Human milk oligosaccharides inhibit growth of group B Streptococcus

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**Streptococcus agalactiae** (group B Streptococcus, GBS) is a leading cause of invasive bacterial infections in newborns, typically acquired vertically during childbirth secondary to maternal vaginal colonization. Human milk oligosaccharides (HMOs) have important nutritional and biological activities that guide the development of the immune system of the infant and shape the composition of normal gut microbiota. In this manner, HMOs help protect against pathogen colonization and reduce the risk of infection. In the course of our studies of HMO-microbial interactions, we unexpectedly uncovered a novel HMO property to directly inhibit the growth of GBS independent of host immunity. By separating different HMO fractions through multidimensional chromatography, we found the bacteriostatic activity to be confined to specific non-sialylated HMOs and synergistic with a number of conventional antibiotic agents. Phenotypic screening of a GBS transposon insertion library identified a mutation within a GBS-specific gene encoding a putative glycosyltransferase that confers resistance to HMOs, suggesting that HMOs may function as an alternative substrate to modify a GBS component in a manner that impairs growth kinetics. Our study uncovers a unique antibacterial role for HMOs against a leading neonatal pathogen and expands the potential therapeutic utility of these versatile molecules.

Group B *Streptococcus* (GBS)** are Gram-positive bacteria that colonize the vaginal epithelium in 15–30% of healthy women. GBS transmission to the newborn is associated with risk of pneumonia, septicemia, and meningitis (1–3). In the United States and other developed countries, implementation of universal antenatal GBS culture screening and administration of intrapartum antibiotic prophylaxis has reduced GBS incidence in the first few days of life; however, it has not had a similar impact on late-onset infections, which now represent approximately one-third of total cases (4). Up to half of all infants with late-onset GBS also develop meningitis, which carries a high incidence (>40%) of neurocognitive sequelae among survivors (5). In a recent meta-analysis, the overall incidence of GBS infection in infants >3 months of age in the Americas and Europe is ~0.53–0.67 cases/1000 births, with an overall case fatality rate of 7–10% (6). The emergence of antibiotic-resistant GBS strains has become an increasing concern (1, 7, 8).

Human milk oligosaccharides (HMOs) are a group of complex carbohydrates that are highly abundant in human milk (10–15 g/liter) but not in infant formula (reviewed in Ref. 9). HMOs are comprised of five monosaccharides: D-glucose (Glc), D-galactose (Gal), GlcNAc, L-fucose, and sialic acid (N-acetylneuraminic acid). The Gal-Glc disaccharide (lactose) backbone can be further elongated by up to 15 Gal-GlcNAc repeats and can be sialylated or fucosylated. Over 150 structurally distinct HMOs have been identified, comprised of neutral (nonsialylated) and acidic (sialylated) forms, and the amount and composition are highly variable between women (reviewed in Ref. 10).

HMOs are not digested by the infant and reach the colon intact, where they serve as metabolic substrates for specific, potentially beneficial bacteria and help shape the infant microbiome. HMOs also act as soluble receptor decoys to prevent attachment of microbial pathogens to the host (11–14). HMOs are partially absorbed and reach the systemic circulation of the central human milk oligosaccharide: LNT, lacto-N-tetraose; LNNT, lacto-N-neotetraose; LNNH, lacto-N-neohexaose; LNPF, lacto-N-fucopentaose I; LNDFHII, lacto-N-difucohexaose II; LNFH, lacto-N-neofucohexoase; LNDFH, lacto-N-neodifucohexose; LNFVP, lacto-N-neofucopentaose; LNFVP, lacto-N-fucopentaose V; THB, Todd-Hewitt broth; SF-RPMI 1640, serum-free RPMI 1640; ANOVA, analysis of variance.

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This article contains supplemental Figs. S1–S3.

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2 The abbreviations used are: GBS, group B Streptococcus; HMO, human milk oligosaccharide; Glc, D-glucose; Gal, D-galactose; UPEC, uropathogenic *Escherichia coli*; pHMO, pooled human milk oligosaccharide; GOS, galacto-oligosaccharide(s); aHMO, acidic human milk oligosaccharide; nHMO, neutral human milk oligosaccharide; LNT, lacto-N-tetraose; LNNT, lacto-N-neotetraose; LNNH, lacto-N-neohexaose; LNPF, lacto-N-fucopentaose I; LNDFHII, lacto-N-difucohexaose II; LNFH, lacto-N-neofucohexoase; LNDFH, lacto-N-neodifucohexose; LNFVP, lacto-N-neofucopentaose; LNFVP, lacto-N-fucopentaose V; THB, Todd-Hewitt broth; SF-RPMI 1640, serum-free RPMI 1640; ANOVA, analysis of variance.
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Infant (15, 16) and appear intact in the urine of breast-fed infants (17, 18), with the potential to exert effects in organs other than the gut and including the urinary tract. Most intriguingly, HMOs also appear in the urine of pregnant women as early as the end of the first trimester (19), suggesting that HMOs might already affect pregnant women and the growing fetus long before birth.

In a previous study, we demonstrated that HMOs regulate the host innate immune response in bladder epithelial cells to prevent invasion and cytotoxicity caused by uropathogenic *Escherichia coli* (UPEC) without any direct interference with bacterial growth (20). To delineate whether HMOs generate similar effects with other neonatal pathogens, we examined the effect of HMOs on GBS, which is commonly found in the urogenital tract of pregnant women. Unexpectedly, we found that HMOs directly inhibit the growth of GBS, a property not shared with UPEC, *Pseudomonas aeruginosa*, or *Staphylococcus aureus*. Further investigation revealed a unique aspect of HMOs that causes a significant GBS growth defect.

Results

**Human milk oligosaccharides inhibit growth of group B Streptococcus**

To evaluate the potential antimicrobial effect(s) of HMO on a group of bacterial pathogens, we resuspended ~10⁵ cfu of each bacterial overnight culture in serum-free tissue culture medium (RPMI) with or without 2 mg/ml of HMOs isolated from pooled human milk (pHMO) and then incubated for 4 h. pHMOs did not affect the growth of UPEC, *P. aeruginosa*, and methicillin-resistant *S. aureus*. However, growth of the GBS test strain (serotype III isolate COH1) was reduced by ~10-fold (p < 0.05) (Fig. 1A). We confirmed that HMOs are bacteriostatic and not bactericidal because they did not kill GBS even at very high concentration (Fig. 1B). pHMO impaired the growth of the three most common GBS serotypes—serotypes III (strain COH1), Ia (strain A909), and V (strain NCTC10/84)—in a dose-dependent manner between 0.25–1.0 mg/ml (Fig. 1C).

**The neutral fraction of HMOs possesses the GBS inhibitory activity**

Galacto-oligosaccharides (GOS) are sometimes used to supplement infant formula to mimic the prebiotic properties of HMOs. However, GOS are structurally different from HMOs and consist of linear chains of two to six galactose residues linked to a single glucose. Unlike pHMOs, GOS did not impact GBS growth (Fig. 2A). Moreover, pHMOs pretreated with proteinase K retained their GBS-inhibitory activity (supplemental Fig. S1). Thus, pHMO-mediated inhibition of GBS growth is neither a nonspecific effect of oligosaccharides nor does it appear to require a protein.

Next, we separated pHMOs into sialylated, acidic HMOs (aHMOs) and non-sialylated, neutral HMOs (nHMOs) (9). Although nHMOs exerted a similar effect as pHMOs, aHMOs did not inhibit GBS growth (Fig. 2B). Consistent with this result, sialidase treatment of pHMOs to remove sialic acid moieties, which was confirmed by HPLC analysis, did not alter the inhibitory properties (Fig. 2C). At 1.0 mg/ml, nHMOs slowed serotype III GBS (COH1) growth by 98.2%, serotype la GBS (A909) growth by 97.0%, and serotype V GBS (NCTC10/84) growth by 96.0% (Fig. 2D). At a lower dose of 0.25 mg/ml, nHMOs inhibited growth of the serotype III and la GBS strains.
by more than 40-fold (p < 0.01), an effect more potent than pHMOs at the same dose (Fig. 1C).

Identification of neutral HMOs that inhibit GBS growth

To identify specific neutral oligosaccharide(s) responsible for GBS-inhibitory activity, we separated nHMOs into 10 fractions via size exclusion chromatography. We evaluated the bacteriostatic activity of each fraction by monitoring the GBS growth rate and found the strongest growth inhibition in the fourth and sixth size fractions (Fig. 3A). Based on HPLC profiles, these fractions primarily contained HMOs with up to eight monosaccharide moieties. Based on this lead, we selected nine commercially available oligosaccharides, each with slight modifications in the core structure known to exist in HMOs: lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), lacto-N-neohexaose (LNnH), lacto-N-fucopentaose I (LNFPI), lacto-N-difucohexaose II (LNDFHII), lacto-N-neooctaose (LnNO), lacto-N-neodifucohexaose (LNnDFH), lacto-N-neofucopentaose (LNnFPV), and lacto-N-fucopentaose V (LNFPV). GBS were challenged with 5 mg/ml of these individual oligosaccharides, and growth was observed to be significantly (60–70%) inhibited by LNT and its fucosylated derivative LNFPI (Fig. 3B).

Most intriguingly, the LNT structural isomer LNnT (Fig. 3C) showed no significant inhibition of GBS growth.

Disruption of GBS glycosyltransferase leads to resistance against HMOs

To identify bacterial factors involved in GBS susceptibility to HMOs, we screened ~1200 serotype III GBS (COH1) transposon Tn917 E mutants from our previously published library (21) for resistance to growth suppression by 2.5 mg/ml of pHMOs. This screen selected for a candidate mutant that exhibited normal growth over 7 h despite exposure to pHMOs (Fig. 4A). Through chromosomal sequence analysis, we identified the transposon insertion in this mutant to lie in the promoter of a gene annotated as gbs0738. Based on the Basic Local Alignment Search Tool (BLAST), gbs0738 encodes a putative glycosyltransferase that belongs to the carbohydrate-active enzymes (CAZY) GT-8 family and is conserved among 18 group B Streptococcus subspecies, with high levels of identity (99%) in strains, including serotype Ia A909 and serotype V NCTC10/84 (supplemental Fig. S2). To confirm that the transposon Tn917E insertion was not accompanied by unlinked mutations in the genome, we created a targeted in-frame insertion mutant in the wild-type strain COH1 to create knockout strain 0738. The targeted 0738 mutant recapitulated the growth profile of the original transposon mutant and was not susceptible to the bacteriostatic effect of pHMO (p ≥ 0.05) (Fig. 4, B and C). This finding confirms that the resistance to HMOs is specifically linked to inactivation of the gbs0738 gene.

Of note, the GT-8 glycosyltransferase shares some sequence homology with LPS glycosyltransferase RfaJ, an important outer membrane biosynthesis protein in Gram-negative bacteria (22). Because GBS is a Gram-positive pathogen lacking an outer membrane, we postulated this glycosyltransferase could promiscuously catalyze the glycosylation of HMOs into polysaccharide components of the GBS capsule or incorporate them into peptidoglycan/glycan-binding proteins (i.e. lectins) in the GBS cell wall. To explore this hypothesis, we first monitored the growth kinetics of a GBS serotype III capsule-deficient mutant (strain HY106 (23)) in the presence of HMOs. We
found that the capsule-deficient mutant still exhibited significant growth inhibition in the presence of nHMO (Fig. 4D), suggesting that the capsule is not targeted by nHMOs. To delineate whether GBS sensitivity to HMOs extends to the activity of other glycosyltransferases, we studied a GBS mutant (ΔagA) encoding a group 1 glycosyltransferase (21) that likewise remained sensitive to nHMO-mediated inhibition (Fig. 4D). Thus, our results suggest that GBS susceptibility to nHMOs is attributed with some specificity to the gbs0738-encoded glycosyltransferase.

**Synergistic activity of HMOs and antibiotics against GBS**

In serious or difficult-to-treat infections, combination antibiotic therapy is often considered to exploit synergistic activities and to reduce the risk of generating antimicrobial resistance. We examined whether prior exposure to HMOs could...
sensitize GBS to different pharmaceutical antibiotics. GBS growth was monitored over 4 h at different concentrations of nHMOs, and the IC\textsubscript{50} was determined to be 0.899 mg/ml (Fig. 5A). We then measured the IC\textsubscript{50} of GBS in the presence or absence of 0.25 mg/ml, a sub-IC\textsubscript{50} of nHMOs, in the presence of the glycopeptide antibiotic vancomycin, the fluoroquinolone antibiotic ciprofloxacin, or the \(\beta\)-lactam antibiotic imipenem. The presence of nHMOs at a sub-IC\textsubscript{50} concentration dramatically reduced the IC\textsubscript{50} of both vancomycin (0.25 mg/ml alone versus 0.00602 mg/ml with nHMOs) and ciprofloxacin (1.37 mg/ml alone versus 0.0021 mg/ml with nHMOs) (Fig. 5, B and C) but did not improve the activity of imipenem (supplemental Fig. S3). Calculation of the interaction index (Fig. 5D) revealed the interaction between nHMO and vancomycin or ciprofloxacin to represent true synergism (I < 0.5).

**Discussion**

A growing body of evidence has documented a variety of protective properties of HMOs against infectious agents. In addition to modulating host immune responses (24,25), oligosaccharides can pose as receptor decoys to prevent adhesion of microbial pathogens to epithelial surfaces (11–14). In a prior study, we demonstrated that HMOs, specifically the sialylated fraction of HMOs, and \(\alpha\)-3'-sialyllactose (\(\alpha\)SL) alone, support host innate defense and reduce invasion of UPEC into bladder epithelial cells. Conversely, in this study we found that certain neutral, non-sialylated forms of HMOs may cause a significant defect in GBS growth. To our knowledge, this is the first study to associate HMOs with a direct antimicrobial effect. Among the four bacterial species tested, the bacteriostatic effect of HMOs was unique to GBS.

Through a combination of mutation and bioinformatics analysis, we identified a conserved putative glycosyltransferase family 8 member (gbs0738) that is required for GBS sensitivity to HMOs. The predicted protein product of this GBS glycosyltransferase does not possess strong identity or similarity to genes from other bacterial species, which may explain why HMO inhibition is specific to GBS and not to other tested organisms (Fig. 1A). This particular family of glycosyltransferases is characterized by the GT-A fold and D\textsubscript{X}D motif. The GBS enzyme (gbs0738) shares high identity with an *E. coli* enzyme within this family of glycosyltransferases that is involved in the biosynthesis of the outer core region of lipo-oligosaccharide, catalyzing the \(\alpha\)-1,2 linkage of donor sugar to their acceptors (26). In *Streptococcus* spp., glycosyltransferases participate in the biosynthesis of capsular polysaccharides, cell wall peptidoglycan, and anchoring of lipoteichoic acid within the cell wall (27, 28). In GBS, glycosyltransferase-encoding *cpsE* is important for the synthesis of the surface polysaccharide capsules (29), and *iagA* is a glycosyltransferase that aids in the anchoring of lipoteichoic acid (21), but neither are essential for GBS growth (21, 29). GBS mutants lacking capsule or IagA, however, remain sensitive to HMOs. Our results suggest that growth inhibition by HMOs may depend on gbs0738-encoded glycosyltransferase to catalyze incorporation of HMO components into the cell wall. Ongoing work aims to biochemically characterize this glycosyltransferase and identify specific HMO substrates and GBS targets.

LNT causes the highest inhibition of GBS growth (Fig. 3B). LNT is one of the most abundant core structures in human milk (0.5–1.5 g/liter in mature human milk) (30). Further, LNT has
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been identified to block *Entamoeba histolytica* binding to epithelial cell surfaces (12). However, LNT has never been proposed to serve any direct antimicrobial function. One intriguing observation is that a slight conformational change of the single 3GlcNAcβ1 residue of LNT to 4GlcNAcβ1 in LNnT nearly abrogates its bacteriostatic effect on GBS (Fig. 3B). Our results suggest that there is a strict 3GlcNAcβ1 conformation requirement for maximal GBS inhibition.

HMOs have been found in the plasma (15, 16) and urine (17, 18) of breast-fed infants. Also, HMOs appear in the urine of pregnant women as early as the end of first trimester. Although the exact concentration of HMOs present in these areas is still uncertain, our results support the notion that lower incidences of GBS infections in breast-fed infants and already in pregnant women could include a contribution from the antibacterial properties of HMOs. Further, our demonstration of HMO synergism with certain pharmaceutical antibiotics suggests their potential utility in adjunctive therapy of GBS infection. Future animal studies as well as human cohort studies on human mother-infant dyads may help identify associations of individual HMOs like LNT with GBS infection risk. It is intriguing to envision the rational development of novel anti-infective strategies based on the natural template of human milk.

**Experimental procedures**

**Bacterial strains, cells, media, and growth conditions**

The wild-type GBS strain COH1 (serotype I and I) and its mutant derivatives HY106 and ΔiagA (21), A909 (serotype la), NCTC10/84 (serotype V), and methicillin-resistant *S. aureus* strain TCH1516 were grown overnight in Todd-Hewitt agar or broths. *S. aureus* strain HY304 was grown overnight in Todd-Hewitt broth (THB). *P. aeruginosa* strain PA14 and UPEC strain CFT073 (O6:K2:H1, ATCC 700928) were grown overnight in Luria-Bertani agar or broth. All bacteria were propagated in standing culture to stationary phase at 37 °C in 5% CO2 unless stated otherwise.

**Human milk oligosaccharide isolations**

pHMOs were prepared as described previously (31) and lyophilized for long-term storage. Milk from 36 different donors was pooled to account for heterogeneity in HMO composition between different women. The human milk donation program has been reviewed by the Institutional Review Board (IRB) Chair at University of California, San Diego and certified as exempt from IRB review under 45 CFR 46.101(b), category 4 because subjects cannot be identified and linked to generated data. Pooled HMOs were separated into aHMO and nHMO by anion exchange chromatography as described previously (31). nHMOs were further separated by size using size exclusion chromatography. pHMOs were disialylated by incubation with neuraminidase from *Vibrio cholerae*. HMO composition was analyzed by high-performance liquid chromatography and mass spectrometry.

**Sources of galacto-oligosaccharides and purified glycans**

Galacto-oligosaccharides were generously provided by Friesland Campina Domo (the Netherlands). Purified glycans were purchased from ELICITYL OligoTech®, which includes LNT, LNnT, LNnH, LNFIPI, LNDFHII, LnNO, LNNDFH, LNNFPV, and LNFPV.

**GBS growth and susceptibility test**

Overnight GBS growth was back-diluted to *A*600 = 0.01 in serum-free RPMI 1640 (SF-RPMI) (Life Technologies) or supplemented with pooled or fractions of HMOs. To measure growth, bacteria were grown in 100 µl of medium at 37 °C. At 600 nm, absorbance was recorded at 30-min interval using the BioScreen instrument (Growth Curves USA). To detect growth in cfu, bacteria were grown in 100 µl of medium at 37 °C in humidified air with 5% CO2 over 2 or 4 h. Recombinant proteinase K (Roche) was used at 0.1 mg/ml.

**GBS COH-1 transposon library**

The transposon library was constructed as described previously (21). To screen for mutants resistant to HMOs, we normalized overnight cultures of transposon mutants to *A*600 of 0.1. Approximately 10 µl of each mutant was cultured in 100 µl of SF-RPMI supplemented with 5 mg/ml of pooled HMOs. Growth was monitored over an 8-h period at 37 °C by measuring optical density at 600 nm at a 30-min interval using a Bioscreen C MBR system.

**GBS COH-1 Δ7038 construct**

To generate a targeted knockout, we cloned the gbs0738 gene to the temperature-sensitive vector PHY304. Briefly, gbs0738 was PCR-amplified with primers Xhol-gbs0738F (5'-CGATCTCGAGTGCTCAGGCACCTACAACTG-3') and HindIII-gbs0738R (5'-CAGTAAGCTTAGCAGGCAAGTTCATCAAGAG-3') to generate a 300-bp amplicon. The purified PCR amplicon was digested with Xhol and HindIII and ligated into previously digested PHY304. The construct was cloned into E. coli DH5α and isolated by mini-prep. Approximately 1 µg of PHY304-gbs0738 DNA was transformed into electro-competent GBS at 1550 V. The transformed bacteria were allowed to recover in 500 µl of THB with 0.25% sucrose at 30 °C for 2 h while shaking at 220 RPM. Bacteria were grown overnight on Todd-Hewitt agar + 2 µg/ml erythromycin at 30 °C. After 2–3 days, colonies were selected and inoculated into 5 ml of THB + 5 µg/ml erythromycin at 30 °C overnight. The next day, cultures were prepared in duplicate in THB + erythromycin at 30 °C and 37 °C to select for bacteria with the targeting vector incorporated into the chromosome. Resultant single colonies from the 37 °C culture were isolated and confirmed for single crossover mutation by PCR using primers M13F (5'-ACACGAGTTTGCTCAGCACTACAAACTG-3') and M13R (5'-GGTTTTCCCAGTCAGGAC-3') as expected 1.67 kb PCR product, whereas the mutant had an expected 6.5-kbp PCR product.

**Statistical analysis**

All experiments were performed in triplicate or quadruplicate and repeated in at least two independent experiments. Error bars represent S.E. (n > 3) from multiple independent experiments. Statistical analysis was performed using Student’s unpaired two-tailed *t* test or one-way ANOVA for multiple comparisons (GraphPad Prism version 5.03). *, *p* < 0.05; **, *p* < 0.01 in *500 nm of*
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