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Milk Oligosaccharides Inhibit Human Rotavirus Infectivity in MA104 Cells

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Abstract

Background: Oligosaccharides in milk act as soluble decoy receptors and prevent pathogen adhesion to the infant gut. Milk oligosaccharides reduce infectivity of a porcine rotavirus strain; however, the effects on human rotaviruses are less well understood.

Objective: In this study, we determined the effect of specific and abundant milk oligosaccharides on the infectivity of 2 globally dominant human rotavirus strains.

Methods: Four milk oligosaccharides—2′-fucosyllactose (2′FL), 3′-sialyllactose (3′SL), 6′-sialyllactose (6′SL), and galacto-oligosaccharides—were tested for their effects on the infectivity of human rotaviruses G1P[8] and G2P[4] through fluorescent focus assays on African green monkey kidney epithelial cells (MA104 cells). Oligosaccharides were added at different time points in the infectivity assays. Infections in the absence of oligosaccharides served as controls.

Results: When compared with infections in the absence of glycans, all oligosaccharides substantially reduced the infectivity of both human rotavirus strains in vitro; however, virus strain–specific differences in effects were observed. Compared with control infections, the maximum reduction in G1P[8] infectivity was seen with 2′FL when added after the onset of infection (62% reduction, $P < 0.01$), whereas the maximum reduction in G2P[4] infectivity was seen with the mixture of 3′SL + 6′SL when added during infection (73% reduction, $P < 0.01$). The mixture of 3′SL + 6′SL at the same ratio as is present in breast milk was more potent in reducing G2P[4] infectivity (73% reduction, $P < 0.01$) than when compared with 3′SL (47% reduction) or 6′SL (40% reduction) individually. For all oligosaccharides the reduction in infectivity was mediated by an effect on the virus and not on the cells.

Conclusions: Milk oligosaccharides reduce the infectivity of human rotaviruses in MA104 cells, primarily through an effect on the virus. Although breastfed infants are directly protected, the addition of specific oligosaccharides to infant formula may confer these benefits to formula-fed infants. *J Nutr* 2017;147:1709–14.

Keywords: human milk oligosaccharides, decoy receptors, rotavirus, infection, 2′-fucosyllactose, sialoglycans

Introduction

Human milk oligosaccharides (HMOs) are a group of structurally complex, unconjugated glycans that are found in human milk. On average, human breast milk contains 5–15 g oligosaccharides/L, making HMOs the third most abundant solid component of breast milk after lipids and lactose (1). The profile of oligosaccharides in human milk is diverse, with ~200 different structures identified to date (2). First described as prebiotic substrates for the infant gut microbiota, HMOs are now recognized to play numerous beneficial roles in the developing neonate. HMOs modulate neonatal immunity by altering host epithelial and immune cell responses in the infant gut and acting as soluble decoy receptors to block the attachment of various microbial pathogens to cell-surface receptors (1). In vitro studies have shown that HMOs prevent binding and infection of cells by a number of diarrheal pathogens such as *Escherichia coli*, *Vibrio cholerae*, and *Salmonella fyris* (3). A population study on mother-infant pairs showed that higher concentrations

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Abbreviations used: GOS, galacto-oligosaccharide; HMO, human milk oligosaccharide; MA104 cells, African green monkey kidney epithelial cells; OSU, Ohio State University; 2′FL, 2′-fucosyllactose; 3′SL, 3′-sialyllactose; 6′SL, 6′-sialyllactose.

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of 2′-linked fucosyloligosaccharides (2′FL) in human milk are associated with protection against diarrhea caused by *Campylobacter* and calicivirus (4).

Diarrheal disease is the second leading cause of death worldwide in children <5 y old. Among the causative agents of diarrhea in this age group, rotavirus is the leading cause of severe dehydrating gastroenteritis and is responsible for >215,000 deaths annually (5). Vaccines against rotavirus were licensed for use in 2006 and have been effective in reducing the incidence of severe rotavirus disease in developed countries (6). The efficacy of rotavirus vaccines, however, is far lower in developing countries, where most cases of severe rotavirus gastroenteritis occur (5, 6). Epidemiologic studies have demonstrated the protective benefits of breastfeeding against severe rotavirus gastroenteritis, with children who are not breastfed showing a 2-fold increased risk of rotavirus-induced diarrhea (7, 8). Rotavirus antigenemia rates are lower in breastfed infants than in infants who are not breastfed (9).

Several milk components have been shown to elicit protection against rotavirus infections. Maternal milk antibodies against rotavirus correlate with protection against infection in neonates, and anti-rotavirus IgA from human breast milk has a neutralizing effect on rotavirus infectivity in vitro (10, 11). In a cohort of mother-infant pairs in Mexico, higher levels of fat globule protein lactadherin were associated with protection against symptomatic rotavirus infection (12). The role of milk oligosaccharides in protection from human rotavirus infections is less well understood. Direct evidence for the binding of rotavirus to HMOs was shown through glycan array studies. The glycan-binding domain (VP8*) of rotavirus capsid protein VP4 demonstrated binding to various HMOs on a shotgun glycan array developed through use of a pool of donor milk samples, with strain-specific differences in binding partners (13). HMOs 3′-sialyllactose (3′SL) and 6′-sialyllactose (6′SL) inhibited the infectivity of porcine rotavirus strain OSU (Ohio State University) both in vitro and in a piglet ileal loop model; however, this effect was not observed when a human rotavirus strain was tested (14). The duration of rotavirus-induced diarrhea with OSU also was reduced in piglets fed a mixture of short-chain galacto-oligosaccharides (GOSs) and long-chain fructo-oligosaccharides; however, the effect of these compounds on human rotavirus strains was not assessed (15). In the present study, we tested the effect of specific and abundant milk oligosaccharides on the infectivity of 2 globally dominant human rotavirus strains in an in vitro infectivity system.

Methods

Cells and viruses. Rotavirus infections were carried out on African green monkey kidney epithelial cells (MA104 cells), a well-established model for rotavirus studies in vitro (16). Two human rotavirus strains, Wa and DS1, representing the globally dominant genotypes G1P[8] and G2P[4], respectively, were tested.

Milk oligosaccharides. The high purity (>95%) oligosaccharides 3′SL and 6′SL (both from IsoSep), 2′FL, and galacto-oligosaccharides [(Vivinal GOS) the latter 2 from FrieslandCampina DOMO] were tested for their effect on rotavirus infectivity. The components of Vivinal GOS have been described previously (17). The oligosaccharides (concentrations of 2.5 and 5.0 mg/mL) were dissolved in serum-free DMEM and filtered by passing the solution through a 0.22-μm filter. Serum-free DMEM without oligosaccharides filtered through a 0.22-μm filter was used as a control in each experiment.

Infectivity assays. The effect of milk oligosaccharides on rotavirus infectivity was assessed through standard fluorescent focus assays on MA104 cells (14, 18). The dilutions of G1P[8] and G2P[4] viruses that yielded ~100–200 focus-forming units/well were tested. Oligosaccharides

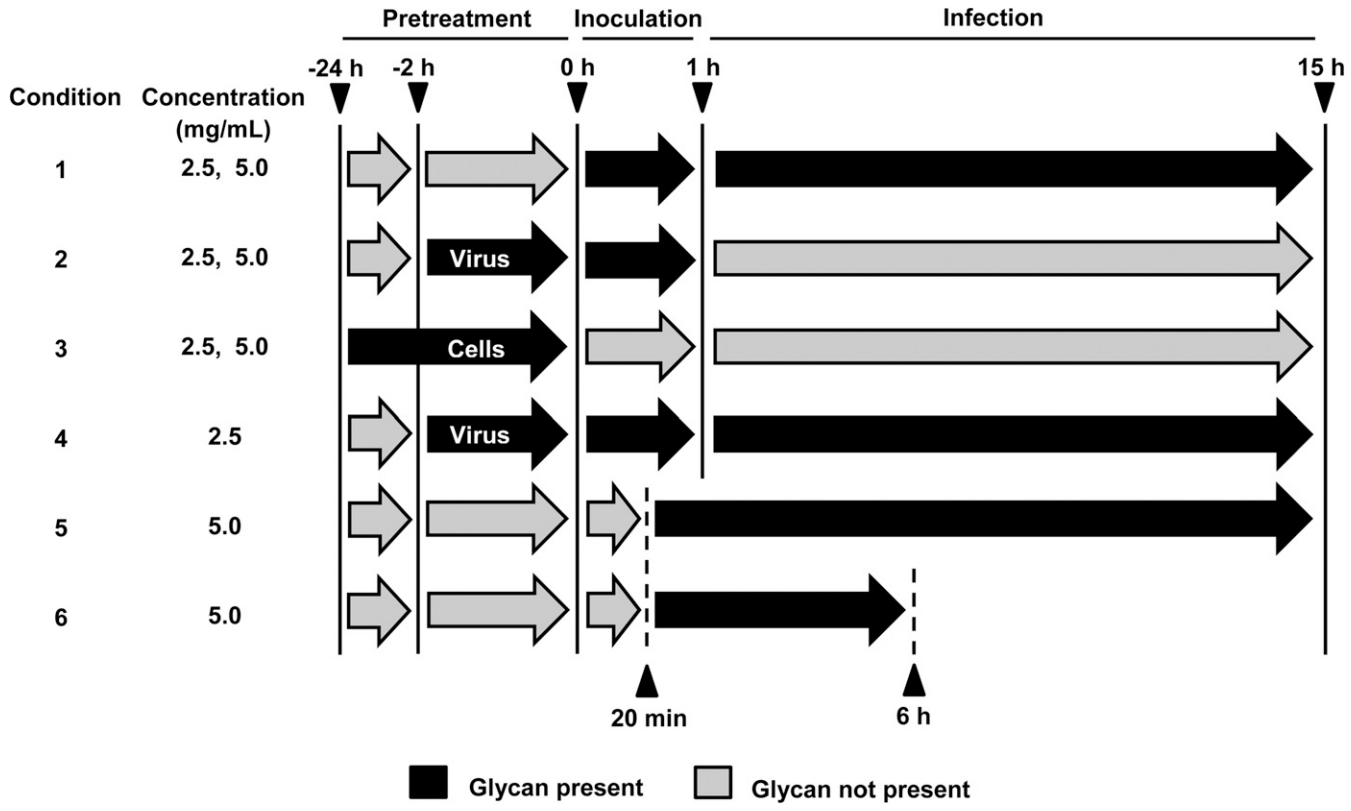

FIGURE 1 Schematic of experimental conditions. Black and gray arrows indicate the presence and absence of oligosaccharides 2′fucosyllactose, galacto-oligosaccharides, 3′sialyllactose, and 6′sialyllactose at various points.

were first added during virus inoculation and during the course of infection to determine the effects on rotavirus infectivity (**Figure 1**, condition 1). Modifications of this protocol were then used to assess specifically the mode of oligosaccharide activity and effect of timing of oligosaccharide addition on virus infectivity (Figure 1, conditions 2–6 and **Supplemental Figure 1**) (18–20). In all of the experiments, infectivity in the absence of oligosaccharides served as the control. Detailed methods for the infectivity assays are provided in the **Supplemental Methods.**

Data analysis. Each experimental condition was tested a minimum of 3 times, with technical replicates for each virus and oligosaccharide concentration included within each assay. The means and SDs from a minimum of 6 data points are represented for each condition in the tables and figures. For each experiment, virus titer measured in the absence of oligosaccharides was considered to be 100% infectivity, and changes in virus titer in the presence of oligosaccharides were expressed as percentage of infectivity compared with no oligosaccharide treatment. For each condition, the percentage of infectivity in the presence of each oligosaccharide was compared with the percentage of infectivity in the absence of oligosaccharides. With the exception of the data presented in **Table 1**, where the effect of the combination of 3′SL + 6′SL was compared with 3′SL and 6′SL individually, no comparisons between different oligosaccharides or between 2 concentrations of the same oligosaccharide were carried out. All of the statistical analyses were performed with use of ANOVA with Dunnett's post hoc test on GraphPad Prism version 6.0 for Windows (GraphPad Software). Multiplicity-adjusted P values were determined and P values <0.01 were considered to be statistically significant.

Results

Effects of milk oligosaccharides on virus infectivity. Two concentrations of each oligosaccharide were tested to determine the effect on rotavirus infectivity (Figure 1, condition 1). Compared with infections carried out in the absence of oligosaccharides, significant reductions in the infectivity of G1P[8] (**Figure 2**A) and G2P[4] (Figure 2B) were observed with all 4 oligosaccharides at 5 mg/mL. At lower oligosaccharide concentrations (2.5 mg/mL), significant reductions in infectivity of G1P[8] and G2P[4] were observed with the sialylated oligosaccharides 3′SL and 6′SL, but not with GOSs or 2′FL. Overall, we observed a 16–40% reduction in infectivity for G1P[8] and a 15–47% reduction for G2P[4] with individual oligosaccharides (**Supplemental Table 1**).

3′SL and 6′SL are the most abundant sialylated HMOs and have been shown to reduce the infectivity of porcine rotavirus OSU in vitro (14). To determine whether the inhibitory effects of 3′SL and 6′SL could be enhanced with a mixture of these oligosaccharides, infectivity assays were carried out with a combination of 3′SL + 6′SL at a ratio similar to that in human breast milk (29%:71%). Although the SL mixture was not more effective at reducing the infectivity of G1P[8] than 3′SL and 6′SL when used separately, the combination of these glycans was more potent at reducing G2P[4] infectivity than the reduction observed with the individual sialylated glycans (Table 1). The SL mixture also significantly reduced G2P[4] infectivity compared with GOSs or 2′FL (data not shown). These results indicate that 3′SL and 6′SL could be more effective at inhibiting human rotavirus infection in combination, but this effect is dependent on the rotavirus genotype.

TABLE 1 Combination of 3′SL and 6′SL enhances reduction of G2P[4] infectivity but not G1P[8] infectivity compared with each glycan alone in MA104 cells[1]

		Infectivity, % of no oligosaccharide control		
Virus	Concentration, mg/mL	3′SL	6′SL	3′SL + 6′SL
G1P[8]	2.5	73 ± 12*	75 ± 7*	68 ± 13*
	5.0	60 ± 9*	63 ± 7*	65 ± 14*
G2P[4]	2.5	54 ± 2*,#	66 ± 5*,#	32 ± 6*
	5.0	53 ± 4*,#	60 ± 12*,#	27 ± 4*

[1] Values are means ± SDs, n = 3. Infectivity in the absence of oligosaccharides was considered to be 100% (G1P[8]: 100% ± 8%, G2P[4]: 100% ± 16%). Data are expressed relative to the no-oligosaccharide control. *Different from control, $P < 0.01$; #different from 3′SL + 6′SL, $P < 0.01$. MA104, African green monkey kidney epithelial cells; 3′SL, 3′-sialyllactose; 6′SL, 6′-sialyllactose.

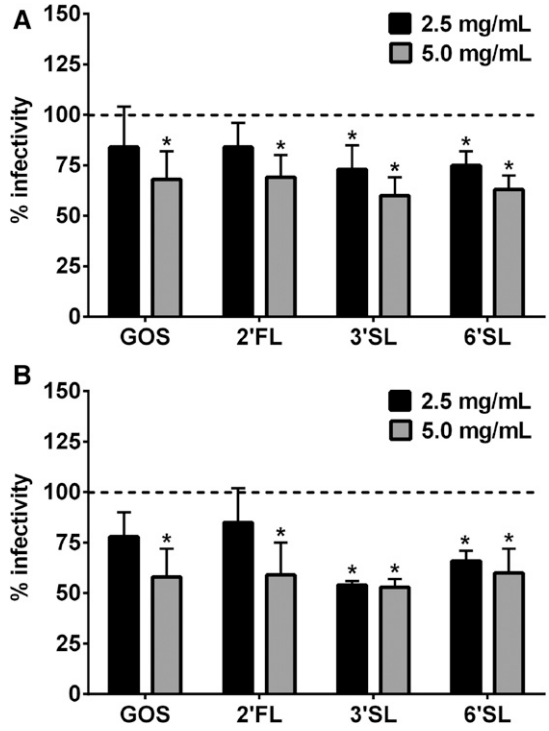

FIGURE 2 Milk oligosaccharides reduce the infectivity of human rotavirus strains in MA104 cells. The effect of milk oligosaccharides (2′FL, GOS, 3′SL, and 6′SL) on the infectivity of 2 clinically relevant human rotavirus strains G1P[8] (A) and G2P[4] (B) was tested (see Figure 1, condition 1 for experimental setup). Data are means ± SDs, $n \geq 3$ independent experiments. Infectivity in the absence of oligosaccharides was considered to be 100% (G1P[8]: 100% ± 8%, G2P[4]: 100% ± 16%) (black dotted line). Data are expressed relative to the no-oligosaccharide control. *Different from control, $P < 0.01$. GOS, galacto-oligosaccharide; MA104, African green monkey kidney epithelial cells; 2′FL, 2′-fucosyllactose; 3′SL, 3′-sialyllactose; 6′SL, 6′-sialyllactose.

Mode of activity. The reduction in rotavirus infectivity can be mediated by the effect of milk oligosaccharides on MA104 cells or directly on the virus. To determine whether the oligosaccharides have a direct effect on the virus, G1P[8] and G2P[4] were preincubated with the oligosaccharides at 37°C for 2 h before infection (Figure 1, condition 2). Alternatively, MA104 cells were treated with milk oligosaccharides overnight at 37°C (Figure 1, condition 3). The cells were pretreated for a longer duration because no differences in infectivity were observed with the pretreatment of cells for 2 h (data not shown), and extended incubation of Caco-2 cells in the presence of milk oligosaccharides has been demonstrated to alter cell surface glycan expression and affect adhesion of enteropathogenic *E. coli* (21). Following pretreatment of virus with oligosaccharides, substantial reductions were observed for G1P[8] infectivity in all but one condition (2.5 mg/mL 2′FL), with a range of 10–39% reduction in infectivity (**Figure 3**A, Supplemental Table 1). Significant reduction in infectivity was observed in the presence of all oligosaccharides at both doses for G2P[4], with a range of 38–67% reduction in

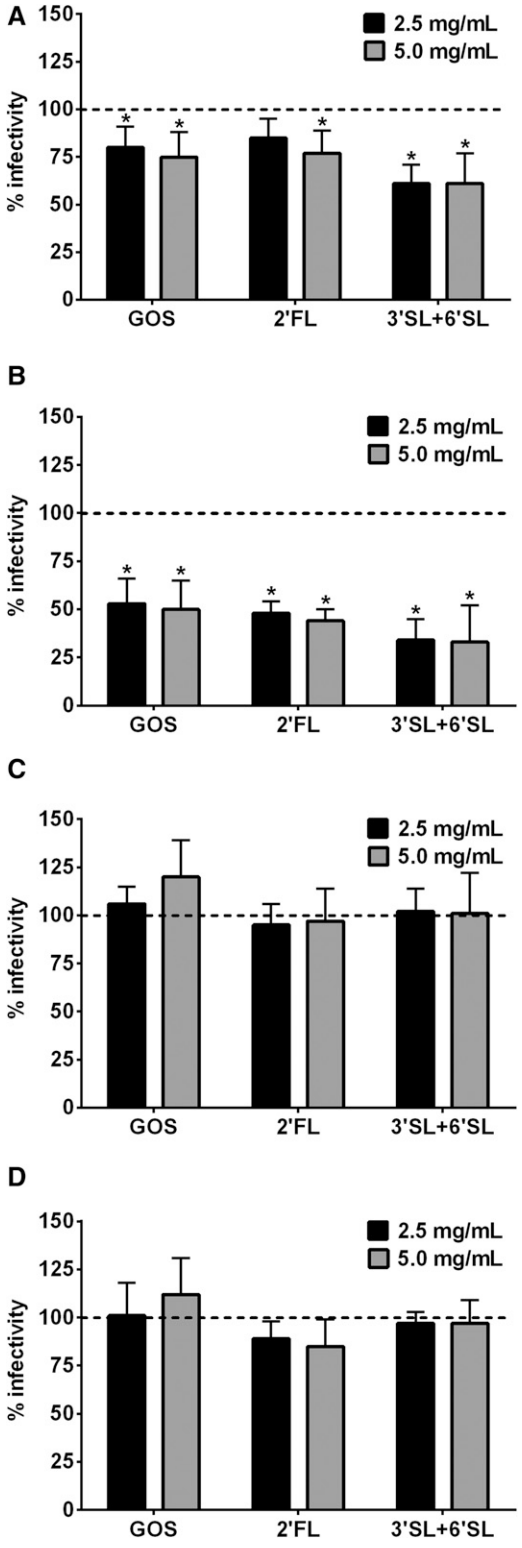

FIGURE 3 Milk oligosaccharides reduce the infectivity of human rotavirus strains through an effect on the virus and not MA104 cells. Preincubation of virus for 2 h in the presence of milk oligosaccharides (2′FL, GOS, 3′SL, and 6′SL) reduces the infectivity of human rotavirus strains G1P[8] (A) and G2P[4] (B) (see Figure 1, condition 2 for experimental setup), whereas preincubation of cells in the presence of milk oligosaccharides for 24 h does not reduce the infectivity of both strains [G1P[8] (C) and G2P[4] (D); see Figure 1, condition 3 for experimental setup]. Data are means ± SDs, $n \geq 3$ independent experiments. Infectivity in the absence of oligosaccharides was considered to be 100% (G1P[8]:100% ± 10%, G2P[4]: 100% ± 9%). (black dotted line). Data are expressed relative to the no-oligosaccharide control. *Different from control, $P < 0.01$. GOS, galacto-oligosaccharide; MA104, African green monkey kidney epithelial cells; 2′FL, 2′-fucosyllactose; 3′SL, 3′-sialyllactose; 6′SL, 6′-sialyllactose.

infectivity across all conditions (Figure 3B, Supplemental Table 1). No reduction in G1P[8] or G2P[4] infectivity was seen when the cells were pretreated with any of the oligosaccharides (Figure 3C, D). Furthermore, the effect of 5 mg/mL 2′FL and 3′SL + 6′SL on virus binding and entry was assessed (Supplemental Figure 1). No significant difference in virus antigen was seen with ELISA following binding of the virus-oligosaccharide mixture to cells at 4°C for 1 h when compared with no-glycan treatment (data not shown). This in part may be the result of the limitations in the sensitivity of ELISA wherein differences in optical density values may not be observed when virus titers differ by 50%. A significant reduction in intracellular virus staining was observed, however, with both oligosaccharides for G1P[8] (**Supplemental Figure 2**A) and G2P[4] (Supplemental Figure 2B) following binding at 4°C. The viruses were allowed to internalize at 37°C for 1 h and the cells were fixed before completing 1 round of virus replication. Overall, our results suggest that milk oligosaccharides reduce human rotavirus infectivity by acting primarily on the virus.

A total of 2.5 mg/mL GOS, 2′FL, or the mixture 3′SL + 6′SL were used for pretreatment of the virus for 2 h and added during infection to determine whether a further reduction in infectivity could be achieved, particularly for lower oligosaccharide concentrations, if viruses were pretreated and oligosaccharides also maintained in the medium during the course of infection (Figure 1, condition 4). The continued presence of oligosaccharides did not significantly enhance reduction for either strain compared with pretreatment alone or the presence of glycan only during infection. The only exception was for G2P[4] and 2′FL, in which the continued presence of the glycan resulted in a 47% reduction in infectivity as compared with a 15% reduction when 2′FL was included only during infection (Supplemental Table 1).

We next wanted to determine whether milk oligosaccharides have an effect on infectivity when added after virus absorption on cells (Figure 1, condition 5). A substantial reduction in infectivity was observed in the presence of all oligosaccharides for G1P[8] infectivity, with reduction ranging from 29% to 62% (**Figure 4**A). The magnitude of reduction was greater with 2′FL and the SL mixture than what was seen with the pretreatment of virus and/or presence of oligosaccharides during infection. For the G2P[4] strain, a significant reduction was observed only with 2′FL (45%, $P < 0.01$; Figure 4B). Because the 15-h infection period may encompass 2 cycles of rotavirus replication, it is possible that the reduction in infectivity was mediated by an effect on newly synthesized virus particles, rather than an effect on virus replication within cells. Indeed, no effect was seen when the infection was stopped at 6 h (Figure 1, condition 6), corresponding to one replication cycle, following the infection of cells for 20 min and the addition of oligosaccharides (data not shown). These findings confirm that milk oligosaccharides can reduce human rotavirus infectivity even after the initial onset of infection.

Discussion

Blocking of microbial attachment to host cells by milk oligosaccharides has been described for a number of enteric pathogens. *Campylobacter jejuni* binds H type 2 histo-blood group antigen (HBGA) expressed on host cell surfaces, and the binding and infection of host cells was inhibited by fucosylated oligosaccharides that show structural similarity to H type 2 HBGA (22).

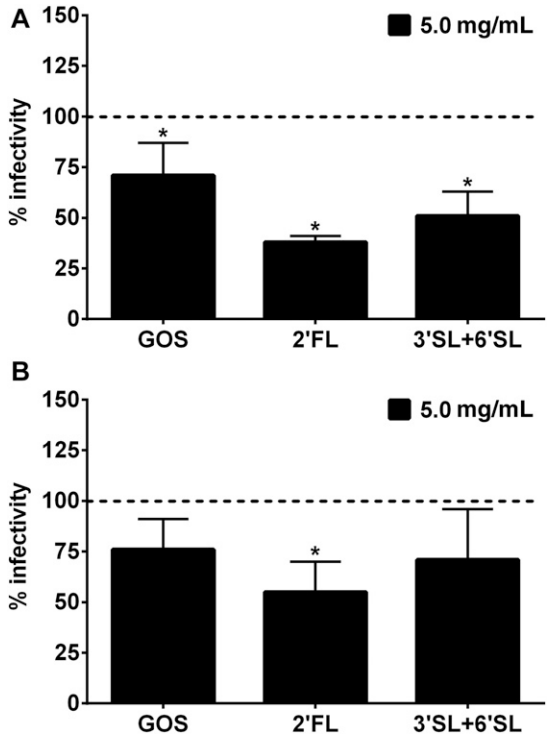

FIGURE 4 Addition of oligosaccharides after virus absorption reduces the infectivity of human rotavirus strains in MA104 cells. Addition of oligosaccharides (2′FL, GOS, 3′SL, and 6′SL) after absorption of G1P[8] (A) and G2P[4] (B) for 20 min reduces infectivity of both strains (see Figure 1, condition 5 for experimental setup). Data are means ± SDs, $n \geq 3$ experiments. Infectivity in the absence of oligosaccharides was considered to be 100% (G1P[8]: 100% ± 10%, G2P[4]: 100% ± 18%) (black dotted line). Data are expressed relative to the no-oligosaccharide control. *Different from control, $P < 0.01$. GOS, galacto-oligosaccharide; MA104, African green monkey kidney epithelial cells; 2′FL, 2′-fucosyllactose; 3′SL, 3′-sialyllactose; 6′SL, 6′-sialyllactose.

GOSs effectively inhibit the adhesion of enteropathogenic *E. coli* to both HEp-2 and Caco-2 cells (23). The binding of norovirus capsid protein to HBGA is well documented. Studies have demonstrated the binding of norovirus capsid protein to HMOs; 2′FL and 3′FL were found to occupy HBGA binding pockets on the norovirus capsid, providing a structural basis for virus inhibition (24, 25). The present study demonstrates the effectiveness of 4 oligosaccharides (2′FL, 3′SL, 6′SL, and GOS) in substantially reducing the infectivity of globally prevalent human rotaviruses G1P[8] (Wa) and G2P[4] (DS1) in vitro. Although the levels of reduction cannot be directly extrapolated to in vivo settings, this study provides a proof of principle that milk oligosaccharides can inhibit the infectivity of clinically relevant human rotavirus strains.

The findings of this study differ from a previous report in which pooled HMOs or specific oligosaccharides were not shown to have an effect on the infectivity of a G1P[8] human rotavirus strain. A significant reduction in infectivity was observed, however, for a G5P[7] porcine rotavirus strain OSU in the presence of 3′SL, 6′SL, and pooled HMOs, but not in the presence of 2′FL (14). The VP8* of porcine rotavirus strain OSU is known to bind sialylated glycans, but binding to nonsialylated HBGA has not been described. Although HMOs did not prevent the onset of porcine rotavirus infection, they did reduce the duration of diarrhea in piglets by having an effect on colonic microbiota and the modulation of cytokine responses induced by infection (15).

The concentrations of milk oligosaccharides tested in this study are comparable to previous studies on animal rotaviruses (14); however, the levels are higher than the physiological concentrations of individual HMOs present in breast milk (1). It should be noted that in the absence of other acidic HMOs, the concentration of 3′SL + 6′SL tested in the present study match the total amount of acidic HMOs present in breast milk (10–20% of total HMOs) (1). Furthermore, fucosylated oligosaccharides account for ~50% of total HMOs (1). The most abundant fucosylated oligosaccharide is 2′FL, which is found at concentrations ≤4.5 mg/mL (26). The concentrations of individual oligosaccharides tested in the in vitro infectivity assays are therefore similar to the concentration of the acidic oligosaccharide fraction present in breast milk and the biological concentration of 2′FL. In addition, higher concentrations may be required in in vitro assays in which the oligosaccharides are added at a single point during infection when compared with physiological conditions in which a child receives multiple feeds during the same 15-h period.

The present study demonstrates that both sialylated and fucosylated oligosaccharides can reduce the infectivity of human rotaviruses. This is interesting in the context of studies on cell-surface receptors for human rotavirus strains. Classically, many human rotavirus strains, including G1P[8], were thought to use internal sialic acid residues on cell surfaces as receptors. A growing body of evidence suggests, however, that HBGAs are important attachment factors for human rotaviruses (27). Specifically, fucosylated glycans, including H-type 1 and Lewis b HBGA, are now recognized as potential binding partners for both G1P[8] and G2P[4] strains (28). All of the milk oligosaccharides tested in the present study could reduce the infectivity of human rotavirus strains G1P[8] and G2P[4]. Compared with infections in the absence of oligosaccharides, the maximum reduction in G1P[8] infectivity was observed with 5 mg/mL 2′FL when added after the onset of infection (62% reduction), whereas the maximum reduction in G2P[4] infectivity was observed with the mixture of 3′SL and 6′SL (5 mg/mL) when added during infection (73% reduction). Although we observed strain-specific differences in the effect of milk oligosaccharides, all glycans appear to act on the virus rather than on the cells. Milk oligosaccharides have been shown to affect the proliferation, maturation, and growth characteristics of human intestinal epithelial cells (29–32); however, our preliminary studies suggest that the action of the oligosaccharides on cells appears to have a limited effect on virus replication. Further studies are required to elucidate whether the reduction in infectivity is mediated by decoy receptor activity of the oligosaccharides' preventing the binding of the virus to cell surface receptors or as the result of an effect on virus entry.

Although single oligosaccharides did not completely block rotavirus infectivity in the transformed monkey kidney epithelial cell line MA104, it is possible that the direct effects of oligosaccharides on the virus, together with other components of intestinal immunity and innate defenses, would result in greater inhibition of infectivity. We have demonstrated the use of human intestinal enteroid cultures as new preclinical models for rotaviruses (33). Testing milk oligosaccharides for their effect on rotavirus infectivity in human intestinal enteroid cultures will allow the evaluation of these compounds in a physiologically relevant human culture system and to elucidate whether, in addition to direct effects on the virus, there are further effects of HMOs on the host intestinal epithelium that affect rotavirus infectivity. Such studies will be important to translate the present findings from the laboratory bench to the human population.

Despite their importance to the developing infant, HMOs are not a component of any commercially available infant formula. The protective effects of milk oligosaccharides are conferred to children who are breastfed, but not all children are breastfed exclusively,

whether because of medical reasons, circumstances, or mothers' personal choice. In these cases, infant formula is used to supplement or replace human milk as a means of nutrition. Infant formulas are produced largely from bovine milk, and oligosaccharides in bovine milk are far less abundant and complex than those in human milk. There is growing interest in the addition of HMOs to infant formula and to determine whether supplementation with specific HMOs confers similar benefits to those of human breast milk. In clinical trials testing milk formula supplemented with 2′FL, infants fed the supplemented formula showed growth and 2′FL uptake that were similar to breastfed infants (26). Although further optimization of effective concentrations and testing in preclinical models and human studies is required, the reduction of rotavirus infectivity by milk oligosaccharides provides an added incentive for the addition of specific oligosaccharides to infant formula and could confer additional benefits to formula-fed infants.

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