Protection from Viral Infections by Human Milk Oligosaccharides: Direct Blockade and Indirect Modulation of Intestinal Ecology and Immune Reactions

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Abstract: Sugar-lectin interactions play an important role in viral infections. Many viruses, such as human immunodeficiency virus (HIV), Ebola, dengue, cytomegalovirus, and hepatitis C, possess glycans that recognize C-type lectins, especially CD209 (DC-SIGN), for infection. Other viruses possess lectins on their surfaces that recognize glycan epitopes on human epithelial cells for infection. Human and avian influenza viruses recognize different glycan epitopes, sialic acid-α2,6 galactose (SA-α2,6Gal) and SA-α2,3Gal, respectively, as their receptors, resulting in different host ranges for these two viruses. We and others have shown that sialogalactosides and fucosyllactoses are receptors for enterovirus 71 and norovirus infections, respectively; human milk oligosaccharides (HMOs) could block enterovirus 71 and norovirus infections. Several lines of evidence also suggest that HMOs cannot only mimic viral receptors and block viral infections, but also raise immune responses through sugar/lectin (galactosides/galactins and sialylglycans/Siglecs) interactions and improve gut ecology by nurturing intestinal cells and/or intestinal microbiota. This review article summarizes how and why HMOs directly or indirectly protect humans from viral infections.

Keywords: Fucosyltransferase, Lactose, Galactose, Glycosyltransferase, Glycan, Human milk oligosaccharides (HMOs), Lectin, Lewis X, Sialic acid, Viral infection.

VIRAL INFECTIONS MEDIATED BY SUGAR-LECTIN INTERACTIONS

Microorganisms possess pathogen-associated molecular patterns (PAMPs) to recognize hosts during infection; human hosts have pattern recognition receptors (PRRs) to detect pathogens for immune responses and defense [1]. The interactions between PAMPs and PRRs include the sugar-lectin interactions that play an important role in the infection of human cells by viruses [2, 3]. Many viruses possess glycan epitopes on their surfaces that recognize receptors (lectins) on human cells. It is well known that human immunodeficiency virus (HIV) possesses glycans (high mannose type) on gp120 that bind dendritic-cell-specific ICAM-3–grabbing non-integrin (DC-SIGN, also called CD209) during infection of human leukocytes [3]. Viruses such as Ebola, dengue, cytomegalovirus, and hepatitis C also possess surface glycans that recognize DC-SIGN or other C-type lectins during infection [3-6] (Table **1**). In contrast, other viruses possess surface lectins that recognize glycans on human epithelial cells. Human and avian influenza viruses recognize different glycan epitopes, sialic acid-α2,6 galactose (SA-α2,6Gal) and SA-α2,3Gal, respectively, as their receptors, resulting in different host ranges for these two viruses [7]. Coxsackievirus A24 recognizes SA-α2,3Gal on intestinal cells as a receptor [8]; we and others have shown that sialogalactosides [9] and sialomucin (CD162) [10] are receptors for enterovirus 71 infection of intestinal cells. Both SA-α2,6Gal and SA-α2,3Gal from human milk oligosaccharides (HMOs) have been shown to block enterovirus 71 infection of DLD-1 intestinal cells [9]. Moreover, norovirus and rotavirus recognize blood group carbohydrates and sialylglycoproteins, respectively, as receptors. Human milk containing secretary blood group carbohydrates such as fucosylated Lewis antigens and sialylgoycoproteins such as lactoadherin have been shown to prevent infection by these viruses [11, 12]. Differential expression of cell surface glycans or lectins and exogenous intake of soluble oligosaccharides from human milk may block or prevent viral infections.

HMOS CONTAIN POLYLACTOSYL OLIGOSACCHARIDES CAPABLE OF NURTURING MICROBIOTA IN THE INFANT GUT

It is well known that infants who are breastfed have fewer respiratory and gastrointestinal infections than those who are fed cow's milk infant formula [12, 13]. The composition and diversity of oligosaccharides in human milk, particularly in colostrum, are very different from those of cow's milk [14-16]. The protective effects of human milk can be

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Table 1. Patterns of Viral Infection by Sugar-lectin Recognition

Patterns	Virus (Epitopes)	Host (Receptors)	References
Glycans recognize lectins:			
	HIV (Glycan on gp120)	Human (CD209)	[2,3]
	Ebola (surface glycoprotein)	Human (CD209), LSECtin	[4,5]
	Dengue (Glycan on E antigen)	Human (CD209, CD299)	[6]
Lectins recognize glycans:			
	Influenza (Hemagglutinin, H1)	Human (SA-α2,6Gal)	[7]
	Avian Flu (Hemagglutinin, H5)	Human (SA-α2,3Gal)	[7]
	Coxsackievirus 24	Human (SA-α2,3Gal)	[8]
	Enterovirus 71	Human (sialylglycans)	[9,10]
	Norovirus	Human (Lewis antigen)	[11]
	Rotavirus	Human (Sialyl-lactadherin	[12]

attributed to HMOs rather than secretory IgA because cow's milk has IgA concentrations similar to that of human milk but 20-fold lower concentrations of oligosaccharides [14, 15].

HMOs are made of lactose (galactose + glucose), fucose, N-acetylglucosamine and N-acetylneuramic acid (sialic acid). Mass spectrometric analysis indicates that HMOs are composed of 2 to 32 oligosaccharides, of which the number of galactose or fucose residues can be up to 15. There is great diversity in the structural isomers seen in HMOs, estimated to include many thousands of oligosaccharides [15]; at present, 115 HMOs have been characterized [16]. Most HMOs are indigestible in the human gastrointestinal tract. However, it is generally accepted that HMOs can be digested by certain bacteria in the gut and are beneficial to the selection of microbiota that promotes better gut ecology. HMOs promote the growth of normal flora such as *Lactobacillus* and *Bifidobacterium* species in the infant gut [14, 17]. LoCascio *et al*. [18] have recently reported that *Bifidobacterium longum* in the infant gut preferentially utilizes short-chain over complex HMOs and is associated with the expression of glycosidases capable of degrading oligosaccharides. These results suggest that the prebiotic effects of HMOs are structure-specific and may vary depending on individual differences in maternal lactation and microbiota of the infant gut [13-19].

STRUCTURES OF HMOS

HMOs containing the lacto-*N*-biose core unit (LNB; Galβ1-3GlcNAc) are called 'type 1' structures; those with a lactosamine (Galβ1-4GlcNAc) core unit are called 'type 2' structures as shown in Fig. (**1**). Oligosaccharides in human milk are predominantly type 1 structures [16, 20]. Both LNB and lactosamine core units are repeatedly elongated from a β1-3 or β 1-4 para linkage to the terminal lactose (Galβ1-4Glc) [20]. More complex oligosaccharides contain additional β1-6 linkages in branched forms and side chain fucosylation *via* α1-2/3/4 linkages and/or sialylation *via* α2-3/6 linkages. Thus, HMOs containing poly-lacto-N-biose or poly-acetyllactosamine with fucosylated and/or sialylated compounds can be composed of hundreds to thousands of oligosaccharides. These compounds, with or without branched adducts, are usually stable and indigestible by human intestinal cells.

HMOs may serve as natural barriers for the prevention of microbial adhesion to cells and infection. Some HMOs have been shown to modulate epithelial glycosyltransferase expression, possibly regulating the surface glycan profiles that affect the susceptibility of human cells to viral infection [21-23]. One study found that the sialic acid in human milk was mostly bound to free oligosaccharides, while most of the sialic acid in infant formulas derived from cow's milk was bound to glycoproteins [24].

HMOS ARE SECRETARY CARBOHYDRATES, CAPABLE OF INTERFERING WITH MICROBIAL ADHESION TO HOST CELLS

HMOs are secreted into breast milk by mammary glands. Approximately 90% of the HMOs in human breast milk are found undigested in infants' feces [17-23]. This suggests that HMOs play other roles beyond their prebiotic effects on microbiota ecology [21]. In fact, there is evidence that HMOs stimulate epithelial cells and afford protection from infections by raising innate immunity and/or nurturing gut ecology [21, 22]. HMOs contain heterogeneously soluble glycans that are present in high concentrations (up to 12 g/l) in human milk, particularly in colostrum. Milk from animals such as cows and goats also contains oligosaccharides but generally 10-fold lower in concentration and less diverse in composition [16]. Varying amounts and compositions of oligosaccharides are found in different mothers in different stages of breast feeding. Structures and quantities of HMOs vary between maternal genotypes, particularly with respect to the content of fucosylated oligosaccharides, which determines whether HMOs are secreted or not.

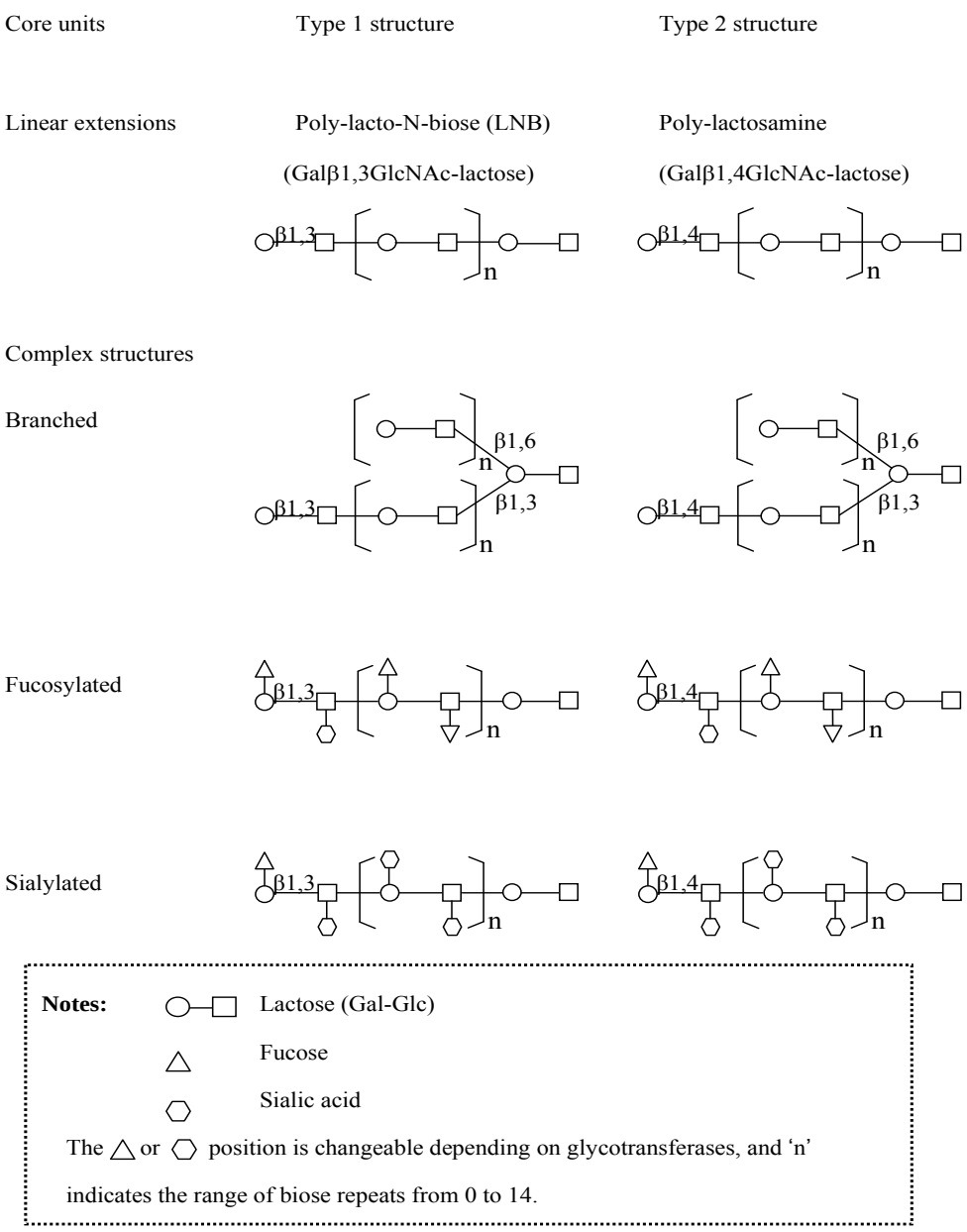

Fig. (1). HMO structures.

BLOOD GROUP CARBOHYDRATES IN HMOS PROTECT INFANTS FROM INFECTIONS

The blood group types A, B, O, and Lewis are determined by carbohydrate compositions. The H antigen is a tetraose carbohydrate consisting of galactose, N-acetylglucosamine, galactose, and fucose attached to a membrane protein or ceramide on human cells, including red blood cells. The blood group A antigen results from a glycosyltransferase that adds N-acetylgalactosamine to the galactose end of the H antigen. The blood group B antigen results from another glycosyltransferase that adds galactose to the galactose end of the H antigen. Blood group O arises from an exon 6 deletion that eliminates glycosyltransferase activity, resulting in the expression of H antigen only, with no A or B antigen expression [25]. Lewis antigens are made of trioses or tetroses determined by the activity of fucosyltransferases (FUT2, FUT3 and FUT4). FUT2 converts the Lewis a antigen (Le^a, Gal-GlcNAc-Fuc) to Lewis b antigen (Le^b, Fuc-Gal-GlcNAc-Fuc) by transferring fucose to the galactose end of Le^a antigen [25,26]. This is the same fucosyltransferase (FUT2) that converts membrane-bound blood group A, B, and H antigens into soluble forms by transferring a fucose residue to the galactose end of the H antigen, allowing humans to secrete blood group antigens into body fluids, including human milk. Most humans (80%) carry the FUT2 gene and are thus capable of secreting blood group antigen; these individuals are referred to as 'secretors' (SeSe/Sese); the remaining 20%, called 'non-secretors' (sese), lack FUT2 expression and thus do not secrete blood group antigens. HMOs from nonsecretors' milk possess no α-2-fucosylgalactosides, but do contain other α-3-fucosyl

Table 2. HMO Functions and Mechanisms of Protection from Infections

Protective Mechanism	HMO Components	Function	References
Anti-microbial effects:			
1. Prebiotics	Lactose, poly-lactosamine poly-lacto-N-biose etc.	Prevent bacterial infections after viral infections	[14–23]
2. Receptor Competition	Secretor HMOs: -- secretory A, B, H, and Lewis b (Le^b) antigens	Compete with viral receptors to block viral infections	[29–32]
	Other fucosylated HMOs: -- Le^y, Le^x, and sLe^x, etc.		
	Other sialylated HMOs: SA α 2,3 galactose SA α 2,6 galactose		
Cell interactions:			
3. Cell-cell Interaction	Sialyllactoses in colostrums	Nurture infant gut and brain cells	[42–46]
4. Immune Regulation	Fucosyl HMOs	Bind or compete selectins to induce anti-inflammation	[47–49]
	Galactosyl or sialyl HMOs	Bind or compete galectins or Siglecs mediated immune responses	[50–52]

galactosides or α-4-fucosyl N-acetylglucosamine residues. Thus, individuals carrying the Le^a antigen will not secrete the A, B or H antigens (ABH non-secretors); the Le^b antigen is only found in secretors [25]. Mothers with different blood group types have different HMO profiles because HMOs have the same glycan structures found in mucosal and epithelial secretions [22, 24-27].

The concentrations and compositions of HMOs are different between human colostrum and mature human milk. The total HMOs are much higher in colostrum than those in mature human milk (22-24 g/l vs. 12-13 g/l), and the concentrations of 2-fucosyllactose and 3-sialyllactose were significantly higher in colostrum [16]. The facts that colostrum has significantly higher concentrations of fucosyllactose and sialyllactose residues and colostrum is known to be important for the protection of infants against certain infections [27, 28], suggest that sialyl and fucosyl HMOs are required for the protection in human infants from infections during early lactation [20, 24]. HMOs with fucosylated and/or sialylated residues might block the binding and infection of bacteria and viruses to epithelial cells [29, 30]. For instance, the blood H2-type antigen (Fuc α1,2Gal β1,4GlcNAc) is a receptor for norovirus infection, and soluble H2 oligosaccharides in milk can prevent norovirus infection [29, 31]. Moreover, individuals with an O blood group were found to be more susceptible to norovirus infection than those with a B blood group [32]. Polylactosyl oligosaccharides (with or without fucosylated or sialylated residues) can also mimic viral receptors and thus inhibit viral infections [29-32]. These glycans function as competitors for receptors and may prevent infection by inhibiting pathogen binding to host epithelial cells. Such glycans are usually stable at room temperature and are intrinsically sweet tasting [33, 34]. These features may allow them to be economically distributed as a drink for use by the general population; glycan supplements would be particularly beneficial to infants, who are more susceptible to infections.

FUNCTIONS AND MECHANISMS OF HMOS ON THE PROTECTION FROM VIRAL INFECTIONS

Protection of infant infections and mortality by human milk has been historically attributed to its nutritional suitability for infants, IgA antibody content, and its lactose-enriched prebiotic effects [13-19]. Many recent studies have found the benefits of human milk to go beyond what can be attributed to nutrient content or prebiotic effects. Table **2** summarizes the 4 major functions of HMOs; the first 2 functions are related to anti-microbial effects and the other 2 functions are related to immune modulation through host cell interactions.

1. HMOs Act as Prebiotics

Human milk is enriched in lactose, poly-acetyllactosamine and poly-lacto-N-biose, which are indigestible by humans but nurture *Lactobacillus* and *Bifidobacillus* species in the gut. These normal flora metabolize lactose and HMOs containing polylactosamine and poly-lacto-N-biose. The metabolites from lactose, polylactosamine, and poly-lacto-N-biose favor the growth of normal flora but inhibit other pathogens by causing acidic conditions, resulting in better microbiota ecology [13-19]. The better microbiota ecology may, therefore, protect from offensive bacterial infections after viral infections.

2. Soluble HMOs Compete with Pathogen Adhesion and Infection

Oligosaccharides are usually soluble but are not necessarily secreted. HMOs are soluble glycans secreted by mammary glands. The secretory pathways of HMOs are not

completely understood, but are presumably similar to those of mucosal glands in the gastrointestinal, respiratory, and urogenital tracts. HMOs become secretory through the transfer of fucose to a galactose or glucosamine residue at the α2 position by fucosyltranferase 2 (FUT2) as described above. Most oligosaccharides in human milk are α2-fucosylated and relatively protective from viral infections [35], and those without FUT2 called non-secretors are usually more susceptible to the infection by viruses that recognize fucosyl oligosaccharides as receptors [36]. In addition to FUT2, other fucosyltransferases (FUT3, FUT5, and FUT6) and sialyltransferases (e.g. ST6Gal) can affect the glycan profiles of human cells, thereby influencing susceptibility to infections by viruses whose entry is mediated by sialyl or fucosyl oligosaccharides [37,38]. For instance, addition of fucose to the α3 position by FUT3 converts type 2 core antigen (Gal β1,4 GlcNAc) to Lewis X antigen (Le^x; Gal β1,4 GlcNAc α1,3 Fuc) and converts blood H2 antigen (Fuc α1,2 Gal β1,4 GlcNAc) to Lewis Y antigen (Le^y; Fuc α1,2 Gal β1,4 GlcNAc α1,3 Fuc) [39]. Lewis X components in human milk are able to bind to DC-SIGN and thereby prevent the capture and subsequent transfer of HIV-1 to CD4+ T lymphocytes [40]. Le^x and Le^y can be sialylated by sialyltransferases at position α2,3 or α2,6, producing sialyl Le^x and sialyl Le^y. sLe^x has been shown to be the common receptor recognized by H5, H6, H7, and H9 avian influenza viruses [41]; sLe^x in milk may therefore be able to inhibit avian influenza virus infections.

3. HMOs May Nurture Intestinal Cell Differentiation

Sialyl oligosaccharides in human milk are more prominent in colostrum [27, 42], which may benefit premature babies by decreasing the incidence of inflammatory bowel diseases *via* its anti-inflammatory effects [43]. Furthermore, sialyl HMOs are high in human milk but much lower in infant formulas. Experimental studies show that a diet rich in sialic acid increases the brain sialic acid levels in infant animals, enhances expression of two learning-related genes, and promotes learning and memory [44]. Since most of HMOs (>90%) are ingestible in gastrointestinal tract, the concentration of HMOs in blood or brain may be too low to directly affect brain development. Thus, sialyl HMOs may directly nurture growth and development of the gastroin-testinal tract that indirectly influence immunity and the brain development of infants. In support of this concept, recent studies demonstrated that HMOs mediate growth signals in intestinal cells through mechanisms other than the epidermal growth factor pathway [45, 46]. Whether the HMOs modulation of intestinal cell differentiation is associated with susceptibility of viral infections remains to be determined.

4. HMOs Act as Ligands for Innate Immune Responses

Recent evidence has shown that HMOs mediate signal cascades in immune and other types of cells. Fucosylated and sialylated oligosaccharides can bind to selectins (P-selectin and E-selectin) on platelets and endothelial cells. Selectins expressed on activated endothelial cells bind to fucose-containing Lewis antigens such as sLe^x on leukocytes, which leads to leukocyte rolling. The leukocytes subsequently transmigrate into subendothelial regions and extravasate [47]. HMOs resemble secretory Lewis antigens and may compete for selectin binding, resulting in anti-inflammatory effects [48, 49]. The backbone of HMOs is formed by poly-*N*-acetyllactosamines, which can bind galactoside-binding lectins (galectins). Galectins are a family of lectins that bind beta-galactosides. At least 12 different galectins have been identified with a variety of functions including regulation of cell growth, proliferation, and apoptosis, as well as immune functions [50]. Expression and interactions of galactosides and galectins may affect gut ecology and leukocyte function that may influence anti-viral defense. Different poly-lactosyl oligosaccharides in HMOs have varied affinities to different galectins, resulting in different immune regulation [51]. Many HMOs also contain sialic acid (α2-3 and/or α2-6) bound to galactose or glucosamine. Sialic acid-containing oligosaccharides have been shown to bind receptors called sialic acid binding immunoglobulin-like lectins (Siglecs). There are at least 12 Siglecs that have been identified. Most of them have two conserved ITIM-like motifs (immunoreceptor tyrosine-based inhibitory motifs) in their cytoplasmic tails, suggesting their involvement in cellular activation or inactivation [52]. HMOs with sialic acid side chains, which may recognize certain Siglecs on intestinal cells and leukocytes, have been proposed to mediate immunoregulatory functions [20, 21].

In summary, HMOs possess antiviral activity that is related to a direct blockade of viral infection or indirect modulation of intestinal ecology and immune responses. In addition to HMOs, glycoproteins in milk such as transferrin which has been shown to have potent activities against hepatitis B virus, human immunodeficiency virus and human cytomegalovirus replication [53,54]. Another mucin glycoprotein with molecular weight of 46-kD in human milk has also been shown to mediate antiviral activity directed against rotavirus [55]. Dietary supplements with HMOs or milk glycoproteins might be suitable for the prevention of infections in infants because of their stability and sweet taste [33, 34]. Further studies on the protective mechanisms of HMOs against viral infections by modulating cell surface expression of sugar epitopes or lectins, or by additional intake of exogenous HMOs might provide better strategies for prevention of viral infections.

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