Gliadin-dependent neuromuscular and epithelial secretory responses in gluten-sensitive HLA-DQ8 transgenic mice

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Verdu EF, Huang X, Natividad J, Lu J, Blennerhassett PA, David CS, McKay DM, Murray JA. Gliadin-dependent neuromuscular and epithelial secretory responses in gluten-sensitive HLA-DQ8 transgenic mice. Am J Physiol Gastrointest Liver Physiol 294: G217–G225, 2008. First published November 15, 2007; doi:10.1152/ajpgi.00225.2007.—Celiac disease is a gluten intolerance caused by a T-cell response against human leukocyte antigen (HLA)-DQ2 and DQ8-bound gluten peptides. Some subjects experience gastrointestinal symptoms in the absence of villous atrophy. Here we investigate the potential mechanisms of gut dysfunction in gluten-sensitive HLA-DQ8 transgenic mice. HLA-DQ8 mice were sensitized and gavaged with gliadin 3×/wk for 3 wk (G/G). Controls included 1) nonsensitized mice gavaged with rice (C); 2) gliadin-sensitized mice gavaged with rice (G/R); and 3) BSA-sensitized mice gavaged with BSA (BSA/BSA). CD3+ intraepithelial lymphocyte, macrophage, and FOX-P3-positive cell counts were determined. Acetylcholine release, small intestinal contractility, and epithelial ion transport were measured. Gut function was investigated after gluten withdrawal and in HLA-DQ6 mice. Intestinal atrophy was not observed in G/G mice. Recruitment of intraepithelial lymphocyte, macrophages, and FOX-P3+ cells were observed in G/G, but not in C, G/R, or BSA/BSA mice. This was paralleled by increased acetylcholine release from the myenteric plexus, muscle hypercontractility, and increased active ion transport in G/G mice. Changes in muscle contractility normalized in DQ8 mice after a gluten withdrawal. HLA-DQ6 controls did not exhibit the abnormalities in gut function observed in DQ8 mice. Gluten sensitivity in HLA-DQ8 mice induces immune activation in the absence of intestinal atrophy. This is associated with cholinergic dysfunction and a prosecretory state that may lead to altered water movements and dysmotility. The results provide a mechanism by which gluten could induce gut dysfunction in patients with a genetic predisposition but without fully evolved celiac disease.

muscle contractility; intestinal ion transport; food sensitivity

CELIAC DISEASE (CD) IS A PERMANENT intolerance to gluten, characterized by specific lesions in intestinal biopsies that improve after gluten withdrawal (15). So far, the only known gene for CD is human leukocyte antigen (HLA)-DQ2/8, which plays an important role in the presentation of gluten to T cells. Genetic (28) and environmental factors may influence the nature of reactivity to gluten and phenotypic expression of disease (12, 21). Studies using rectal gluten challenge in patients with autoimmune disorders have identified gluten-sensitive patients who exhibit a deranged immune response to gluten with no atrophic enteropathy (22, 30). Others have shown that a proportion of patients with normal small-bowel mucosal morphology and positive IgA-class antireticulin or antigliadin antibodies have immunohistochemical markers of CD latency, indicating gluten sensitivity (18). Finally, it has been reported that patients who do not fit all of the criteria for diagnosis of CD exhibit gluten-dependent functional bowel symptoms (2, 8, 25) and mild histopathological abnormalities, such as intraepithelial lymphocytosis, in the context of HLA-DQ2 markers (33, 34).

Sensitization to gliadin can be induced in BALB/c mice, and this strategy has been used successfully to test the potential for intranasal tolerization protocols (23). A model of gluten sensitivity also exists in transgenic mice that express the human major histocompatibility class II molecules (DQ8) associated with CD. Previous work in DQ8 mice has investigated the T-cell response in the model and demonstrated that gliadin-sensitized mice develop systemic IgG anti-gliadin antibodies, but no tissue transglutaminase (Ttg) antibodies, suggesting absence of autoimmune reaction. The type of immune response observed was HLA-DQ mediated, since proliferation of T cells incubated with gliadin was inhibited by anti-DQ8 monoclonal antibodies and did not occur in transgenic negative controls (6). The absence of enteropathy in these gluten-sensitive mice may reflect the appropriate regulatory response and hence explain the absence of widespread inflammation and classic mucosal injury (6). Thus the model allows investigation of the physiopathological changes due to gluten sensitization that may occur in the absence of villous atrophy in a genetically predisposed host. The importance of DQ8 expression to contribute to sensitivity to gluten was also shown in a study using HLA-DQ8 transgenic nonobese diabetic mice, in which a percentage of sensitized mice developed blistering pathology similar to that seen in dermatitis herpetiformis, an autoimmune skin disorder associated with gluten sensitivity (20).

In this study, we use HLA-DQ8 mice to investigate the effects of gliadin sensitization on innate immune markers and on neuromotor and epithelial cell secretory function in the gut to provide a potential mechanism to explain gluten-induced symptoms in humans without CD.

MATERIALS AND METHODS

Mice. Transgenic mice expressing HLA-DQ8 (HLA-DQA1*0301; HLA-DQB1*0302) genes in the absence of endogenous mouse class II genes were used (7). The mice were bred in a conventional specific pathogen-free colony at McMaster University and maintained at least

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two generations before breeding on a gluten-free diet (Bio-Serve, Frenchtown, NJ). Transgenic HLA-DQ6 mice (courtesy of Dr. J. Murray, Mayo Clinics) were used as controls for the DQ8 transgene. Mice were used at the age of 8–14 wk. All experiments were conducted with approval from the McMaster University Animal Care Committee.

**Experimental groups.** All mice were continuously fed a gluten-free diet. For sensitization, mice were injected intraperitoneally with 500 μg of gliadin (gliadin from wheat; Sigma-Aldrich, Oakville, Ontario, Canada) dissolved in 0.02 mM acetic acid in 50 μg of Complete Freund’s Adjuvant (CFA; Sigma-Aldrich). Starting 1 wk later, gliadin challenge was performed 3×/wk by intragastric gavage, for 3 wk, using 2 mg of gliadin dissolved in 0.02 mM acetic acid. Control groups consisted of 1) nonsensitized mice (injected with adjuvant only) that were subsequently challenged with rice cereal (2 mg/0.02 mM acetic acid); 2) gliadin-sensitized mice subsequently challenged with rice cereal (2 mg/0.02 mM acetic acid); and 3) BSA-sensitized mice subsequently challenged with BSA (2 mg/0.02 mM acetic acid).

**Overall design.** To investigate the immune and gut functional consequences of gliadin sensitization, four groups of mice were used: HLA-DQ8 mice, nonsensitized mice challenged with rice (n = 5); gliadin-sensitized mice challenged with rice (n = 5); BSA-sensitized mice challenged with BSA (n = 5); and gliadin-sensitized mice challenged with gliadin (n = 5). Mice were killed 24 h after the last oral challenge. The longitudinal muscle myenteric plexus preparation (LMMP) (32) was obtained from 4-cm-long jejunal sections for ACh release measurement. To test the sensitization procedure, serum was collected for anti-gliadin IgG antibodies and Ttg antibodies. Sections (1 cm long) were obtained from the proximal jejunum, starting at the ligament of Treitz for histology evaluation by hematoxylin and eosin (H&E) stain. CD3⁺, macrophage, and FOX-P3 cell examination by immunohistochemistry was performed in paraffin blocks.

To investigate the functional consequences of changes in ACh release in the intestinal muscular apparatus, additional mice (n = 8/group) were used to investigate in vitro contractility of jejunal strips in response to pharmacological stimulation in the presence and absence of tetrodotoxin (TTX) and after electrical field stimulation (EFS) at neural parameters. Because only neural parameters were used for EFS, TTX administration was not applied after this stimulation. In a third set of experiments, ion transport was investigated, using a group of gliadin-sensitized and -challenged mice (n = 8) and nonsensitized mice challenged with rice cereal (n = 8). Again, controls consisted of gliadin sensitized, challenged with rice (n = 8) and BSA sensitized, challenged with BSA (n = 8). Twenty-four hours after the last oral challenge, jejunal tissues were obtained and mounted in Ussing chambers. To test whether changes in ion transport in transgenic mice were dependent on chloride secretion, we performed experiments in five HLA-DQ8 mice using chloride-free buffer or Krebs.

To investigate the dependence on oral gliadin for the altered muscle contractility and ion transport observed in DQ8 mice, additional groups of DQ8 gliadin-sensitized and -challenged mice were studied. After 3 wk of gliadin challenge, one group was allocated to gluten-free chow (n = 5), while gliadin challenges continued for 3×/wk for 3 wk in the other group (n = 6). A control group of nonsensitized mice (CFA only) fed rice were also studied. Muscle contraction and ion transport were measured 24 h after the last gliadin or rice challenge.

The importance of the DQ8 trangene for the responses observed after gliadin sensitization and challenge was examined in experiments...
performed in HLA-DQ6 haplotype controls. One group of HLA-DQ6 (n = 5) was sensitized and challenged with gliadin following the protocol already described. A second group (n = 5) was used as nonsensitized (CFA only) controls and was gavaged with rice.

**Anti-gliadin and Ttg antibodies.** For determination of specific anti-gliadin IgG and Ttg IgA antibodies, serum was obtained at death, and antibodies were detected using kits from Inova Diagnostics (San Diego, CA). Detection antibodies consisted of biotinylated rat anti-mouse IgG and IgA, respectively (Sigma-Aldrich).

**Light microscopy and histological evaluation.** Cross sections from the proximal small intestine were preserved in 10% formalin and then processed and stained with H&E. Specimens were examined under light microscopy for villus-to-crypt ratio, presence of polymorphonuclear and mononuclear inflammatory infiltrates, and epithelial damage. FOX-P3 cell staining was counted in the lamina propria in five villi randomly chosen using two different sections per mouse. A mean value was obtained and expressed as number of positive cells per five villi. Intraepithelial lymphocytes (IELs)/20 enterocytes in five randomly chosen villous tips were counted according to the method of Biagi et al. (5) and expressed as IEL/100 enterocytes. Positive cells for F4/80 staining were counted in the lamina propria in five villi randomly chosen using two different sections per mouse. A mean value was obtained and expressed as number of macrophage-positive cells per five villi. Slides were examined in a blinded fashion by two investigators (E. F. Verdu and X. Huang).

**Immunohistochemistry for FOX-P3 cells in lamina propria.** FOX-P3 is a transcription factor that functions as regulator of the development of CD4+ regulatory T cells. Immunostaining for FOX-P3-positive cells was performed on paraffin sections. The primary monoclonal antibody mouse anti-mouse (1:200; BioLegend, Vineyard, Ontario, Canada) was used with M.O.M Basic Immunodetection system (Vector Laboratories Canada, Burlington, Ontario, Canada). This system provides a blocking reagent and a biotinylated anti-mouse IgG reagent. A streptavidin/horseradish peroxidase (1:300; Dakocytomation, Mississauga, Canada) was used for signal enhancement and detection. Antibodies were visualized by 3-amino-9-ethylcarbazole substrate chromogen (Dakocytomation) and counterstaining with Mayer’s hematoxylin. Negative controls were performed in the absence of primary antibody.

**Immunohistochemistry for CD3+ cells.** Immunostaining for CD3+ cells was performed on paraffin sections using a modified method described previously (3). Rabbit anti-mouse CD3 (1:300; Dako A/S) was used as primary antibody, followed by biotinylated swine anti-rabbit (1:300; Dako A/S) and streptavidine peroxidase conjugate (1:600; Dako A/S). The antibodies were visualized using 3-amino-9-ethylcarbazole and counterstaining with Mayer’s hematoxylin. Negative controls were performed in the absence of primary antibody.

**Immunohistochemistry for macrophages.** Immunostaining for macrophages was performed on paraffin sections using a monoclonal antibody recognizing the F4/80 antigen (4). The primary antibody rat

![Fig. 2](http://ajpgi.physiology.org/)

**Fig. 2.** Immunohistochemistry showing increased number of CD3+ intraepithelial lymphocytes (IEL) in a gliadin-sensitized and -challenged mouse. A: nonsensitized mouse gavaged with rice. B: gliadin-sensitized mouse challenged with rice. C: BSA-sensitized mouse challenged with BSA. D: gliadin-sensitized mouse challenged with gliadin. Bar graph (means ± SD) depicts quantification of CD3+–positive cells/20 enterocytes in 5 villi. Statistical significance: P < 0.05 (ANOVA for multiple comparisons).
neural crest, and the loss of neural crest derivatives leads to abnormal development of the gut. The authors also discuss the role of reactive oxygen species (ROS) in the development of gut inflammation in gliadin-sensitized mice. They present evidence that gliadin exposure induces oxidative stress, which contributes to the development of enteropathy in these mice. The study concludes with a discussion of the potential therapeutic implications of these findings for the treatment of celiac disease.

The research presented in this article highlights the importance of studying the role of gliadin exposure in the development of gut inflammation and the potential therapeutic implications of these findings. It provides a comprehensive overview of the current understanding of the mechanisms underlying gliadin-induced gut inflammation and the potential for developing new therapeutic strategies for the treatment of celiac disease.
RESULTS

Markers of gliadin sensitization. Antigliadin antibodies (IgG) were found in sera from all gliadin-sensitized mice. Antibody levels in gliadin-sensitized and -challenged mice were $0.9/11006^{0.02}$ (outer diameter); antibody levels were similar in gliadin-sensitized mice challenged with rice (outer diameter: $1.0/11006^{0.01}$). Nonsensitized mice and BSA-sensitized mice had undetectable levels of antigliadin antibodies. Ttg antibodies (IgA) were undetectable in all groups.

Markers of regulatory immune response. In agreement with Black et al. (6), who have previously reported increased IL-10 production by T lymphocytes from HCD4/DQ8 mice incubated with gliadin, we found evidence for a regulatory immune response in DQ8 mice sensitized and challenged with gliadin. FOX-P3-positive cell counts in the lamina propria were increased in gliadin-sensitized and -challenged mice, but not in controls and in DQ8 mice sensitized and challenged with BSA (Fig. 1).

Markers of innate immune activation. No intestinal atrophy was observed in H&E-stained slides under light microscopy. An increase in cellularity was noted in the lamina propria of gliadin sensitized and challenged mice compared with the rest of the groups. No significant infiltrations with mononuclear or polymorphonuclear cells were observed in the muscularis propria or in the region of the myenteric plexus in any group. To quantify cellular changes in the lamina propria, CD3^{+} IEL counts and F4/80-positive cell counts were performed, showing increased counts in gliadin-sensitized and -challenged mice (Figs. 2 and 3).

Gliadin-sensitized and -challenged DQ8 mice exhibit increased ACh release from LMMP. Figure 4 shows increased ACh release after EFS, but not after KCl stimulation, in gliadin-sensitized and -challenged mice compared with nonsensitized controls. Increased ACh release was not observed in control mice and in mice that had been sensitized and challenged with BSA.

Gliadin-sensitized and -challenged DQ8 mice exhibit neuromuscular dysfunction. To investigate the functional consequences of increased ACh release, contractility experiments of whole muscle strips were performed. The results show that gliadin-sensitized and -challenged mice displayed significantly higher maximal tension compared with gliadin-sensitized mice challenged with BSA, BSA-sensitized mice challenged with BSA, and nonsensitized mice. Increased maximal tension was evident in gliadin-sensitized and -challenged mice after stimulation with EFS at neural parameters and after $10^{-6}$ M carbachol (Figs. 5 and 6). To examine whether responses to carbachol were neurally mediated, we tested differences in muscle contraction before and after administration of TTX in the muscle bath. TTX administration abolished the neurogenic component of contraction and led to an overall attenuation in contractility after carbachol in all groups. After TTX, gliadin-sensitized and -challenged mice still demonstrated increased muscle hypercontractility compared with nonsensitized controls, but not compared with gliadin-sensitized mice challenged with BSA and BSA-sensitized and -challenged mice (Fig. 6).

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**Fig. 4.** ACh release from longitudinal muscle myenteric plexus preparation (LMMP) after electric field stimulation (EFS) and KCl in nonsensitized controls challenged with rice, gliadin-sensitized mice challenged with rice, and BSA-sensitized mice challenged with BSA. Gliadin-sensitized and -challenged mice exhibited a marked increase in ACh release from jejunal LMMP after EFS, but not after KCl stimulation. Values are means ± SD; n, no. of mice. Statistical significance: $P < 0.05$ (ANOVA for multiple comparisons). *$P = 0.01$ vs. all groups.

**Fig. 5.** Maximal peak of contraction ($g/mm^2$) of whole intestinal jejunal strips in nonsensitized controls challenged with rice, gliadin-sensitized mice challenged with rice, and BSA-sensitized mice challenged with BSA. Muscle contractility after EFS was increased in gliadin-sensitized mice challenged with gliadin. Values are means ± SD; n, no. of mice. Statistical significance: $P < 0.05$ (ANOVA for multiple comparisons).
Table 1. Baseline and stimulated secretory response

<table>
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<tr>
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<th>Nonsensitized</th>
<th>Gliadin-Rice</th>
<th>BSA-BSA</th>
<th>Gliadin-Gliadin</th>
</tr>
</thead>
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<tr>
<td>Baseline $I_{sc}$, μA/cm²</td>
<td>29.9±10</td>
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<td>Carbachol $I_{sc}$, μA/cm²</td>
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<tr>
<td>Conductance, mS/cm²</td>
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<td>19.0±8</td>
<td>17.4±6</td>
<td>22.5±4.7</td>
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<tr>
<td>Forskolin $I_{sc}$, μA/cm²</td>
<td>73.6±30</td>
<td>57.2±23</td>
<td>55.2±24</td>
<td>80.0±40</td>
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Values are means ± SD; n = 8 mice/group. $I_{sc}$, short circuit current. See MATERIALS AND METHODS for explanation of groups.

DISCUSSION

The main objective of this study was to determine the presence of functional changes in the gut of mice with gluten sensitivity, but lacking the severe atrophic enteropathy of CD. We confirm the lack of chronic enteropathy development in the model (20), as assessed by light microscopy. In conjunction with previous results (6, 20, 27), activation of both innate and adaptive arms of the immune system is present in DQ8 mice sensitized and challenged with gliadin. We found increased CD3+ IEL and macrophage counts in lamina propria in gliadin-sensitized and -challenged mice. Also, staining for the T regulatory transcription factor FOX-P3 (24) was increased in gliadin-sensitized and -challenged mice. This is consistent with the previous report of increased IL-10 production by T cells from gliadin-sensitized HLA-CD4/DQ8 mice incubated with gliadin (6) and further supports that the generation of a regulatory immune response may be involved in precluding widespread inflammation and atrophic enteropathy in transgenic mice. However, our results indicate that neither widespread
inflammation, nor the presence of atrophy is required for the generation of gliadin-induced gut dysfunction. Moreover, the generation of gut dysfunction as assessed in this study seems to depend on immune changes induced by gliadin in DQ8 mice, since the functional abnormalities were not observed in mice after sensitization and challenge with BSA, in which immune markers were not increased.

EFS induced a threefold increase in ACh release from the myenteric plexus from sensitized mice challenged with gliadin vs. nonsensitized controls. In contrast, KCl stimulation, which depolarizes neuronal membranes and releases all of the available pool of ACh, was similar in all groups. These changes were not observed in nonsensitized mice and in mice that had been sensitized and challenged with BSA. These results suggest a functional change leading to altered neuronal release of ACh, rather than impaired synthesis or storage of the neurotransmitter, which is specific to gliadin.

The induction of cholinergic dysfunction by gliadin sensitization and challenge in DQ8 mice provides an explanation for the observed altered muscle contraction and ion transport. We performed in vitro contractility experiments in whole jejunal strips. Results using TTX suggest that, in addition to the neural component, a muscle component underlies in vitro muscle hypercontractility in gliadin-sensitized and -challenged mice. Our results also show that BSA-sensitized and -challenged mice and nonsensitized controls had similar contractile responses to EFS or carbachol. However, when tissues were stimulated in vitro by the addition of BSA to the organ bath, an immediate contractile response was observed in sections from mice that had been sensitized to BSA. The contractile responses weakened with subsequent BSA challenges in the organ bath, suggesting mast cell involvement (data not shown). Taken together, the results indicate that the hypercontractile response after EFS or carbachol in gliadin-sensitized and -challenged mice is sustained and does not require in vitro stimulation with antigen. We suggest that mainly neural dysfunction is responsible for this sustained hypercontractility. This is supported by the finding that the difference in muscle contraction between gliadin-sensitized mice challenged with gliadin and gliadin-sensitized mice challenged with BSA is abolished after administration of TTX.

Water movement is regulated through epithelial ion transport, which is controlled by intrinsic and extrinsic factors, with one of the main regulatory systems being cholinergic nerves. Our results suggest that gliadin sensitization and challenge may lead to a prosecretory state in the jejunum and that this effect is neurally mediated. In contrast, BSA-sensitized and -challenged mice lacked these changes in nerve-mediated ion transport. In conjunction with the in vitro contractility experiments, the results suggest that enteric responses to gluten ingestion in sensitized DQ8 mice result in immune responses and functional changes on the neuromuscular apparatus that differ from those induced by BSA and classic allergic sensitization (26). Furthermore, the muscle hypercontractility and ion transport increases observed after gliadin sensitization and challenge in DQ8 mice were not observed in HLA-DQ6 mice. The results suggest the consequences of gliadin sensitization and challenge on secretomotor function are related to the HLA background.

It has recently been suggested that the presence of HLA-DQ2 status predicts the symptomatic response to a gluten-free diet in a subset of IBS patients (33). Gluten withdrawal for 3 wk completely normalized muscle hypercontractility in previously sensitized and challenged DQ8 mice. $I_{sc}$ responses after stimulation of electric current tended to decrease, but were still

### Table 2. Secretory response to ETS, carbachol, and forskolin using Cl-free buffer

<table>
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<tr>
<th></th>
<th>Krebs</th>
<th>Cl−-Free Buffer</th>
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<tbody>
<tr>
<td>ETS $I_{sc}$</td>
<td>29.9±10</td>
<td>6.0±4*</td>
</tr>
<tr>
<td>Carbachol $I_{sc}$</td>
<td>90.3±29</td>
<td>11±3*</td>
</tr>
<tr>
<td>Forskolin $I_{sc}$</td>
<td>80.8±38</td>
<td>29±6*</td>
</tr>
</tbody>
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Values are means ± SD in μA/cm²; $n$ = 5 mice/group. ETS, electric transmural stimulation. *P < 0.0001 vs. Krebs.
elevated compared with nonsensitized controls. Previous studies have demonstrated long-term cAMP-induced changes in $I_{sc}$ after experimental colitis (1, 14). Also, barrier abnormalities improve but do not completely return to normal in a proportion of patients with CD after 1 yr of gluten-free diet (9). Thus it is possible that $I_{sc}$ responses require a longer period of gluten withdrawal to normalize.

In animal models of nematode infection, macrophages play an important role in neural dysfunction (13). A role for the innate immune system in the initial steps of gliadin sensitization has been proposed (11, 17, 19, 29, 31). Thus we hypothesize that the innate immune response to gliadin that involves both recruitment of IELs and macrophages may promote neuronal dysfunction. This hypothesis is currently being tested in a follow-up study after inhibition of macrophage recruitment.

In conclusion, our results demonstrate that gliadin sensitivity in HLA-DQ8 mice leads to changes in gut secretory and neuromotor function that are not explained by the presence of chronic atrophic enteropathy. We propose that, in HLA-DQ8 mice, gliadin sensitization and challenge induce macrophage recruitment, leading to the development of altered neurotransmitter release, altered contraction, and a hypersecretory state. The DQ8 mouse model provides a tool to investigate early mechanisms linked to gut sensitivity in a genetically predisposed host before the establishment of a chronic lesion. These results may provide an explanation for the gluten sensitivity reported in some patients with gut functional symptoms who respond to gluten withdrawal in the absence of fully evolved CD (8, 10, 19, 33).

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GRANT

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