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Human milk oligosaccharides protect against enteropathogenic *E.coli* (EPEC) attachment in vitro and EPEC colonization in suckling mice

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Abstract

Breastfeeding reduces the risk of enteric bacterial infections in newborns in part due to human milk oligosaccharides (HMO), complex glycans that are present in human milk, but not in infant formula. Enteropathogenic *Escherichia coli* (EPEC) are attaching/effacing pathogens that cause serious diarrheal illness with potentially high mortality in infants. We isolated HMO from pooled human milk and found that they significantly reduce EPEC attachment to cultured epithelial cells. In suckling mice, administration of HMO significantly reduced colonization with EPEC compared to untreated controls. These data suggest an essential role for HMO in the prevention of EPEC infections in human infants.

Keywords

enteropathogenic E. coli; human milk oligosaccharides; bacterial attachment; breastfeeding

Introduction

Human milk oligosaccharides (HMO) are highly abundant in human breast milk, but not in infant formula. In addition to serving as prebiotics that are selectively metabolized by specific bacteria and shape the infant's intestinal microbiome, HMO may provide specific anti-infective functions by interfering with bacteria-host interactions (1). One liter of mature human milk contains 5–15 g of unbound oligosaccharides (in addition to lactose), which often exceeds the amount of total milk proteins. The building blocks of milk oligosaccharides are the five monosaccharides D-glucose (Glc), D-galactose (Gal), N-acetylglucosamine (GlcNAc), L-Fucose (Fuc), and sialic acid (Sia; N-acetyl neuraminic acid [Neu5Ac] in humans and both Neu5Ac and N-glycolyl neuraminic acid [Neu5Gc]

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in most other species). Enteropathogenic *Escherichia coli* (EPEC) can contaminate food and water supplies and cause serious diarrheal illness with potentially high mortality in infants, especially in developing countries (2). EPEC is classified as an attaching/effacing (A/E) pathogen due to its ability to adhere to intestinal epithelium, efface microvilli, and induce characteristic, actin-filled membranous pedestals (3). Initial attachment leads to the formation of distinct microcolonies and is mediated by the plasmid-encoded type IV bundle-forming pili (4). Intimate adherence is a key feature of a number of important enteric pathogens (5), including enterohaemorrhagic *E. coli* and *Campylobacter jejuni*. Attachment of *Campylobacter jejuni* to human intestinal mucosa *ex vivo* can be inhibited by HMO, particularly fucosylated oligosaccharides (6). The goal of the present study was to determine whether HMO block the attachment of EPEC to intestinal epithelial cells in culture and protect against EPEC infection in neonatal mice.

Materials and Methods

Bacteria and reagents

EPEC strain 2348/69 (serotype O127:H6) was used for these studies (7). HMO were isolated and purified from pooled human milk from 41 different donors as previously described (8).

Cell culture and attachment assays

EPEC was grown overnight at 37°C in Luria-Bertani (LB) broth, diluted 1:20, and grown for an additional 2 h. After washing with PBS, bacterial density was estimated by OD600, and bacteria were resuspended in Dulbecco´s modified eagle medium (DMEM) containing different amounts of HMO or GOS (galactooligosaccharides) and incubated at 37°C for different times before addition to epithelial cells. The human epithelial cell lines HeLa (ATCC CCL-2) and HEp-2 (CCL-23) cells were cultured in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin. For infection experiments, cells were seeded into 6-well plates and grown to confluence in antibiotic-free medium. The human colon epithelial cell line T84 (ATCC CCL-248) was seeded onto 42 mm^2 permeable filter supports (EMD Millipore, Billerica, MA) in 6-well plates and grown to confluence over 10-14 days to reach a minimum transepithelial resistance of 1,000 Ω cm^2. One day before infection, the medium was changed to serum-free DMEM. Subsequently, medium was replaced by medium containing bacteria pre-incubated with HMO, GOS or neither, and plates were placed onto a rocking shaker (Boekel Scientific, Feasterville, PA; 15° angle, 18 rpm) for 1 h at 37°C. To assay attached bacteria, cells were washed four times with warm PBS to remove non-adherent bacteria, and incubated with 0.1% Triton X-100 in phosphate buffered saline (PBS) for five minutes. Cells were scraped off and lysates were transferred to 1.5 ml microtubes. After vigorous vortexing, serial dilutions of the lysates were plated onto Tryptic Soy Agar plates. CFU were counted after overnight incubation. All cell culture experiments were repeated at least three times.

Mice and infection protocols

Wild-type C57BL/6J mice (6-8 week old) were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in our Animal Facility. The resulting newborn mice (7 days) were infected by oral gavage with 10^5 CFU/mouse of EPEC strain 2348/69 in PBS, with or

without preincubation with HMO or GOS (15mg/ml) for 1 hour before infection. For some experiments pups were treated with HMO (15mg/d), GOS (15mg/d) or vehicle (PBS) three times daily on the day before and after infection. To ensure that preinucbation with HMO or GOS did not alter EPEC viability, aliquots were plated onto MacConkey agar without showing any differences in EPEC growth.

To determine bacterial numbers in the intestine, mice were sacrificed on indicated time points and small intestine and colon were collected, weighed, and homogenized in 5 ml PBS. Homogenates were plated onto MacConkey agar and CFU were counted after overnight incubation. The detection limit of the CFU assay was 10^3 CFU/g organ. To confirm identity of single colonies, PCR analysis was done for EPEC gene *EspB* (7). All experiments were at least repeated three times; overall 90 mice were used. All animal studies were reviewed and approved by the University of California, San Diego Institutional Animal Care and Use Committee.

Statistical analysis

CFU counts from in vivo experiments were log10 transformed, and means and SEM of the mean were calculated from the log values. Mice without detectable bacteria in small intestine or colon were assigned a log10 value equivalent to half of the detection limit of the CFU assay (10^3 CFU/g). Results from males and females were combined, as no significant differences were observed between the genders in bacterial colonization or mucosal responses after infection. Data from bacterial attachment assays *in vitro* are expressed as mean ± SEM. CFU data are either shown as % of initial inoculum or counts were log10 transformed (see above). Differences between groups were evaluated by one-sample t-test or Wilcoxon rank-sum test with $p<0.05$ considered significant.

Results

HMO block EPEC attachment to epithelial cells

As first step to determine whether HMO can block EPEC attachment to epithelial cells, we used *in vitro* assays. To better mimic the dynamic conditions in the intestine, where intestinal motility and bulk fluid movements compromise attachment opportunities, we developed a new infection model that mimics the situation by rocking the culture plates, reducing the bacterial inoculum, and shortening the infection times. EPEC (3×10^6 CFU) were preincubated with HMO (10 mg/ml) or medium (DMEM) alone for 1 h, added to different epithelial cell monolayers (HeLa, HEp-2 and T84 cells), and incubated for 1 h to allow attachment. HMO preincubation significantly reduced EPEC attachment in all three cell lines (Fig. 1A). The effect was dose-dependent (Fig. 1B) and even more prominent when EPEC were preincubated with HMO for 2 h (Fig. 1C). In contrast, galactooligosaccharides (GOS), which are structurally distinct from HMO and are currently added to some infant formula, were used as control and did not reduce EPEC attachment to any of the epithelial cell lines (Fig. 1B,C). Subsequently, we first preincubated epithelial cells with HMO and then added EPEC to the cells. Under these conditions HMO were no longer able to interfere with EPEC attachment (Fig. 1D), suggesting that HMO bind

to critical bacterial adhesion molecules to block attachment rather than act directly on epithelial cells.

Oral HMO administration to suckling mice attenuates EPEC infection

Next we sought to determine the physiological importance of the attachment blocking effects of HMOs. Adult mice are not readily infectable with human EPEC strains (9) and were less relevant for the present studies. Instead, we established a new model of EPEC infection in newborn mice. Testing of different bacterial inocula indicated that an inoculum of 10^5 CFU/mouse was optimal for achieving stable infection over at least 3-4 days (Fig. 2A). After an initial decline in bacterial numbers in the colon, numbers rebounded and reached stable levels after 18-24 h. Compared to the low infection rates in adult mice, newborn mice displayed 10^3-10^5 fold greater bacterial colonization in small intestine and colon after 3 days (Fig. 2B). Together, these data show that newborn mice were actively and selectively colonized by EPEC, making this model suitable for evaluating the physiological impact of HMO on infection. HMO (15 mg/day) were orally administered to newborn mice on the day of oral infection with EPEC, and administration was continued throughout the infection period. HMO-treated pups showed significantly lower EPEC colonization than PBS-treated animals. By comparison, administration of GOS to pups had no significant effect on EPEC colonization (Fig. 2C).

Discussion

In this study we show that HMO, the third most abundant solid component of human milk next to lipids and protein, reduce EPEC attachment to human epithelial cells and significantly protect against EPEC infection in newborn mice. Intimate adherence to the intestinal epithelium is important for EPEC pathogenicity (10), so interference in this essential step by HMO is likely to attenuate infection and disease induction. It has been reported that the bundling protein of EPEC has the properties of an N-acetyllactosamine-specific lectin (11), which might be responsible for EPEC binding to intestinal epithelial host cells. Because N-acetyllactosamine forms the backbone of most HMO (Bode (1), we speculate that HMO prevent the lectin from binding to glycans on the intestinal epithelial surface. Future studies will have to reveal which of the more than 150 different HMO are responsible for the observed effects. Our data also show that HMO attenuate intestinal EPEC colonization of infant mice. Our murine model uses the human pathogen EPEC instead of the mouse-specific A/E pathogen, *Citrobacter rodentium* (12), making it likely that the present insights can be applied to humans. Thus, we would predict that HMO protect the breast-fed infants from EPEC infection, thus providing an explanation for the lower incidence of EPEC infections in breast-fed compared to formula-fed infants (13).

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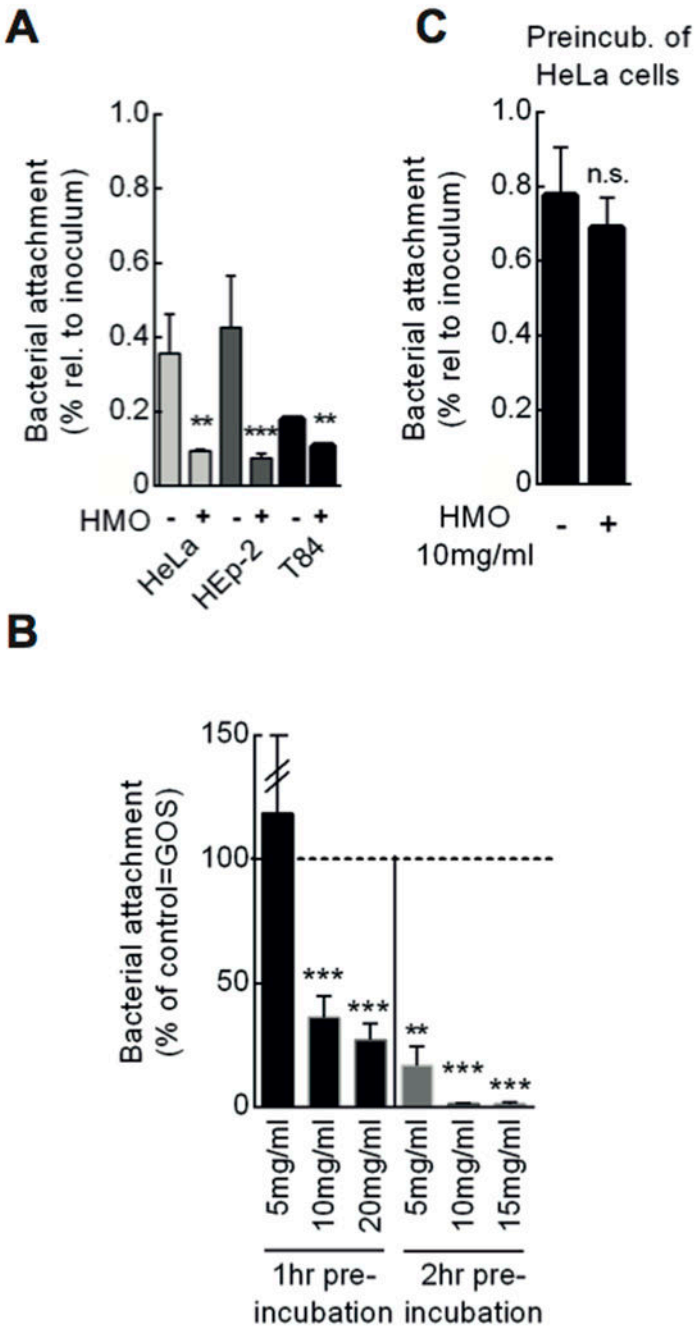

Figure 1. HMO block adherence of EPEC in vitro.

A. Cultures of 80-90% confluent HeLa, T84 or HEp-2 cells in 6-well plates were infected with EPEC (3 x 10^6 CFU) previously incubated with HMO (10mg/ml) or medium for 1 h, then bacterial attachment (% of inoculum) was assessed, data are shown as mean ± SEM. Indication of significant difference by Mann-Whitney-Test compared to medium alone, ** $p \leq 0.01$, *** $p \leq 0.0001$. **B.** HeLa cells were infected with EPEC (3 x 10^6) for 1 h previously incubated with different amounts of HMO or GOS (control) for indicated time (1 or 2 h), bacterial attachment was assessed thereafter. Data are shown as mean ± SEM, indication of

significant difference by one-sample *t* test compared to control, ** $p \leq 0.01$, *** $p \leq 0.0001$. **C.** HeLa cells were preincubated with HMO (10mg/ml) for 1 h, and then washed and EPEC was added for 1 h, then bacterial attachment was assessed, data are shown as mean ± SEM compared to initial inoculum (3×10^6). All cell culture experiments were at least performed 3 times.

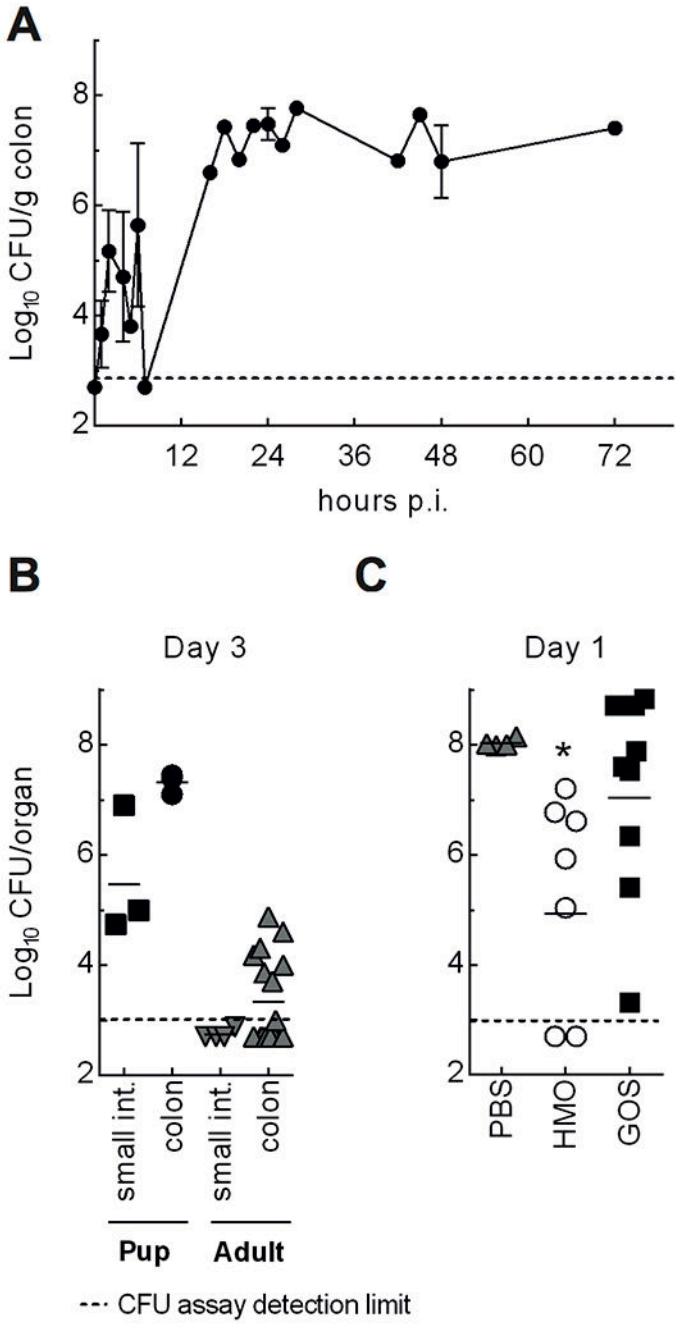

Figure 2. HMO attenuates infection with EPEC in mouse pups.

A. 7 day old C57BL/6J mouse pups (n = 51; n=2-5 per group and time point) were infected orally with EPEC (10^5) and bacterial load of the colon was assessed via CFU assay at indicated time points, data are shown as mean ± SEM. **B**. Colonization level of colon and small intestine on day 3 of infection with EPEC in mouse pups (7 days old, n = 3) compared to adult mice (6-8 weeks of age, n = 15) infected with EPEC (10^5 vs. 5 x 10^8 CFU/mouse respectively). C. Mouse pups were orally treated with HMO (15mg/day, n = 7), GOS (15mg/day, n = 10) or PBS (vehicle, n = 4) three times daily on the day before and after infection

with EPEC and infection levels were assessed after 24 h in the colon via CFU assay, *, $p \leq 0.05$ by rank-sum test.