Do the Binding Properties of Oligosaccharides in Milk Protect Human Infants from Gastrointestinal Bacteria?¹,²

David S. Newburg³

Department of Biochemistry, Shriver Center for Mental Retardation, Waltham, MA 02254 and Harvard Medical School, Boston MA 02115

ABSTRACT The oligosaccharide fraction of human milk, the third most abundant solid constituent, consists of hundreds of structures, many of them fucosylated. Oligosaccharides may bear structural homology to cell surface glycoconjugates used as receptors by pathogens, thus protecting nursing infants. The ability of human milk to protect against heat-stable enterotoxin of Escherichia coli in suckling infants has been attributed to neutral fucosylated oligosaccharides of milk. The same phenomenon has been found in human T84 cells, allowing the mechanism of inhibition by the oligosaccharide to be studied in vitro. The oligosaccharide binds to the extracellular domain of guanylyl cyclase, thereby inhibiting the binding of stable toxin. The protective oligosaccharide is a large structure present in too low a concentration to be routinely measured directly; however, its concentration in milk may be inferred by measuring smaller, more plentiful, structurally homologous oligosaccharides. The adhesion by invasive pathogenic strains of Campylobacter to their enterocyte target is also inhibited by human milk fucosyloligosaccharides. Because Campylobacter binds H-2 type oligosaccharide structures, the concentration of protective oligosaccharide may also be inferred from the total oligosaccharide profile. The relationship between oligosaccharide profile heterogeneity in human milk and the incidence of specific gastrointestinal bacterial disease in infants consuming these milks could indicate the significance of these oligosaccharides to infant health. The efficacy of synthetic analogs of active oligosaccharides will confirm their clinical relevance and define minimum structural features essential for activity. J. Nutr. 127: 980S–984S, 1997.

KEY WORDS: · human milk · oligosaccharides · Campylobacter jejuni · Escherichia coli · enteric disease

HUMAN MILK OLIGOSACCHARIDES

The human milk oligosaccharide fraction was described in 1933 (Polonovsky and Lespagnol 1933). The several laboratories reporting quantitative data on this fraction are in general agreement that it represents over 12 g/L of mature milk and approximately 22 g/L of colostrum. This consensus is the more convincing because of the very disparate methods of analysis that were used for its quantification (Newburg and Neubauer 1995). Thus, the oligosaccharide fraction of human milk is the third largest solid component, after fat and lactose. However, despite having a higher concentration in human milk than protein, a fraction of widely recognized importance, oligosaccharides were long believed not to have biological significance. Their presence was attributed to the accident of a high concentration of lactose occurring in the presence of large amounts and numbers of glycosyltransferases, the enzymes responsible for the synthesis of complex carbohydrate moieties of glycoproteins and glycolipids (Kobata 1978).

Close to 90 major milk oligosaccharides have been isolated and their structures determined. The oligosaccharides generally have a lactose moiety at the reducing end of the molecule and often contain a fucose and/or sialic acid moiety at the nonreducing end. The number of potential structural permutations for the oligosaccharides is astronomical. Using state-of-the-art time-of-flight mass spectrometry, Stahl et al. (1994) detected approximately 900 fucosyloligosaccharides containing up to 32 sugars and up to 15 fucose residues. With such a large array of structures, it becomes increasingly plausible that among them might be molecules that are biologically active.

Other considerations also argue for the likelihood of biological activity. Cell surfaces contain a large number of oligosaccharide moieties, primarily in the form of glycolipids and glycoproteins, that function in intracellular communication. These normally participate in binding to other cells and to hormones and other humoral effectors; however, an essential first step for many pathogens is binding to a specific extracellular receptor.
Because the milk oligosaccharides seem to be synthesized by glycosyltransferases similar to those that synthesize glycoprotein and glycolipid cell surface components (Kobata 1978), it is reasonable to postulate that some of these oligosaccharides could have structural homology to cell surface carbohydrates. These could act as analogs or homologs of host cell surface receptors for pathogens, thereby inhibiting the ability of the pathogen to bind to the homologous cell surface glycoconjugate. Infants, whose stomachs are less acidified than those of adults and whose immune systems are not mature, may need additional protection from enteric pathogens; oligosaccharides, a major milk fraction composed of water-soluble cell surface analogs that can inhibit enteropathogen binding to host cell receptors, could serve such a protective function.

This concept is supported by several examples. Andersson et al. (1986) reported that specific oligosaccharides obtained from human milk can inhibit binding of *Streptococcus pneumoniae* to its receptor. Similarly, Cravioto and co-workers (1991) described a milk oligosaccharide that inhibits adherence of enteropathogenic *E. coli* to their receptors. We found that a fucosylated oligosaccharide inhibits the toxicity of stable toxin of *E. coli* in vivo (Newburg et al. 1990) and that another fucosylated oligosaccharide inhibits binding of invasive strains of *Campylobacter jejuni* to its host cell (Ruiz-Palacios et al. 1992). Details of these latter two examples follow.

**A FUCOSYLOLIGOSACCHARIDE ACCOUNTS FOR THE PROTECTION AGAINST HEAT-STABLE ENTEROTOXIN BY HUMAN MILK**

Enterotoxigenic *E. coli* are pathogens that cause gastroenteritis in adult travelers to many areas of the globe and in infants indigenous to these regions. Two major enterotoxins can be responsible for the secretory diarrhea that follows infection: heat-stable enterotoxin (ST) and heat-labile enterotoxin. Heat-labile enterotoxin resembles cholera toxin and the toxin of *C. jejuni* in that it binds to the monosialyl ganglioside 1 (GM1) on the cell surface as an essential step in its pathogenesis, and the GM1 of human milk inhibits its diarrheagenic effect (Ottaess et al. 1983).

In 1983, Cleary et al. reported that human milk contained a low-molecular-weight, nonprotein inhibitor of the action of ST. In subsequent work, a human milk oligosaccharide fraction was isolated that, at the concentrations found in milk, inhibits ST in vivo in the suckling mouse model. The protective oligosaccharide is neutral and binds to lectin 1 of ulex europaeus, indicating a fucosylated oligosaccharide. These conclusions are supported by the successful isolation of this activity by different isolation schemes and by the strong and unambiguous inhibition of ST activity in two different in vivo test systems (Newburg et al. 1990).

However, in the studies with suckling mice, the mechanism of protection of the oligosaccharide could not be determined, and the protective efficacy of the oligosaccharide in human intestinal tissue was untested. The relevance of the inhibitor to human cells and its mechanism of inhibition were investigated in T84 cells, an in vitro model for ST action on intestinal epithelium. These cells express many of the features of human intestinal epithelium, including tight junctions, desmosomes and microvilli on the apical surface. These cells also express guanylyl cyclase.

**MECHANISM OF INHIBITION**

Guanylyl cyclase is a transmembrane protein found on the microvillus membrane. It contains a transmembrane domain, an extracellular region that includes the binding site for ST, an intracellular domain that includes a region with protein kinase activity, and a region with guanylyl cyclase activity. Stable toxin binds to the extracellular region, which causes the activation of guanylyl cyclase in the intracellular domain, leading to elevated production of cGMP from GTP. The elevated cGMP causes an inhibition of chloride channel function, resulting in a net flux of fluid to the lumen of the intestine across the microvillus membrane. This is thought to account for the mechanism of secretory diarrhea caused by stable toxin of *E. coli*.

The ability of the human milk oligosaccharide to inhibit this process was tested on membrane preparations of T84 cells; none of the oligosaccharide fractions alone changed basal guanylyl cyclase activity. Stable toxin alone caused a significant elevation of guanylyl cyclase activity, consistent with its known mechanism of action. The total oligosaccharide fraction and the ulex-binding fucosylated oligosaccharide fraction inhibited ST-stimulated guanylyl cyclase activity by 70–80% in a dose-dependent manner, whereas the non-ulex binding, mainly nonfucosylated, oligosaccharide fraction did not inhibit. These data indicate that the oligosaccharide most likely acts by preventing toxin-mediated activation of guanylyl cyclase (Crane et al. 1994).

Because the oligosaccharide and the toxin both seem to act extracellularly, the most likely mechanism of protection by the oligosaccharide would be the inhibition of ST binding to the extracellular docking domain of guanylyl cyclase. Therefore, the ability of the milk oligosaccharides to inhibit the binding of ST to T84 cell membranes was measured. The total oligosaccharide fraction inhibited 125I-labeled ST binding by 17%, and the fucosylated oligosaccharide fraction at half the concentration inhibited ST binding by 27%.

The ability of the oligosaccharide to inhibit ST activation of guanylyl cyclase persisted even after oligosaccharide-treated cells or cell membranes were washed to remove free oligosaccharides prior to adding ST; this inhibition was equivalent to that seen in controls in which oligosaccharides and ST were present together. These results led to the conclusion that the active component of the milk oligosaccharide fraction binds to the ST receptor rather than to the toxin.

These data suggest the following mechanism whereby the human fucosylated oligosaccharide inhibits ST activity in mammalian intestines. The active human milk fucosyloligosaccharide inhibits the action of ST by docking with a carbohydrate recognition domain in the extracellular region of guanylyl cyclase. This active oligosaccharide may bind allosterically to the extracellular portion of guanylyl cyclase proximal to the ST binding site rather than competing directly for the ST binding site itself. This bound oligosaccharide would interfere with the ability of extracellular ST to cause an elevation of intracellular guanylyl cyclase activity in enterocytes. Without the elevation of cGMP, chloride channels are able to function normally, allowing the resorption of fluid from the intestinal lumen and thereby preventing the secretory problem normally induced by exposure to ST. This may account for the observed inhibition of ST-induced secretory diarrhea in vivo by human milk oligosaccharides and represents a novel protective mechanism for human milk oligosaccharides.

**ISOLATION OF THE ACTIVE FUCOSYLOLIGOSACCHARIDE**

The first step towards studying the details of these phenomena is to isolate and identify the protective oligosaccharide so that its essential structural features can be defined and related to function.
The *E. coli*–bound fucosylated oligosaccharide fraction was resolved by semipreparative HPLC into more than 30 distinct fractions. Only one of these displayed reproducible, robust, inhibitory activity. Therefore, this fraction was resolved by reversed-phase HPLC chromatography into seven distinct peaks, each of which was purified to homogeneity. When tested at 2.5 times their original concentrations in milk, only one of these showed inhibition, and this was comparable to that of the control human milk sample. Testing was at this higher level to take into account probable losses in the many steps of its purification from pooled human milk. This single fucosylated oligosaccharide peak is present in human milk at approximately 40 parts per billion (approximately 20 pmol/L) and may be responsible for the protective activity of human milk against ST-induced enteric fluid loss in infant mice.

**GENETIC HETEROGENEITY OF MILK OLIGOSACCHARIDES**

The isolation of this active oligosaccharide peak provided valuable clues to its structure. Adsorption to *A. citrus* affinity columns was a definitive step in the separation of the active fraction from other oligosaccharides, indicating that the active oligosaccharides must contain fucosyl 1-2 linkages, the major binding determinants for this lectin. It seems to be a large structure of more than 10 sugar residues.

Fucosyl 1-2 linkages are synthesized in the mammary gland by fucosyl α1-2 transferases. Fucosyltransferases in milk probably reflect fucosyltransferases active in the Golgi apparatus of the acinar cells of the breast epithelium. Milk fucosyltransferase activity varies among individuals; furthermore, the activity changes over the course of lactation. Such differences in transferase activities could reflect qualitative as well as quantitative differences in fucose-containing oligosaccharides of milk from different individuals and from different stages of lactation (Wiederschain and Newburg 1995). For example, human milk oligosaccharide profiles are characteristic of the individual's Lewis blood group type and secretor status, which reflect genetic differences in fucosyltransferases (Vivere et al. 1990).

**OLIGOSACCHARIDE MEASUREMENT IN HUMAN MILK**

The complex structure and low concentration of the protective oligosaccharide fraction in milk, which contains large amounts of related fucosyloligosaccharides, make it unlikely that levels of the protective oligosaccharide can be measured directly by a simple routine assay. However, structural features that some oligosaccharides have in common, such as fucosyl α1-2 linkages, are thought to be synthesized by the same or closely related fucosyltransferases. Therefore, the relative amount of the protective oligosaccharide in the milk of an individual may be related to the amount of structurally related smaller, more plentiful fucosyloligosaccharides. We developed a sensitive method for routine identification and quantification of milk oligosaccharides and tested the method by measuring individual differences of oligosaccharide profiles in 50 random human milk samples (Newburg et al. 1996). The profiles consisted of more than 12 oligosaccharide peaks composed of tri- to octasaccharides. Variations in oligosaccharide profiles of individual samples were related significantly to variations in fucosyltransferase levels. This implies that lactating mothers may differ genetically in their ability to produce protective oligosaccharides. If the protective oligosaccharide makes a significant contribution toward protecting the infant from disease, such genetic variation among lactating women may influence their breast-fed infants’ susceptibilities to specific enteric diseases.

To test whether the ST inhibitory oligosaccharide in milk protects infants from gastrointestinal bacteria, the heterogeneity of milk oligosaccharide profiles will be tested for any relationship to the history of ST-associated enteric disease in the infants consuming this milk. Synthetic oligosaccharide analogs will be evaluated for their ability to inhibit ST of *E. coli* in vivo, to identify simplified structures suitable for large-scale synthesis. This synthetic material will be utilized in detailed studies on the mechanism by which fucosyloligosaccharides inhibit ST docking to guanyl cyclase and to test whether an ST-binding inhibitor is efficacious in humans.

**CAMPYLOBACTER**

In many of the developing regions of the world, *Campylobacter jejuni* is responsible for much of the bacterial diarrhea that threatens infants. The pathogenesis of *Campylobacter* diarrhea involves a series of complex events, including toxin production and adherence to gut mucosa followed by its invasion. Thus, the inhibition of attachment to mucosal surfaces would disrupt an essential early step in the pathogenesis of *Campylobacter* infection. The binding of invasive (pathogenic) strains of *Campylobacter* to their target epithelial cells is inhibited in vitro by human milk, and specifically by the isolated fucosylated oligosaccharides of human milk. Infection by *Campylobacter* in a BALB/c mouse intestinal colonization model is also inhibited by these oligosaccharides. Five days after inoculation, mouse gut colonization by *Campylobacter* was $1.1 \times 10^{10}$ colony-forming units per gram of feces in controls, whereas feces of similar mice fed oligosaccharide contained only $1.2 \times 10^5$ colony-forming units per gram.

The full structure of the milk oligosaccharide that inhibits *Campylobacter* has not been determined. However, a bacterial binding immunoblot assay using a panel of defined albumin-linked carbohydrate chains was developed; *Campylobacter* bound to immobilized oligosaccharides with the H-2 type structure (Fucα1-2Galβ1-4GlcnAc), and less to those of the H-1 type (Fucα1-2Galβ1-3GlcnAc) and the Lewis’ type [Fucα1-2Galβ1-3(Fucα1-4)GlcnAc] (Cervantes et al. 1995). Structures with these moieties are found in the human milk oligosaccharides.

These data suggest that the human milk inhibitor of *Campylobacter* binding could be α1-2 fucosylated oligosaccharides that compete with the receptors present in epithelial cells; the human milk fucosylated oligosaccharides inhibit the bacterium in vitro and in vivo. Again, the relevance of these findings to the protection of infants from *Campylobacter* infection by human milk can be tested by relating oligosaccharide profiles in milk samples to the incidence of *Campylobacter* diarrhea in infants consuming those milk samples. The efficacy of synthetic analogs of α1-2 fucosyloligosaccharides in preventing *Campylobacter* infection in humans will also address this issue.

**PROTECTION OF THE HUMAN INFANT**

Many studies have demonstrated that breast-feeding protects against enteric disease (Feachem and Koblinsky 1984). In one example from Lehore, Pakistan, Hanson et al. (1991) analyzed breast-feeding protection of neonates by the same type of statistical treatment as one would use for an antibiotic drug, i.e., efficacy in preventing enteric disease in a population at risk. Breast-feeding was most efficacious among rural and urban poor and during early lactation; however, it also protected upper middle-class infants, whose level of hygiene and...
Oligosaccharides in human milk that inhibit enteropathic agents

<table>
<thead>
<tr>
<th>Structure</th>
<th>Pathogen</th>
<th>Mechanism of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal(1→3)GlcNAc(1→3)Gal(1→4)Glc</td>
<td>Streptococcus pneumoniae</td>
<td>Inhibits S. pneumoniae adhesion in vitro</td>
</tr>
<tr>
<td>Fucosylated pentasaccharide</td>
<td>Enteropathogenic Escherichia coli</td>
<td>Inhibits E. coli adhesion in vitro</td>
</tr>
<tr>
<td>Fucosylated oligosaccharide</td>
<td>Campylobacter jejuni (invasive)</td>
<td>Inhibits C. jejuni adhesion in vivo</td>
</tr>
<tr>
<td>Fucosylated oligosaccharide</td>
<td>Stable enterotoxin of E. coli (ST)</td>
<td>Inhibits ST binding to receptor in vitro</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibits ST-induced diarrhea in vivo</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Binds to toxin receptor</td>
</tr>
</tbody>
</table>

1 Newburg (1996).

Economic status are more relevant to typical Western populations.

Protection of neonates by breast-feeding may be attributed to many factors, including decreased exposure to pathogens, some aspects of breast-feeding per se, and the human milk components. Protection from environmental pathogens by human milk components may include several tiers of defense, perhaps compensating for the lack of a highly acidic stomach to act as a barrier to enteric pathogens and for the immaturity of the immune system. First, human milk provides a complement of nutrients that optimize development of the intestinal mucosa and immune system. In addition, many major milk components, which may be sources of nutrients, may also be capable of protecting against disease. This second tier of defense includes multifunctional and broad spectrum inhibitory activities, such as lactoferrin, free fatty acids and monoglycides. Many of these materials can protect against a wide range of pathogens. Most widely recognized of the milk protective components is a third tier of defense, the antibodies, particularly the secretory immunoglobulin A that is so important to mucosal immunity. Wider interest is now emerging on a possible fourth tier of protection, i.e., cell surface homologs that can inhibit pathogen binding to their host cell receptors. These are, for the most part, nonnutrient glycoconjugates such as glycolipids, glycoproteins, mucins, glycosaminoglycans and the oligosaccharides. Although the relative contributions of these different defense mechanisms, either individually or in concert, are unknown, recognition of the potential for the milk oligosaccharides to contribute significantly to the defense of the infant is increasing. Other bioactive constituents of milk may also contribute toward protection. These several tiers of defense have the potential to work synergistically. Upon exposure of the nursing dyad to a pathogen, constitutive protective factors may control pathogens early in the infection, while the mother is mounting an immune response. Specific antibodies may then work in concert with the constitutive factors to overcome the infection.

Many examples of human milk glycoconjugate protective components are now known, and prominent among them are the oligosaccharides. These are summarized in Table 1. The oligosaccharides inhibit the adherence of S. pneumoniae, enteropathogenic E. coli and invasive C. jejuni and also inhibit the toxicity of the heat-stable enterotoxin of E. coli. Two types of mechanisms may account for such protection. In one, exemplified by Campylobacter, the nonreducing end of the protective milk oligosaccharide, because of its homology to a cell surface receptor, may bind to the pathogen, thereby inhibiting the binding of the pathogen to its cell surface receptor. A different, unexpected mechanism, exemplified by ST, is that oligosaccharides may bind to the cell surface receptor and thereby inhibit the ability of the pathogen to affect the host cell.

The quantity and types of oligosaccharides in milk seem to be characteristic to each species. Therefore, common artificial diets for infants would not be expected to contain oligosaccharides that are qualitatively or functionally comparable to those of human milk, because they would be unlikely to resemble human cell surface receptors. The milks of other species may contain oligosaccharides that protect infants of that species against species-specific pathogens.

Many aspects of the inhibitory oligosaccharides in relation to protection of infants require further study: heterogeneity of milk glycoconjugates as a function of maternal genetics, stage of lactation, nutritional status, disease, and exposure to environmental agents should be investigated. The persistence and modification of milk oligosaccharides during transit through the infant’s gastrointestinal tract may be highly relevant to their clinical significance. Details on the mechanisms by which these milk components inhibit pathogenesis require more investigation, including structure-function relationships. To directly address the issue of whether the binding properties of oligosaccharides in milk protect infants from gastrointestinal bacteria, a measure of the levels of the protective oligosaccharides in individual milk samples, such as the oligosaccharides profiles discussed above, must be related to the incidence of diarrhea associated with specific pathogens in a nursing population at risk for this disease. The efficacy of synthetic neoglycoconjugates containing essential structural features of the milk oligosaccharides would strongly support the clinical relevance of the human milk oligosaccharides.

LITERATURE CITED


