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Diabetes progression and alterations in gut bacterial translocation: prevention by diet supplementation with human milk in NOD mice

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Abstract

Impaired intestinal barrier function occurs before type 1 diabetes (T1D) onset with a possible contribution of microbial translocation. Breastfeeding is associated with enhanced mucosal intestinal integrity and T1D protection. Our aim was to study the potential of human milk (HM) to prevent diabetes onset and modulate the translocation of gut bacteria susceptible to breastfeeding or associated to diabetes onset. We show that HM intake can prevent T1D in nonobese diabetic mice independently of bifidobacteria colonization. Prior to diabetes onset, HM mice harbored splenic bacterial counts and plasma lipopolysaccharides level similar to control mice but exhibited a reduced expansion of *Anaerotruncus* sp. in pancreas and *Lactobacillus johnsonii* and *Barnesiella* in Peyer's patches (PP). Surprisingly, pancreas and PP bacterial expansion did not correlate with their own gut localization but with ileal *Escherichia coli* and cecal HM-susceptible bacteria (the promoted *L. murinus* and *Bacteroides vulgatus*, and the repressed *B. fragilis* and *E. coli*), respectively. Besides, higher colonic *B. vulgatus* counts induced by HM intake were associated with low islet infiltration and pancreatic *E. coli* expansion. On another hand, splenic dendritic cells (DCs) were identified as negative covariate of PP *Barnesiella*, suggesting a possible HM contribution to preserving splenic DCs through the reduction of *Barnesiella* translocation. Fecal *B. vulgatus* also negatively correlated with PP *Barnesiella* expansion, indicating that the mouse coprophagic behavior likely added to HM effect. Our findings provide evidence that HM has a multilevel impact and cooperates with some gut bacteria for controlling bacterial translocation at the earliest stage of insulitis.

Keywords: Human milk; Type 1 diabetes; Bacterial translocation; NOD mice; Microbiota

1. Introduction

Type 1 diabetes (T1D) is the result of insulin-producing beta cells destruction or dysfunction in the Langerhans islets within the pancreas. Genetic susceptibility is a critical factor in the initiation of autoimmunity, with the majority of risk found in the HLA class II region [1]. Precipitating events such as exposure to environmental factors are thought to initiate or aggravate beta cell impairment [2,3]. The hypothesis is supported by the dramatic increase in incidence of T1D during the last decades in developed countries, as well as the lack of complete concordance in monozygotic twins [4–7]. Children aged 1–5 years are particularly affected, underlining the role of early life exposure [8].

Compelling evidence from cohorts of children living in North Europe highlights the impact of intestinal microbiota on T1D [9,10]. Evolution of the relative abundance of *Firmicutes* and *Bacteroidetes* differed over time, with a decrease in *Firmicutes* and an increase in *Bacteroidetes* in genetically at-risk children who progressed to T1D as compared to age- and HLA-matched healthy children [11]. Changes in the microbiome of children during progression from seroconversion to multiple islet autoantibodies (insulin autoantibodies, glutamic acid decarboxylase 65 autoantibodies, insulinoma antigen 2 autoantibodies) to T1D onset were further documented [9]. Progression to T1D onset was associated with reduced community diversity and an increase in inflammation-associated organisms and pathways [9]. Moreover, Finnish infants have a distinct gut microbiome as compared to their genetically similar counterparts in Russian Karelia, the latter being characterized by a six times lower disease incidence [12]. During the first year of life, breastfed infants from Finland and Estonia had an increased abundance of *Bacteroides*, while Russian infants had a bacterial microbiota dominated by *Bifidobacterium*.

The long-term protective effect of breastfeeding is questioned by such observation, all infants being breastfed for at least 1 year. Epidemiological studies found a weak but positive protective association between exclusive breast-feeding and T1D risk [13]. Duration of breastfeeding seems to be determinant for the protective effect [14]. A shorter duration or lack of breastfeeding is a risk factor for the development of T1D later in life [15]. But exposure to infectious agents during infancy is as well a critical factor. Analysis of association between T1D, breastfeeding and daycare attendance used as a proxy of infection shows an increased risk of T1D in weaned children and a

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decreasing risk of T1D with increasing breastfeeding duration [16]. The findings suggest that breastfeeding provides immunological support to fight off diabetogenic infections while allowing the low immune stimulation found in daycare environments to prevent the development of autoimmunity and T1D. The immunological support likely involves modulation of some bacterial determinants within the intestinal microbiome related to breastfeeding. Among the possible regulation induced by human milk (HM), control of a compromised permeability of the epithelial lining could reverse the passage of toxins, antigens and bacteria that enter the body and trigger the initiation and development of T1D [17].

To address the role of the bacterial translocation in mechanisms of autoimmunity and its modulation by breastfeeding, animal models are useful. Intestinal microbiota is a critical modulator for T1D development in genetically susceptible murine models [18,19]. Diabetes incidence is higher in cleaner nonobese diabetic (NOD) mice colonies partly because it prevents natural colonization with segmented filamentous bacteria that correlates with disease prevention [20–22]. Moreover, treatment of NOD mice with vancomycin from birth to weaning reduces diabetes incidence and leads to an increase abundance of the mucin degrader *Akkermansia muciniphila* [23]. Switching from a conventional chow-based diet to a gluten-free diet reduces T1D incidence. Resistance to T1D progression is associated with the replacement of *Barnesiella* by *A. muciniphila* [24]. Besides, *A. muciniphila* transfer to NOD mice prone to T1D delayed diabetes development [25]. NOD mice susceptibility to the bacteria colonizing the gut — more specifically during the weaning period — provides a model for assaying the possible protection induced by HM against diabetes with respect to change to the gut bacteria translocation. In this way, it was shown that enhanced gut bacteria translocation to the pancreatic lymph nodes measured by 16S rDNA contributed to T1D onset [26].

In the present study, HM was introduced in the otherwise not protective diet after weaning to determine whether HM can prevent diabetes and modulate gut bacterial translocation. The study focused on a panel of bacteria already known to be susceptible to breastfeeding (bifidobacteria, lactic acid bacteria) and/or to be implicated in T1D development (*Akkermansia, Bacteroides, Barnesiella*, enterobacteria) [25,27,28]. Gut bacterial translocation was studied prior T1D onset by quantifying by real-time quantitative polymerase chain reaction (qPCR) in Peyer's patches (PP), spleen and pancreas the distribution of the panel of bacteria after 2 weeks of HM intake and its association with body weight, insulitis grade, endotoxemia, spleen dendritic cells (DCs) and glycemia. In a subset of animals, the panel of bacteria was as well quantified in mouse feces over time up to diabetes onset.

2. Materials and methods

2.1. HM collection

Healthy lactating mothers were enrolled in the study by the Regional Lactarium-Hôpital Jeanne de Flandre–CHRU de Lille. Before sample collection, they received oral and written information and gave written informed consent to the protocol approved by the Ethics Committee of the Hospital. Milk samples (around 20 ml per sample) from 122 donations (87 donors) collected between day 1 and day 10 after birth were pooled in a single batch and centrifuged at 15,000×*g* for 1 h at 4°C. The fat layer was discarded to avoid the confounding effect of a high-fat diet on bacteria. The defatted milk supernatant was filtered through sterilized Whatman paper (GR1 30MM, GE Healthcare, Buy, France) to remove the last fat remains. The defatted milk supernatant (HM) was aseptically allocated to sterilized bottles and kept frozen at −20°C until use. Protein content was analyzed by Lowry and Dubois methods, respectively [29,30]. Results (5.26 mg protein/ml, 59.2 mg carbohydrates/ml supernatant) gave values within the range of individual samples. Taking into account the measured protein and sugar content, the energy content was estimated at 0.25 kcal/ml of supernatant close to the 0.30 kcal/ml of HM prior 10 days postpartum [31]. Bacteria established in mice were searched for in the pool by culture and qPCR (Appendix Table A1). Live lactobacilli, enterococci, and *Aerococcus viridans* were grown, their counts being close to the enumeration carried out by qPCR. Except for *A. viridans* (4.33 log cfu/ml), none belonged to the species recovered in mice (*Lactobacillus murinus*, *L. johnsonii*, *L. reuteri*, *Enterococcus faecalis*). No bifidobacteria and *Bacteroides* were grown. However, *Bifidobacterium longum* (3.80 log cfu/ml) (but not *B. pseudolongum*), *Bacteroides dorei* (2.31 log cfu/ml) and *B. vulgatus* (3.64 log cfu/ml) were quantified by qPCR. *Akkermansia*, *Anaerotruncus*, *Barnesiella*, *B. fragilis* and *Escherichia coli* were not detected by either method.

2.2. Animals and study design

Experiments were carried out in accordance with the EU directive 2010/63/EU and approved by the Ethical Committee for Animal Experimentation (CEEA 242012) (Lille, France). Four-week-old NOD/LTJ female mice were purchased from Charles Rivers Laboratories (Saint-Germain-Nuelles, France). They were housed in type IV cages (16 per group, 4 per cage) and were fed with sterile RO3 diet (UAR, Epiney-sur-Orge, France). Energy intake was estimated on a daily basis by weighting the pellets before administration and on the next morning. The mean calorie intake was 11.64±1.68 kcal/day/mouse fed exclusively RO3 diet and 10.93±1.64 kcal/day/mouse when RO3 diet was supplemented with HM.

2.2.1. Longitudinal study

Diabetes onset occurs in NOD mice between 14 up to 31 weeks of age. Defatted HM (HM mice, $n=16$) or sterile water (control mice, $n=16$) was administrated from week 5 up to week 32 [20]. The purpose of a long dietary intervention (27 weeks) was to prevent T1D onset in mice older than 25 weeks. Administration from 4 up to 20 weeks is usually not protective against late T1D onset [32]. Bottles were changed every evening, the remaining volumes being recorded on the morning (Appendix Fig. A1a). Calorie intake related to HM volume ranged between 1.5 and 1.75 kcal/day/mouse. Mice were weighed once a week, and feces were collected. At the end of the survey period (32 weeks), the mice still alive were euthanized by halothane inhalation.

2.2.2. Topological study

In NOD mice, progression to diabetes is divided into early infiltration of the islets from 4 to 7 weeks, development of inflammation from 8 ti 11 weeks, cytotoxicity and clinical diabetes from 12 weeks. We assume that changes in bacterial translocation occur prior the development of inflammation, re in 8–11-week-old NOD mice. Therefore, we administrated to 5-week-old mice for 2 weeks either defatted HM (HM mice, $n=8$) or water (control mice, $n=8$) [33]. On week 7 (end of the early infiltration stage), 200 μl of HM or water was administrated through oral gavage 2 h prior sacrifice. Mice were dosed on the morning ($n=8$) or on the afternoon ($n=8$). Blood and organs were aseptically removed in the following order to avoid cross bacterial contamination: spleen, pancreas, PP, small intestine (distal portion), cecum and colon (whole tissue scrapings). PP collection was carried out according to Pastori and Lopalco [34].

2.3. Glucose monitoring

Diabetes onset was monitored every week by Contour TS glycometer system (Bayer HealthCare, Lyon, France) for blood glucose measurement at the tail. Glucosuria measured using Multistix strips (Siemens Healthcare Diagnostics, Saint-Denis, France) was considered positive when reading was scored 3+ (estimated at 55 mM glucose/L urine). Mice were considered diabetic following two consecutive blood glucose readings above 14 mmol/L and two consecutive scores 3+.

2.4. Histological examination

Half the pancreas was fixed in 4% paraformaldehyde (VWR, Fontenay-sous-Bois, France) overnight for histological examination. Nonconsecutive paraffin-embedded sections were stained with hematoxylin and eosin. The number of islets was counted, and sections were evaluated randomly and blindly. Insulitis scores were graded as 0, no mononuclear cell infiltration; 1, peri-insulitis; 2, mononuclear cell infiltration in <50% of the islet area; or 3, mononuclear cell infiltration in ≥50% of the islet area (Appendix Fig. A2, Appendix Table A2).

2.5. Splenic DCs counting

A fraction of spleen was placed in HEPES buffer with 2 mg/ml collagenase (Sigma Aldrich, Lyon, France) and incubated for 2 h at 37°C for DCs isolation. CD11c-positive cells were isolated using MACS MicroBead Technology (Miltenyi, Paris, France). Splenocytes and CD11c-positive cells were stained with trypan blue and enumerated using Malassez cell. Counts were expressed as percentage of splenocytes in spleen.

2.6. Bacterial enumeration

A metagenomic approach was not suitable for an accurate quantification of bacteria in internal organs with the possible environmental DNA contamination. Thus, we focused on selected bacteria after a thorough analysis of the literature (Appendix Table A1).

Feces and organs samples suspended in 2 ml prereduced Ringer solution (Solabia, Pantin, France) supplemented with cysteine HCl (0.03%) (VWR) were analyzed by culture and nonculture techniques. Culture techniques were used to determine whether spleen, pancreas and PP harbored viable bacteria. Intestinal fractions and feces were as well subjected to culture to qualify the bacteriological handling. A fraction of the suspension was diluted for culture-dependent enumeration. Another fraction was kept frozen until total DNA extraction. After thawing, aliquots corresponding to 40 mg of