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HUMAN MILK GLYCANS PROTECT INFANTS AGAINST ENTERIC PATHOGENS

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■ **Abstract** Breastfed infants have lower morbidity and mortality due to diarrhea than those fed artificially. This had been attributed primarily to the secretory antibodies and prebiotic factors in human milk. Oligosaccharides are the third largest component of human milk. They were initially considered to be functionless by-products of glycoprotein and glycolipid synthesis during milk production. However, in the past few decades it has become apparent that the human milk oligosaccharides are composed of thousands of components, at least some of which protect against pathogens. Oligosaccharide protection against infectious agents may result in part from their prebiotic characteristics, but is thought to be primarily due to their inhibition of pathogen binding to host cell ligands. Most human milk oligosaccharides are fucosylated, and their production depends on enzymes encoded by the genes associated with expression of the Lewis blood group system. The expression of specific fucosylated oligosaccharides in milk thus varies in relation to maternal Lewis blood group type, and is significantly associated with the risk of infectious disease in breastfed infants. Specific fucosylated moieties of oligosaccharides and related glycoconjugates (glycans) are able to inhibit binding and disease by specific pathogens. This review presents the argument that specific glycans, especially the oligosaccharides, are the major constituent of an innate immune system of human milk whereby the mother protects her infant from enteric and other pathogens through breastfeeding. The large input of energy expended by the mother in the synthesis of milk oligosaccharides is consistent with the human reproductive strategy of large parental input into rearing relatively few offspring through a prolonged period of maturation. These protective glycans may prove useful as a basis for the development of novel prophylactic and therapeutic agents that inhibit diseases caused by mucosal pathogens.

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INTRODUCTION: PROTECTION BY BREASTFEEDING

A relationship between breastfeeding and health of the infant has been noted from the times of the first recorded use of human milk substitutes, going back thousands of years (61). A more recent example is a study in Chicago in the 1930s in which more than 20,000 mother-infant dyads were systematically studied to determine disease incidence in infants in relation to mode of infant feeding (15). Infants consuming their mother's milk were found to have a several-fold lower risk of diarrheal disease, and a lower risk of respiratory infections as well as other types of infectious disease, including otitis media. The findings of this large, early study were generally replicated by subsequent studies conducted over several decades. Recent randomized clinical trials of breastfeeding promotion demonstrate that when breastfeeding rates increase, there is a proportional decrease in infant diarrhea (24, 30).

MECHANISMS OF BREASTFEEDING PROTECTION

Prebiotics

In the 1950s, based on advances in understanding of infant nutrition, many thought the improved composition of milk substitutes justified a shift to the use of artificial

feeding. This perspective was reinforced by the reports that formula-fed infants typically gained more weight than did breastfed infants (25). However, the higher morbidity and mortality due to diarrhea and respiratory infections observed in artificially fed infants suggested a potent ability of breastfeeding to protect infants against infectious disease. This observation naturally led to heightened interest in identifying mechanisms whereby breastfeeding might protect against disease, including psychosocial factors, avoidance of contaminated food and water, superior nutrition, and human milk components that might directly or indirectly protect breastfed infants.

In 1905 (56), the difference in the composition of intestinal microflora was documented between breastfed and precociously weaned infants. Breastfed infants were noted to have predominance of lactobacilli, and, in particular, *Lactobacillus bifidus* (now known as *Bifidobacterium bifidum*), which was cultured from the feces of breastfed infants. In contrast, adults and precociously weaned infants had a predominance of *Escherichia coli*. Breastfeeding was hypothesized to mediate protection of the infant through milk components that specifically supported colonization by *L. bifidus*. *L. bifidus*, in turn, acidified the gut, bound to potential sites for colonization, and thereby inhibited pathogens from colonizing the gut and causing disease. It was not until 1974 that a glycan from human milk that stimulated the growth of *L. bifidus* was isolated and posited to be the heretofore undefined "bifidus factor" (17). Other types of indigestible oligosaccharides from other sources have since been found to stimulate colonization by *B. bifidum* and several lactobacilli. When fed in the diet, such materials stimulate gut colonization by beneficial microbes, presumably promoting health, and are known as prebiotics. Beneficial microorganisms that are introduced directly in the diet are known as probiotics. Human milk, through its oligosaccharides, acts as a prebiotic. Breastfeeding may also introduce probiotics into the digestive tract of the nursing infant, thereby protecting the infant from infectious disease (17).

Secretory Antibodies

In 1892, the ability of milk to transfer immunity from the mother to her offspring was reported (11), and in 1903 this property was attributed to milk antibodies (49). When significant amounts of secretory antibodies (sIgA) were found in human milk in 1961 (18), they were assumed to be the major agents whereby milk protected nursing infants. The very high concentrations of sIgAs in colostrum seemed consistent with human milk being a medium through which the maternal adaptive immune system could transmit mucosal protection to the infant gut, augmenting the gift of maternal antibody through prenatal transfer of serum antibodies across the human placenta. This seemed an effective mechanism to protect infants from pathogens to which the mother had prior exposure. However, upon exposure of the mother-infant dyad to a new pathogen, the prolonged lag between exposure and the appearance of the sIgA in the milk seemed enough time for the pathogen to establish a profound infection in the infant. Thus, other forms of interim protection seemed necessary to fully account for protection of infants by breastfeeding.

Multifunctional Agents

Other protective components found in human milk include multifunctional agents. These are milk constituents that function as a source of nutrients and that also function, either directly or through partial digestion products, as pathogen inhibitors. For example, lysozyme and lactoferrin, major sources of protein, are bacteriostatic or bacteriolytic. Free fatty acids and monoglycerides released from human milk triglycerides in the stomachs of breastfed infants and peptides released upon partial digestion of lactoferrin and other milk proteins exhibit a wide array of antibacterial, antiviral, and antiprotozoal activities (35). These multifunctional agents in human milk had been thought to slow the process of infection in nursing infants until sufficient sIgA appeared in the milk they were consuming, after which the multifunctional agents could act synergistically with antibody protection.

Multifunctional components may affect disease processes other than acute diarrhea. For example, a different form of multifunctional component from milk that inhibits tumors was recently described. The major protein found in human milk, α-lactalbumin, under acidic conditions in the presence of oleic acid, conditions that occur in the stomachs of breastfed infants, forms an alternate structural conformer. This α-lactalbumin-oleic acid complex, named HAMLET (human α-lactalbumin made lethal to tumor cells), seems to inhibit a wide array of tumors by triggering an apoptosis-like event (54). Current understanding of protective components of milk is that each may have a very specific mode of inhibition with regard to pathogens, location in the infant gut, host state, and timing. Some may exist only transiently or conditionally. However, the multiplicity of agents and their synergy result in potent protection by milk (16, 22, 32, 35, 45, 48).

Oligosaccharides and Other Glycans

The human milk oligosaccharides are complex carbohydrate structures attached to a lactose moiety at the reducing end. Human milk also contains glycoconjugates in which complex carbohydrate structures are attached at their reducing end to lipid to form glycolipids, or are attached to macromolecules to form glycopeptides, glycoproteins, glycosaminoglycans, or mucins. All of these are glycans. Recently, the oligosaccharides and other glycans of human milk have been gaining increasing attention as a major class of anti-infective agents in human milk. This review presents the argument that these milk glycans, by inhibiting the ability of pathogens to bind to their host cell receptors, represent a heretofore-underappreciated major mechanism whereby human milk affords robust protection to the nursing infant.

HUMAN MILK OLIGOSACCHARIDES AND OTHER GLYCANS

Although lactose was first isolated from milk in 1633, it was 1933 before the nonlactose carbohydrate fraction of human milk was isolated and the 1950s before the oligosaccharides were systematically characterized (40). Most human milk

oligosaccharides are derived from 12 core structures made up of glucose, galactose, and *N*-acetylglucosamine. Four types of terminal fucose linkages and three types of sialic acid linkages can give rise to a vast array of compounds. Hundreds, and perhaps many thousands, of human milk oligosaccharides are thought to exist (51). Of these, well over a hundred structures have now been isolated and characterized. Almost all have lactose on the reducing end, and most have at least one fucose or sialic acid on the nonreducing terminus. Because these glycan structures are analogous to glycan moieties of the more complex glycoconjugates, such as glycolipids, glycoproteins, mucins, and glycosaminoglycans, it was originally thought that the milk oligosaccharides were an accidental by-product of having large amounts of active glycosyltransferases needed for glycoconjugate synthesis. This notion was reinforced by the resistance of glycans to digestion (breastfed baby stool was a known rich source of human milk glycans) and by the lack of any known biological functions. However, over the course of lactation, the production of milk represents a major commitment of maternal resources. A milk component that represents significant maternal calories but with no nutritional or other biological role would be inconsistent with the biological frugality that usually ensues from many generations of intermittent marginal nutritional status. That these milk oligosaccharides and glycoconjugates, that is, these human milk glycans, had a function seemed a reasonable hypothesis (38). Furthermore, because the milk oligosaccharides and glycans are made by the same types of glycosyltransferases that are responsible for the synthesis of human cell surface glycans, they would be expected to have structural moieties in common. Most enteric pathogens use cell surface glycans to identify and bind to their target cells as the critical first step in pathogenesis. Thus, soluble glycans from milk could competitively inhibit the ability of pathogens to bind to receptors in the gut and thereby protect the breastfed infant from diarrhea (Figure 1).

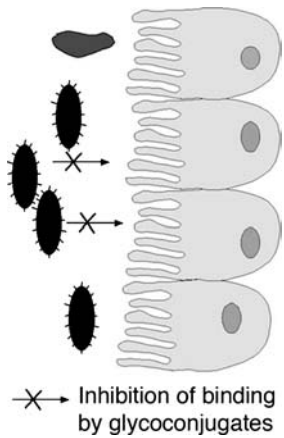

Figure 1 If human milk glycoconjugates contain epitopes that bind to specific pathogens, they can compete for the pathogen binding sites and block pathogens from binding to their host cell receptors in the mucosa.

In 1983, two observations suggested that human milk glycans could serve to protect against disease. In one, the human milk ganglioside fraction was reported to inhibit the activity of labile toxin of *E. coli*, as well as that of cholera toxin (44). Both of these toxins were known to bind to the ganglioside GM1, a monosialylated glycosphingolipid. These data implied that a human milk glycolipid that binds to a pathogen might inhibit the ability of the pathogen to bind to the intestinal mucosa of infants, thereby protect nursing infants from the diarrhea induced by the pathogen, in this case, labile toxin of *E. coli* or its homologue, cholera toxin. The other observation was that a factor in human milk inhibited the ability of stable toxin (ST) of *E. coli* to induce diarrhea in vivo. This human milk component did not partition into organic solvents, was dialyzable, was stable to heat, protease, acid or alkaline pH, but was destroyed by strong acid digestion that hydrolyzes glycosidic linkages, which suggested that the protective molecule could be an oligosaccharide or glycoconjugate, i.e., a glycan. Other indications that the human milk glycans might participate in the protection of breastfed infants included reports in 1986 that human milk glycans inhibit *Streptococcus pneumoniae* binding to human cells (1), and in 1991 that they inhibit binding by enteropathogenic *E. coli* (9).

FUCOSYLATED INHIBITORS OF PATHOGENS

Many enteric pathogens are inhibited by human milk, and for several, fucosylated glycans proved to be the active components.

Stable Toxin of *Escherichia coli*

To investigate whether the factor in human milk that inhibits ST was an oligosaccharide, fractions of human milk were tested, at the concentrations found in milk, for their ability to inhibit ST in vivo. The ST-protective factor was isolated and characterized as follows: Pooled human milk was separated into its components, and the only component that inhibited ST was the oligosaccharide fraction. Of the subfractions, only the neutral oligosaccharides inhibited (Table 1). The activity was specific to the neutral oligosaccharides that bound *Ulex europaeus; U. europaeus*

TABLE 1 Stable toxin (ST) protective activity: charcoal fraction separated by charge

ST	Lethality	Percent
ST + saline	32/50	61
ST + acidic oligosaccharides	34/60	57
ST + neutral oligosaccharides	13/59*	22
ST + milk aq_4	14/52*	27

*Value differs significantly from control value, $p < 0.0003$.

lectin avidly binds α1,2-linked fucose structures, which strongly suggested that the ST-protective factor in milk was a fucosylated oligosaccharide. Diluted to the volume of milk from which it was isolated, the Ulex-adherent fucosylated oligosaccharide fraction was as effective as human milk itself in preventing mortality in suckling mice due to ST-induced diarrhea (41).

The mechanism whereby the oligosaccharide inhibits ST was studied in T84 cells, an immortal line of human enterocytes. These cells express guanylate cyclase, whose extracellular domain is the receptor for ST, and whose intracellular activity produces cyclic GMP. Elevated cyclic GMP causes loss of chloride and bicarbonate transport, ultimately leading to the efflux of fluid and electrolytes. In vivo, the result is secretory diarrhea. In the presence of the protective fucosyloligosaccharides of human milk, ST is unable to stimulate production of cyclic GMP, either in intact T84 cells or in isolated membrane preparations. The mechanism of this protection appears to be binding by the oligosaccharide to the T84 extracellular domain of guanylate cyclase, thereby blocking its binding by ST. This prevents the ST-induced loss of chloride ion homeostasis and secretory diarrhea (8).

The *U. europaeus*–binding fucosyloligosaccharide fraction was resolved into more than 30 components by semipreparative high-performance liquid chromatography. Of these fractions, only one displayed consistent, robust activity at the concentration found in human milk. This fraction was further subfractionated into seven components; when purified to homogeneity only one of these inhibited the diarrheagenic activity of stable toxin in the suckling mouse. We estimate that this subfraction is active at a concentration of approximately 30 parts per billion (0.000003%), the concentration at which it is found in human milk (37).

Campylobacter

Campylobacter is a bacterium carried by birds, including domestic fowl, and is the single largest cause of bacteria-induced diarrhea worldwide. In nitrocellulose-immobilized neoglycoproteins, campylobacter has high avidity for the fucosylated antigen H-2 (Fucα1,2Galβ1,4GlcNAc); this specificity is confirmed by inhibition of campylobacter binding by monoclonal antibodies against the H-2 epitope. *Campylobacter jejuni*, which normally does not bind to Chinese hamster ovary cells, bound avidly when the cells were transfected with a human α1,2-fucosyltransferase gene that caused overexpression of the H-2 fucosylated antigen (Figure 2, main panel).

This binding was inhibited by ligands that bind to H-2, e.g., *Ulex europaeus* agglutinin, *Lotus tetragonolobus* lectins, and anti-H-2 mAbs, or by H-2 soluble mimetics, e.g., H-2 neoglycoproteins, human milk fucosylated oligosaccharides, and 2′-fucosyllactose (2′-FL) (Table 2), which compete with cell receptors (Figure 2, upper right inset). Thus, the fucosylated H-2 antigen was established as the host cell surface antigen that is the determinant of susceptibility to campylobacter infection, and human milk oligosaccharides inhibit campylobacter binding to this critical determinant (46).

TABLE 2 Inhibition of campylobacter binding to Chinese hamster ovary α1,2-fucosyltransferase (CHO-FUT1) cells by α1,2-fucosyl ligands and homologues (IC_{50})*

α1,2-Fucosyl ligands	
Ulex europaeus 1	0.4 mg/ml
Lotus tetragonolobus	1.9 mg/ml
Dolicholus biflorus (negative control)	>3.2 mg/ml
Anti-H-2	1:20
Anti-H-1	<1:10
Anti-A (negative control)	<1:10
α1,2-Fucosyl homologues	
H-2-BSA	0.024 μmol/ml
BSA (negative control)	>0.28 μmol/ml
2′-Fucosyllactose	5.2 μmol/ml
Lactose (negative control)	>23 μmol/ml
Human milk neutral oligosaccharide	1.1 μmol/ml

*Adapted from (43), with permission from the American Society for Biochemistry and Molecular Biology.

Human milk contains the H-2 epitope in many forms, including 2-fucosyllactose, the most prevalent of the milk oligosaccharides. The relevance of the presence of H-2 epitope in milk to protection from campylobacter-induced disease was assessed in three models: Human milk oligosaccharides inhibited campylobacter colonization in mice in vivo, and inhibited invasive, pathogenic campylobacter from binding to human intestinal mucosa ex vivo. Inhibition by 2-linked glycans was also assessed in transgenic animals. Female mice were transfected to carry the human α1,2-fucosyltransferase gene (*FUT1*) with a whey acidic protein (WAP) promoter that induces the expression of 2-linked fucose antigens in mammary gland during lactation, and thus, in milk. As a control, nontransgenic mice of the parental strain were used. Their pups were challenged with one inoculum of *C. jejuni*. Up to 90% of nontransgenic litters remained colonized throughout the 15-day follow-up period. In contrast, colonization of transgenic mice was transient, even when the inoculum was quite high (Table 3). Thus α1,2-linked fucosylated glycoconjugates expressed in milk strongly protect against campylobacter infection in vivo, confirming that the main intestinal ligands for campylobacter are the H-2 histo-blood group antigens, and that milk fucosyloligosaccharides containing α1,2-linked fucose, including 2′-FL, strongly inhibit this binding.

Noroviruses

The calicivirus family of enteric viruses is now recognized as a major cause of diarrhea in humans, and especially in infants. This family includes rabbit hemorrhagic disease virus and the human caliciviruses, including Norwalk virus and the various Norwalk-like viruses, which are now designated as noroviruses (NVs).

The first indication that these viruses bind to fucosylated glycans was that rabbit hemorrhagic disease virus specifically attaches to rabbit epithelial cells through H histo-blood group fucosylated oligosaccharides (47). There is no animal model for NVs, as they infect only humans, and thus the model of choice is expression of viral capsid proteins, which self-aggregate into virus-like particles whose binding characteristics to human cells or proteins are identical to the whole virus. Virus-like particles of NVs bind to human gastro-duodenal epithelial cells derived from individuals of secretor phenotype, but not from nonsecretor phenotype (28). Nonsecretors lack α1,2-fucosyltransferase activity encoded by the *FUT2* gene, which suggests that the α1,2-fucose epitope is essential for NV binding. This specificity was confirmed by loss of ability of target tissues to bind after treatment with α1,2-fucosidase, and by specific blocking of NV binding by competitive inhibitors of H binding: H-1 and H-3 monoclonal antibodies, glycans containing α1,2-linked fucose, and human milk from a secretor. Furthermore, when Chinese hamster ovary cells—to which NVs do not typically bind—are transfected with α1,2 fucosyltransferase cDNA, which causes them to express α1,2-fucosyl glycans, the result is attachment of Norwalk virus-like particles (28). NVs also bind to the fucosylated glycans of human saliva. The expression of fucosylated glycans of human saliva depends upon the Lewis and ABO blood group types of the donor.

Binding specificities of other strains of NVs were tested through their ability to bind to human saliva. Multiple patterns of binding have been found: three strains (387, NV, and MOH) bind secretors and one (strain 207) binds both nonsecretors and secretors, but binds more strongly to nonsecretor saliva. Of the three secretor-binding strains, 387 recognizes all secretors (A, B, and O blood group types), NV recognizes A and O, and MOH recognizes A and B. This implies that NV uses host cell surface fucosylated glycans to infect, but that the ability of a specific strain to infect a given individual will depend upon the binding specificity of the strain of NV and the specific types of glycans expressed by an individual. This concept was tested in 77 NV-challenged volunteers. Seventy-five percent of the saliva samples from secretors but none from the nonsecretors bound NV capsids. Secretors were almost 40 times more likely than nonsecretors to become infected with NV, a finding that strongly suggests that susceptibility to NV infection depends on secretor status (27).

The same individual variation in glycan expression that occurs on the intestinal mucosa and saliva also occurs in milk. However, the presence of an NV-binding epitope in milk would be expected to inhibit binding of the virus to its host, as this soluble glycan would compete for binding with the host cell surface glycan, and thereby inhibit the ability of the virus to attach to its host cell surface receptor (Figure 1). The ability of human milk to block binding of the most dominant strain of NV (VA387, genogroup II) to its receptor was tested (Figure 3). The milk from 54 of the 60 mothers (23) blocked this common human enteropathogen; only the milk from the six nonsecretors failed to block binding, which indicated that the binding seemed to be inhibited by α1,2-linked fucose epitopes. In conclusion,

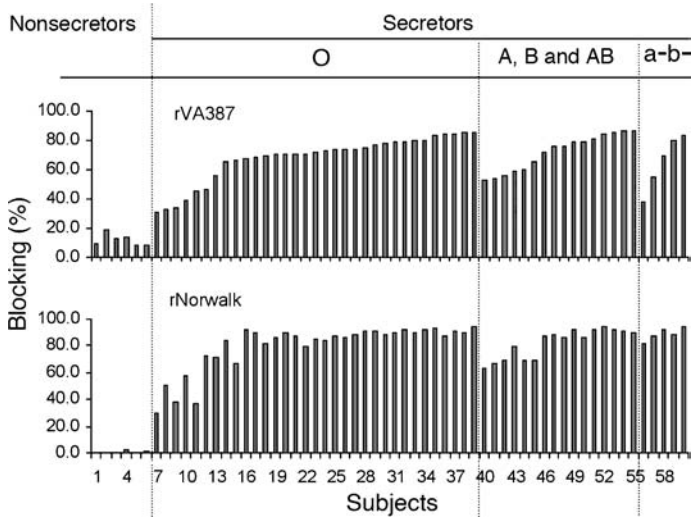

Figure 3 Inhibition of norovirus binding by human milk. Milk of secretors of all blood group types was effective at blocking binding, whereas milk from nonsecretors did not block binding. [Adapted from (23) and reprinted with permission from The University of Chicago Press]

most human milks contain glycans that specifically block NV binding to histo-blood group antigen receptors, which may provide protection to infants from NV infection.

INNATE IMMUNITY

We hypothesize that these fucosylated glycans are an essential part of the innate immune system of human milk whereby the mother protects her infant. If this were so, three characteristics are expected: (*a*) The glycans would be constitutively expressed in milk independently of the history of maternal exposure to pathogens. (*b*) The active glycans would need to be resistant to digestion in the gut so that they could be present at the site of infection. (*c*) Variation in the presence of these fucosylated glycans in milk should have a measurable consequence on the risk of disease by the relevant pathogens in breastfed infants. Each of these three criteria was investigated.

Constitutive Expression

Secretor and nonsecretor individuals are defined by the presence or absence of α1,2-linked fucosylated epitopes in their secretions, including saliva, tears, and milk. Thus, variation in milk glycan expression was typically thought of in terms

of homozygous recessive (nonsecretors, no glycans with α1,2-linked fucose), homozygous dominant (secretors, high levels of α1,2-fucosylated glycans), and heterozygotes (secretors, intermediate levels of α1,2-linked fucosylated glycans) (14, 58). To this was added a fourth phenotype (55). The distribution of these phenotypes differs among populations (12), and oligosaccharide expression changes over the course of lactation (7, 12).

When individual oligosaccharides are measured in milk from a population of lactating mothers over the entire course of lactation, yet more complexity emerges (4). Nonsecretors do indeed produce milk that lacks α1,2-linked fucosyloligosaccharides, but after approximately a half-year of lactation, 2-linked fucosyloligosaccharides gradually become a significant component of the milk. Secretors produce milk that contains 2-linked fucosyloligosaccharides over the entire course of lactation, but the absolute and relative amounts of the individual oligosaccharides change over the course of lactation: The ratio of α1,2-linked fucosyloligosaccharides to α1,3- and α1,4-linked fucosyloligosaccharides declines exponentially (from a fivefold ratio in the first month to about 1:1 at the end of one year). However, among secretors, mothers who produce the highest amount of α1,2-linked oligosaccharides at any point in lactation tend to produce the highest amounts at other points of lactation, and likewise those who produce the lowest amounts tend to do so throughout the course of lactation. Thus, the pattern of oligosaccharide expression relative to the rest of the population tends to remain constant. This is consistent with genetic variation as the basis of variable expression of oligosaccharides in milk. In secretors, expression of the α1,2-linked fucosyloligosaccharides decreases over the course of lactation, with a reciprocal increase in expression of α1,3/4-linked fucosyloligosaccharides. In nonsecretors, expression of α1,3/4-linked oligosaccharides decreases over the course of lactation with a reciprocal increase in α1,2-linked oligosaccharides late in lactation. This inverse relationship implies reciprocal control of synthesis of these oligosaccharides, perhaps through reciprocal control of expression of fucosyltransferase genes, such as the secretor and Lewis genes that control the individual differences in expression of Lewis blood group type.

Synthesis of Oligosaccharides in Mammary Gland

All secretors express the secretor gene (*FUT2*) for the production of α1,2-fucosyltransferase. Of these, those of the Lewis a−b− blood group phenotype (Le^{a-b-}) do not express the Lewis gene (*FUT3*), which encodes the α1, 3/4-fucosyltransferase, while individuals of Le^{a-b+} blood group phenotype express the *FUT3* as well as *FUT2* genes. Just as differences in the expression of these two genes are invoked to explain the Lewis blood group system of fucosylated glycolipid expression in erythrocytes, the expression of those same genes may also explain the variation in expression of fucosylated oligosaccharides in milk. If so, the Lewis blood group type of the mother should strongly relate to fucosyloligosaccharide expression in her milk. The data are

consistent with this hypothesis: Milk of the Le^{a-b+} mothers contained more lacto-*N*-fucopentaose II (LNF-II) and 3-fucosyllactose (3-FL), oligosaccharides that are exclusively fucosylated with α1,3- or α1,4-linkages, than milk from Le^{a-b-} mothers, which contained more LNF-I (H-1) and 2′-FL (H-2), oligosaccharides that are exclusively fucosylated with α1,2-linkages. The pattern of oligosaccharides varied among individual milk samples; in each, the pattern was summarized as a ratio of the concentration of 2-linked fucosyloligosaccharides to the concentration of fucosyloligosaccharides containing no fucosyl α1,2 linkages. Of the secretors tested, milks with the highest ratios were produced primarily by Le^{a-b-} mothers; those with the lowest ratios were produced exclusively by Le^{a-b+} mothers. Thus, the genetic polymorphisms expressed as maternal Lewis blood group types are expressed in their milk as varied fucosyloligosaccharide ratios (Figure 4).

These results can be explained according to the schema in Figure 5. The expression of the secretor gene (*FUT2*) accompanied by the lack of expression of the Lewis gene (*FUT3*) results in milk with a preponderance of α1,2-linked oligosaccharides (LNF-I, LDFH-I, 2′-FL, LDFT) for both the type 1 and the type 2 pathways. The lack of expression of the *FUT2* secretor gene with the expression of the *FUT3* Lewis gene conversely results in a preponderance of α1,3- and α1,4-linked oligosaccharides (LNF-II, LNF-III, 3-FL), and the permutations of partial expression explain the apparent continuum of oligosaccharide expression that is exhibited among most of the population. These two fucosyltransferases compete for the same substrate, explaining the reciprocal control of the expression of these two classes of fucosyloligosaccharides in human milk. This also offers a rationale for using a ratio of 2-linked to 3- and 4-linked fucosyloligosaccharides as a surrogate measure of the genotypic status of the mother (42). Unfortunately, data suggest that even this complex schema only represents milk oligosaccharide expression to a first approximation. For example, we found some 2-linked fucosyloligosaccharide in milks of all donors from a Mexican population, including milk produced by nonsecretor mothers who do not express the secretor gene; however, nonsecretors did not express 2-linked fucosyloligosaccharides in their milk until late in lactation. This suggests some involvement of *FUT1* in the production of milk oligosaccharides during some stages of lactation, or the presence of partial secretors in our population. Similarly, other members of the fucosyl α1,3 transferase family (*FUT4,5,6,7*, and *9*) may be involved in synthesis of the α1,3-linked fucosyloligosaccharides. This area is ripe for investigation using the modern tools of molecular biology. The current data support the conclusion that expression of oligosaccharides in milk depends on the genotype of the mother and is produced constitutively.

Resistance to Digestion During Transit Through the Gut

For human milk glycan inhibition of enteric pathogens to be biologically relevant, the glycans must remain intact during transit through the alimentary canal and into

the intestinal regions where infections occur. It was expected that at least some oligosaccharides would survive because the carbohydrate linkages in the complex glycans are entirely different from those in the carbohydrates we use as nutrients, minimizing the potential for digestion by the mammalian enzymes of the gut, and the transit time for breastfeeding infants is quite fast, on the order of a few hours, minimizing the opportunity for bacterial digestion. Furthermore, breastfed infant stools were a known source of some human milk oligosaccharides, but stable isotope-labeled milk oligosaccharides are also absorbed from the gut (43). The survival of the human milk glycans in the gut can be studied by measuring the human milk oligosaccharide profile in the milk being consumed, in the feces, and in the urine of individual breastfed infants. If the pattern of oligosaccharides in the feces and urine is the same as that in the milk being consumed, this is taken as evidence that the oligosaccharides are surviving transit through the gut. High-performance liquid chromatography of the neutral oligosaccharides revealed a very close match between patterns of relative amounts of oligosaccharides in milk, feces, and urine of breastfed infants. The concentrations of the total oligosaccharides, however, differed; total milk oligosaccharides were about 1% as high in urine as was found in the original milk, but approximately tenfold higher in feces than in milk (5). The higher concentration in feces is consistent with the digestion and absorption of the other milk components during passage through the alimentary canal, leaving the indigestible glycans at higher concentrations in the gut than originally found in milk. Some absorption of intact oligosaccharides would account for the human milk oligosaccharides found in the urine, and the similarity of the patterns to those of milk suggests that there is little, if any, selective absorption of specific components, nor destruction of specific components from the circulation. Daily milk consumption and stool output was not measured in this study, but if average intake and output are assumed for purposes of estimating the absolute amount of ingestion and excretion of oligosaccharides, over 90% of the oligosaccharides seem to survive transit through the gut, and less than 1% are lost in the urine. The higher concentration of milk glycans in stools than in milk strongly supports their role in protecting breastfed infants from pathogens, as most of the protection by oligosaccharides is evident at concentrations typical of human milk.

Human Milk α1,2-Linked Fucosyloligosaccharides and Risk of Diarrhea

Although specific human milk oligosaccharides and glycans could inhibit specific pathogens when tested as isolated compounds in vitro and in vivo, the relevance to protecting infants was untested. It was possible that they would be ineffective in the complex matrix of milk or intestinal contents, or irrelevant in the context of the many other protective components in milk discussed earlier. The variation in glycan expression of human milk provided the opportunity to test the efficacy of these pathogen inhibitors in a human population (31, 42). Data and banked samples were analyzed from 93 breastfeeding mother-infant pairs who were

prospectively studied from birth to two years of age with infant feeding and diarrhea data collected weekly. A single milk sample collected from mothers 1–5 weeks postpartum was analyzed for oligosaccharide content. Infants who developed diarrhea associated with ST of *E. coli* were consuming milk with significantly lower 2-linked to 3/4-linked oligosaccharide ratios than those of the remaining infants. Similarly, campylobacter diarrhea occurred less often in infants whose mother's milk contained high levels of 2′-FL, the specific 2-linked fucosyloligosaccharide that inhibits campylobacter. Norovirus diarrhea occurred less often in infants whose mother's milk contained high levels of lacto-*N*-difucohexaose (LDFH-I), a 2-linked fucosyloligosaccharide that contains an epitope that inhibits NV binding in vitro. Moderate-to-severe diarrhea of all causes occurred less often in infants whose milk contained high levels of total 2-linked fucosyloligosaccharide as a percent of milk oligosaccharide (Figure 6).

Host cell receptors for other pathogens can be glycans whose terminal moieties contain nonfucosylated epitopes. Binding by these pathogens likewise may be inhibited by milk glycans whose terminal moieties resemble their gut glycan receptors. Rotavirus is an example.

Rotavirus Inhibition by Human Milk Lactadherin

Infection by rotavirus is responsible for much of the diarrhea in infants around the world, and especially for diarrhea of young children in developed countries. The ability of rotavirus to infect MA-104 cells in culture is inhibited by human milk, and this inhibition is due to a mucin-associated 46 kDa milk glycoprotein named lactadherin. As shown in Figure 7, the inhibition is dose-dependent; furthermore, after sialic acid is removed from lactadherin, its ability to inhibit rotavirus is

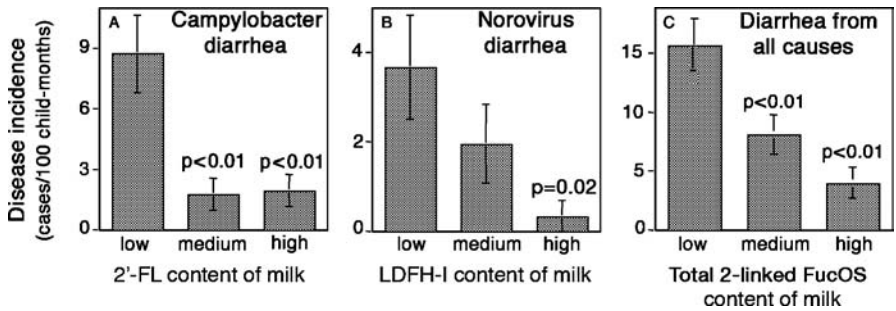

Figure 6 Diarrhea in nursing infants and content of 2′-fucosylated oligosaccharides in the milk consumed. *Panels A–C*: Content of milk refers to specific oligosaccharides as a percent of total oligosaccharide; low, medium, and high groups are tertiles of n = 31 infants each. *Panel C*: Diarrhea from all causes refers to all moderate-to-severe diarrhea. 2′-FL, 2′-fucosyllactose; LDFH-I, lacto-*N*-difucohexaose I; FucOS, fucosylated oligosaccharides. [Adapted from (31), with permission from Elsevier]

essentially lost, which suggests that the glycan portion of the molecule may be responsible for inhibition and that a specific terminal sialic acid may be required for inhibition to occur. Lactadherin from human milk also inhibits rotavirus (EDIM strain) gastroenteritis in mice. Most pathogenic strains of rotavirus bind to a host cell glycoprotein receptor that contains a terminal sialic acid, and inhibition of this binding is thought to be the mechanism whereby lactadherin inhibits rotavirus (62).

The clinical relevance of variable expression of lactadherin in maternal milk was examined in a study conducted on rotavirus diarrhea in 200 infants in Mexico City (34). Infants were enrolled from birth, monitored regularly by enzyme immunoassay for rotavirus in stools, serology for induction of antirotavirus antibody in serum, and for feeding and stool patterns. Milk samples were obtained from the mothers weekly until four weeks post partum and monthly thereafter. The sample collected immediately before an episode of rotavirus infection was assayed for lactadherin, butyrophilin, mucin, and sIgA. Thirty-one infants were infected with rotavirus in the absence of other confounding pathogens. Of these, 15 infants exhibited symptoms of diarrhea, while 16 had no symptoms. The median concentration of lactadherin in maternal milk samples obtained 4–41 days before the infection was 48 μg/ml in the asymptomatic group and 29 μg/ml in the symptomatic group. Although these medians did not differ significantly, logistic regression analysis adjusted for age at infection and sIgA concentration revealed a significant difference between the groups in regard to concentration of milk lactadherin levels (Figure 8). Thus, variation in the concentration of the glycan, lactadherin, that inhibits rotavirus in vitro and in vivo is a determinant in the risk of diarrhea in nursing infants who are infected with rotavirus. This inhibition by lactadherin during nursing provides the dual purpose of preventing diarrhea when the infant is most vulnerable while still permitting the infant to raise antibodies to rotavirus, allowing for protection from rotavirus after weaning. No significant association between symptom status and concentrations of butyrophilin, mucin, or sIgA was found. This suggests that the milk glycans make a major contribution to the protection of breastfed infants from diarrheal disease that, in some cases, is at least as important as the secretory antibodies. We conclude that the human milk glycans are a major component of an innate immune system of human milk that plays a significant role in the ability of the mother to confer protection to her infant against disease.

Milk Glycan Inhibition of Other Pathogens

The human milk glycans that inhibit specific pathogens described above are representative of a growing family of such milk glycans that have been found to inhibit key steps of pathogenesis of individual pathogens in vitro, ex vivo, or in vivo (Table 4). In most cases, the detailed chemical structure of the specific inhibitor has not been fully defined. Although each of these milk glycans is capable of inhibiting only a specific pathogen or family of pathogens, their presence in the

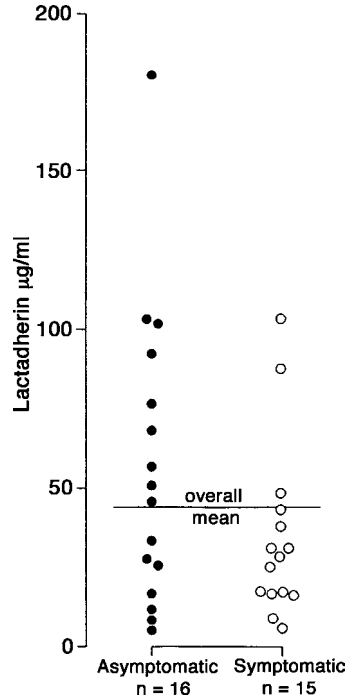

Figure 8 Breastfed infants infected with rotavirus but who remained asymptomatic were consuming milk with significantly higher content of lactadherin than milk consumed by infants who were infected and exhibited diarrhea; $p = 0.01$. [Reprinted from (34), with permission from Elsevier]

aggregate may represent an armamentarium that may be able to protect infants from many, and perhaps most, of the common enteric diseases, and perhaps many other diseases, of childhood.

PERSPECTIVE

In the arts, the mother/infant nursing dyad represents the essence of human kindness. Viewed through the lens of sociobiology, breastfeeding is considered the apex of altruism in the animal kingdom, wherein the mother gives of her own body to protect and nurture her progeny, but for the purpose of maximizing her own reproductive potential (19, 60). Two defining features of humans are a large brain and slow maturation; this combination optimizes the efficiency and duration of learning, allowing transmission across generations of large amounts of information needed for maintaining a complex culture. For this to occur, the optimum

TABLE 4 Human milk glycoconjugates that inhibit pathogens

Glycoconjugate	Pathogen	Reference	Typical concentration[a]
GM1	Labile toxin, cholera toxin	(44)	180 μg/liter
GM3	Enteropathogenic *Escherichia coli*	(20)	13 mg/liter
Gb_3	Shiga toxin	(36)	100–150 μg/liter
Sulfatide	Human immunodeficiency virus	(59)	100 μg/liter
Chondroitin sulfate	Human immunodeficiency virus	(39)	6 mg/liter
Lactadherin	Rotavirus	(62)	100 μg/liter
Mucin	S-fimbriated *E. coli*	(50)	1 g/liter
Mannosylated glycopeptide	Enterohemorrhagic *E. coli*	(2)	60 mg/liter
Oligosaccharides	*Streptococcus pneumoniae*	(1)	0.2–10 g/liter
	Enteropathogenic *E. coli*	(9)	3 g/liter
	Listeria monocytogenes	(6)	3 g/liter
Fucosylated oligosaccharides	*Campylobacter jejuni*	(46)	1–25 mg/liter
	Vibrio cholerae	(46)	1–25 mg/liter
	Stable toxin	(41)	40 μg/liter
Macromolecule-associated glycans	Noroviruses	(23)	370 mg/liter
	Pseudomonas aeruginosa	(26)	370 mg/liter
Sialyllactose	Cholera toxin	(21)	200 mg/liter
	E. coli	(53, 57)	200 mg/liter
	P. aeruginosa	(10)	200 mg/liter
	Aspergillus fumigatus conidia	(3)	200 mg/liter
	Influenza virus	(13, 29)	200 mg/liter
	Polyomavirus	(52)	200 mg/liter
	Helicobacter pylori	(33)	200 mg/liter

[a]Typical concentration of active fraction or component of milk; calculated by D.S.N. from published and unpublished data.

reproductive strategy is to rear relatively few offspring who are intensively nurtured by the parents over this extended maturation. Perhaps the most significant component of this huge parental investment in the survival of their offspring is the protection afforded by breasts that, in addition to providing balanced nutrients, are optimized to provide both innate and adaptive immune products that synergistically protect the infant from a vast array of virulent and often lethal pathogens that emerge from the environment.

Human Milk Protection

The substantial redundancy and synergy that occurs among the protective components in milk is consistent with the central role of milk in supporting the human reproductive strategy. The prebiotic effect changes the gut microflora, altering the gut environment in such a way as to make it much less accessible to many types of pathogens. Likewise, the inhibitory effects of multifunctional components of milk, including anti-inflammatory and immunomodulatory components, tend to be broad in their spectrum of inhibitory activity, much like some components of the innate immune system in infants, such as the toll-like receptors of the gut. More specific, and probably more robust, inhibition is provided by glycans and the pathogen-specific secretory antibodies. The additive effects and any synergy of these milk components results in profoundly less risk of disease in breastfed infants than in those fed artificially. The many protective milk components, working in concert with the developing immune system of the infant, may afford redundant protection adequate to overcome genetic variation in the expression of some milk glycans (16, 22, 32, 35, 45, 48).

Glycans

Inhibitory glycans act as competitive inhibitors, preventing the pathogen from approaching and docking onto its host cell, thereby inhibiting this essential first step of pathogenesis. This mechanism of inhibition is entirely distinct from that of the current families of antibiotics in use. The antimicrobials in use today inhibit metabolic processes of bacteria that differ from those of mammals, but leave the niche of the microbe intact. Any mutation that allows the microbe to thrive in the presence of antibiotic is selected for, leading inevitably to antibiotic-resistant strains. In contrast, antiadhesion antimicrobials inhibit the ability of a pathogen to identify, bind to, and infect its host cell, making the niche of the pathogen effectively unavailable. Naturally occurring glycans (oligosaccharides and glycoconjugates) of human milk are antimicrobials that inhibit adhesion by specific pathogens. Glycans from maternal milk have been consumed by infants for millennia, protecting them from diarrheal disease without inducing resistance in human enteropathogens.

FUTURE DIRECTIONS

The use of these human milk glycans as active ingredients in infant foods, or as novel antimicrobials for older children and adults, is currently limited by our ability to synthesize large enough quantities in pure enough form to be able to perform clinical trials for efficacy and safety. Chemical synthesis would require expensive clean-up steps, and chemienzymatic synthesis also appears to be quite expensive. The creation of genetically modified microbes that would produce specific human milk oligosaccharides in high yield is a promising approach to allow enough food-grade oligosaccharides to become available for testing.

A second potential limitation to the use of human milk glycans to inhibit disease is that genetic differences in the expression of key cell surface glycans affect the susceptibility of different populations to a given disease. Thus, a mechanism to define the glycan cell surface phenotype of different populations and individuals would allow rational design of a mixture of human milk glycans that are most relevant to protecting specific populations from specific mixes of endemic pathogens. Solving these two impediments would allow orally administered synthetic human glycans to be tested in human populations for their ability to protect the target populations from enteric and other diseases that are endemic to their region. The success of this line of investigation might allow a new generation of antimicrobial agents to be used therapeutically and prophylactically against enteric disease. This might be useful as a supplement to benefit children weaned from their mother's milk. With inexpensive local production and distribution, these antimicrobials could be delivered to those children most in need of such protection, thereby assisting the international campaign against infant mortality due to diarrhea.

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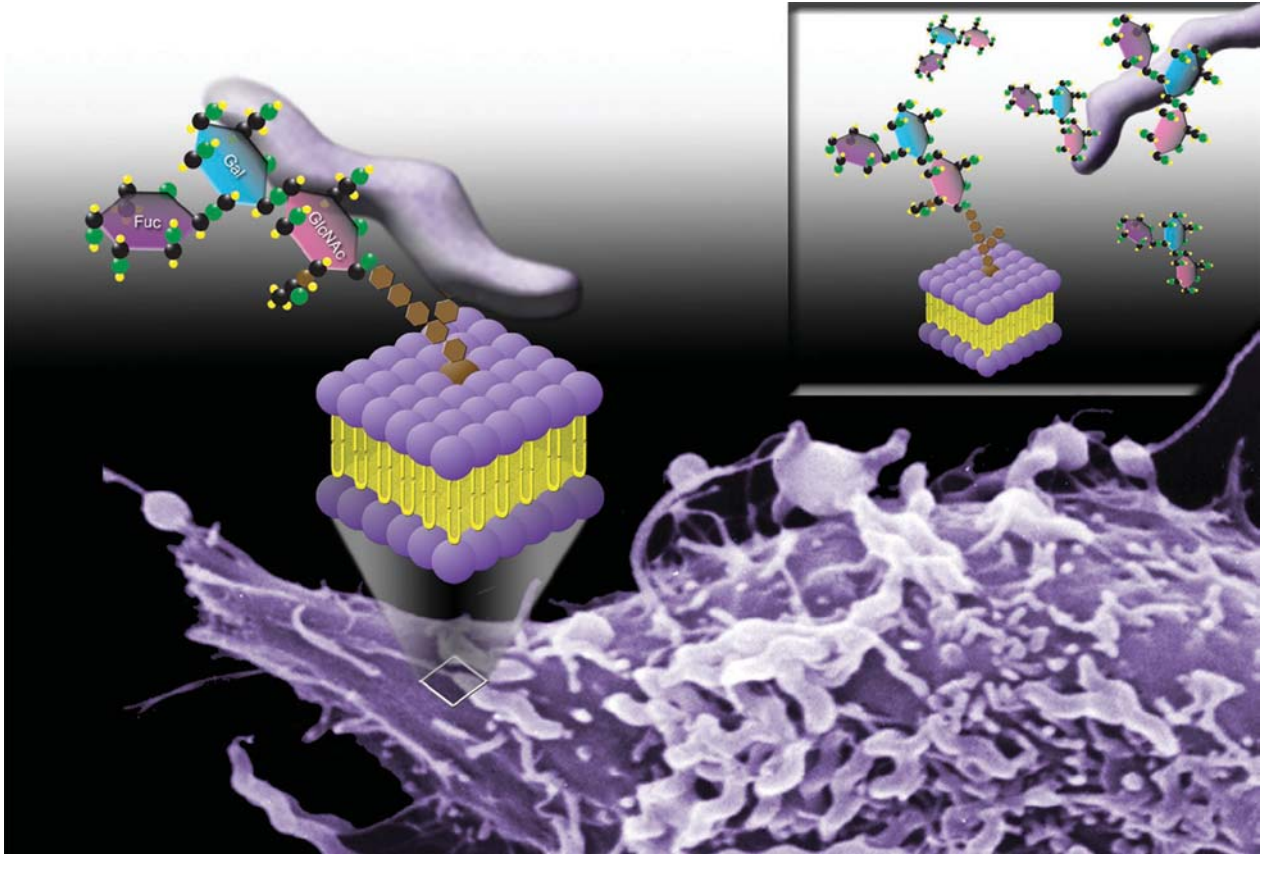

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Figure 2 Scanning electron micrograph of a Chinese hamster ovary cell FUT1 transfect, whose expression of H(O) antigens on the cell surface allows attachment by *Campylobacter jejuni.* The sugars that comprise H-2-antigen (Fucα1,2Galβ1, 4GlcNAc) tether campylobacter to cell surface glycoconjugate (*left*). Campylobacter binding to these glycoconjugates is inhibited by fucosyloligosaccharide H-2 homologues present in human milk (*right*). [Artwork by Lynn Doucette. Reprinted from (35), with permission from the American Society for Nutritional Sciences]

TABLE 3 Pups colonized by campylobacter (%)[a]

Inoculum (CFU/ml)	10^4	10^8	10^9	10^8
Days after infection	Transgenic (FUT1)			Nontransgenic
1	40	30	90	100
3	40	60	50	90
5	10	40	20	70
7	0	0	0	70
9	0	10	10	67
11	0	0	0	70
13	0	0	0	70
15	0	0	0	90

[a]Mouse pups fed milk from transgenic dams carrying the FUT1 gene with the whey acidic protein promoter cleared colonization five to nine days after challenge with campylobacter. Control pups fed from nontransgenic dams were unable to clear campylobacter colonization. [Adapted from (46), with permission from the American Society for Biochemistry and Molecular Biology]

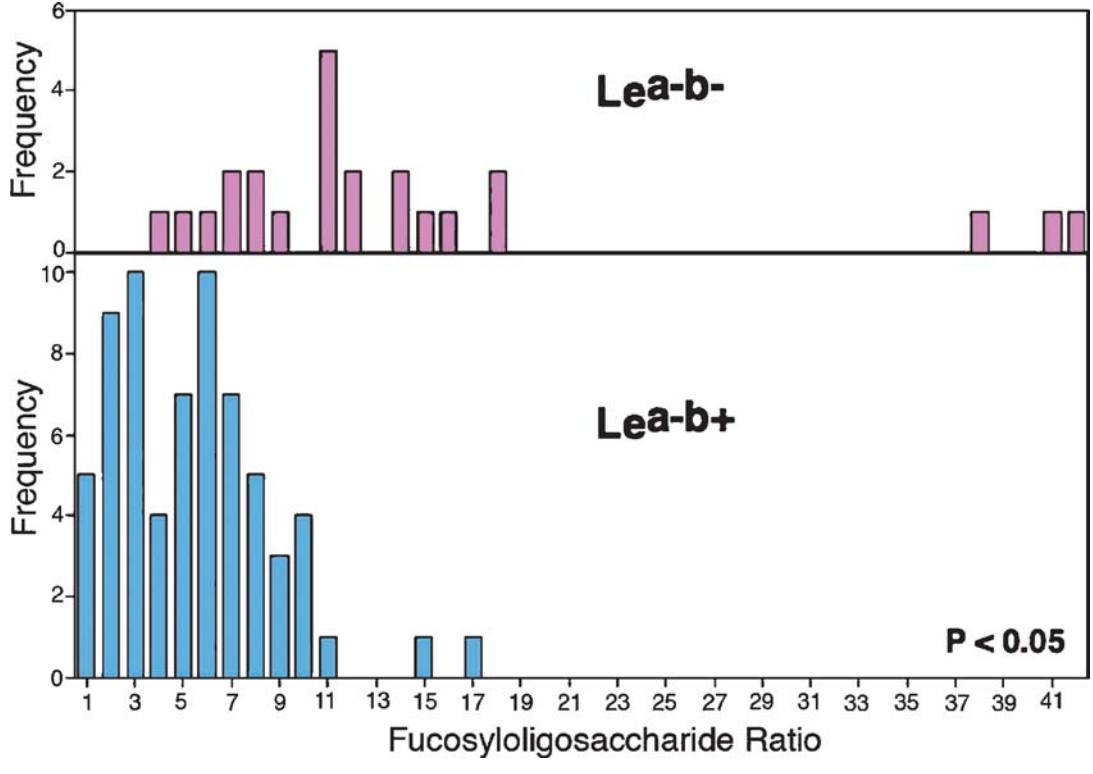

Figure 4 Distribution of the ratios of α1,2-linked fucosyloligosaccharides to those containing only α1,3/4 linkages. Mothers whose Lewis blood group type is Le^{a-b+} have significantly lower ratios than those of mothers whose blood group type is Le^{a-b-}. [Reprinted from (42), with permission from Oxford University Press]

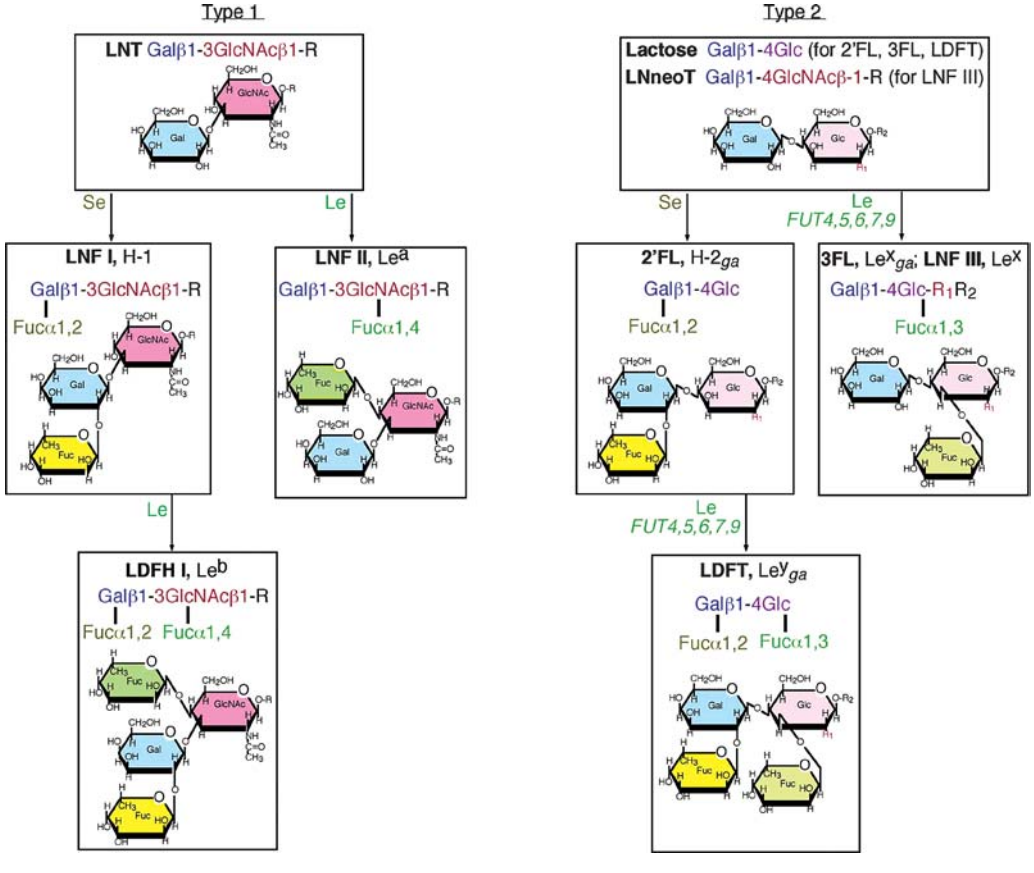

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Figure 5 Synthetic pathways of principal fucosyloligosaccharides of human milk. α1,2 Linkages are synthesized by α1,2 fucosyltransferases of *FUT2*, the secretor gene (Se). α1,3/4 Linkages are synthesized by α1,3/4 fucosyltransferase of *FUT3*, the Lewis gene (Le), and for type 2 oligosaccharides, by the family of α1,3 fucosyltransferases expressed by *FUT4,5,6,7,* and *9*. The core type 1 structure, lacto-*N*-tetraose (LNT), is Galβ1,3GlcNAc on the terminal end of lactose (−R). The core for the most abundant type 2 structures in milk is lactose for 2′-FL, 3-FL, and LDFT, and lacto-*N*-neotetraose (LNneoT, Galβ1,4GlcNAc on a lactose terminus) for LNF-III. [Reprinted from (42), with permission from Oxford University Press]

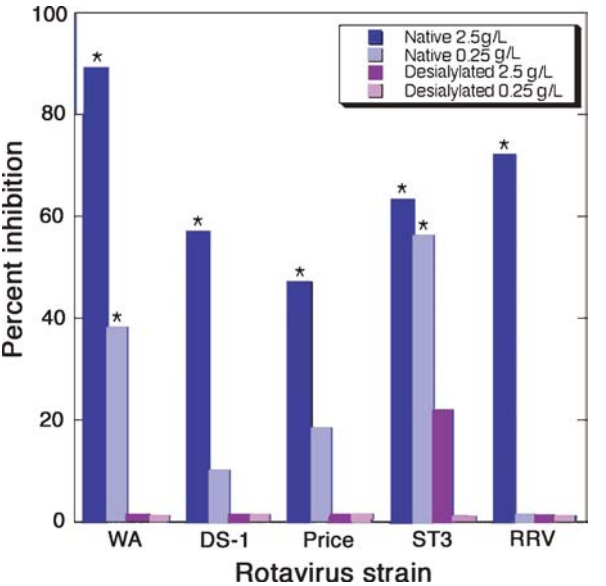

Figure 7 Inhibition of rotavirus infection by lactadherin from human milk is dose dependent. Loss of sialic acid from lactadherin results in loss of this inhibitory activity.

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ERRATA

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