (TNF-α) for 30 s, and 72°C for 40 s. Gene expression levels were calculated using the ΔΔCt method (where Ct is threshold cycle) (26) and presented as fold changes after normalization to the L-32 housekeeping gene. The following primer sequences were used: L-32, forward 5’-CCTCAGCCCTTTGAGGC-3’ and reverse 5’-GCCCTTGAATCTTCTTACGAAACC-3’; E-cadherin, forward 5’-GCACATATGAGCTCTCATC-3’ and reverse 5’-CCTTCAGACACTACACATG-3’; COX-2, forward 5’-ACCGGACTGTTCTAT-3’ and reverse 5’-GCTTCAGCCTTTGTA-3’; and TNF-α, forward 5’-CATCTTCTCAAAATTCGTAGTCAA-3’ and reverse 5’-TGGAAGTAGACAGTGTCACACC-3’.

**Evaluation of oxidative stress.** To evaluate the effects of the different treatments on intestinal redox status, intracellular total glutathione (GSH) and protein carbonyls (PCs) were measured (1) to estimate the level of intestinal antioxidant defenses and the extent of oxidized proteins, respectively. The activities of two enzymes involved in the cellular detoxification, namely, acylpeptide hydrolase (APEH) and glutathione-S-transferase (GST), were measured as previously described (2).
(20 μl/mg tissue) containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 10 mM CaCl2, 0.2 mM NaN3, 0.01% Triton X-100, and a protease inhibitor cocktail. After centrifugation, supernatants were collected, and their protein content was determined. A total of 10 μg protein was electrophoresed in a 12% SDS-polyacrylamide gel copolymerized with gelatin or casein (0.1%) as a substrate. At the end of the electrophoresis, gels were washed three times for 15 min each with 2.5% Triton X-100 to remove SDS and to allow the partial renaturation of the proteins and recovery of their activity. Gels were then incubated in zymography buffer [50 mM Tris·HCl (pH 7.5) containing 5 mM CaCl2] for 18 h at 37°C. Individual MMPs were identified by the degradation of their preferential substrate, as visualized upon Coomassie blue staining, and by their apparent molecular weight.

Serum antibodies. On day 30, serum was collected and IgG/IgA antibodies toward gliadin and tTG were assayed by ELISA. In brief, gliadin or tTG was coated onto U-bottomed microtiter plates at 0.5 μg/well overnight at 4°C. Plates were blocked with PBS containing 2% BSA. After samples had been washed with PBS containing 0.05% Tween 20, 1:10 dilutions of serum were added to individual wells and incubated for 2 h at room temperature. The presence of antibodies was detected with peroxidase-conjugated rabbit anti-mouse isotype-specific antibodies (Dako), and the reaction developed with 1 mg/ml o-phenylendiamine/HCl substrate and 0.06% H2O2. Results are expressed as absorbance values at 450 nm after subtraction of the blank background.

Statistical analysis. Differences among various treatment groups in the morphology data were determined by the Kruskal-Wallis nonparametric test followed by Dunn’s multiple-comparison test. Differences between groups in the cytokine assays were established by Student’s t-distribution. Differences in the parameters of oxidative stress and caspase-3 activity were determined by one-way ANOVA followed by the Tukey test. Differences in antibody levels were determined by a Mann-Whitney test. P < 0.05 was selected to denote a significant difference.

RESULTS

Gliadin intake in association with Indo alters the small intestinal architecture in DQ8 mice. DQ8 mice on a gluten-free diet were challenged with Indo, which is a nonspecific COX inhibitor, and wheat gliadin for 10 days (Fig. 1A). The control group received Indo + wheat Alb/Glob. SDS-PAGE analysis of main protein bands showed no cross-contamination between the two preparations (Fig. 1B). Examination of the biopsy specimens through a stereomicroscope showed that villi of the jejunal mucosa from treated mice were shorter than those from mice belonging to the control group (Fig. 1C). By histology, villus blunting was observed when gliadin was intragastrically administered during the treatment with Indo but not when Alb/Glob was used (Fig. 1D), showing a good correlation between the stereomicroscopic and histological observations. Furthermore, Indo + gliadin also increased crypt depth (Fig. 1D). The statistical relevance of gliadin as a specific food molecule capable of causing the onset of the mucosal lesion was then examined. As shown in Fig. 2, top, mice that received wheat gliadin along with Indo showed significant villus shortening compared with the other experimental groups. Moreover,

Fig. 3. Innate immunity induced in the small intestine of enteropathic mice. A: immunofluorescence staining for the F4/80 marker in the jejunal mucosa from mice treated with Indo + Alb/Glob (left) or Indo + gliadin (right). B: immunofluorescence staining for the CD86 marker in the jejunal mucosa from mice treated with Indo + Alb/Glob (left) or Indo + gliadin (right). Nuclei are shown in red after ToPro-3 staining. Original magnification: ×40. C: statistical assessment of F4/80+ (top) and CD86+ (bottom) cells in the two experimental groups. Bars represent median values; n = 5. D: top: TNF-α mRNA levels evaluated by real-time PCR in the two experimental groups. Values were normalized to L-32 mRNA and are presented as fold changes in gene expression [in arbitrary units (AU)]. Bars represent median values. Bottom: basal interferon (IFN)-γ levels secreted in vitro from mesenteric lymph node (MLN) cells. Bars represent mean values ± SD. Results are representative of three independent experiments. *P < 0.05; **P < 0.01.

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the increase in crypt depth registered in gliadin-treated mice was also significant (Fig. 2, middle). On the other hand, IEL numbers were similar among all of the experimental groups (Fig. 2, bottom). Interestingly, immunofluorescence analysis revealed an increased number of cells expressing F4/80, a marker of mature macrophages (Fig. 3), together with higher expression of the costimulatory CD86 molecule in LP cells of Indo + gliadin-treated mice (Fig. 3B); both differences were significant (Fig. 3C). In line with these results, enteropathic mice showed increased transcript levels of LP TNF-α, a cytokine mainly produced by activated macrophages (Fig. 3D, top). To further investigate the inflammatory status of the small intestine, MLN cells were isolated and analyzed in vitro. We found that cells from mice treated with Indo and gliadin secreted basal levels of IFN-γ higher than cells from mice administered Alb/Glob (Fig. 3D, bottom). However, no increases in the basal secretion of IL-4 or IL-10 were observed (data not shown). These data indicate that gliadin intake, in the absence of COX activity, was sufficient to unbalance the immune homeostasis of the small intestine by introducing an inflammatory status in DQ8 mice.

Gliadin intake and specific inhibition of COX-2 are mandatory for generating the intestinal lesion in DQ8 mice. Indo belongs to the nonsteroidal anti-inflammatory drug family, molecules that are able to inhibit COX-2 activity by specifically suppressing gene transcription (49). Moreover, Indo has been implicated in downregulating E-cadherin mRNA, a modulator of intestinal barrier function in gluten-sensitized DQ8 mice (33). To assess the effect of Indo in our model, we quantitatively examined transcript levels of both E-cadherin and COX-2 in the small intestine of treated mice. The analysis of mRNA indicated that administration of subenteropathic doses of Indo alone (1.5 mg/100 ml and 3.0 mg/100 ml) or in combination with Alb/Glob or gliadin did not influence the transcription of E-cadherin (Fig. 4A, top). Interestingly, subenteropathic doses of Indo only in combination with wheat proteins significantly reduced transcript levels of COX-2 (Fig. 4A, bottom). To further investigate the role of COXs, mice were treated with NS-398, a specific competitive inhibitor of COX-2 (35). We found that 7 days of treatment with NS-398 during gliadin challenge also induced enteropathy, as evidenced by significant villus shortening and increased crypt depth (Fig. 4B). Cotreatment with NS-398 and Alb/Glob was unable to produce similar alterations of the mucosal architecture. Taken together, these data clearly indicate that inhibition of COX-2 activity is required to generate the gliadin-driven mucosal lesion in DQ8 mice.

Increased apoptosis and MMP-2/MMP-9 activities are involved in the onset of the gliadin-specific enteropathy. To dissect the molecular mechanisms elicited by gliadin that are directly responsible for the mucosal lesion, apoptosis and MMP activities of the small intestinal mucosa were determined in the various experimental groups. Caspase-3 activity was significantly increased in Indo-treated mice after gliadin intake (Fig. 5A). Interestingly, the increase in apoptosis was associated with enhanced expression of tTG (Fig. 5A, inset). This result was in line with reported observations in CD (17, 22, 23). Substrate zymography was performed to analyze the involvement of MMP activity in the induced mucosal damage. As shown in the casein zymogram (Fig. 5B, left), which is useful for detecting collagenases, no specific increase of MMP activity was observed, with the exception of MMP-7, which was enhanced in both Indo-treated groups. In the gelatin zymogram, gliadin intake further enhanced the activity of MMP-9 (85 kDa) and the overall expression of MMP-2, with the appearance of the MMP-2 latent form (pro-MMP-2, 72 kDa; Fig. 5B, right).

Fig. 4. Cooperation between gliadin intake and cyclooxygenase (COX)-2 inhibition in generating the intestinal lesion. A: E-cadherin and COX-2 mRNA levels evaluated by real-time PCR in DQ8 mice challenged with wheat proteins in the presence of Indo. Values were normalized to L-32 mRNA and are presented as fold changes in gene expression (in AU). Bars represent median values. ++, 3.0 mg/100 ml Indo (double dose). B: morphometric analysis of the small intestine from DQ8 mice challenged with wheat proteins and cotreated with NS-398, a specific inhibitor of COX-2 activity. Bars represent median values; n = 5. *P < 0.05; **P < 0.01.
High oxidative stress conditions are associated with the intestinal lesion. As antioxidant and detoxifying defenses are unpaired in CD patients (2, 48), these parameters were examined in our model to further dissect the mechanisms responsible for the onset of enteropathy. Gliadin intake was associated with a significant increase of PC levels (Fig. 6A) and a specific reduction of total GSH content in the small intestine (Fig. 6B, top). GST activity was also reduced compared with untreated mice (Fig. 6B, middle), but its level was similar to that in mice treated with Alb/Glob or Indo alone (data not shown). Among detoxifying enzymes, APEH (also called oxidized protein hydrolase) was specifically decreased after gliadin treatment (Fig. 6B, bottom). Taken together, these data indicate that the combined action of gliadin and Indo specifically triggered oxidative stress conditions at the intestinal level, similar to those evidenced in CD patients.

Prolonged gliadin intake induced tTG autoantibodies in Indo-treated mice. To assess whether chronic administration of gliadin in enteropathic DQ8 mice could elicit other signs of mucosal alterations observed in CD, we extended our administration protocol to 30 days. However, as prolonged Indo treatment at 1.5 mg/100 ml was associated with a high mortality rate (data not shown), we halved the dose of Indo. This new regimen drastically reduced the number of dead mice during the long treatment but still induced a significant intestinal lesion on both days 10 and 30 (Fig. 6A, top and middle). Interestingly, on day 30, an increased number of neutrophils infiltrating the intestinal LP was found (Fig. 7A, bottom), whereas the IEL number remained unchanged (data not shown). Moreover, enhanced expression of tTG in enteropathic mice was still detected on day 30 (Fig. 7B). In contrast, at this time, neither enhanced basal levels (Fig. 8A) nor gliadin-specific cell-mediated induction of IFN-γ (data not shown) were found in isolated MLN cells. Notably, the analysis of humoral responses showed significant levels of serum anti-tTG (IgG) and anti-gliadin (IgA) antibodies in long-term enteropathic mice (Fig. 8B). No significant differences were observed among mice treated with Alb/Glob and Indo alone (data not shown). Importantly, after gliadin withdrawal, a significant recovery of villus height together with a trend in the reduction of crypt depth were reported in 30-day Indo-treated mice (Fig. 9A).
On the other hand, reversal of the mucosal lesion was not associated with a reduction of caspase-3 activity (Fig. 9B). Furthermore, oxidative stress conditions were restored only for total GSH content (Fig. 9B), whereas GST activity (Fig. 9B) and PC levels (data not shown) were unchanged.

**DISCUSSION**

In the present study, we showed, for the first time, that the oral intake of gliadin, in association with specific inhibition of COX-2, caused alteration of the small intestinal architecture in DQ8 mice followed by the development of an antigen-specific immunity. Various in vivo strategies have been tested so far to reproduce CD (5, 20, 28, 29). In particular, the existence of a linkage between the acquisition of gluten sensitivity and expression of DQ8 (2, 5, 33) was confirmed in DQ8 transgenic mice on a NOD background, which develop dermatitis herpetiformis after gluten exposure (28). The DQ8-restricted gluten sensitivity, along with the T helper (Th)1 phenotype of the...
Gliadin-specific intestinal immunity (44), potentially made this model useful to analyze celiac-type responses. However, none of these models develop enteropathy. To address this issue, we recently explored the strategy of blocking intestinal COXs, enzymes involved in the synthesis of prostaglandins from arachidonic acid. In particular, PGE₂ has a crucial role in intestinal immune homeostasis (3, 13, 47). We previously applied a protocol of Indo administration in mucosally immunized DQ8 mice. Interestingly, an intestinal lesion was generated, characterized by a reduction of villus height without influencing the epithelial cell turnover, because crypt cell proliferation and enterocyte apoptosis did not increase (11). In the present study, we showed that intestinal adaptive immunity toward gliadin was not an essential prerequisite to trigger the mucosal damage in this model. In particular, we found that the simultaneous specific inhibition of COX-2 and gliadin intake were sufficient conditions to generate an even more marked lesion in the small intestine. Most important, the peculiarity of wheat gliadin as a food molecule capable of generating such a deleterious response was underscored by the finding that the Alb/Glob fraction, the soluble protein fraction from wheat, was unable to generate any type of damage. In particular, even if both protein fractions were found to reduce transcript levels of COX-2, in this context only gliadin was able to induce the lesion. Furthermore, the induced enteropathy represented a reversible process: gliadin withdrawal caused a recovery of the mucosal architecture in Indo-treated DQ8 mice, similarly to CD.

Gliadin peptides exhibit intrinsic cytotoxicity by reducing F-actin content (15), causing cytoskeleton rearrangements through the zonulin pathway (16) and reducing cell viability and detoxifying enzyme activities in Caco-2 cells (2). However, these activities are not observed in DQ8 mice fed a gluten-containing diet (2), suggesting that gluten alone is incapable of reproducing the typical oxidative stress signs of CD in this mouse model. Notably, we found that gliadin intake along with inhibition of COXs triggered the production of oxidative stress markers in DQ8 mice. In particular, total GSH content and APEH activity decreased, whereas PCs increased, in line with our findings in intestinal biopsies from CD patients (2). On the other hand, the reported reduction of GST, a well-known phase 2 enzyme that has a key role in detoxification mechanisms, was essentially related to Indo administration. APEH is an enzyme expressed in the intestinal mucosa that participates in the degradation of oxidized proteins (46) or cleaves N-formyl peptides derived from bacteria (36), potent proinflammatory chemoattractants for phagocytes. Importantly, gliadin intake specifically elicited an inflammatory status in the small intestine of Indo-treated mice, characterized by an increased number of LP F4/80⁺ cells and high basal levels of IFN-γ in MLNs. Therefore, the significant decrease of APEH activity in our animal model confirmed the hypothesis that APEH is involved in severe intestinal inflammation (9).

To identify the molecular mechanisms mediating the intestinal lesion, we mainly focused on the degree of apoptosis occurring in the small intestine and on the MMP activity pattern that is engaged in tissue remodeling. We found that in enteropathic mice, gliadin intake drastically increased caspase-3 activity, one of the key executers of apoptotic cell death (42). Interestingly, we also detected a parallel increased expression of tTG. The expression of this enzyme has been associated with apoptotic activity. In fact, tTG is induced and activated during apoptosis in the liver and other tissues, forming cross-linked protein polymers that may help keep intracellular components inside dying cells (18, 37). In addition, tTG overexpression is described in untreated CD patients (17, 22, 23). In line with our findings, gliadin-induced inflammation has recently been related to an increased production of tTG in the LP of gluten-fed BALB/c mice (41). MMP activity has been recently been related to an increased production of tTG in the LP of gluten-fed BALB/c mice (41). MMP activity has been associated with tissue remodeling but also with changes in epithelial barrier function and mucin production (21). Importantly, MMPs are activated in CD as a consequence of the gluten-related inflammatory response (8). We (11) have previously found increased activities of MMP-1, MMP-2, and MMP-7 in immunized DQ8 mice after Indo treatment. A different MMP activation pattern was elicited here, essentially characterized by enhanced activities of MMP-2 and MMP-9. Different inflammatory conditions are elicited by the two...
protocols (COX-2 inhibition in the presence of an ongoing gliadin-specific adaptive response to gliadin vs. gliadin uptake in naïve mice temporally associated with COX-2 inhibition) that could explain the reported discrepancies in the MMP activation pathway. Within the large family of zinc-dependent MMPs, the two gelatinases MMP-2 and MMP-9 have the unique ability to degrade collagen type IV, which is a major component of the basement membrane and differ from other MMPs in their molecular structures (40). Whereas MMP-9 is absent from healthy tissues, its expression and activity are increased in several animal models of colitis (30, 43). It is noteworthy that the increased activities of MMPs in enteropathic mice were consistent with MMP activation in the celiac duodenal mucosa and with the described association between MMP activity and oxidative stress conditions (34).

Notably, prolonged intake of gliadin in Indo-treated DQ8 mice induced the production of anti-gliadin and anti-tTG antibodies, a hallmark of the HLA-restricted adaptive immune response of CD. Most important, Alb/Glob was unable to induce these antibodies, strengthening the role of gliadin in the onset of HLA-mediated autoimmunity in CD. More specifically, the increased expression of tTG induced by gliadin intake in Indo-treated mice could be mainly responsible of the observed humoral response. In contrast, no signs of the canonical gliadin-specific Th1 T-cell mediated response (38) were reported in enteropathic mice on day 30. This could be a consequence of the prolonged COX-2 inhibition that favors differentiation of Th2-like T cells (6). Furthermore, this could also explain why, different from day 10, the basal levels of intestinal IFN-γ on day 30 were not higher than those found in control mice. Long-term enteropathy in DQ8 mice was also associated with an increased number of neutrophils, whereas IELs remained unchanged. Neutrophilic infiltration has been described in duodenal biopsy specimens from patients with CD (14). On the contrary, the unchanged number of IELs reported in enteropathic DQ8 mice was in apparent contrast with normal findings in CD, suggesting that apoptotic mechanisms mediated by IELs (31) are not operative in our model. Studies to evaluate if the administration of IL-15, known to induce IEL massive expansion (39), could exacerbate the intestinal lesion in our model are currently planned.

Taken together, our data point out a role of IFN-γ production and related oxidative stress to drive the early phase of the mucosal lesion by activating MMPs and inducing apoptosis. This condition can be reproduced in DQ8 mice by deregulating the cell-mediated immunity to gliadin (11) or by gliadin challenge in the absence of COX-2 activity. We speculate that the initial inflammatory phase paves the way for developing the antigen-specific machinery of the adaptive immune response needed for perpetuating the enteropathy and inducing autoimmune responses. Therefore, this model could be instrumental in further investigations of the pathogenesis of CD and to analyze possible strategies to block gluten toxicity.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


