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Probiotics and colostrum/milk differentially affect neonatal humoral immune responses to oral rotavirus vaccine

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ABSTRACT

Breast milk (colostrum [col]/milk) components and gut commensals play important roles in neonatal immune maturation, establishment of gut homeostasis and immune responses to enteric pathogens and oral vaccines. We investigated the impact of colonization by probiotics, *Lactobacillus rhamnosus GG* (LGG) and *Bifidobacterium lactis Bb12* (Bb12) with/without col/milk (mimicking breast/formula fed infants) on B lymphocyte responses to an attenuated (Att) human rotavirus (HRV) Wa strain vaccine in a neonatal gnotobiotic pig model. Col/milk did not affect probiotic colonization in AttHRV vaccinated pigs. However, unvaccinated pigs fed col/milk shed higher numbers of probiotic bacteria in feces than non-col/milk fed colonized controls. In AttHRV vaccinated pigs, col/milk feeding with probiotic treatment resulted in higher mean serum IgA HRV antibody titers and intestinal IgA antibody secreting cell (ASC) numbers compared to col/milk fed, non-colonized vaccinated pigs. In vaccinated pigs without col/milk, probiotic colonization did not affect IgA HRV antibody titers, but serum IgG HRV antibody titers and gut IgG ASC numbers were lower, suggesting that certain probiotics differentially impact HRV vaccine responses. Our findings suggest that col/milk components (soluble mediators) affect initial probiotic colonization, and together, they modulate neonatal antibody responses to oral AttHRV vaccine in complex ways.

1. Introduction

Acquisition of commensal microbiota after birth promotes maturation and regulation of the neonatal immune system, and establishment of gut homeostasis [1–3]. This critical stage is decisive for imprinting of neonatal immune responses. Initial microbial colonization in infants depends on the mode of delivery and the type of feeding. *Lactobacilli* and *Bifidobacteria* species are common in breast fed infants, in contrast to more diverse flora belonging to *Bacteriodes*, *Atopobium*, *Clostridium*, and *Enterococci* in formula fed infants [4–6]. The lower rate of gastrointestinal infections in breastfed infants compared to formula-fed infants may be attributed, not only to breast milk antibodies, but also to differences in gut microbiota. Breast milk or colostrum/milk (col/milk) promotes colonization by commensals and provides maternal antibodies and various biological soluble mediators such as CD14 (sCD14), cytokines, growth factors, and lactoferrin [7–10]. Recently, we reported that sow col/milk contains large amounts of TGFβ (T regulatory) and IL-4 (T helper 2) cytokines, and sCD14, similar to that in human breast milk. Besides acting locally in the gut, these soluble mediators were also transferred to the serum of suckling neonatal pigs [9,10], suggesting that they may influence commensal colonization and immune responses to vaccines and infections. The impact of breast milk and its components on generation of the microenvironment to promote colonization by selected commensals (*Bifidobacteria* and *Lactobacilli*), and their combined effect on subsequent immunologic maturation of the neonatal gut and oral vaccines, is largely undefined.

Rotavirus (RV) is a leading cause of viral diarrhea in infants and children. Currently available RV vaccines are effective against severe RV gastroenteritis in developed countries (>80% efficacy), but for unknown reasons, they show reduced efficacy (~50%) in impoverished countries [11]. *Lactobacilli* and *Bifidobacteria* spp. are reported to reduce the severity of RV diarrhea and RV shedding in children, although mechanisms are undefined [12,13]. Colonization by certain probiotics, which were selected based on their ability to reduce infectious diarrhea may also act as adjuvants to enhance the efficacy of HRV vaccines [14].

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Piglets resemble human infants in gastrointestinal physiology, anatomy and development of mucosal immune responses [15,16]. The gnotobiotic (Gn) piglets, devoid of microflora and sow col/milk, are a unique animal model to investigate initial interactions between col/milk components and the probiotics that commonly colonize breast fed neonates. These initial interactions imprint neonatal immunity, which may also affect immune responses to oral AttHRV vaccines. For this study, our major objectives were: (a) to investigate whether col/milk influences dual *Lactobacilli rhamnosus* GG (LGG) and *Bifidobacterium animalis* subsp. *lactis* (Bb12) colonization, persistence and distribution in the gut; and (b) to determine if LGG + Bb12, without sow col/milk (mimick formula fed infants) or in association with col/milk (mimick breastfed infants) enhance antibody responses to an oral AttHRV Wa strain (G1P1A [8]) vaccine that is genotypically similar to the current HRV vaccine (RotaRix, G1P [8]). In addition, this study also highlights the role of probiotics in modulating antibody responses in the presence of passive HRV-specific col/milk antibodies.

2. Materials and methods

2.1. Probiotic strains

The probiotics LGG strain ATCC 53103 (ATCC, Manassas, VA, USA) and Bb12 (Christian Hansen Ltd., Horsholm, Denmark) were used to colonize the Gn pigs. The LGG and Bb12 were propagated overnight at 37 °C in anaerobic conditions in Man–Rogosa–Sharpe broth with and without 0.05% cysteine hydrochloride, respectively. The CFU[1] were enumerated as previously described [17].

2.2. Sow colostrum and milk

Colostrum and milk were collected from RV-field exposed seropositive, non HRV-vaccinated lactating sows and were pooled and centrifuged (1850 × *g*, 30 min) to remove fat and cellular fractions. The whey fraction was collected for further use and will be referred to as col/milk supplement for this study. The sow colostrum and milk whey were sterilized by treating with 0.05% β-propiolactone (BPL, Sigma) for 1 h and then agitated at 37 °C for 2 h to break up BPL and make it safe for *in vivo* use. The pooled, treated col/milk samples were retested to verify sterility by culturing in non-selective media under aerobic and anaerobic conditions.

2.3. Experimental design

All experimental procedures were approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC protocol number: 2010A0088). Gn piglets were surgically derived as previously described [18] and were divided into two major groups: one group was initially fed ($n = 16$) sterile sow col/milk for the first 6 days of life and the other was fed ($n = 20$) ultra high temperature processed commercial cow milk (Parmalat) at derivation and throughout the study. Sow col/milk fed pigs received sow colostrum for the first two days of life and subsequently sow milk for 4 days, followed by Parmalat for the duration of the experiment (Fig. 1A). Piglets from each major group were assigned randomly to one of the following four groups: 3× AttHRV vaccinated and probiotic colonized (Vac + Pro, $n = 5$; Vac + Pro + Col/milk, $n = 4$); 3× AttHRV vaccinated only (Vac, $n = 5$; Vac + Col/milk, $n = 4$); probiotic colonized only (Pro, $n = 5$; Pro + Col/milk, $n = 4$); and negative controls (Cont, $n = 5$, Col/milk, $n = 4$). Cell culture adapted AttHRV Wa (propagated in a rhesus monkey kidney cell line, MA 104) was diluted in antibiotic free minimum essential medium (Sigma) and used as vaccine at a dose of 5×10^7 FFU[2] [19]. Vaccinated pigs were orally inoculated with the first dose at 6 days of age and then received two additional doses at 10-day intervals (post-inoculation day [PID] 10 and PID20). All pigs received 5 ml of 100 mM $NaHCO_3$ before each vaccine/mock dose to reduce gastric acidity. Probiotic groups were sequentially colonized orally at day 3 of age with Bb12 and at day 5 of age with LGG + Bb12 (1:1) at a dosage of 10^5 CFU/pig/time-point in 0.1% peptone water (Fig. 1A). Pigs were euthanized at PID27 and intestinal tissues (ileum and duodenum), spleen and blood were collected aseptically as previously described [19].

2.4. Probiotic shedding/colonization and enumeration

Rectal swabs were collected for measuring probiotic colonization and shedding on post-bacterial colonization days (PBCD) 3, 6, 11, 14, 19 and 25. To assess bacterial colonization of the gastrointestinal tract, sections of small intestinal and large intestinal tissues were collected at euthanasia (PID27) and were rinsed and homogenized. Enumeration of probiotics in rectal swab fluids and gut tissues was performed as previously described [17]. The total LGG and Bb12 colonies in rectal swab fluids and tissues were enumerated collectively as total CFU. However, the specific colonization of LGG and Bb12 was determined by real time qPCR on DNA extracted from representative rectal swab fluids and tissues using species specific primers and probes (LGG; courtesy of Dr. Gloria Solano-Aguilar, USDA, and Bb12 [20]). Probiotic counts in the gut tissues included bacteria in the mucus layer, epithelial surface and in the tissue and will be referred to as mucosa associated bacteria.

2.5. Isolation of mononuclear cells (MNCs) and assessment of B cell responses

The MNCs were isolated from blood, spleen, duodenum and ileum at PID27 as previously described [21,22]. The HRV specific and total immunoglobulin (Ig) A and IgG in serum and intestinal contents were detected as previously described [17,22]. The HRV specific IgA and IgG antibody secreting cells (ASC) were measured as previously described [17,21]. The frequency of B cells was measured by detecting $CD21^+CD3^-$ cells. Briefly, 1×10^6 MNCs were stained with anti-porcine CD3-SpectralRed and CD21-*Phycoerythrin* antibodies (Southern Biotech, Birmingham, AL) for 15 min at 4 °C. Stained cells were washed and samples were acquired and analyzed using Accuri C6 flow cytometer and C6 flow sampler software, respectively.

2.6. TGFβ (TGFβ1) and IL4 cytokine ELISA

TGFβ and IL4 cytokine concentrations in BPL treated and untreated colostrum and milk and in the serum of piglets at early time-points were measured by using porcine TGFβ and IL4 specific coating and detection antibody pairs (Biosource) as previously described [23].

2.7. Statistical analysis

Statistical analysis was done by SAS version 9.3 (SAS Institute, Cary, NC, USA). Mean probiotic fecal shedding, probiotic tissue colonization, total Ig and RV specific antibodies were log transformed and compared using one-way analysis of variance (ANOVA-general linear model), followed by Duncans multiple range test. Frequencies of $CD21^+CD3^-$ lymphocytes, RV specific ASC, and serum TGFβ

[1] Colony forming units.

[2] Fluorescent focus-forming units.

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