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Duodenal Bacteria From Patients With Celiac Disease and Healthy Subjects Distinctly Affect Gluten Breakdown and Immunogenicity

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BACKGROUND & AIMS: Partially degraded gluten peptides from cereals trigger celiac disease (CD), an autoimmune enteropathy occurring in genetically susceptible persons. Susceptibility genes are necessary but not sufficient to induce CD, and additional environmental factors related to unfavorable alterations in the microbiota have been proposed. We investigated gluten metabolism by opportunistic pathogens and commensal duodenal bacteria and characterized the capacity of the produced peptides to activate gluten-specific T-cells from CD patients. **METHODS:** We colonized germ-free C57BL/6 mice with bacteria isolated from the small intestine of CD patients or healthy controls, selected for their in vitro gluten-degrading capacity. After gluten gavage, gliadin amount and proteolytic activities were measured in intestinal contents. Peptides produced by bacteria used in mouse colonizations from the immunogenic 33-mer gluten peptide were characterized by liquid chromatography tandem mass spectrometry and their immunogenic potential was evaluated using peripheral blood mononuclear cells from celiac patients after receiving a 3-day gluten challenge. **RESULTS:** Bacterial colonizations produced distinct gluten-degradation patterns in the mouse small intestine. *Pseudomonas aeruginosa*, an opportunistic pathogen from CD patients, exhibited elastase activity and produced peptides that better translocated the mouse intestinal barrier. *P aeruginosa*–modified gluten peptides activated gluten-specific T-cells from CD patients. In contrast, *Lactobacillus* spp. from the duodenum of non-CD controls degraded gluten peptides produced by human and *P aeruginosa* proteases, reducing their immunogenicity. **CONCLUSIONS:** Small intestinal bacteria exhibit distinct gluten metabolic patterns in vivo, increasing or reducing gluten peptide immunogenicity. This microbe–gluten–host interaction may modulate autoimmune risk in genetically susceptible persons and may underlie the reported association of dysbiosis and CD.

Keywords: Celiac Disease; Gluten Metabolism; Intestinal Microbiota; Intestinal Inflammation.

Gluten-related disorders are increasingly prevalent conditions[1] that encompass all diseases triggered by dietary gluten, including celiac disease (CD), a T-cell–mediated enteropathy, dermatitis herpetiformis, gluten ataxia, and other forms of non-autoimmune reactions.[2] Gluten proteins, predominantly gliadins in wheat, are resistant to complete degradation by mammalian enzymes, which results in the production of large peptides with immunogenic sequences, such as the 33-mer in α-gliadin. Overall, this specific peptide contains 6 copies of 3 different epitopes (PYPQPQLPY, PQPQLYPQ, PFPPQPQLPY) to which most celiac patients react.[3,4] Partially digested gluten peptides translocate the mucosal barrier and are deamidated by human transglutaminase 2 (TG2), the CD-associated autoantigen.[5] This process converts glutamine residues to glutamate and increases peptide binding affinity to HLA-DQ2 or DQ8 heterodimers in antigen-presenting cells, initiating the T-cell–mediated inflammation characteristic of CD.[6] Up to 40% of most populations express the susceptibility genes for CD; however, only 2%–4% will develop the disease, possibly due to additional unknown environmental triggers.[7] As with other autoimmune and inflammatory diseases, intestinal dysbiosis characterized by abundance of Proteobacteria and decreases in *Lactobacillus* has been described in some CD patients.[8–10] There is little mechanistic insight regarding the association between dysbiosis and gluten-specific T-cell responses, and the functional relevance of these associations in CD remain unclear.

Abbreviations used in this paper: **ASF, altered Schaedler flora; CD, celiac disease; CFU, colony-forming unit; LC-MS/MS, liquid chromatography tandem mass spectrometry; PT, pepsin-trypsin; PBMC, peripheral blood mononuclear cell; SFU, spot-forming units; TG, transglutaminase.**

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The human gastrointestinal tract is colonized by bacteria with in vitro gluten-degrading capacity.[11,12] This has prompted the hypothesis that bacteria could reduce gluten immunogenicity by producing enzymes that effectively cleave proteolytic-resistant sequences in gluten peptides.[13] Here we show a complex scenario in which gluten metabolism in the small intestine of gnotobiotic mice is differentially affected by opportunistic pathogens and commensal bacteria. We demonstrate that *Pseudomonas aeruginosa*, isolated from the duodenum of CD patients, produces, through its elastase activity, a multitude of peptides that activate gluten-specific T-cells in HLA-DQ2.5^{+} CD patients. Conversely, *Lactobacillus* spp from healthy subjects, degrade *P aeruginosa*–modified peptides and decrease their immunogenic potential. We identify a microbe–dietary–host interaction that may modulate autoimmune risk in genetically susceptible persons and that could be targeted to reduce the rising incidence of these conditions.

Materials and Methods

Mice

C57BL/6 germ-free mice were generated by axenic 2-cell embryo transfer technique, as described previously,[14] and maintained in flexible film isolators at the McMaster University Axenic Gnotobiotic Unit. Germ-free status was evaluated weekly by a combination of culture and culture-independent techniques.[14,15] We used mice colonized with an 8 strain-murine microbiota (altered Schaedler flora [ASF])[16] as controls. All mice had unlimited access to a gluten-free autoclaved mouse diet (Harlan, Indianapolis, IN) and water. All experiments were carried out in accordance with the McMaster University animal utilization protocols.

Origin of Bacterial Strains

We previously sequenced and isolated a collection of bacterial strains with in vitro gluten-degrading capacity from the small intestine of CD patients and non-celiac controls.[17,18] Briefly, duodenal biopsies were incubated in specific gluten media (MCG-3)[11] for 48 hours under anoxic and microaerophilic conditions. Bacteria were selected based on production of a proteolytic halo and lack of growth in the same media without gluten. Most of the strains were classified within the phylum *Firmicutes* (88%), mainly from the genera *Lactobacillus*. Strains were also classified into Actinobacteria (8%), Proteobacteria (3%), and Bacteroidetes (1%).[17] For the experiments in this study, 3 bacterial groups of interest were chosen (Supplementary Table 1). *P aeruginosa* X-46.1 was selected as an opportunistic pathogen only isolated from CD patients,[17] and a member of Proteobacteria, a group previously associated with CD.[8–10] *Staphylococcus* spp was selected because alterations in this group have been described in CD patients.[19] *Lactobacillus* spp from healthy subjects were selected because it constitutes a core resident group in the human small intestine[18,20] that is involved in gluten metabolism in vitro[11] and is altered in CD patients.[21,22]

16S Sequencing

DNA was extracted from small intestinal samples of colonized mice as described previously.[23] Extracted DNA underwent amplification for the hypervariable 16S ribosomal RNA gene v3 region and sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA). Generated data were analyzed as described previously. Briefly, sequences were trimmed using Cutadapt software, version 1.2.1, aligned using PANDAseq software, version 2.8, operational taxonomic units selected via AbundantOTU, and taxonomy assigned against the Greengenes reference database.[24,25]

QPQLPY-Peptide Quantification

The amount of QPQLPY-peptide, a key motif in the major immunogenic epitope within the 33-mer peptide from α-gliadin, was measured with the competitive G12 ELISA GlutenTox Kit (Biomedal, Spain) according to the manufacturer's instructions.[26] For animal studies, total small intestinal content was flushed at sacrifice with 3 mL extraction solution provided by the kit.

Degradation of QPQLPY Peptides by Intestinal Washes

Intestinal contents were collected from colonized mice at sacrifice and diluted 1:5 with phosphate-buffered saline and incubated at 37°C with 7 mg pepsin-trypsin (PT)-gliadin (Supplementary Materials) for 30 minutes, 2 hours, and 4 hours. After incubations, remaining QPQLPY-peptides were quantified by the G12 antibody using the GlutenTox ELISA Kit.[26]

Cleavage of Gluten-Derived Tripeptides

Peptidase activity against gluten-derived tripeptides was performed as described previously.[27] Five synthetic analogs—Z-YPQ-pNA, Z-QQP-pNA, Z-PPF-pNA, Z-PFP-pNA, and Z-QPQ-pNa—were chosen as representative gliadin-derived substrates (Biomatik). Twenty millimolars of each peptide was incubated with the small intestinal washes of *P aeruginosa*-, *Lactobacillus* spp- or *Staphylococcus* spp-colonized mice, or with single bacteria cell cultures at the same concentration found in the small intestine of mice (10^4 colony-forming units [CFU]) in 50 mM ammonium bicarbonate buffer (pH 8.0). Enzyme activity was determined by the proteolytic removal of the paranitroanilide group, which was monitored spectrophotometrically at 405 nm.

Proteolytic Activity in Gluten Media

Degradation of gluten proteins in solid media was measured using bioassays on agar plates containing 1% gluten.[28] Small intestinal contents of mice were diluted 1:5 with phosphate-buffered saline and incubated at 37°C in gluten-agar media for 24 hours. Plates were evaluated by measuring the diameter of the halo formed. Trypsin diluted in saline was used for construction of a standard curve.

Liquid Chromatography Tandem Mass Spectrometry Analysis of 33-mer–Derived Peptides

Degradation of 33-mer peptide was performed using liquid chromatography tandem mass spectrometry (LC-MS/MS). The reaction mixtures (100 μL) containing 10 μL bacterial culture (10^4 CFU) and 60 μM of the 33-mer peptide in phosphate-buffered saline (pH 7.3), were incubated at 37°C for 4 hours. Reactions were stopped by incubation at 100°C for 10 minutes, and resultant products subjected to LC-MS/MS. LC-MS/MS data