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Intestinal Microbiota Modulates Gluten-Induced Immunopathology in Humanized Mice

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Celiac disease (CD) is an immune-mediated enteropathy triggered by gluten in genetically susceptible individuals. The recent increase in CD incidence suggests that additional environmental factors, such as intestinal microbiota alterations, are involved in its pathogenesis. However, there is no direct evidence of modulation of gluten-induced immunopathology by the microbiota. We investigated whether specific microbiota compositions influence immune responses to gluten in mice expressing the human *DQ8* gene, which confers moderate CD genetic susceptibility. Germ-free mice, clean specific-pathogen-free (SPF) mice colonized with a microbiota devoid of opportunistic pathogens and Proteobacteria, and conventional SPF mice that harbor a complex microbiota that includes opportunistic pathogens were used. Clean SPF mice had attenuated responses to gluten compared to germ-free and conventional SPF mice. Germ-free mice developed increased intraepithelial lymphocytes, markers of intraepithelial lymphocyte cytotoxicity, gliadin-specific antibodies, and a proinflammatory gliadin-specific T-cell response. Antibiotic treatment, leading to Proteobacteria expansion, further enhanced gluten-induced immunopathology in conventional SPF mice. Protection against gluten-induced immunopathology in clean SPF mice was reversed after supplementation with a member of the Proteobacteria phylum, an enteroadherent *Escherichia coli* isolated from a CD patient. The intestinal microbiota can both positively and negatively modulate gluten-induced immunopathology in mice. In subjects with moderate genetic susceptibility, intestinal microbiota changes may be a factor that increases CD risk. *(Am J Pathol 2015, 185: 2969–2982; http://dx.doi.org/10.1016/j.ajpath.2015.07.018)*

Celiac disease (CD) is an immune-mediated enteropathy triggered by gluten in individuals with genetic risk. Proteolytic-resistant gluten peptides are deamidated by transglutaminase 2 (TG2) in the small-intestinal lamina propria, increasing their binding affinity to the CD-associated HLA-DQ2 or DQ8 molecules, leading to T-cell activation.[1,2] CD also requires an innate immune response, characterized by up-regulation of stress markers on epithelial cells as well as up-regulation and activation of intraepithelial lymphocytes (IELs).[3,4] There has been a rapid rise in CD prevalence over the past 50 years.[5] This, in conjunction with the fact that only 2% to 5% of genetically susceptible individuals will develop CD, argues for environmental modulators of CD expression.[6]

The intestinal microbiota plays an important role in mucosal immune maturation and homeostasis as evidenced from seminal studies using germ-free and gnotobiotic mice.[7,8] Clinical and animal studies also suggest that altered

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colonization early in life increases susceptibility to chronic inflammatory diseases and food sensitivities.[9–11] Indeed, alterations in intestinal microbial composition have been described in CD patients, some of which normalize after treatment with a gluten-free diet.[12] Clinical studies have also proposed a link between antibiotic use and elective caesarean section and CD development.[13–15] However, recent studies in families with high genetic risk for CD (positive family history or homozygous for HLA-DQ2.5) have not been able to identify an environmental determinant, including the timing and dose of gluten introduction to an infant's diet.[16,17] Microbial factors were not directly investigated, and results may not apply to the general population or individuals with moderate genetic risk for CD (HLA-DQ2 heterozygous or HLA-DQ8).

Whether altered colonization instigates CD in an individual at moderate risk of developing CD remains unclear.[18] Therefore, we investigated whether microbial colonization modulates host responses to gluten using transgenic HLA-DQ8 mice on the nonobese diabetic background (NOD/DQ8).[19] We used complementary strategies to investigate host responses to gluten under different microbial conditions. Clean specific-pathogen-free (SPF) mice, strictly monitored for the absence of a variety of pathobionts and Proteobacteria, were protected from gluten-induced immunopathology when compared to germ-free and conventional SPF mice. Perinatal disruption of the microbiota leading to Proteobacteria expansion in conventional SPF mice further enhanced severity of responses to gluten; whereas specific pathobiont supplementation to clean SPF mice reversed the protective effect of the benign microbiota.

Materials and Methods

Mice and Colonization Procedures

Female and male germ-free, clean SPF and conventional SPF NOD AB° DQ8 (NOD/DQ8) transgenic mice maintained on a gluten-free diet were used for experiments.[20] Germ-free mice were generated by two-stage embryo transfer, as previously described,[21] and bred and maintained in flexible film isolators in McMaster's Axenic Gnotobiotic Unit. Clean SPF mice originated from germ-free mice that were naturally colonized by co-housing with female mouse colonizers harboring altered Schaedler flora and bred for three generations in individually ventilated cage racks. Pathogen contamination and microbiota diversification in mouse cecum contents of clean SPF mice was evaluated every 2 weeks in cage sentinels and at the end of the study in the experimental mice by PCR for *Helicobacter bilis*, *H. ganmani*, *H. hepaticus*, *H. mastomyrinus*, *H. rodentium*, *Helicobacter spp*., *H. typhlonius*, and *Pneumocystis murina*. Mouse serum was also tested for murine viral pathogens by multiplexed fluorometric immunoassay/enzyme-linked immunosorbent assay (ELISA)/indirect fluorescent antibody tests. Germ-free status was monitored in sentinels and, at the end of the study in the experimental mice, by immunofluorescence (SYTOX Green; Invitrogen, Burlington, ON, Canada), anaerobic and aerobic culture, as well as PCR technique.

Additional experiments were performed in germ-free and clean SPF C57BL/6 mice. For pathobiont supplementation experiments, 8- to 12-week-old clean SPF NOD/DQ8 mice were orally fed with 10^8 colony-forming units of *Escherichia coli* ENT CAI:5, isolated from fecal microbiota of a CD patient,[22] three times a week, 1 week before the start of sensitization and once a week during the sensitization and challenge period. Conventional SPF mice were bred and maintained in a conventional SPF facility at McMaster University. All experiments were conducted with approval from the McMaster University Animal Care Committee.

Gluten Sensitization and Challenge

NOD/DQ8 mice were sensitized with 500 μg of sterilized pepsin-trypsin digest of gliadin (PT-gliadin) and 25 μg of cholera toxin (Sigma-Aldrich, St. Louis, MO) by oral gavage once a week for 3 weeks, as previously described.[19] PT-gliadin was prepared as previously described.[19] In antibiotic experiments, mice were sensitized at 3 weeks of age, following weaning. For all other experiments, 8- to 12-week-old mice were used for sensitizations. Following PT-gliadin sensitization, gluten-treated mice were challenged by oral gavage with 2 mg of sterile gluten (Sigma-Aldrich) dissolved in acetic acid three times a week for 2 weeks. Nonsensitized control mice received cholera toxin alone during the sensitization phase and acetic acid alone during the challenge phase. NOD/DQ8 mice were weaned and maintained on a gluten-free diet. In additional experiments, C57BL/6 mice were sensitized with PT-zein and cholera toxin, once a week for 3 weeks, and challenged with zein dissolved in acetic acid three times a week for 2 weeks. All preparations were tested for lipopolysaccharide contamination using the E-Toxate kit (Sigma-Aldrich). Mice were sacrificed 18 to 24 hours following the final gluten or zein challenge.

Microbial Analysis

Fecal and cecal samples were collected and flash frozen on dry ice. DNA was extracted from samples as previously described.[23] Extracted DNA underwent amplification for the hypervariable 16S rRNA gene v3 region and sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA). Generated data were analyzed as described previously.[23] Briefly, sequences were trimmed using Cutadapt software version 1.2.1,[24] aligned using PANDAseq software version 2.8,[25] operational taxonomic units selected via AbundantOTU,[26] and taxonomy assigned against the Greengenes reference database.[27] α-Diversity was calculated using Quantitative Insights Into Microbial Ecology (QIIME),[28] and heat maps were generated using R (R Foundation for Statistical