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OLIGOSACCHARIDES IN HUMAN MILK: Structural, Functional, and Metabolic Aspects

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■ **Abstract** Research on human milk oligosaccharides (HMOs) has received much attention in recent years. However, it started about a century ago with the observation that oligosaccharides might be growth factors for a so-called bifidus flora in breast-fed infants and extends to the recent finding of cell adhesion molecules in human milk. The latter are involved in inflammatory events recognizing carbohydrate sequences that also can be found in human milk. The similarities between epithelial cell surface carbohydrates and oligosaccharides in human milk strengthen the idea that specific interactions of those oligosaccharides with pathogenic microorganisms do occur preventing the attachment of microbes to epithelial cells. HMOs may act as soluble receptors for different pathogens, thus increasing the resistance of breast-fed infants. However, we need to know more about the metabolism of oligosaccharides in the gastrointestinal tract. How far are oligosaccharides degraded by intestinal enzymes and does oligosaccharide processing (e.g. degradation, synthesis, and elongation of core structures) occur in intestinal epithelial cells? Further research on HMOs is certainly needed to increase our knowledge of infant nutrition as it is affected by complex oligosaccharides.

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INTRODUCTION

Recently, human milk (HM), compared with milk from other species, was considered to be unique in terms of its content of complex oligosaccharides (10, 16, 17, 25, 29, 31, 56). Today, we are aware of one exception, which is the milk of elephants. Its composition and the interpretation of the data are discussed elsewhere (22). For many years, oligosaccharides have been discussed only in terms of their role in the development of a specific intestinal flora in breast-fed infants. Today, there is striking evidence that free oligosaccharides, as well as glycoproteins, are potent inhibitors of bacterial adhesion to epithelial surfaces, an initial stage of infective processes. Therefore, these oligosaccharides are considered to be soluble receptor analogs of epithelial cell surface carbohydrates. Whether they also function as ligands for selectins influencing inflammatory reactions is currently of great research interest as well.

STRUCTURAL DIVERSITY OF OLIGOSACCHARIDES

Biological functions of oligosaccharides are closely related to their conformation. The monomers of milk oligosaccharides are D-glucose (Glc), D-galactose (Gal), N-acetylglucosamine (GlcNAc), L-fucose (Fuc), and sialic acid [N-acetylneuraminic acid (NeuAc)]. With few exceptions, oligosaccharides isolated so far carry lactose at their reducing end (see Table 1). An elongation is achieved by an enzymatic attachment of GlcNAc residues linked in ß1-3 or in ß1-6 linkage to a Gal residue followed by further addition of Gal in a ß-1-3 or ß-1-4 bond. Thus, a large number of core structures can be formed. Further variations occur due to the attachment of lactosamine, Fuc, and/or NeuAc residues at different positions of the core region and of the core elongation chain (10, 50).

The addition of Fuc is dependent on the actions of at least three different fucosyltransferases in a genetically determined process. The α1-2-fucosyltransferase is found in ~77% of Caucasians who are classified as secretors. Therefore, oligosaccharides in milk from women with secretor status are characterized by the presence of 2-fucosyllactose (Fucα1-2Galß1-4Glc), of lacto-N-fucopentaose I (Fucα1-2Galß1-3GlcNAcß1-3Galß1-4Glc) (Table 1 and 2), and of more-complex oligosaccharides all possessing Fucα1-2Galß1-3GlcNAc residues.

Another fucosyltransferase that is Lewis gene dependent attaches Fuc residues in α1-4 linkages to a subterminal GlcNAc residue of type 1 chains. Therefore, in

TABLE 1 Core structures of oligosaccharides in human milk[a]

Component	Structure
Lactose	Galβ1-4Glc
Lacto-*N*-tetraose (type 1)	3Galβ1-4Glc 3GlcNAcβ1 Galβ1
Lacto-*N*-neo-tetraose (type 2)	3Galβ1-4Glc Galβ1-4GlcNAcβ1
Lacto-*N*-hexaose	3Galβ1-4Glc 3Galβ1-4GlcNAcβ1 Galβ1-4GlcNAcβ1 3Galβ1-4Glc 3Galβ1-4GlcNAcβ1 3GlcNAcβ1 Galβ1 Galβ1-4GlcNAcβ1 3/6Galβ1-4Glc Galβ1-4GlcNAcβ1 Galβ1-4GlcNAcβ1 3/6Galβ1-4Glc 3GlcNAcβ1 Galβ1
Lacto-*N*-octaose	3Galβ1-4Glc 3Galβ1-4GlcNAcβ1 3Galβ1-4GlcNAcβ1 Galβ1-3/4GlcNAcβ1 3Galβ1-4GlcNAcβ1 Galβ1-3/4GlcNAcβ1 3/6Galβ1-4Glc Galβ1-3/4GlcNAcβ1
Lacto-*N*-decaose	3Galβ1-GlcNAcβ1 3Galβ1-4GlcNAcβ1 3/6Galβ1-4Glc Galβ1-3/4GlcNAcβ1 Galβ1-3/4GlcNAcβ1 Galβ1-4GlcNAcβ1 3/6Galβ1-4GlcNAcβ1 3GlcNAcβ1 3/6Galβ1-4Glc Galβ1 3GlcNAcβ1 Galβ1
Lacto-*N*-dodecaose	$(Galβ1-4GlcNAcβ1)_{0/1}$ 3/6Galβ1-4GlcNAcβ1 $(Galβ1-4GlcNAcβ1)_{0/1}$ 3/6Galβ1-4Glc 3/6Galβ1-3/4GlcNAcβ1 Galβ1-3/4GlcNAcβ1

[a]Adapted from Reference 10 by permission of the publisher.

milk of nonsecretors, the major fucosylated oligosaccharide is lacto-N-fucopentaose II (Galß1-3[Fucα1-4]GlcNAcß1-3Galß1-4Glc; see Table 2), which is the "Lewis a" blood group determinant. This characteristic component is found in ~20% of the population. If both fucosyltransferases, the secretor gene and the Lewis gene-dependent form are present, one of the major milk oligosaccharides is lacto-N-difucohexaose I (Fucα1-2Galß1-3[Fucα1-4]GlcNAcß1-3Galß1-4Glc).

TABLE 2 Oligosaccharides in term and preterm human milk

Trivial name	Abbreviation	Structure
Lactose	Lac	Galβ1-4Glc
2′-Fucosyl-lactose[a]	2′-Fuc-lac	Fucα1-2Galβ1-4Glc
3-Fucosyl-lactose	3-Fuc-lac	Galβ1-4Glc 3 \| 1 Fucα
3′-Sialyl-lactose 3-fucosyl-lactose	3′NeuAc-3-Fuc-lac	NeuAcα2-3Galβ1-4Glc 3 \| 1 Fucα
Difucosyl-lactose[a]	Fuc_2-lac	Fucα1-2Galβ1-4Glc 3 \| 1 Fucα
Lacto-*N*-tetraose (type 1)	LNT	Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Lacto-*N*-neo-tetraose (type 2)	neo-LNT	Galβ1-4GlcNAcβ1-3Galβ1-4Glc
Lacto-*N*-fucopentaose I[a]	LNFP I	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Lacto-*N*-fucopentaose II	LNFP II	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 \| 1 Fucα
Lacto-*N*-fucopentaose III	LNFP III	Galβ1-4GlcNAcβ1-3Galβ1-4Glc 3 \| 1 Fucα
Lacto-*N*-fucopentaose V	LNFP V	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 3 \| 1 Fucα
Lacto-*N*-difuco-hexaose I[a]	LNDFH I	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 \| 1 Fucα

(*Continued*)

TABLE 2 (*Continued*)

Trivial name	Abbreviation	Structure
Lacto-*N*-difuco-hexaose II	LNDFH II	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 3 \| \| 1 1 Fucα Fucα
Sialylα(2-3)lactose	NeuAcα(2-3)lac	NeuAcα2-3Galβ1-4Glc
Sialylα(2-6)lactose	NeuAcα(2-6)lac	NeuAcα2-6Galβ1-4Glc
Sialyl-lacto-*N*-tetraose a (LST a)	NeuAc-LNT	NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Sialyl-lacto-*N*-tetraose b (LST b)	NeuAc-LNT	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 6 \| 2 NeuAcα
Sialyl-lacto-*N*-tetraose c (LST c)	NeuAc-LNT	NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc
Sialyl-fucosyl-lacto-*N*-tetraose I	NeuAc-fuc-LNT I	—[b]
Sialyl-fucosyl-lacto-*N*-tetraose II	NeuAc-fuc-LNT II	—[b]
Disialyl-lacto-*N*-tetraose	$NeuAc_2$LNT	NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc 6 \| 2 NeuAcα

[a]Lewis b-active components: 2′ fucosyl-lactose, difucosyl-lactose, lacto-*N*-fucopentaose I, lacto-*N*-difuco-hexaose I.
[b]Not determined yet.

In ~5% of the population who belong to blood group Lewis $a^- b^-$, a third type of milk oligosaccharide, carrying fucose α1-3 linked to GlcNAc in type 2 chains, was found. The major oligosaccharide in the milk of these donors is lacto-N-fucopentaose III (Galß1-4[Fucα1-3]GlcNAcß1-3Galß1-4Glc; see Table 2).

In addition to fucosyltransferases, several sialyltransferases may add NeuAc at different positions in milk oligosaccharides (see Table 2). The various actions of fucosyltransferases and sialyltransferases led to the identification and subsequent characterization of >100 different oligosaccharide structures in HM.

CONCENTRATIONS OF OLIGOSACCHARIDES

Table 3 summarizes our recent results on oligosaccharides in term and preterm milk (21, 31, 33). The major components are mono- and difucosyllactose,

TABLE 3 Oligosaccharides in human milk and cow's milk

Component	Amount (g/liter)	
	Human milk[a]	Cow's milk[b]
Lactose	55–70	40–50
Oligosaccharides		
Lacto-*N*-tetraose	0.5–1.5	Traces
Lacto-*N*-fucopentaose I	1.2–1.7	—
Lacto-*N*-fucopentaose II	0.3–1.0	—
Lacto-*N*-fucopentaose III	0.01–0.2	—
Lacto-*N*-difucohexaose I	0.1–0.2	—
NeuAc(α2-6)lactose	0.3–0.5	0.03–0.06
NeuAc(α2-3)lactose	0.1–0.3	
NeuAc-lacto-*N*-tetraose a	0.03–0.2	Traces
NeuAc-lacto-*N*-tetraose c	0.1–0.6	Traces
$NeuAc_2$-lacto-*N*-tetraose	0.2–0.6	Traces
Oligosaccharides (total)	5.0–8.0	Traces

[a]Data are obtained from References 17 and 31.
[b]Data are obtained from A Kobata (1972, *Methods Enzymol.* 28:262) and J Parkkinen & J Finne (1987, *Methods, Enzymol.* 138:289).

lacto-N-tetraose (LNT) (mainly type 1), and their mono- and difucosylated derivatives as well as fucosylated lacto-N-hexaoses and lacto-N-octaoses. Among the sialylated components we isolated sialyllactose, the three isomeric forms of LNT (LST a, b, and c), and disialyl-LNT. Because of the fucosylation of these oligosaccharides, there are many more components in milk that need further characterization. There seem to be no differences in the qualitative or quantitative aspects of term and preterm milk. Compositional changes of oligosaccharides in term milk also occur during lactation with the largest amounts being found at early stages (22; Table 4). So far, differences between term and preterm milk have not been observed.

There are also reports from other groups that show a similar trend for the total concentration of HMO (9, 10a, 32a, 51). However, it should be mentioned that data obtained from different groups should be compared with caution for the following reasons. First, there is still no routine method available for investigating the pattern or the total amount of HMOs. Each method has its own characteristic separation behavior, which makes a direct comparison with other methods rather difficult. Second, in some papers the exact method is not given. Therefore, it cannot be evaluated whether all or at least most of the lactose has been removed from the oligosaccharide fraction. If the quantitation is based on the dry weight of a fraction, then the values may be far too high because of residual lactose. The same holds for residual proteins, which may contaminate fractions with rather complex acidic and neutral oligosaccharides. Third, the quantitation of complex components requires

TABLE 4 Concentrations of inividual oligosaccharides (g/liter) in human milk during the first weeks of location

	HM 1						HM 2		HM 3					HM 4				
Days postpartum	**2**	**5**	**7**	**9**	**12**	**19**	**7**	**10**	**3**	**5**	**8**	**11**	**14**	**3**	**4**	**13**	**15**	**19**
LNT	0.50	1.30	1.60	1.51	1.54	1.42	1.28	1.23	0.12	0.20	0.16	0.45	0.56	0.47	0.47	0.80	0.77	0.62
LNFP 1	Traces	Traces	Traces	Traces	Traces	Traces	Traces	Traces	1.45	1.39	1.36	1.02	0.98	2.00	1.45	1.50	1.64	1.48
LNFP II	0.20	0.16	0.87	1.12	1.03	0.97	0.43	0.56	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]
NeuAcα2-6lac	0.33	0.42	0.43	0.48	0.47	0.47	0.86	0.53	0.38	0.41	0.56	0.51	0.46	0.39	0.31	0.42	0.40	0.36
NeuAcα2-3lac	0.23	0.10	0.18	0.11	0.22	0.10	0.30	0.25	0.27	0.25	0.30	0.22	0.18	0.23	0.11	0.12	0.14	0.18
LST c	0.17	0.34	0.20	0.15	0.12	0.10	0.44	0.30	0.46	1.01	0.74	0.66	0.54	0.51	0.52	0.22	0.17	0.14
LST a	0.10	0.19	0.15	0.17	0.06	0.16	—	—	—	0.03	0.05	—	0.03	0.11	0.05	0.08	0.05	0.07
NeuAc2LNT	0.47	0.34	0.21	0.17	0.29	0.19	0.26	0.17	0.20	0.25	0.38	0.50	0.41	0.59	0.38	0.28	0.15	0.21
Total	2.00	2.85	3.64	3.71	3.73	3.41	3.57	3.04	2.44	3.12	3.14	3.06	2.87	3.70	2.85	2.97	2.82	2.61
Lactose	40.18	55.26	58.12	58.84	57.23	58.66	54.36	57.23	48.44	46.64	56.33	58.66	55.07	44.32	44.85	54.72	54.54	55.26

[a]HM, Human milk. Milk from four mothers was analyzed.
[b]Not detectable.

several chromatographic steps with different systems (e.g. ion-exchange chromatography, gel filtration, etc) leading to very distinct methodologies for different laboratories, although they all may address the same question.

With regard to the total amount of oligosaccharides, it is important that some oligosaccharides may occur in concentrations as high as those of milk proteins like lactoferrin, α-lactalbumin, and secretory IgA (Table 3).

The major component among complex oligosaccharides is LNT (0.5–1.5 g/liter), followed by lacto-N-fucopentaose I or II. LNT and its monofucosylated derivatives add up to ~50%–70% of the total complex carbohydrates. Of the sialylated components, the content of sialyllactose (NeuAcα2-6Lac and NeuAcα2-3Lac) is highest, ~1.0 g/liter, followed by isomers of monosialylated LNT and disialylated LNT. The total amount of complex oligosaccharides in mature milk is between 5 and 8 g/liter. In cow's milk, only small amounts of oligosaccharides are detectable, with sialyllactose being the major component.

OLIGOSACCHARIDES AS PREBIOTICS

Breast-Fed Vs Formula-Fed Infants

At the beginning of this century, Tissier & Moro reported that in feces of breast-fed infants, unlike those of bottle-fed infants, bifidobacteria are the predominant microorganisms (32, 52). This observation has been confirmed by several research groups but not by others (for a review, see 17). According to Rolles et al (41), the normal pattern of bacterial colonization of the intestine may be more complex than previously reported. Studies using gas liquid chromatography as a specific method to identify anaerobic microorganisms led to the conclusion that neither bifidobacteria nor lactobacilli were predominant in most normal infants born at full term, whether they were breast-fed or bottle-fed (27).

There seem to be several reasons why an increasing number of studies are not confirming the presence of a specific bifidus flora in breast-fed infants: (*a*) methodical problems in identifying different bacterial species, for example microbiological assays vs gas chromatography; (*b*) environmental factors such as mode of delivery, for example vaginal delivery vs cesarean section; (*c*) treatment with antibiotics; (*d*) preterm delivery; (*e*) mode of collection and storage of feces samples (considering the slower proliferation of apathogenic bifidobacteria); or (*f*) use of infant formulas which are in part adapted to HM. These and other factors might explain the differing results of many reports. A thorough review of this topic is given by Adlerberth (1).

N-Acetylglucosamine-Containing Oligosaccharides as Growth Factors for *Bifidobacterium bifidum*

The predominance of *Bifidobacterium bifidum* in the intestinal flora of breast-fed infants led Moro in 1900 to the conclusion that HM contains a growth factor for

these microorganisms (32). György et al (1954), using a "bifidum mutant" called *Bifidobacterium bifidum* subspecies *pennsylvanicum*, which was isolated from feces of breast-fed infants, found that gynolactose, a mixture of ~10 oligosaccharides containing GlcNAc, is the growth-promoting bifidus factor. However, it should be mentioned that the bifidus strain that has been used as a laboratory strain does not regularly occur in feces of breast-fed infants. Meanwhile, clinical studies have also been performed to identify the components in breast milk, which stimulate the growth of a nonpathogenic microflora. Besides a certain whey protein-to-casein ratio, lactoferrin with and without added iron, nucleotides, and, very recently, deglycosylated bovine whey proteins have been investigated. However, there has been no evidence that any of these components markedly affect the intestinal flora. The question of whether HM oligosaccharides are the responsible growth factors cannot be answered yet, as no clinical data are available. However, such studies can now be performed as the synthesis of at least some oligosaccharides is feasible today.

OLIGOSACCHARIDES AS NONSPECIFIC DEFENSE MECHANISMS

There is increasing evidence that oligosaccharides and glycoconjugates in HM have a direct inhibitory effect on certain virulence-related abilities of pathogenic microorganisms (37, 17, 45–47, 53, 57). Although the exact pathophysiological mechanism of the genesis of diarrhea is not fully known yet, it seems that the ability of microorganisms to adhere to the mucosal surface is essential for the spreading of these bacteria (e.g. *Escherichia coli*, *Helicobacter jejuni*, *Shigella* strains, *Vibrio cholerae*, and *Salmonella* species) in the duodenum (6, 30). Bacterial adhesion in general involves a ligand-receptor interaction between structures on the bacterial surface and complementary structures on the mucosal surface of the host. Some of the best-characterized adhesins of bacteria are those of *E. coli*, which possesses type 1 fimbriae (mannose sensitive), S fimbriae (sensitive to sialylated galactosides), or colonization factors [a heterogeneous group with various receptor specificities (6, 14, 24, 30, 35)]. The various ligand specificities of *E. coli* strains could explain the differences in intestinal colonization of breast-fed versus formula-fed newborns: The free oligosaccharides and glycoproteins of HM, which are present in large amounts and great variety, might prevent intestinal attachment of microorganisms by acting as receptor analogs competing with epithelial ligands for bacterial binding. To illustrate the similarities between oligosaccharides in HM and the receptors for pathogenic microorganisms, some receptor ligands naturally occurring in term and preterm milk are listed in Table 5 (see also Table 2).

Good examples of the distinct specificity of microorganisms are influenza A, B, and C viruses, which recognize either NeuAc(α2-6)Lac, NeuAc(α2-3)Lac (both major components of HM), or the 9-O-acetylated form of NeuAcα2-3R.

TABLE 5 Oligosaccharides, naturally occurring in human milk, as receptors for microbes[a]

Receptors	Microorganisms
Mannose-containing glycoproteins	*Escherichia coli* (type 1 fimbriae)
Fucosylated oligosaccharides	*E. coli* (heat-stable enterotoxin)
Fucosylated tetra- and pentasaccharides	*E. coli*
Sialyl(α2-3)lactose and glycoproteins	*E. coli* (S-fimbriae)
Sialyl(α2-3)galactosides in mucins	*E. coli* (S-fimbriae)
Neutral oligosaccharides (LNT, neo-LNT)	*Streptococcus pneumoniae*
Gal(ß1-4)GlcNAc or Gal(ß1-3)GlcNAc	*Pseudomonas aeruginosa*
Fucα1-2Gal epitopes	*Candida albicans*
Sialyl-lactose	*Helicobacter pylori*
Sialyl-lactose	*Streptococcus sanguis*
Sialyl-lactose and sialylated glycoproteins	*H. pylori*
Sialylated glycoproteins (α2-3-linked)	*Mycoplasma pneumoniae*
Sialylated poly-*N*-acetyllactosamine	*M. pneumoniae*
Sialylated (α2-3)poly-*N*-acetylactosaminoglycans	*Streptococcus suis*
Sialyl(α2-6)lactose	Influenzavirus A
Sialyl(α2-3)lactose	Influenzavirus B
9-O-Ac of NeuAc(α2-3)R	Influenzavirus C

[a]For References, see 47.

About 70% of otitis media cases in newborn infants are caused by infections with pneumococci or *Haemophilus influenzae.* In 1986, Andersson et al were able to show that the adhesion of these microorganisms to specific carbohydrate structures of pharyngeal or buccal epithelial cells was inhibited by HM (3). In a similar way, neutral oligosaccharides of HM could protect the intestinal tract of newborns from infection by *V. cholerae* (13).

In 1983, Parkkinen et al showed that sialylated HM oligosaccharides abolished the binding activity of *E. coli* strains that cause meningitis and neonatal sepsis in newborns (36).

Because S-fimbriated *E. coli* strains exert a receptor specificity for sialylated galactosides, one might speculate that HM, with its high content of components with these epitopes, is responsible for there being fewer infections observed in breast-fed infants than in bottle-fed ones. In 1987, Ashkenazi & Mirelman revealed that proteolytic digestion of the nonimmunoglobulin fraction of HM had no effect on the inhibitory activity of adherence of enterotoxigenic *E. coli* strains to the guinea pig intestinal tract whereas oxidization of the carbohydrates by sodium periodate led to a loss of inhibitory activity (5). Although there has been no definitive proof of an influence of HMO on microbial cell interactions, it is surprising that with each suckling the infant's orogastrointestinal

tract is rinsed with 100 to 500 mg of HMO. This large amount makes it very likely that HMO have local effects on the mucosal cell surface or even within the cell.

IMMUNOMODULATORY EFFECTS OF BREAST MILK OLIGOSACCHARIDES

Adhesion of leukocytes to the endothelium and extravasation at sites of inflammation are multistep processes. Low-avidity binding by siaylated and fucosylated oligosaccharides and members of the selectin (CD62) family results initially in slowing down (rolling) of passing leukocytes. This is then followed by firm adhesion and transmigration mediated by activated ß$_1$ and ß$_2$ integrins and members of the immunoglobulin supergene family, particularly VCAM-1 and ICAM-1. The migration of leukocytes such as neutrophils, lymphocytes, and monocytes to sites of inflammation is therefore determined by the expression of adhesion molecules, which in turn is regulated by signals mediated by surface costimulatory molecules and by cytokines (12, 15, 23, 48, 49, 54).

There is increasing evidence that the complex multicellular processes of inflammation, hemostasis, and thrombosis are closely linked. It has been established that inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) can induce a prothrombotic state. This results from increased monocyte and endothelial cell tissue factor expression and pathway activation and from reduced fibrinolytic activity secondary to increased levels of plasminogen activator inhibitor 1 (38). Conversely, key components of the coagulation pathways, such as thrombin, have now been shown to contribute to inflammation by inducing release of IL-6 and IL-8 from monocytes and endothelial cells.

Recently, it has become apparent that platelets are pivotal in regulating this hemostatic/inflammatory axis. Platelets express surface adhesion molecules responsible for primary hemostasis and accelerate the conversion of prothrombin to thrombin (4). In addition, platelets can secrete proinflammatory mediators such as IL-1 and express surface molecules capable of modulating inflammatory processes (e.g. CD40L).

The capacity for platelets to influence the hemostatic/inflammatory axis may rely on direct contact with inflammatory cells. Heterotypic aggregation of platelets with neutrophils (and other leukocytes) has been observed in vitro and in blood taken from healthy volunteers. These interactions are mediated by platelet CD62P expression and leukocyte β_2 integrins (38, 39). Indirect evidence that these complexes may themselves have a physiological function is provided by studies, which have shown changes in the numbers of or capacity to form these heterotypic cell complexes in clinical conditions, in which thrombosis and inflammation are prominent features. How platelet-leukocyte complexes contribute to these conditions has yet to be elucidated.

There is now good evidence that sialyl and fucosyl lactosamines such as sialyl Lewis x and sialyl Lewis y are critical epitopes during lectin-ligand binding steps. Similar carbohydrate structures or their precursors are abundant in breast milk and may therefore influence adhesive events in vivo (13, 53). In our current studies, all HMO samples were tested for their capacity to induce the endothelial adhesion molecules ICAM-1, VCAM-1, and E-selectin (15a). None was found to affect either resting endothelial cells or TNF-stimulated cells. Also, neutral oligosaccharides did not influence neutrophil adhesion at rest. However, when added to TNF-α-stimulated human umbilical vein endothelial cells, there was a dose-dependent inhibition of neutrophil adhesion. We did not detect any consistent changes in lymphocyte adhesion under these conditions. Adhesion in the presence of acidic oligosaccharides was usually minimal, although in some experiments there was enhancement of neutrophil adhesion.

An examination of the neutrophils from whole blood stained with CD42b-phycoerythrin and CD11b-fluorescein isothiocyanate allowed the detection of two populations of cells: free neutrophils and platelet-neutrophil complexes (PNCs). ADP stimulation increased the intensity of CD11b staining in both PNCs and free neutrophils. Addition of defatted breast milk led to a dose-dependent increase in PNC levels and an increase in neutrophil CD11b expression. This occurred in the presence or absence of ADP. Experiments using HMO also showed increased PNCs, although low levels of endotoxin were detected in the samples used. To summarize our present studies, we observed the following:

1. This study investigated the capacity for different breast milk fractions to influence the processes of adhesion involved in endothelial cell-leukocyte interactions and in platelet-neutrophil complex formation. Both adhesion models require selectins, integrins, and members of the immunoglobulin superfamily. We found that purified neutral oligosaccharides had the capacity to inhibit binding of neutrophils to TNF-stimulated endothelium. The model examined adhesion under low-flow conditions. Therefore, it is difficult to be certain exactly how these sugars were operating. It is probable that selectin-mediated adhesion was most affected, but other studies are required to examine this further.
2. We also found that defatted human milk and purified oligosaccharides enhanced the formation of PNCs. Again, both selectins and integrins are involved in PNC formation. How the oligosaccharides are mediating this effect is unclear. They may act to bridge CD62P to its ligands on neutrophils, but more probably, through the cross-linking of neutrophil selectins, they activate a signaling cascade that leads to increased integrin expression and activation. More work is required to understand these processes in more detail.
3. Our results indicate that human milk oligosaccharides can influence cellular adhesion, but further work is required before we can be confident that the oligosaccharides are operating alone and are not acting in concert

with nonoligosaccharide contaminants present in the samples as a result of the purification process.

METABOLIC ASPECTS OF OLIGOSACCHARIDES

Renal Excretion of Oligosaccharides in Infants

As reported above, several in vitro studies have shown that oligosaccharides are potent inhibitors of bacterial adhesion to epithelial surfaces, an initial stage of infective processes. A prerequisite for this mechanism to function in the urinary tract is the intestinal absorption and renal excretion of potentially active milk components.

Recent data obtained by high-pH anion exchange chromatography with pulsed amperometric detection in combination with fast atom bombardment-mass spectrometry (19) are in agreement with those of Lundblad, who found small oligosaccharides in urine of breast-fed infants (26). Besides 2′-fucosyllactose, 3-fucosyllactose, and difucosyllactose, other complex oligosaccharides, such as LNT, lacto-N-fucopentaose I and II, difucosyl-LNT, difucosyl-lacto-N-hexaose, sialyl-LNT, and disialyl-LNT were identified in HM-fed infants (44). Fucosylated oligosaccharides were virtually absent in urine of formula-fed infants, whereas sialylated components occurred in both groups. These results indicate that nutrition has a significant impact on the urinary oligosaccharide composition. Recently, Kunz et al verified these data by orally administering [^{13}C]Gal to lactating women. After in vivo labeling of lactose and oligosaccharides, they demonstrated that the [^{13}C] enrichment of the infants' urine is caused by very specific milk-derived oligosaccharides (20; see below).

The presence of oligosaccharides that are typical for HM in urine of breast-fed infants might be explained by intestinal absorption of intact oligosaccharides from breast milk, where they occur in major amounts. Assuming that HM is the source of specific urinary oligosaccharides, renal excretion of these components would account for ∼1% of the daily intake.

Studies with ^{13}C-Labeled Galactose in Lactating Mothers and Their Infants

As mentioned above, it seems likely that, among other components, milk oligosaccharides play an important role in an infant's defense against bacterial and viral adhesion, particularly within the gastrointestinal tract (17, 21, 53, 57). A prerequisite for systemic effects is that milk oligosaccharides are absorbed and distributed via the blood to different organs and cells. The availability of suitable methods of studying oligosaccharide metabolism in the infant would be a distinct advantage in attaining our goal of increased knowledge of this process.

Currently, a dietary influence on the biosynthesis of carbohydrates in the mammary gland is not considered to be of any importance. However, we hypothesized

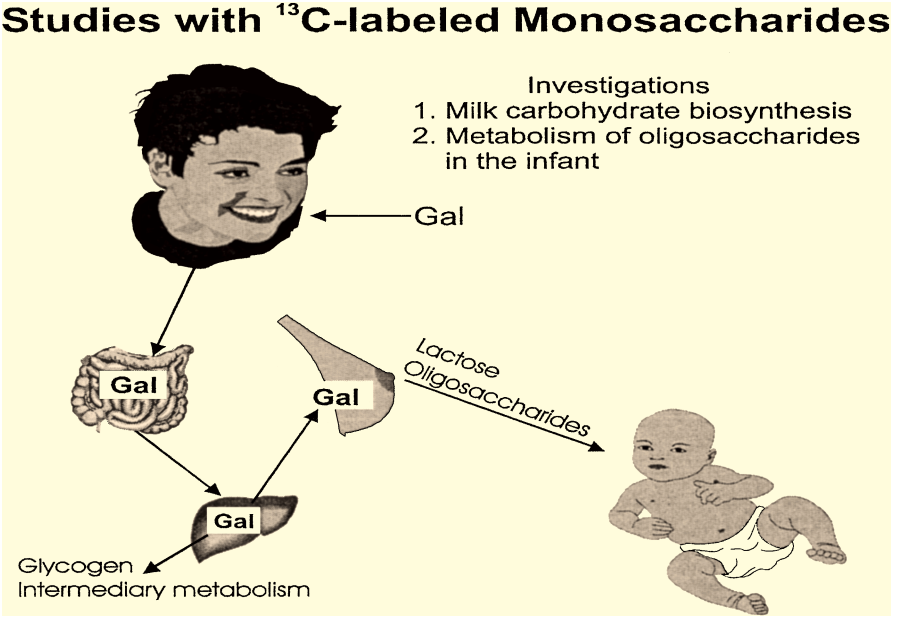

Figure 1 Scheme of our studies with ^{13}C-labeled monosaccharides ([^{13}C]Gal and [^{13}C]Glc) to investigate questions with regard to milk carbohydrate biosynthesis and oligosaccharide metabolism in the infant.

that lactating women have an exogenous demand for Gal because they may produce up to several liters of milk with a high lactose and oligosaccharide content per day. One of the major constituents of these carbohydrates is Gal and not Glc (6, 7; see Table 1 and 2).

Therefore, we investigated whether an oral administration of a ^{13}C-labeled Gal bolus to lactating mothers leads to a specific labeling of milk lactose and oligosaccharides in the human mammary gland (Figure 1). If the ^{13}C enrichment of milk carbohydrates would have been high enough, we should then have been able to follow some metabolic pathways of milk oligosaccharides in the infant (22a).

So far, 15 breast-feeding women (third to fourth month of lactation) received, immediately after their breakfasts, an oral Gal bolus (25 g of Gal plus 2 g of [^{13}C]Gal; D-Gal; 1-^{13}C, 99%). During the following 24–36 h, they collected 5–10 ml of milk at each nursing as well as urine (at 4-h intervals). Most of the mothers also collected breath at each nursing. The milk volume was determined by weighing the infants immediately before and after suckling. We also collected urine from two infants between 24 and 36 h after the Gal bolus was given to their mothers.

The ^{13}C enrichment in breath, urine, and whole milk and in milk fractions with fat, protein, and carbohydrates was determined after total combustion by

isotope ratio mass spectrometry (IR-MS) (Delta S; Finnigan, Bremen, Germany). To identify ^{13}C-labeled components, protein- and fat-free milk was separated into individual fractions by Sephadex G25 gel filtration chromatography and characterized by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), high-performance thin-layer chromatography (silica-HPTLC), fast atom bombardment mass spectrometry, and ^{13}C nuclear magnetic resonance spectroscopy (18, 20, 34).

To exemplify the potential of applicating stable isotopes to lactating mothers and monitoring the metabolic fate in their infants, we report the data from one mother-infant pair.

IR-MS (expressed as $\delta^{13}C_{PDB}$[‰]), which is the deviation from the baseline ratio of $^{13}C + {}^{12}C$ in the milk sample, corrected against the internationally used standard Per Dee Belemmite, of whole milk from all women revealed maximal ^{13}C enrichment during the first 2–8 h after the oral intake of [^{13}C]Gal, followed by a continuous decrease of $\delta^{13}C_{PDB}$[‰]. An example is given in Figure 2, which shows the $\delta^{13}C_{PDB}$ values for whole milk and for the milk fat, protein, and carbohydrate fractions of one woman. In milk, the maximum $\delta^{13}C_{PDB}$ was reached within 8 h after administration of the Gal bolus, and the value then rapidly declined in the following hours. The ^{13}C enrichment observed in milk carbohydrates was highest, followed by that in milk proteins. However, the high $\delta^{13}C_{PDB}$[‰] in the protein fraction is partly caused by the residual lactose. Only a small amount of ^{13}C enrichment was detectable in fat. The cumulative ^{13}C enrichment over the first peak was about 7% of the oral ^{13}C dose.

To identify ^{13}C-enriched components, the carbohydrate fraction was separated by Sephadex G25 gel filtration chromatography into individual subfractions, which were investigated by HPAEC-PAD, silica-HPTLC, and IR-MS. As can be seen in Figure 3, the highest $^{13}C_{PDB}$ values were found in lactose, followed by neutral and, to a lesser extent, acidic oligosaccharides.

^{13}C nuclear magnetic resonance spectroscopy of the isolated lactose from milk samples collected over a 36-h period after the Gal intake demonstrated that a ^{13}C atom was present only at the C_1 position of Gal and at the C_1 position of Glc. Moreover, in milk from some women, there seemed to be a preferential labeling of Gal compared with Glc immediately after Gal intake. Because the orally given [^{13}C]Gal was labeled only at the C_1 atom, the data indicate that a significant part of dietary Gal is directly transported to the lactating mammary gland without being metabolized by the liver (6, 7).

To answer the question of whether milk oligosaccharides are absorbed in the infant's intestinal tract, we collected urine from five infants and analyzed it by the method described above. In Figure 4, the ^{13}C enrichment in the milk of one mother and in the urine of her infant is shown. Compared with the milk, the maximal $\delta^{13}C_{PDB}$ of the infant's urine was delayed, with -10.7‰ as the highest ^{13}C enrichment (in mother's milk, 34.3‰).

To further investigate which urinary component contributed to the ^{13}C enrichment, deproteinated urine was separated by gel filtration into six fractions that were

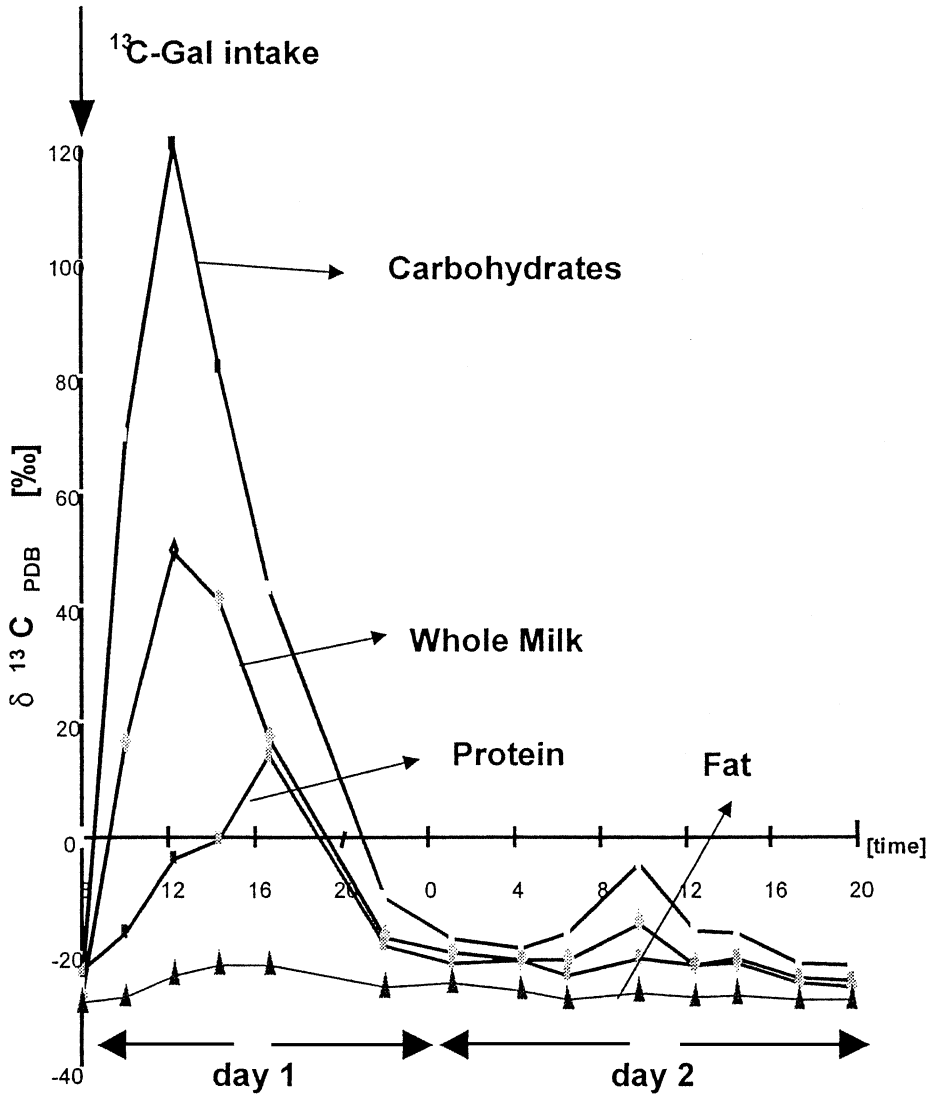

Figure 2 ^{13}C enrichment (expressed as $\delta^{13}C_{PDB}$[‰]) of whole milk and milk fractions from one woman during the first 36 h after administration of a single oral Gal bolus (25 g of Gal plus 2 g of [^{13}C]Gal; D-Gal; 1-^{13}C, 99%).

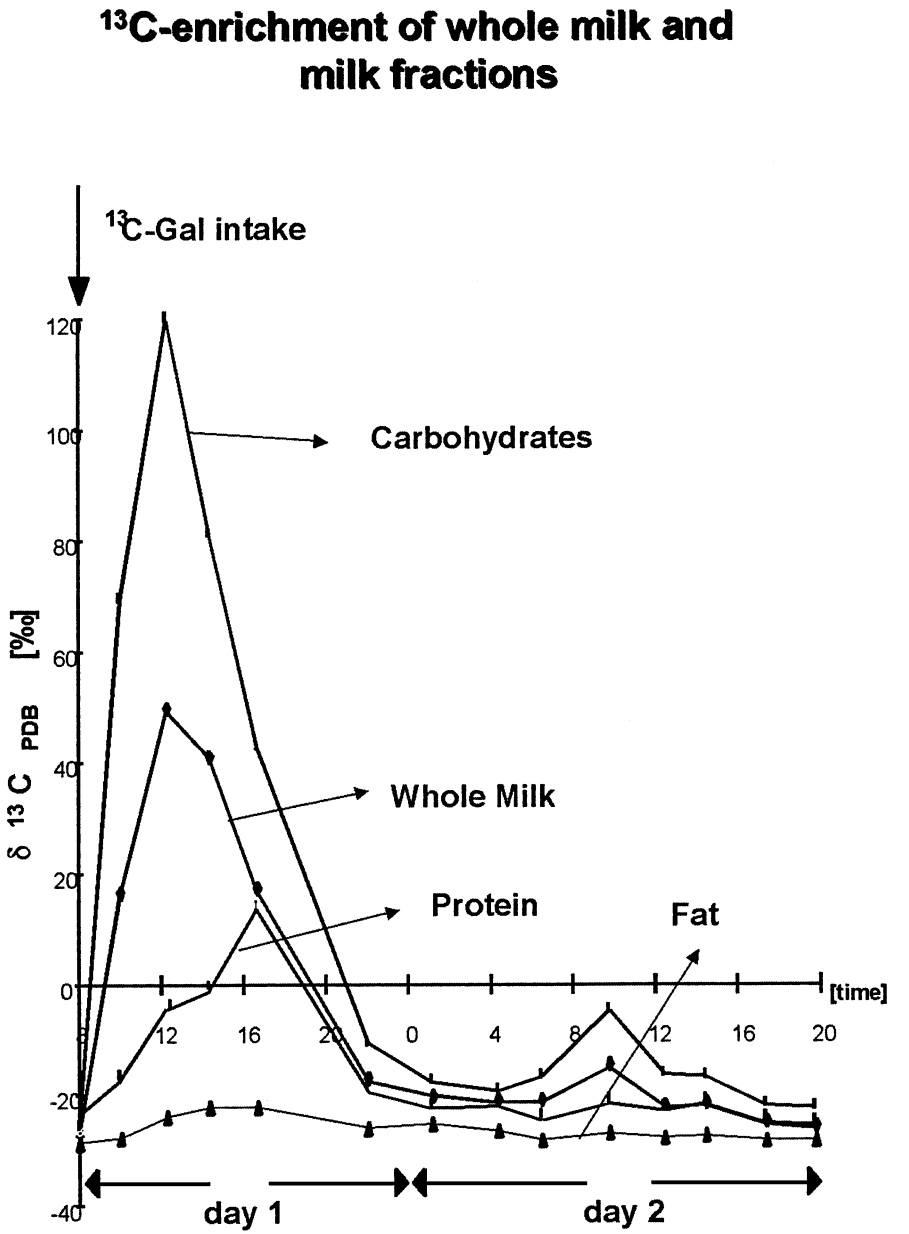

Figure 3 ^{13}C enrichment in fractions after Sephadex G25 chromatography of milk carbohydrates. $\delta^{13}C_{PDB}$ values are shown from the fractions with lactose and neutral and acidic oligosaccharides from 10 milk samples collected over a 32-h period after the Gal bolus was given.

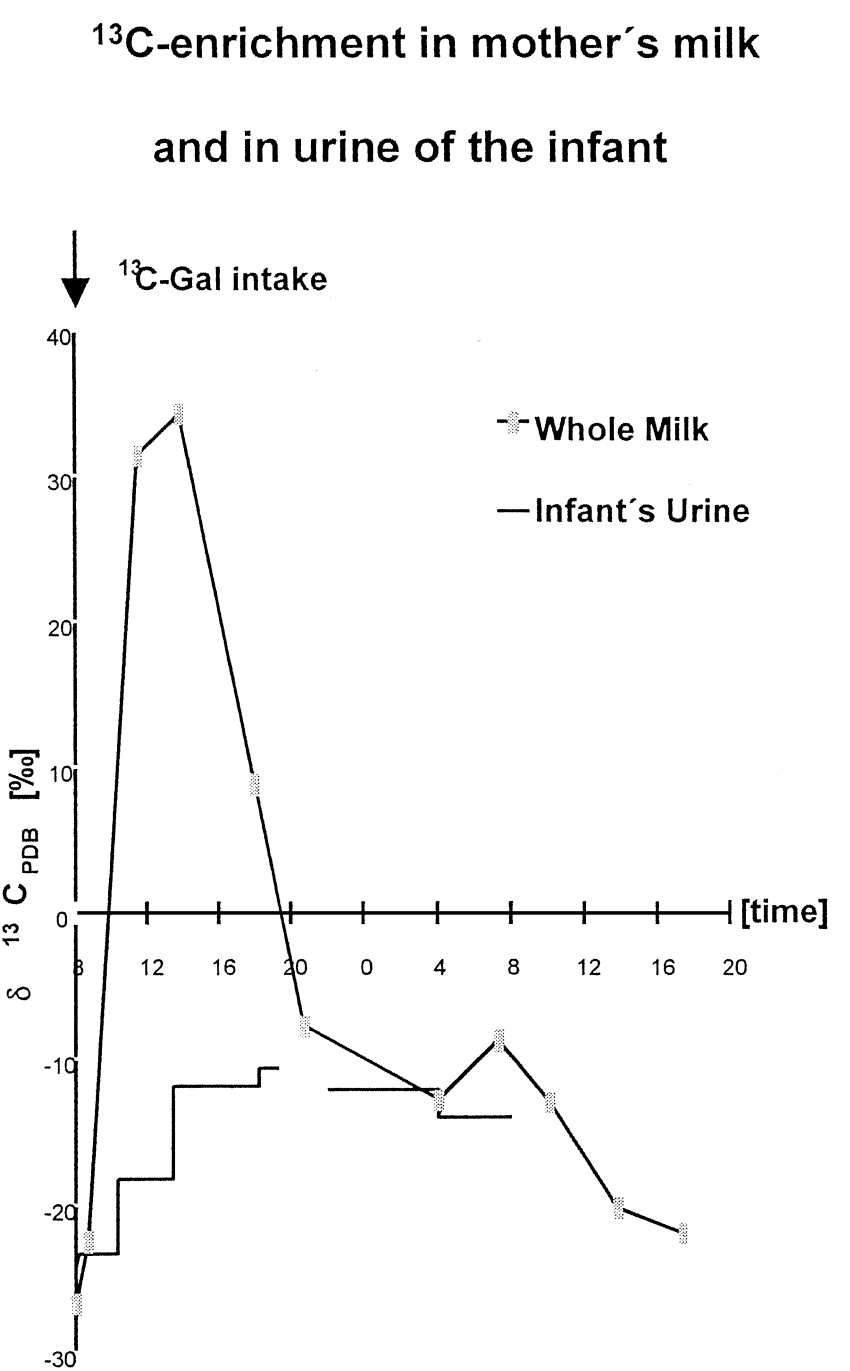

Figure 4 ^{13}C enrichment in mother's whole milk and in urine of her infant.

then subjected to IR-MS directly or after further chromatographic purifications. IR-MS analysis of lactose and these oligosaccharides revealed that the highest $\delta^{13}C_{PDB}$ value was found for lactose, followed by fucosyl lactose (Fuc-Lac), LNT, Fuc-LNT, and Fuc_2-Lac (listed in order of decreasing amounts of ^{13}C enrichment).

Our data demonstrated that part of the orally administered Gal was directly incorporated into milk lactose, neutral oligosaccharides, and acidic components. Moreover, there seemed to be a preferential labeling of Gal compared to Glc within the lactose moieties. The metabolic fate of in vivo-labeled lactose and oligosaccharides in the recipient infant is currently being investigated. So far, by determining the amount of specific oligosaccharides in milk and their excretion via urine in the infant, we found that ~0.5– 1.0% of the LNT and lacto-N-fucopentaose 1 could be detected in urine. The data confirm our previous studies comparing the urinary oligosaccharide profiles of breast-fed and bottle-fed infants (44). The conclusion is that some milk oligosaccharides are absorbed without being digested in the gastrointestinal tract. Hence, a variety of physiological effects of milk oligosaccharides may be possible not only locally in the infant's gastrointestinal tract but also after being absorbed. Therefore, an influence of oligosaccharides on leukocyte endothelial interactions may be of significant importance with regard to immunological reactions.

Oligosaccharides and Brain Development

The biological significance of the unique oligosaccharide composition of HM remains to be elucidated. In addition to their possible functions in neonatal host defense and inflammatory events, oligosaccharides, along with lactose, may play a role in postnatal brain development (7). Many newborn mammals undergo a period of rapid postnatal brain development that requires large amounts of glycolipids, which are components of the cell membranes of neurons and myelin.

Galactocerebroside, with Gal as its polar head group, is the predominant glycolipid in myelin. The liver may not be capable of providing all the Gal needed by the young mammal during this period of myelination and brain development. Thus, a possible role of milk oligosaccharides in which Gal is a main component is ensuring that Gal levels in the infant do not become limiting during this time. A prerequisite for this mechanism is that oligosaccharides are not completely excreted via feces, but are to some extent absorbed in the digestive tract, as we discussed above. The conclusion that can be drawn from these studies is that some oligosaccharides were absorbed without being digested. Therefore, one cannot exclude the possibility that beside their local action, oligosaccharides may have systemic effects as well, e.g., on brain glycoconjugate composition. This suggestion was confirmed by Carlson & House (8), who compared the effects of intraperitoneal and intragastric applications of NeuAc on rat brain composition. They found that both the oral and intraperitoneal routes resulted in significantly more cerebral and cerebellar glycolipid and glycoprotein NeuAc than did glucose injection. Furthermore, the advantage of oral dose of NeuAc-lactose, the major acidic fraction in

HM, over application of free NeuAc for brain composition in rats has been shown by Witt et al (55). A further indication that dietary carbohydrates may be important for normal brain composition is the observation that in patients with classic galactosemia, exogenous Gal may be important for the maintenance of a correct ratio of UDP-Glc to UDP-Gal in some cells (11). The impairment of UDP-Gal concentration in affected subjects could be, in parts, responsible for the altered biosynthesis of brain glycolipids in these subjects (40).

Because the oligosaccharide pattern in the milk of elephants is even more complex than that of human milk, it is fascinating that the two species show similar patterns of postnatal ontogeny; they both grow slowly, have relatively large and highly developed central nervous systems developing mainly after birth, are highly intelligent, and exhibit a high degree of learned behavior (22). The degree of encephalization is considerably higher in humans and elephants than in nonhuman primates (e.g. rhesus monkeys), reflecting the differences in milk oligosaccharide concentrations. Thus, we speculate that lactose-derived oligosaccharides, and in particular their Gal moieties, may play a role in the development of the infant brain.

IMPLICATIONS FOR FUTURE RESEARCH

It is well known that HM contains a variety of immunological and anti-inflammatory components. Recent studies indicated that lactose-derived oligosaccharides could function as protective factors on a nonimmunological basis. Although it is certainly necessary to clearly identify the components of nonspecific immune defense, HM oligosaccharides could have an influence through various mechanisms. First, they affect the growth of the intestinal flora and second, they prevent the attachment of pathogenic microorganisms to intestinal cells by acting as receptor analogues of mucosal adhesion molecules. In addition to this, they might function as ligands for selectins influencing inflammatory processes by reducing leukocyte binding to endothelial cells.

Recent results confirm that lactose-derived oligosaccharides in infants survive the gastrointestinal passage and the absorptive processes and are excreted via urine. Therefore, some carbohydrate epitopes from HM might circulate in the infant's blood for a period of time before they are excreted. Hence, the prevention of inflammatory bowel diseases or their chronic manifestation in breast-fed infants by carbohydrate interactions with mucosal leukocytes might be possible.

Today, extensive efforts are made to develop new drugs based on carbohydrates with nonimmunological functions. These are investigated with the aim to decrease the interactions of, for example, leukocytes with endothelial cells, thereby preventing an overreaction during inflammation. Further studies are needed to investigate the influence of carbohydrate epitopes from HM on the interaction of leukocytes with endothelial cells and on the attachment of pathogenic microorganisms to the intestinal cell. With this in mind, the current discussion on supplementation of infant formulas with oligosaccharides could be substantiated by studies looking at

the functions of individual components and at the metabolism of in vivo-labeled oligosaccharides by administering stable isotopes to lactating mothers and/or their infants.

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