Human-Milk Glycans That Inhibit Pathogen Binding Protect Breast-feeding Infants against Infectious Diarrhea[1,2]

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ABSTRACT Breast-feeding is a highly effective strategy for preventing morbidity and mortality in infancy. The human-milk glycans, which include oligosaccharides in their free and conjugated forms, constitute a major and an innate immunologic mechanism by which human milk protects breast-fed infants against infections. The glycans found in human milk function as soluble receptors that inhibit pathogens from adhering to their target receptors on the mucosal surface of the host gastrointestinal tract. The α1,2-linked fucosylated glycans, which require the secretor gene for expression in human milk, are the dominant glycan structure found in the milk of secretor mothers, who constitute the majority (~80%) of mothers worldwide. In vitro and in vivo binding studies have demonstrated that α1,2-linked fucosylated glycans inhibit binding by campylobacter, stable toxin of enterotoxigenic *Escherichia coli*, and major strains of caliciviruses to their target host cell receptors. Consistent with these findings, recently published epidemiologic data demonstrate that higher relative concentrations of α1,2-linked fucosylated glycans in human milk are associated with protection of breast-fed infants against diarrhea caused by campylobacter, caliciviruses, and stable toxin of enterotoxigenic *E. coli*, and moderate-to-severe diarrhea of all causes. These novel data open the potential for translational research to develop the human-milk glycans as a new class of antimicrobial agents that prevent infection by acting as pathogen anti-adhesion agents. J. Nutr. 135: 1304–1307, 2005.

KEY WORDS: • *oligosaccharide* • *anti-adhesion agents* • *innate immunity* • *receptor analogs* • *histo-blood groups*

Breast-feeding is one of the most cost-effective strategies known to medicine and public health for the prevention of morbidity and mortality caused by infectious disease in infancy and early childhood (1–6). Whereas human milk is widely accepted as the ideal food for young infants, human milk could also be considered the model "nutriceutical," that is, a food that conveys immunologic and other health benefits. Significant protection by feeding human milk has been demonstrated in relation to diarrheal diseases, respiratory-tract infections, bacteremia and meningitis, and necrotizing enterocolitis (1–6). The protection against infection afforded to breast-fed infants appears to occur through a variety of complementary acquired and innate defense factors found in human milk, including oligosaccharides and their glycoconjugates (3–7).

Human-milk oligosaccharides are carbohydrates that contain lactose at the reducing end and, typically, a fucose or a sialic acid at the nonreducing end. These oligosaccharides can be free or conjugated and expressed as glycoproteins, glycolipids, or other structures. The conjugated and unconjugated forms of oligosaccharides are together classified as glycans. The importance of the human-milk glycans is suggested by their prominence: free, unconjugated oligosaccharides alone constitute the third-most common solid component of human milk after lactose and lipid (6). Oligosaccharide concentrations are ~10 g/L in mature milk (8,9). Less than 100 different oligosaccharides have been isolated from human milk and characterized to date, but there is evidence from time-of-flight mass spectrometry of >900 distinct oligosaccharides in human milk (10). The human-milk oligosaccharides range in size from 3 to 32 sugars; most of these are fucosylated, including 1 to 15 fucoses (6,10). The concentrations of total and specific oligosaccharides in human milk have been shown to vary between

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mothers, diurnally, by infant gestational age, and over the course of lactation (8,9,11,12). Human-milk glycans are found in infant feces largely intact, because they tend to withstand the digestive processes of the gastrointestinal tract (13). Their resistance to digestion allows them to remain available to protect the mucosal surface of the gastrointestinal tract.

Initially, the milk glycans were thought to lack biological function, but it is now widely accepted that they function as immunologic or anti-infective agents (3,5,7,9,14–17). Some of the glycans appear to function as probiotic agents, i.e., they selectively stimulate the growth of beneficial bacteria in the intestine (18). However, an even more important role for human-milk glycans is that of pathogen-binding agents (3–7,9,11,14–17,19). This review describes the human-milk glycans as inhibitors of pathogen binding. We focus on the α1,2-linked fucosylated glycans, which predominate in human milk, and the evidence that the α1,2-linked fucosylated human-milk glycans are associated with protection of breast-fed infants against diarrhea caused by campylobacter, caliciviruses, stable toxin of *Escherichia coli,* and moderate-to-severe diarrhea in general (3,5,9,14–17,19).

Human-milk glycans as pathogen-binding inhibitors

Human-milk glycans are innate anti-adhesion agents that protect the breast-fed child by preventing pathogens from adhering to host ligands (5–7). The glycans of human milk have structural homology to host cell receptors and thus function as "receptor decoys," such that pathogens bind to human-milk glycans instead of to the host cell-surface glycans. Alternatively, human-milk glycans can inhibit pathogens by competitive binding with the host cell-surface receptor. An example of this is milk oligosaccharide binding to the guanylin cyclase receptor, which blocks binding to that site by the stable toxin of enterotoxigenic *E. coli* (19).

Host susceptibility

Pathogens infect their target host tissues through a series of steps that begin with attachment to cell-surface glycan binding sites. For many enteric pathogens, host susceptibility to infection is related to cell-surface expression of ABH(O) and Lewis-secretor blood group antigens (5,15,17,20–24). Individuals who are O blood group, for example, are known to have greater susceptibility than others to cholera and to Norwalk virus (NV), a major calicivirus (20,21). Genetic polymorphisms that determine blood group type also result in varied expression of these same antigens in the gastrointestinal tract and in human milk (5,9,15). The fucose terminus of cell-surface glycans or human-milk glycans may be connected by an α1,2 linkage catalyzed by a fucosyltransferase encoded by the secretor gene (*FUT2*)[4] or by an α1,3 or α1,4 linkage catalyzed by fucosyltransferases encoded by the Lewis gene (*FUT3*) family. The synthesis pathway is shown in **Figure 1**. Binding to an α1,2-linked fucosylated host cell-surface receptor is a critical step in the pathogenesis of campylobacter, cholera, major caliciviruses, and other enteric pathogens (5,17,24).

α1,2-linked fucosylated human-milk glycans

The oligosaccharides that are most commonly found in human milk are fucosylated. The most common of the fucosylated oligosaccharides in the milk of secretor mothers are 2′-fucosyllactose (2′-FL) and lacto-*N*-fucopentaose-I (LNF-I) (**Fig. 2**), both of which contain an α1,2-linked fucose. Other major fucosylated oligosaccharides are 2 that contain an α1,2-linked fucose, and 2 that do not include an α1,2-linked fucose, and the 2 precursors (type 1 and type 2) of these structures. As shown in Figure 1, all eight of these oligosaccharides are homologs of the Lewis-secretor histo-blood group antigens (9,16). In vitro and in vivo studies indicate that the α1,2-linked fucosylated oligosaccharides are especially important inhibitors of major diarrhea-causing pathogens, as described below.

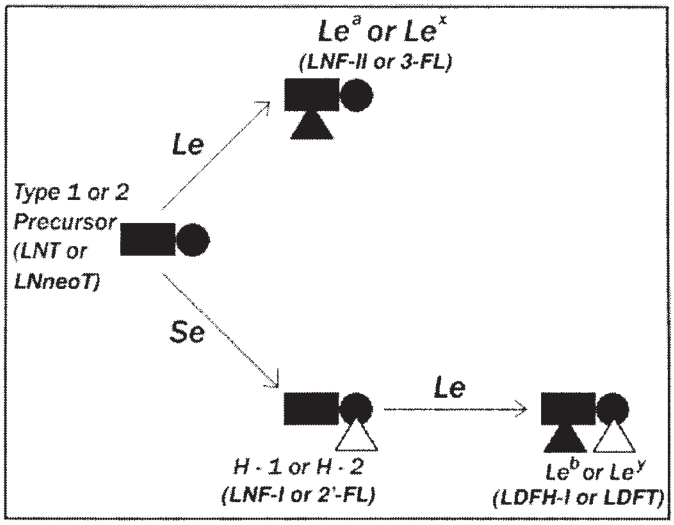

FIGURE 1 The human-milk oligosaccharide synthesis pathway. Beside the arrows along the pathway, Se indicates the secretor gene (*FUT2*) and Le indicates the Lewis gene (*FUT3*). Precursors and products along the pathway are indicated by the geometric figures with Lewis epitope names (indicated along with the biochemical names).

A seminal early observation was that human milk contains a nonimmunoglobulin, low-molecular-weight component absent from formula or bovine milk that protects suckling mice from ST-induced diarrhea (19). All of the ST-protective activity was localized to the neutral oligosaccharide fraction that bound to *Ulex europaeus*, suggesting that the ST protective factor includes an α1,2-linked fucose. In the presence of protective fucosyloligosaccharides of human milk, ST is unable to stimulate production of cyclic GMP. Binding by oligosaccharide to the extracellular domain of guanylate cyclase blocks binding by ST and prevents the ST-induced loss of chloride ion homeostasis that results in secretory diarrhea.

Similarly, campylobacter binding to HEp2 cells is inhibited by fucosylated carbohydrate moieties containing the H-2 blood group epitope (17). *Campylobacter jejuni*, which normally does not bind to Chinese hamster ovary (CHO) cells, binds avidly when the cells are transfected with a human α1,2-fucosyltransferase gene that causes overexpression of H-2 antigen. Binding between *C. jejuni* and these transfected CHO cells is inhibited, however, by ligands that bind to H-2, including anti-H-2 mAbs, H-2 neoglycoproteins, and 2′-FL, which compete with cell receptors. Human-milk oligosaccharides also inhibit campylobacter colonization of mice in vivo and inhibit invasive, pathogenic campylobacter from binding to human intestinal mucosa ex vivo. Protection against campylobacter is thus limited to the α1,2-linked H-2 fucosylated glycoconjugates in milk, consistent with the finding that the main intestinal ligands for campylobacter are the H-2 histo-blood group antigens (17).

[4] Abbreviations used: CHO, Chinese hamster ovary; *FUT2,* secretor gene; *FUT3,* Lewis gene; LNF-I, lacto-*N*-fucopentaose I; NV, Norwalk virus; ST, stable toxin; 2′-FL, 2′-fucosyllactose.

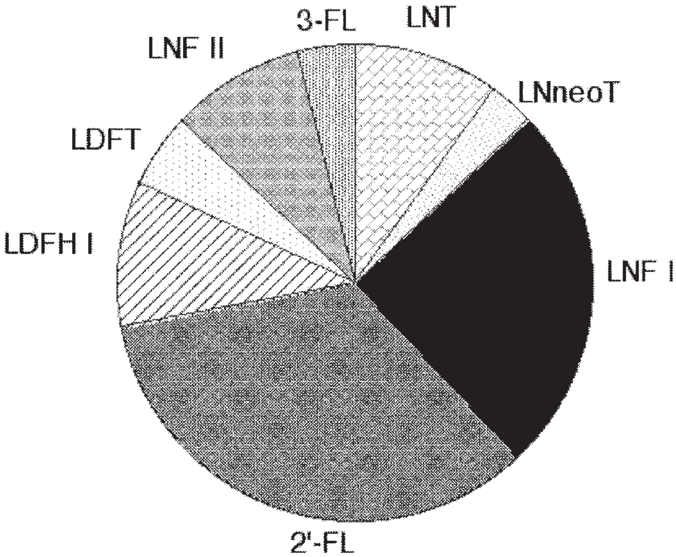

FIGURE 2 Quantity of fucosylated oligosaccharides in human milk. LNT, lacto-*N*-tetraose; LNneoT, lacto-*N*-neo-tetraose; LDFT, lactodifucotetraose; LDFH I, lacto-*N*-difucohexaose I.

Noroviruses (previously known as Norwalk-like viruses), a major genus of calicivirus, recognize human histo-blood group antigens as receptors. In 50 NV-challenged volunteers, saliva samples from 68% of secretors, but only 6% of nonsecretors bound NV capsids ($P < 0.001$), indicating that susceptibility to NV infection depends on secretor status and, thus, on the presence of α1,2-linked fucose in the gastrointestinal tract (22). However, different strains of noroviruses recognize different receptors defined by the ABO, Lewis, and secretor blood types. Of 60 Mexican and U.S. mothers, the milk samples of all 54 secretor mothers, but none of the 6 nonsecretor mothers were able to block the secretor-binding strains of norovirus (NV and VA387) from binding to saliva samples (14). Conversely, all 6 nonsecretor, Lewis positive milk samples blocked binding by the Lewis epitope-binding (VA207) strain, whereas variable blocking was exhibited by the 54 secretor milk samples to this strain. Binding of the MOH strain of norovirus to A and B antigens was not inhibited by any of the samples. These data suggest that secretor and Lewis but not A or B antigens are present in human milk and block binding to host receptors by the majority of clinically relevant norovirus strains (14).

Protection of breast-fed infants

We used the innate variation in milk oligosaccharide expression to examine the effectiveness of naturally occurring human-milk glycans to protect breast-fed infants against diarrhea (9,16). Data and samples were analyzed from 93 breast-feeding mother–infant pairs who were prospectively studied during 1988–1991 as part of our ongoing program project. Mother–infant pairs were followed from birth up to 2 y postpartum with weekly collection of infant stool and infant feeding and illness data. About three-quarters of study mothers were Lewis a-b+, one-quarter were Lewis a-b-, two-thirds were O blood type, and one-third were A, B, or AB blood types.

A single milk sample per mother obtained 2–5 wk postpartum was analyzed. The concentrations of individual oligosaccharides were assayed by HPLC. As shown in Figure 2, the α1,2-linked fucosylated oligosaccharide comprised 73% (50–92%) of total oligosaccharide (9,16). The mean concentration of 2′ -FL in milk was 3.8 ± 1.0 (mean ± SD) mmol/L (34% of total fucosylated oligosaccharides) but varied greatly among mothers. Poisson regression was used to analyze continuous oligosaccharide values in maternal milk in relation to incidence of infant disease. A *t* test was used to compare rates of disease by oligosaccharide concentration categories defined by tertiles (low, intermediate, high) of 31 subjects each. Specific and total α1,2-linked milk oligosaccharides were analyzed as a concentration (mmol/L) and as a percentage of milk oligosaccharide.

In the 93 study children, consumption of high levels of 2′ -FL as a percentage of milk oligosaccharide was associated with protection against campylobacter diarrhea (Poisson regression, $P = 0.004$) (16). Consumption of high levels of LDFH-I as a percentage of milk oligosaccharide was associated with protection against calicivirus diarrhea (Poisson regression, $P = 0.012$) (16). Consumption of high levels of total 2-linked oligosaccharide as a percentage of milk oligosaccharide was associated with protection against moderate-to-severe diarrhea of all causes ($P < 0.001$) (**Fig. 3**). Further, the children who contracted ST-associated diarrhea while breast-feeding were consuming milks with a low content of 2-linked oligosaccharides: the ratio of α1,2- to α1,3/4-linked fucosylated oligosaccharides in their mothers' milk was 3.9 ± 0.7 SE ($n = 4$), significantly lower than that of milks being consumed by infants who were infected with ST-*E. coli* but did not develop diarrhea (7.6 ± 1.0, $n = 43$) or uninfected controls (7.5 ± 1.0, $n = 46$) ($P < 0.01$) (**Fig. 4**). Thus, we found higher consumption of 2-linked oligosaccharides to be associated with protection against campylobacter, calicivirus, and ST-associated diarrhea and moderate-to-severe diarrhea overall (9,16).

Summary and implications

Human-milk α1,2-linked fucosylglycans inhibit binding by campylobacter, cholera, stable toxin of *E. coli*, and major strains of caliciviruses in vitro and in vivo. A population-based study has shown that high levels of specific 2-linked fucosylglycans in maternal milk are associated with lower risk of diarrhea from campylobacter, caliciviruses, and ST of *E. coli*, and that high levels of all 2-linked fucosylglycans in maternal

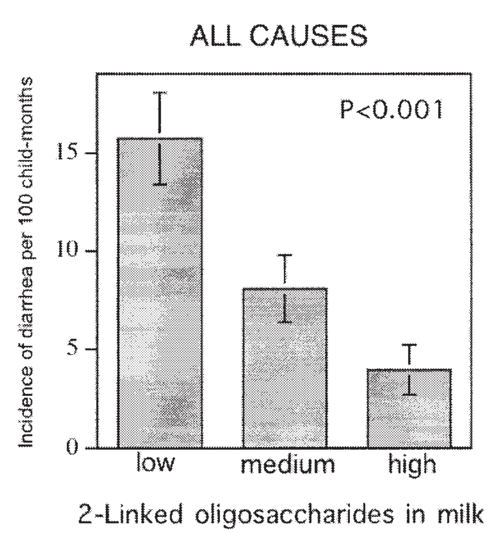

FIGURE 3 *A–C*. Incidence of diarrhea in breast-fed infants in relation to the relative quantity of 2-linked oligosaccharide in maternal milk. [Reprinted from the Journal Pediatrics, Vol. 145 (3), Morrow et al., Human milk and diarrhea in breast-fed infants, pages 297–303, copyright 2004, with permission from Elsevier.]

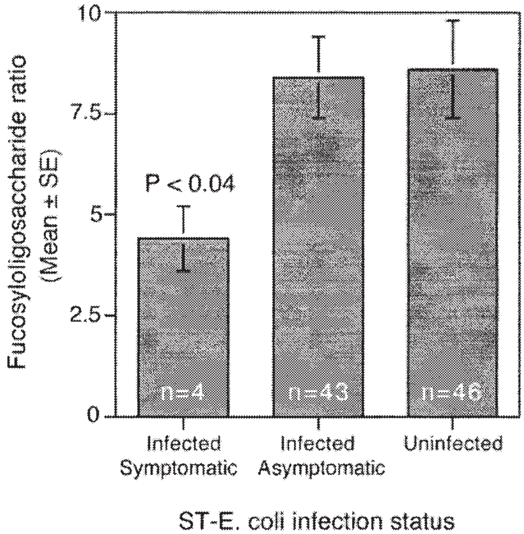

FIGURE 4 Two-linked oligosaccharide ratios in maternal milk relation to symptomatic and asymptomatic ST infection of breast-fed infants. [Reprinted from Glycobiology, Vol. 14 (3), Newburg et al., Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breast-fed infants, pages 253–263, copyright 2003, with permission from the Society for Glycobiology.]

milk are associated with lower risk of all moderate-to-severe diarrhea in breast-fed infants (9,16). Thus, the human-milk glycans that strongly inhibit pathogen binding in the laboratory appear to account for much of the protection of breast-fed infants against enteric disease.

Not all pathogens bind to the histo-blood group antigens and the homologous milk oligosaccharides synthesized by the fucosyltransferases. Some pathogens, including rotavirus, *Haemophilus influenzae*, and others, bind to sialic acid-containing receptors (25–27). Lactadherin, a 46-kDa sialylated glycoprotein found in varying concentrations in human milk, has been shown to bind to rotavirus and prevent symptomatic infection in infants (27). Human-milk glycans include diverse protective structures.

Breast-feeding conveys natural anti-infective compounds to the child and is the most effective intervention currently known for preventing morbidity and mortality, caused by infectious disease in young children (2). The soluble glycans found in human milk inhibit pathogens from binding to their host cell-surface glycans and are associated with significant protection from diarrhea in breast-fed infants. Diarrhea is a leading cause of morbidity and mortality in developing countries, accounting for 22% of all deaths in children under 5 y of age (2). Diarrhea remains endemic in developing countries despite improved hygiene, potable water, and sanitation, suggesting the need for additional interventions. This is a promising line of research, with the potential to develop a novel class of antimicrobial agents that have widespread application to improving child health.

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