Research Article

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The gut microbiota contributes to a mouse model of spontaneous bile duct inflammation

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Background & Aims: A strong association between human inflammatory biliary diseases and gut inflammation has led to the hypothesis that gut microbes and lymphocytes activated in the intestine play a role in biliary inflammation. The NOD.c3c4 mouse model develops spontaneous biliary inflammation in extra- and intrahepatic bile ducts. We aimed to clarify the role of the gut microbiota in the biliary disease of NOD.c3c4 mice.

Methods: We sampled cecal content and mucosa from conventionally raised (CONV-R) NOD.c3c4 and NOD control mice, extracted DNA and performed 16S rRNA sequencing. NOD.c3c4 mice were rederived into a germ free (GF) facility and compared with CONV-R NOD.c3c4 mice. NOD.c3c4 mice were also co-housed with NOD mice and received antibiotics from weaning.

Results: The gut microbial profiles of mice with and without biliary disease were different both before and after rederivation (unweighted UniFrac-distance). GF NOD.c3c4 mice had less distended extra-hepatic bile ducts than CONV-R NOD.c3c4 mice, while antibiotic treated mice showed reduction of biliary infarcts. GF animals also showed a reduction in liver weight compared with CONV-R NOD.c3c4 mice, and this was also observed in antibiotic treated NOD.c3c4 mice. Co-housing of NOD and NOD.c3c4 mice indicated that the biliary phenotype was neither transmissible nor treatable by co-housing with healthy mice.

Conclusions: NOD.c3c4 and NOD control mice show marked differences in the gut microbiota. GF NOD.c3c4 mice develop a milder biliary affection compared with conventionally raised NOD.c3c4 mice. Our findings suggest that the intestinal microbiota contributes to disease in this murine model of biliary inflammation.

Lay summary: Mice with liver disease have a gut microflora (microbiota) that differs substantially from normal mice. In a normal environment, these mice spontaneously develop disease in their bile ducts. However, when these mice, are raised in an environment devoid of bacteria, the disease in the bile ducts diminishes. Overall this clearly indicates that the bacteria in the gut (the gut microbiota) influences the liver disease in these mice.

Introduction

NOD.c3c4 mice spontaneously develop biliary inflammation in intrahepatic and extra-hepatic bile ducts [1]. The NOD.c3c4 model is developed on the NOD background [2]; a genetic background with increased susceptibility to autoimmune phenotypes similar to that seen in human biliary diseases [3]. The regular NOD mice also develop diabetes [4]. In contrast, NOD.c3c4 mice do not develop diabetes [2] and have been used as a model of the human biliary disease, primary biliary cirrhosis (PBC), as it develops autoantibodies and lymphocytic infiltrates similar to

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PBC [2]. The pathogenesis of this biliary disease in NOD.c3c4 mice has not been completely clarified, but it is considered to be immune mediated [5]. The NOD.c3c4 mouse is the only known mouse model that spontaneously develops dilatation and inflammation of the common bile duct [6]. These features are hallmarks of the human biliary disease primary sclerosing cholangitis (PSC) and as such this mouse model can also be used to model aspects of PSC.

Inflammatory biliary diseases are strongly associated with gut diseases [7,8]. This clinical association has led to the hypothesis that gut microbes, and activated lymphocytes in the intestine, play a role in biliary inflammation [7]. The hypothesis that gut microbiota could play an important role in bile duct disease is experimentally supported by studies showing that small bowel bacterial overgrowth in rats leads to bile duct inflammation that can be treated with antibiotics [9,10]. Several independent studies on human cholangiopathies have recently demonstrated that patients with PSC have a different gut microbiota compared to healthy individuals [11–14], and in line with this, manipulation of the gut microbiota using antibiotics in PSC patients has shown beneficial effects [15]. Another study demonstrated that germ free (GF) multidrug resistance knock out (*Mdr2*$^{-/-}$) mice developed more severe liver disease compared with conventionally raised (CONV-R) *Mdr2*$^{-/-}$ mice [16]. The *Mdr2*$^{-/-}$ mouse is a common mouse model of PSC [6], as it develops periductal fibrosis due to regurgitation of bile into portal tracts [17]. The NOD.c3c4 model differs from the *Mdr2*$^{-/-}$ model as the biliary disease of the NOD.c3c4 model is largely immune driven, but with minimal fibrosis [1].

In the present study, we explored the role of the intestinal microbiota in the biliary inflammation observed in NOD.c3c4 mice. We found significant differences between the gut microbiota of NOD.c3c4 mice and NOD control mice. In experiments with rederivation into a GF environment we found that GF NOD.c3c4 mice were protected from biliary disease compared with CONV-R NOD.c3c4 mice. Also, when NOD.c3c4 mice were treated with antibiotics we saw a milder liver phenotype corroborating the GF results. Collectively, the present results suggest that intestinal bacteria contribute to disease in this murine model of biliary inflammation.

Materials and methods

Mice

NOD.c3c4 and NOD mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All CONV-R mice were housed in a Minimal Disease Unit (MDU) at the animal facility at Oslo University Hospital Rikshospitalet, Oslo, Norway.

NOD.c3c4 and NOD mice housed in the MDU facility, were harvested at 10 weeks of age, and cecal content and mucosa was sampled. NOD.c3c4 and NOD strains were then rederived into a new MDU facility by caesarean sections, and after three generations, sampling of cecal content and mucosa was repeated. These rederived NOD.c3c4 mice were then rederived as axenic mice at the Core Facility for Germfree Research at the Karolinska Institutet, Stockholm, Sweden by caesarean sections, housed in a GF environment and regularly monitored to ensure their GF status. GF and CONV-R NOD.c3c4 mice were sampled at 9 weeks and 18 weeks of age. GF NOD mice were housed at a GF facility at the University of Gothenburg, Sweden. In co-housing experiments age- and gender-matched CONV-R NOD.c3c4 mice and NOD mice were co-housed in a MDU facility from the age of 4 weeks (after weaning) for 4 weeks. CONV-R NOD.c3c4 mice were treated with non-absorbable antibiotics; ampicillin 1.0 g/L (Bristol-Myers Squibb, Solna, Sweden) and Neomycin 0.5 g/L (Fisher Scientific, Geel Belgium) in drinking water from weaning for 4 weeks. The number of animals in each experiment was determined by power calculations and experience from similar experiments.

All animal experiments were approved by the Norwegian National Animal Research Authority (project license no FOTS 6809/14) and/or the Ethics Committee on Animal Care and Use in Gothenburg and Stockholm, Sweden. The animal experiments were performed in accordance with the European Directive 2010/63/EU and The Guide for the Care and Use of Laboratory Animals, 8th edition (NRC 2011, National Academic Press). All mice had *ad libitum* access to water and standard rodent diet.

Tissue collection and extraction of primary lymphocytes from liver

Mice were sacrificed at the indicated age, and whole body weight as well as the weight of the liver, spleen and cecum were recorded. Dilatation of the common bile duct (CBDD) was measured. Collection of blood, serum, liver tissue, and extraction of primary lymphocytes from perfused livers were also performed as described in the Supplementary material. Cecal content and mucosal samples were taken from the cecum with sterile equipment, and immediately snap-frozen in liquid nitrogen and stored at −80 °C until DNA extraction.

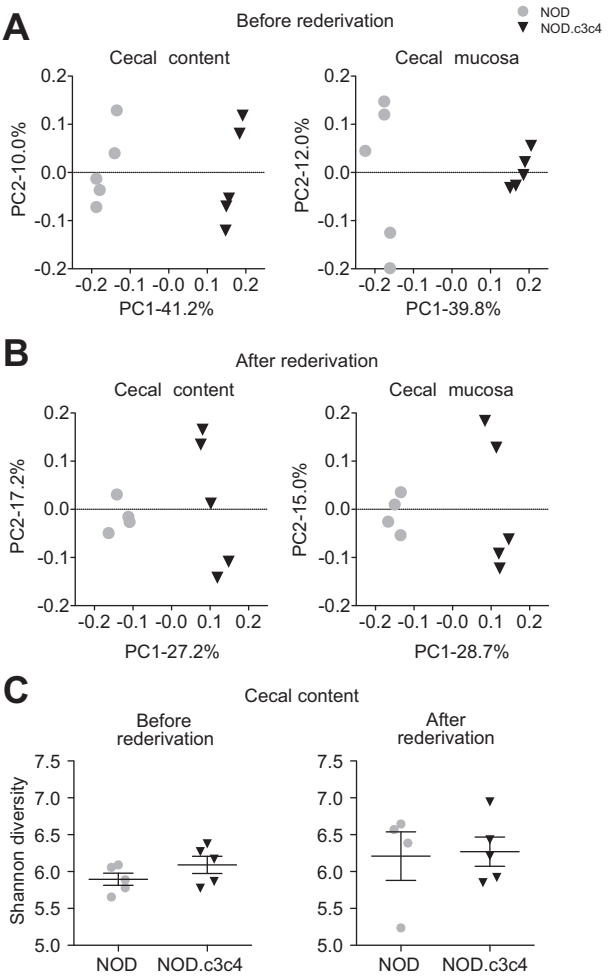

Fig. 1. NOD.c3c4 mice have a distinct global bacterial composition compared with NOD control mice. Principal coordinate plot based on unweighted UniFrac distances illustrating separation of the NOD (n = 4–5) and NOD.c3c4 mice (n = 5) in cecal content and mucosa (A) before (p <0.01) and (B) after rederivation into another conventional animal facility (p <0.02). (C) Intra-individual diversity measured by Shannon Diversity Index in cecal content of NOD (n = 4–5) and NOD.c3c4 mice (n = 5) was similar. Data in (A) and (B) compared using the *anosim* function in QIIME. Data in (C) are presented as mean ± SEM, unpaired Student's t test used for comparison.