Review

Structures and application of oligosaccharides in human milk

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Abstract: Comparative study of the oligosaccharide profiles of individual human milk revealed the presence of three different patterns. Four oligosaccharides containing the Fucα1-2Gal group were missing in the milk of non-secretor, and three oligosaccharides containing the Fucα1-4GlcNAc group were missing in the milk of Lewis negative individuals. Disappearance of some major oligosaccharides in these samples led to the finding of five novel minor oligosaccharides, which were hidden under the missing oligosaccharides. Following these studies, structures of many novel milk oligosaccharides were elucidated. At least 13 core oligosaccharides were found in these oligosaccharides. By adding α-fucosyl residues and sialic acid residues to these core oligosaccharides, more than one hundred oligosaccharides were formed. All these oligosaccharides contain lactose at their reducing termini. This evidence, together with the deletion phenomena found in the milk oligosaccharides of non-secretor and Lewis negative individuals, suggested that the oligosaccharides are formed from lactose by the concerted action of glycosyltransferases, which are responsible for elongation and branching of the Galβ1-4GlcNAc group in the sugar chains of glycoconjugates on the surface of epithelial cells. Therefore, oligosaccharides in human milk could include many structures, starting from the Galβ1-4GlcNAc group in the sugar chains of various glycoconjugates. Many lines of evidence recently indicated that virulent enteric bacteria and viruses start their infection by binding to particular sugar chains of glycoconjugates on the target cell surfaces. Therefore, milk oligosaccharides could be useful for developing drugs, which inhibit the infection of bacteria and viruses.

Keywords: blood types, Bifidus factor, enteric bacteria, human, milk, oligosaccharides

Introduction

Milk contains various oligosaccharides in addition to glycoproteins, glycopeptides and glycolipids, many of which are found specifically in this particular secretion. These components occur in large amounts in the milk secreted at early stages of lactation. Among them, casein and lactose are important nutritional ingredients, while IgA works as a component of transfer immunity for babies.[1] Lactoferrin plays a role to suppress the growth of bacteria by binding to the iron in milk.[2,3]

However, the physiological roles of other minor components, such as oligosaccharides in milk, are mostly unknown. Among the milk of mammals, human milk is unique because it contains a large number of oligosaccharides.

In 1933, Polonovski and Lespagnol found a new oligosaccharide containing nitrogen in human milk, and named it "gynolactose".[4] Over two decades later, Polonovski and Montreuil found that gynolactose is actually a mixture of more than ten oligosaccharides, and nitrogen is included only in part of them.[5]

Independent to their study, Kuhn's group in the Max Planck Institute in Heidelburg reached to the

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Abbreviations: 2′-FL: 2′-fucosyllactose; 3-FL: 3-fucosyllactose; Fuc: L-fucose; Gal: D-galactose; 6′-GalL: 6′-galactosyllactose; GalNAc: N-acetyl-D-galactosamine; Glc: D-glucose; GlcNAc: N-acetyl-D-glucosamine; LD: lactodifucotetraose; Le^a: Lewis a; Le^b: Lewis b; Le^x: Lewis x; LND-I: lacto-N-difucohexaose I; LND-II: lacto-N-difucohexaose II; LNF-I: lacto-N-fucopentaose I; LNF-II: lacto-N-fucopentaose II; LNF-III: lacto-N-fucopentaose III; LNF-V: lacto-N-fucopentaose V; LNH: lacto-N-hexaose; LNnH: lacto-N-neohexaose; LNT: lacto-N-tetraose; LNnT: lacto-N-neotetraose; Neu5Ac: N-acetylneuraminic acid; Type I chain: Galβ1-3GlcNAcβ1-; Type II chain: Galβ1-4GlcNAcβ1-.

Table 1.　Structures of human milk oligosaccharides found until 1965

Names of oligosaccharides	Structures	References
2′-Fucosyllactose (2′-FL)	Galβ1-4Glc 2 ↑ Fucα1	13
3-Fucosyllactose (3-FL)	Galβ1-4Glc 3 ↑ Fucα1	14
Lactodifucotetraose (LD)	Galβ1-4Glc 2 3 ↑ ↑ Fucα1 Fucα1	15
Lacto-*N*-tetraose (LNT)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc	16
Lacto-*N-neo*tetraose (LN*n*T)	Galβ1-4GlcNAcβ1-3Galβ1-4Glc	17
Lacto-*N*-fucopentaose I (LNF-I)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 2 ↑ Fucα1	18
Lacto-*N*-fucopentaose II (LNF-II)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 ↑ Fucα1	19
Lacto-*N*-difucohexaose I (LND-I)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 2 4 ↑ ↑ Fucα1 Fucα1	20
Lacto-*N*-difucohexaose II (LND-II)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 3 ↑ ↑ Fucα1 Fucα1	21
3′-Sialyllactose (3′-SL)	Galβ1-4Glc 3 ↑ Neu5Acα2	22
6′-Sialyllactose (6′-SL)	Galβ1-4Glc 6 ↑ Neu5Acα2	23
LST-a	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 3 ↑ Neu5Acα2	24
LST-b	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 6 ↑ Neu5Acα2	24
LST-c	Galβ1-4GlcNAcβ1-3Galβ1-4Glc 6 ↑ Neu5Acα2	25

same conclusion by investigating the chemical entity of Bifidus factor.

It has been known since 1900 that the feces of breast-fed babies are more acidic than those of artificially nourished babies.[6),7)] These papers drew widely scientist's notice, when Grulee *et al.* reported that breast-feeding was strongly associated with a lower incidence of diarrhea, otitis media, and respiratory diseases[8)] in suckling babies. Paul György, who was the Professor of Pediatrics in Pennsylvania University, considered that this is because *Lactobacillus bifidus* becomes a predominant intestinal flora of babies fed with human milk. This bacterium digests lactose, and produces large amounts of lactic acid and acetic acid. The acidic condition in the intestine of babies suppresses the growth of many other microorganisms, and may protect babies from harmful intestinal infection.

Schönfeld[9)] reported that the whey fraction of human milk contains a growth-promoting factor for *Lactobacillus bifidus* var. pennsylvanics, and named it **Bifidus factor**. In collaboration with György, Kuhn started a series of systematic investigations to elucidate the chemical entity of Bifidus factor,[10)–12)] and found many oligosaccharides in human milk.

Until 1965, structures of fourteen oligosaccharides shown in Table 1 were determined by Kuhn's group and Montreuil's group.[13)–25)]

All of these oligosaccharides contain lactose at their reducing termini. An interesting finding was

that some of these oligosaccharides showed haptenic activities of blood group determinants:[21),26)] 2′-FL and LNF-I showed haptenic activity of H determinant, LND-I showed activity of Le[b] determinant, and LNF-II showed activity of Le[a] determinant. These findings of human milk oligosaccharides significantly contributed to the elucidation of the structures of H and Lewis blood group determinants.[27)]

Elucidation of the biochemical basis of non-secretor phenomenon and Lewis-negative phenomenon based on the finding of correlation of the occurrence of fucose-containing oligosaccharides with blood types of the donors

In 1967, Grollman and Ginsburg[28)] found an interesting fact that 2′-FL was not detected in the milk samples obtained from individuals of non-secretor blood type. Non-secretor individuals express ABO blood types on the surface of their erythrocytes according to their genetic background of ABO locus, but not in the glycoproteins secreted from the epithelial cells of mucous glands. In order to extend this interesting finding further, Kobata et al.[29)] devised a new technique to fingerprint the oligosaccharides by using small amount of milk samples. As shown in Fig. 1A, the fourteen milk oligosaccharides in Table 1 were successfully fractionated by using approximately 10 ml of milk samples.

By analyzing around 50 human milk samples, three types of oligosaccharide profiles were found to occur in human milk. Approximately 80% of human milk contained all fourteen oligosaccharides as shown in Fig. 1A. Approximately 15% of human milk lacked four oligosaccharides as shown in Fig. 1B, and remaining 5% lacked three oligosaccharides as shown in Fig. 1C. The missing oligosaccharides were indicated in Fig. 1B and C by dotted lines. The small grey spots, detected at the positions of missing oligosaccharides, are minor oligosaccharides hidden under the major oligosaccharides.

Important evidence was that all mothers whose milk gave the profile Fig. 1B were non-secretors, and
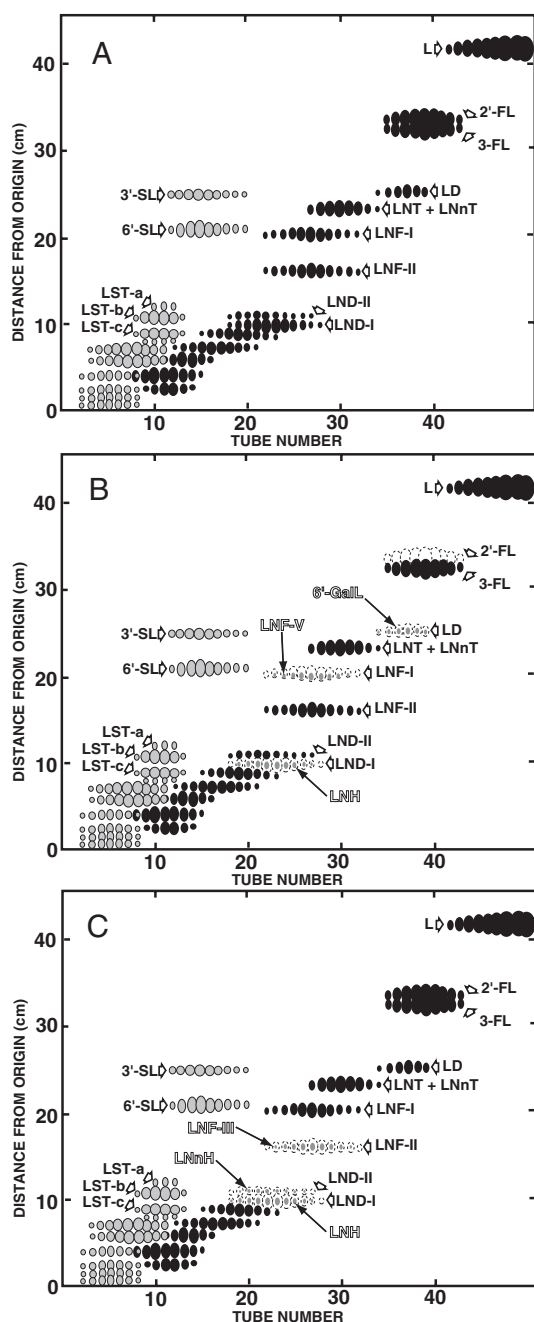

Fig. 1. Three typical fingerprinting patterns obtained from the oligosaccharide fraction of milk sample, collected from a single donor. The fraction numbers as indicated by "TUBE NUMBER" in abscissa were obtained by Sephadex G-25 column chromatography of human milk oligosaccharide fraction. Aliquot of the fractions were spotted at the origin of a sheet of a filter paper, and subjected to chromatography using ethylacetate/pyridine/acetic acid/water (5:5:1:3) as solvent. Black spots represent oligosaccharides visualized by alkaline-AgNO₃ reagent, and hatched ones encircled by black line represent those detected by both alkaline-AgNO₃ reagent and thiobarbituric acid reagent. Spots shown by dotted lines were missing in the pattern. Grey spots detected at the positions of missing oligosaccharides are minor oligosaccharides hidden under the major oligosaccharides. A, the pattern obtained from milk samples of Le[a+b+] individuals; B, the pattern obtained from milk samples of Le[a+b−] (non-secretor) individuals; C, the pattern obtained from milk samples of Le[a−b−] individuals. Revised from the figure in Glycoconj. J. **17**, 443–464 (2000) with kind permission from Springer Science+Business media.

Table 2. Structures of ABO blood group antigenic determinants

H Antigenic Determinant [*]	Galβ1-3/4GlcNAcβ1- 2 \| Fucα1
A Antigenic Determinant	GalNAcα1-3Galβ1-3/4GlcNAcβ1- 2 \| Fucα1
B Antigenic Determinant	Galα1-3Galβ1-3/4GlcNAcβ1- 2 \| Fucα1

*Those containing Galβ1-3GlcNAcβ1- and Galβ1-4GlcNAcβ1- are discriminated by the name of H-1 and H-2, respectively.

Table 3. Contents of P_I and P_{II} in human milk

Donors	Blood type	Secretor status	Oligosaccharides	
			P_I	P_{II}
			nmol/ml milk	
B.J.	A_2	secretor	13	4
E.K.	A_2	secretor	3	2
E.W.	A_1	secretor	0.4	0.2
M.C.	A_1	secretor	8	4
M.R.	A_1	secretor	0.2	0.3
K.C.	A_1	secretor	6	1
B.B.	A_1	non-secretor	<0.1	<0.1
H.S.	O	secretor	<0.1	<0.1
M.S.	B	secretor	<0.1	<0.1

those who gave the profile Fig. 1C were all Lewis negative, lacking both Le^a and Le^b antigens.

The four oligosaccharides: 2′-FL, LD, LNF-I, and LND-I, which were missing in the milk of non-secretor individuals, contain the Fucα1-2Gal group in common. Namely, the secretory organs of non-secretor individuals probably lack the fucosyltransferase responsible for formation of the disaccharide group. The three oligosaccharides: LNF-II, LND-I, and LND-II, which were missing in Lewis negative individuals, all contain the Fucα1-4GlcNAc group, indicating that these mothers may lack another fucosyltransferase responsible for formation of the Fucα1-4GlcNAc group.

These estimations were proven by enzymatic studies.[30),31)]

Finding of minor oligosaccharides, which contain blood group A determinant, in human milk

In Table 2, the structures of H antigenic determinant, which is the major antigen of human O-blood type, and blood type A and B antigenic determinants are summarized. As easily speculated from the structures of the three antigenic determinants, blood types A and B antigenic determinants are formed by adding GalNAc residue and Gal residue to H antigenic determinant, respectively. Although oligosaccharides containing blood type A and B antigenic determinants were not included among the milk oligosaccharides listed in Table 1, an α-N-acetylgalacosaminyltransferase (A-enzyme) and an α-galactosyltransferase (B-enzyme) were found to occur in the milk of blood type A and B individuals, respectively.[32),33)] These enzymes catalyze the addition of an α-GalNAc residue and an α-Gal residue to the C-3 position of the Gal residue of the Fucα1-2Gal group, respectively. GalNAcα1-3(Fucα1-2)Galβ1-

4Glc and GalNAcα1-3(Fucα1-2)Galβ1-3GlcNAcβ1-3Galβ1-4Glc, which were formed respectively from 2′-FL and LNF-I by the catalysis of partially purified A-enzyme, showed haptenic activity of blood type A.[34)] Occurrence of these haptenic tetrasaccharide (P_I) and hexasaccharide (P_{II}) in the milk samples of blood type A secretor individuals was confirmed as summarized in Table 3. The amounts of both haptenic oligosaccharides in blood type A milk samples varied by individuals from 0.2 to 13 nmol per ml of milk. These values are from one-hundredth to one-thousandth of those for LNT, LNF-I and LNF-II.

Structures of the minor oligosaccharides detected in the milk samples obtained from non-secretor individuals and Lewis negative individuals

As named by white letters in Fig. 1B and C, five novel oligosaccharides in total were found in the milk samples of non-secretor and Lewis negative individuals. Structures of these oligosaccharides were elucidated as summarized in Table 4.[35)–39)]

Among them, LNF-III served as an important haptenic oligosaccharide to investigate the functional role of Le^x antigen.[40)] LNH and LNnH served as useful models of branched core structures in the later studies of many blood group related antigens.[41),42)]

Before the structures of these oligosaccharides were elucidated, lactose, LNT and LNnT were considered as the cores of human milk oligosaccharides. Branched hexasaccharides: LNH and LNnH were newly added as cores, and a large number of fucosyl and sialyl derivatives of these newly added cores were found in human milk as shown in Tables 5–7.[38),43)–55)]

Table 4. Novel oligosaccharides isolated from milk of non-secretor and Lewis negative individuals

Names	Structures	References
6 '-Galactosyllactose (6' -GalL)	Galβ1-6Galβ1-4Glc	35
Lacto-*N*-fucopentaose V (LNF-V)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 3 Fucα1	36
Lacto-*N*-fucopentaose III (LNF-III)	Galβ1-4GlcNAcβ1-3Galβ1-4Glc 3 Fucα1	37
Lacto-*N*-hexaose (LNH)	Galβ1-4GlcNAcβ1 ＼ 　　　　　　　6 Galβ1-4Glc Galβ1-3GlcNAcβ1 ／ 3	38
Lacto-*N-neo*hexaose (LN*n*H)	Galβ1-4GlcNAcβ1 ＼ 　　　　　　　6 Galβ1-4Glc Galβ1-4GlcNAcβ1 ／ 3	39

Table 5.   Human milk oligosaccharides with lactose, LNT and LN*n*T as cores

Cores	Names	Structures	References
Lactose			
	3' -Galactosyllactose (3' -GalL)	Galβ1-3Galβ1-4Glc	43
	3-Fucosyl-3' -SL (3F-3' -SL)	Neu5Acα2-3Galβ1-4Glc 3 Fucα1	44
LNT			
	Sialyl-LNF-II (S-LNF-II)	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 Fucα1	45
	Sialyl-LNF-I (S-LNF-I)	Neu5Acα2 | 6 Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc	45
	Disialyl-LNT (DS-LNT)	Neu5Acα2 | 6 Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	46
	Disialyl-LNF-II (DS-LNF-II)	Neu5Acα2 | 6 Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 Fucα1	47
	Disialyl-LNF-V (DS-LNF-V)	Neu5Acα2 | 6 Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc 3 Fucα1	47
LN*n*T			
	Lacto-*N-neo*-difucohexaose II (L*n*ND-II)	Galβ1-4GlcNAcβ1-3Galβ1-4Glc 3　　　　　3 Fucα1　　Fucα1	43
	3-Fucosyl-LST c (3F-LST c)	Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc 3 Fucα1	48

Table 6. Human milk oligosaccharides with LNH as a core. Numbers in the parentheses indicate those of references

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Galβ1-4GlcNAcβ1\                    Neu5Acα2                           Fucα1                              Fucα1
               6Galβ1-4Glc                |                                |                                  |
Galβ1-3GlcNAcβ1/3                         6                                3                                  3
2           (42)          Galβ1-4GlcNAcβ1\              Galβ1-4GlcNAcβ1\              Galβ1-4GlcNAcβ1\
|                                        6Galβ1-4Glc                 6Galβ1-4Glc                 6Galβ1-4Glc
Fucα1                     Galβ1-3GlcNAcβ1/3           Galβ1-3GlcNAcβ1/3           Galβ1-3GlcNAcβ1/3
                                          (38)         6          (50)        3   4          (53)
                                                       |             Neu5Acα2  Fucα1
                                                     Neu5Acα2
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        Fucα1                  Neu5Acα2                    Fucα1                   Neu5Acα2
          |                      |                          |                       |
          3                      6                          3                       6
Galβ1-4GlcNAcβ1\     Galβ1-4GlcNAcβ1\          Galβ1-4GlcNAcβ1\        Galβ1-4GlcNAcβ1\
              6Galβ1-4Glc      6Galβ1-4Glc                6Galβ1-4Glc              6Galβ1-4Glc
Galβ1-3GlcNAcβ1/3    Galβ1-3GlcNAcβ1/3         Galβ1-3GlcNAcβ1/3       Galβ1-3GlcNAcβ1/3
         (51)        3   (54)                  3   (50)                2   4   (50)
                     |                         |                       |   |
                   Neu5Acα2                  Neu5Acα2                 Fucα1 Fucα1
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        Fucα1                                              Galβ1-4GlcNAcβ1\                 Fucα1
          |                                                              6Galβ1-4Glc          |
          3                                                Galβ1-3GlcNAcβ1/3                   3
Galβ1-4GlcNAcβ1\          Galβ1-4GlcNAcβ1\                 3   4   (53)            Galβ1-4GlcNAcβ1\
              6Galβ1-4Glc              6Galβ1-4Glc         |   |                                6Galβ1-4Glc
Galβ1-3GlcNAcβ1/3        Galβ1-3GlcNAcβ1/3              Neu5Acα2 Fucα1            Galβ1-3GlcNAcβ1/3
2           (42)         6                                                        3   6   (49)
|                        |               (54)                                     |   |
Fucα1                  Neu5Acα2 Neu5Acα2                                        Neu5Acα2 Neu5Acα2
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        Fucα1                                              Neu5Acα2                          Fucα1
          |                                                  |                                  |
          3                                                  6                                  3
Galβ1-4GlcNAcβ1\          Neu5Acα2                Galβ1-4GlcNAcβ1\              Galβ1-4GlcNAcβ1\
              6Galβ1-4Glc    |                                6Galβ1-4Glc                   6Galβ1-4Glc
Galβ1-3GlcNAcβ1/4            6                    Galβ1-3GlcNAcβ1/3           Galβ1-3GlcNAcβ1/3
4           (38)  Galβ1-4GlcNAcβ1\               4           (50)            3   6   (49)
|                             6Galβ1-4Glc        |                           |   |
Fucα1            Galβ1-3GlcNAcβ1/3              Fucα1                       Neu5Acα2 Neu5Acα2
                 3   6   (52)
                 |   |
               Neu5Acα2 Neu5Acα2
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        Fucα1                                              Neu5Acα2                          Neu5Acα2
          |                                                  |                                  |
          3                                                  6                                  6
Galβ1-4GlcNAcβ1\                                 Galβ1-4GlcNAcβ1\              Galβ1-4GlcNAcβ1\
              6Galβ1-4Glc                                    6Galβ1-4Glc                   6Galβ1-4Glc
Galβ1-3GlcNAcβ1/4                                Galβ1-3GlcNAcβ1/3           Galβ1-3GlcNAcβ1/3
2   4   (63)                                     2           (42)            3   4   (53)
|   |                                            |                           |   |
Fucα1 Fucα1                                    Fucα1                       Neu5Acα2 Fucα1
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For the structural studies of these novel milk oligosaccharides, many new sensitive analytical techniques, such as tritium-labeling,[56] sequential exoglycosidase digestion,[57),58] and sensitive methylation analysis suitable for the amino sugar-containing oligosaccharides[59] were established. These new techniques play important roles in the later structural study of the sugar chains of various glycoconjugates. In addition to the physicochemical analyses such as NMR and mass-spectrometry, recycling chromatography devised by Donald *et al.*[43] was found to be effective for the study of milk oligosaccharides.

Looking at the structures of these oligosaccharides silhouettes several interesting structural rules. As shown in Table 6, the Fucα1-2Gal group is not evenly distributed to the two Galβ1-GlcNAc groups of the branched arms of LNH, but is rather limited to the arm extending from C-3 position of the branching Gal.[42] Finding of the Fucα1-3Gal group in a fucosylated LNH is also noteworthy.[49] This group is limited to the Type II chain in contrast to the Fucα1-2Gal group. Many fucosyltransferases have been cloned in recent years.[60] However, no enzyme to catalyze the formation of the Fucα1-3Gal group has been reported.

Several oligosaccharides containing both Fucα1-2Gal group and sialic acid were detected. It was confirmed that these oligosaccharides could not work as the substrate of A-enzyme (Kobata *et al.*, unpublished data). Therefore, sialylation of the sugar chains may inhibit the conversion of H-antigen to A-antigen.

Many novel cores were found in the human milk oligosaccharides

As described already, structural study of human milk oligosaccharides get started by the interest of their relation to blood group antigens. The development of research as introduced so far and the possibility of important functions of these oligosaccharides aroused the interest of many researchers, and the structures of remaining larger oligosaccharides were investigated. As shown in Table 8, we found four novel core oligosaccharides: *para*-Lacto-*N*-hexaose,[61] *para*-Lacto-*N-neo*hexaose,[61] Lacto-*N*-octaose,[62] and Lacto-*N-neo*octaose[62] in human milk. Other groups found three additional novel core

Table 7.   Human milk oligosaccharides with LNnH as a core

Structures	References
Fucα1 ― 3 ― Galβ1-4GlcNAcβ1 ―6 Galβ1-4Glc; Galβ1-4GlcNAcβ1 ―3, with Fucα1 ― 3 on lower branch	55
Neu5Acα2 ―6― Galβ1-4GlcNAcβ1 ―6 Galβ1-4Glc; Galβ1-4GlcNAcβ1 ―3	38
Neu5Acα2 ―6― Galβ1-4GlcNAcβ1 ―6 Galβ1-4Glc; Galβ1-4GlcNAcβ1 ―3, 6―Neu5Acα2	47
Fucα1 ―3― Galβ1-4GlcNAcβ1 ―6 Galβ1-4Glc; Galβ1-4GlcNAcβ1 ―3, 6―Neu5Acα2	44
Fucα1―2, Fucα1―3 on Galβ1-4GlcNAcβ1 ―6 Galβ1-4Glc; Galβ1-4GlcNAcβ1 ―3, 6―Neu5Acα2	44
Neu5Acα2 ―6(3)― Galβ1-4GlcNAcβ1 ―6 Galβ1-4Glc; Galβ1-4GlcNAcβ1 ―3, 6(3)―Neu5Acα2, 3―Fucα1	49

oligosaccharides: *iso*-Lacto-*N*-octaose,[63] *para*-Lacto-*N*-octaose,[55] and Lacto-*N*-decaose.[64] Quite recently, Amano *et al.*[65] reported the occurrence of an isomeric decasaccharide as another core in milk oligosaccharides. Therefore, at least 13 core oligosaccharides have been found for human milk oligosaccharides until now.

All of these core oligosaccharides contain lactose at their reducing termini. Based on this evidence, all human milk oligosaccharides are considered to be produced by the action of glycosyltransferases of epithelial cells, which are responsible for formation of the sugar chains of glycoproteins and glycolipids in the cells. Namely, by the action of iGnT[66] on lactose, which is synthesized in a large amount by the action of β4GalT-I and α-lactalbumin, GlcNAcβ1-3Galβ1-4Glc is formed as shown in Fig. 2. All linear core oligosaccharides might be formed by the concerted actions of β4GalT[67] and iGnT as shown in the left hand side of Fig. 3. The sugar chains, produced by the concerted action of the two glycosyltransferases, are all *neo*-type, which has only the repeating structures of the Galβ1-4GlcNAcβ1-3 group. However, when the Galβ1-3GlcNAcβ1-3 group is formed by the action of β3GalT[68] instead of β4GalT, extension of the sugar chain might stop there.

Core milk oligosaccharides with branched structures might be produced by the action of IGnT[69] as shown in Fig. 2, and in the right hand side of Fig. 3.

The thirteen core oligosaccharides, thus formed, are further elongated to the fucosylated and/or sialylated oligosaccharides by the actions of fucosyltransferases and/or sialyltransferases. In Tables 5–7, and Tables 9 and 10, all sialylated and fucosylated oligosaccharides, reported so far, are summarized. Many fucosyl derivatives of Lacto-*N*-decaose and its

Table 8.   Various core structures found in human milk oligosaccharides

Names	Structures	References
para-Lacto-*N*-hexaose	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc	61
para-Lacto-*N*-neohexaose	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc	61
Lacto-*N*-octaose	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1 ⁶\\ Galβ1-4Glc Galβ1-3GlcNAcβ1 / 3	62
Lacto-*N*-neooctaose	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1 ⁶\\ Galβ1-4Glc Galβ1-4GlcNAcβ1 / 3	62
iso-Lacto-*N*-octaose	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1 ⁶\\ Galβ1-4Glc Galβ1-3GlcNAcβ1 / 3	63
para-Lacto-*N*-octaose	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc	55
Lacto-*N*-decaose	Galβ1-4GlcNAcβ1 ⁶\\ Galβ1-4GlcNAcβ1 ⁶\\ Galβ1-3GlcNAcβ1 / 3 Galβ1-4GlcNAcβ1 Galβ1-4Glc Galβ1-3GlcNAcβ1 / 3	64
	Galβ1-4GlcNAcβ1 ⁶\\ Galβ1-4GlcNAcβ1 ⁶\\ Galβ1-4GlcNAcβ1 / 3 Galβ1-4GlcNAcβ1 Galβ1-4Glc Galβ1-3GlcNAcβ1 / 3	65
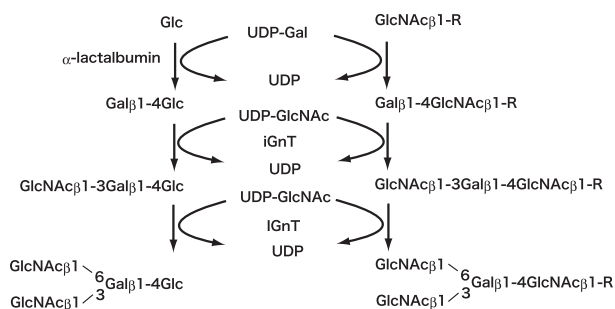
Fig. 2.   Elongation of lactose and *N*-acetyllactosamine group of the sugar chain of glycoconjugates by the action of iGnT and IGnT. R represents the aglycon moieties of glycoconjugates. Revised from the figure in Chang Gung Medical J. **26**, 620–636 (2003).

isomer were reported by Chai *et al.*,[76] and Amano *et al.*[65] Although only one tetra-fucosyl derivative was reported for *para*-Lacto-*N*-octaose,[55] many fucosyl and/or sialyl derivatives will be found for this core oligosaccharide in the future.

Important evidence is that Lacto-*N*-octaose and Lacto-*N*-neooctaose were not detected by mass-spectrometric analysis.[55] This evidence should be explained in the future for the sound development of mass-spectrometric methods.

Various problems related to the activity of Bifidus factor in milk

The problem of the chemical entity of Bifidus factor had been considered to be solved, when *Lactobaccilus bifidus* var. pennsylvanicus was found to request GlcNAc as a growth factor and only the milk oligosaccharides containing GlcNAc residue as Bifidus factor. However, the project is developing now to a new aspect by the finding that human milk oligosaccharides show a growth activity working specifically for various Bifidus strains as described below.

It was found that most of human milk oligosaccharides are not degraded by the action of exoglycosidases in digestive tract and reach to intestine.
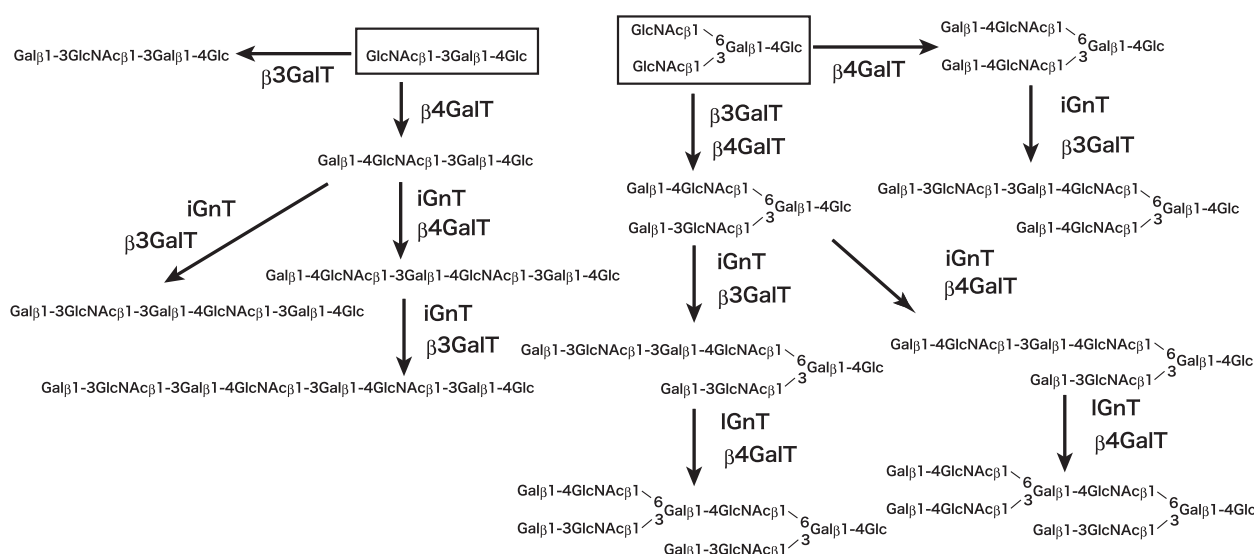
Fig. 3. Biosynthetic pathways leading to various core oligosaccharides from GlcNAcβ1-3Galβ1-4Glc and GlcNAcβ1-6(GlcNAcβ1-3)Galβ1-4Glc.

Because of this characteristics, these oligosaccharides are now classified as water-soluble fibers.[77]–[81] Accordingly, these oligosaccharides must first be digested to their monosaccharide constituents in order to be used by intestinal flora as nutrients. However, it is not so easy for bacteria in intestine to digest human milk oligosaccharides, because most of the β-galactosidases of intestinal bacteria hydrolyze the Galβ1-4GlcNAc group but not the Galβ1-3GlcNAc group, which amply occurs at the non-reducing termini of human milk oligosaccharides.[58]

The research group of Kenji Yamamoto found that a lacto-N-biosidase, reported by Sano et al.[82] in the culture fluid of Streptomyces sp. 142, was widely found in Lactobaccilus bifidus strains, but not in other intestinal bacteria.[83] This endoglycosidase effectively releases the Galβ1-3GlcNAc group from non-reducing termini of various oligosaccharides. They also found a transporter in the membrane of L. bifidus strains. This transporter specifically transports Galβ1-3GlcNAc from culture medium into cells.[84]

Meanwhile, Kitaoka et al.[85] found in the culture fluid of Bifidobacterium bifidum JCM1254 an enzyme, which converts the Galβ1-3GlcNAc to Galα1-PO4 and GlcNAc, and named it lacto-N-biose phosphorylase. An interesting evidence is that this enzyme also works on the Galβ1-3GalNAc, which is released from O-linked sugar chains by the action of endo-α-N-acetylgalactosaminidase,[86] and convert it

to Galα1-PO4 and GalNAc, but not on the Galβ1-4GlcNAc group. They successfully cloned the gene of this enzyme from Bifidobacterium longum JCM1217, and further found a series of genes of enzymes, which are responsible for the metabolism of monosaccharides constructing human milk oligosaccharides, in the operon containing the gene of lacto-N-biose phosphorylase. Based on this finding, they proposed the metabolic pathway of human milk oligosaccharides by the bacterium.

These results indicated that L. bifidus strains are equipped with a series of special catabolic mechanism, which specifically uses the oligosaccharides having the Galβ1-3GlcNAc group in their termini for metabolic sources. Therefore, human milk oligosaccharides, which are enriched in the Galβ1-3GlcNAc group in their non-reducing termini, specifically work as nutritional sources for L. bifidus strains.

A trial to apply human milk oligosaccharides as drugs to prevent bacterial and viral infection

As already described, human milk oligosaccharides are synthesized by the concerted action of a series of glycosyltransferases, which are responsible for formation of the sugar chains of glycoproteins and glycolipids produced by the epithelial cells of mammary gland. Accordingly, human milk oligosaccharides may be useful for elucidating the structures of potential ligands for cell adhesion molecules.[87] Actually, LNF-III was effectively used to elucidate the physiological role of Le[x] antigen.[40]

Table 9. Human milk oligosaccharides containing *para*-Lacto-*N*-hexaose (*para*-LNH) and *para*-Lacto-*N-neo*hexaose (*para*-LN*n*H) as cores

Cores	Structures	References
para-LNH	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc 3 | Fucα1	64
	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc 4 | Fucα1	64
	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc 4 3 | | Fucα1 Fucα1	61
	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc 2 4 3 | | | Fucα1 Fucα1 Fucα1	70
	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc 4 3 3 | | | Fucα1 Fucα1 Fucα1	64
	6S | Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc 2 3 | | Fucα1 Fucα1	71
	6S | Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc 4 3 | | Fucα1 Fucα1	71
	6S | Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc 2 4 3 | | | Fucα1 Fucα1 Fucα1	71
para-LN*n*H	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc 3 3 | | Fucα1 Fucα1	61
	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc 3 3 3 | | | Fucα1 Fucα1 Fucα1	64

It was known that the infection of many bacteria and viruses starts by binding to particular sugar chains of glycoconjugates on the surface of cells, which are constructing the mucous epithelium of digestive tracts and respiratory tracts. Therefore, human milk oligosaccharides might be useful for elucidating the structure of target sugar chain of each bacterium or virus on the surface of epithelial cells.
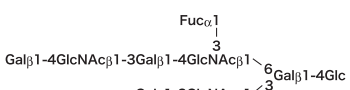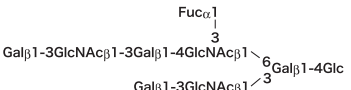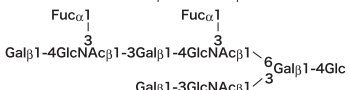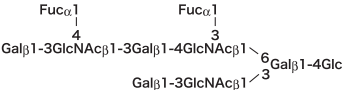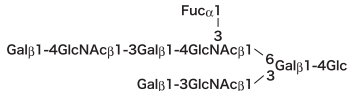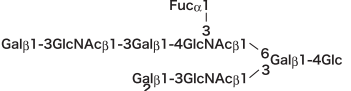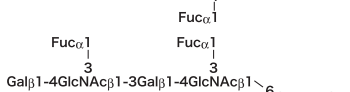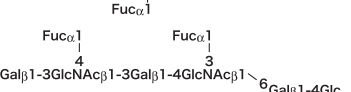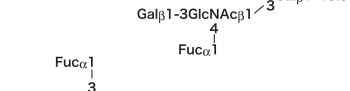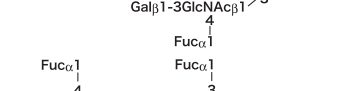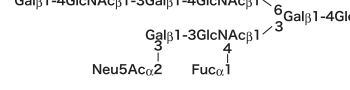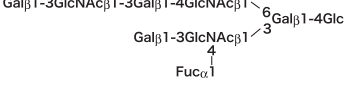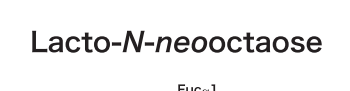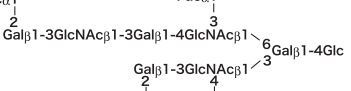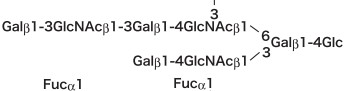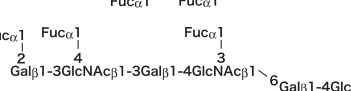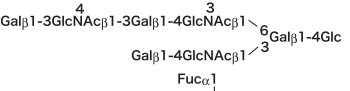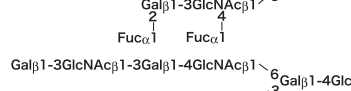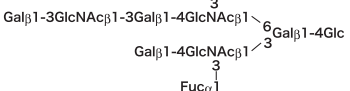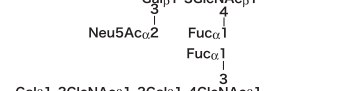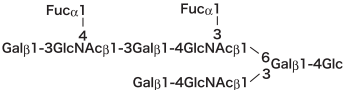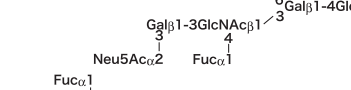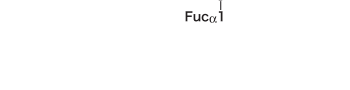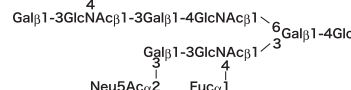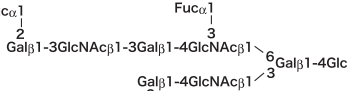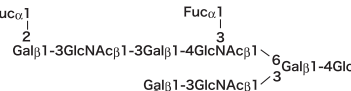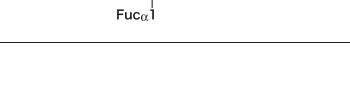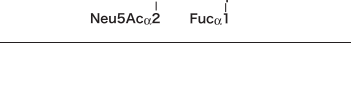
After entering into 1990th, several research groups started to report the phenomenon that some of the human milk oligosaccharides inhibit attachment of intestinal bacteria to the surface of the epithelial cells of intestine.[88)–90)] Based on these findings, Newburg started to investigate the possibility that human milk oligosaccharides may be useful for developing new drugs to protect suckling babies from bacterial and viral infections.[91)]

Newburg reviewed that addition of various oligosaccharides and glycoconjugates to milk prevents babies from infectious diseases.[92)]

One of the toxins produced by enterotoxigenic *E. coli* is called stable toxin (STa), because of its stability to organic solvent and heat. STa induces secretory diarrhea by activating guanylate cyclase of the surface of intestinal epithelial cells.[93)] It was known that T84 cells, established by Dharmsathaphorn, differentiate in culture and express various features of intestinal epithelium including formation of microvillae and transmembrane guanylate cyclase.[94)–96)]

Table 10. Human milk oligosaccharides containing Lacto-*N*-octaose, Lacto-*N-neo*octaose, and *iso*-Lacto-*N*-octaose as coses. The numbers in the parentheses indicate the numbers of references

Lacto-*N*-octaose

```
                      Fucα1
                        |
                        3
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (72)
                                  6Galβ1-4Glc
               Galβ1-3GlcNAcβ1 / 3
```

```
        Fucα1         Fucα1
          |             |
          3             3
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (62)
                                  6Galβ1-4Glc
               Galβ1-3GlcNAcβ1 / 3
```

```
                      Fucα1
                        |
                        3
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (62)
                                  6Galβ1-4Glc
               Galβ1-3GlcNAcβ1 / 3
                        4
                        |
                      Fucα1
```

```
        Fucα1         Fucα1
          |             |
          3             3
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (62)
                                  6Galβ1-4Glc
               Galβ1-3GlcNAcβ1 / 3
                        4
                        |
                      Fucα1
```

```
      Fucα1
        |
        3
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (75)
                                  6Galβ1-4Glc
               Galβ1-3GlcNAcβ1 / 3
                      3         4
                      |         |
                  Neu5Acα2    Fucα1
```

Lacto-*N-neo*octaose

```
                      Fucα1
                        |
                        3
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (62)
                                  6Galβ1-4Glc
               Galβ1-4GlcNAcβ1 / 3
```

```
        Fucα1         Fucα1
          |             |
          4             3
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (62)
                                  6Galβ1-4Glc
               Galβ1-4GlcNAcβ1 / 3
```

```
                      Fucα1
                        |
                        3
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (62)
                                  6Galβ1-4Glc
               Galβ1-4GlcNAcβ1 / 3
                        3
                        |
                      Fucα1
```

```
        Fucα1         Fucα1
          |             |
          4             3
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (63)
                                  6Galβ1-4Glc
               Galβ1-4GlcNAcβ1 / 3
                        3
                        |
                      Fucα1
```

```
    Fucα1             Fucα1
      |                 |
      2                 3
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (63)
                                  6Galβ1-4Glc
               Galβ1-4GlcNAcβ1 / 3
                        2
                        |
                      Fucα1
```

iso-Lacto-*N*-octaose

```
                      Fucα1
                        |
                        3
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (73)
                                  6Galβ1-4Glc
               Galβ1-3GlcNAcβ1 / 3
```

```
        Fucα1         Fucα1
          |             |
          4             3
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (74)
                                  6Galβ1-4Glc
               Galβ1-3GlcNAcβ1 / 3
```

```
                      Fucα1
                        |
                        3
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (74)
                                  6Galβ1-4Glc
               Galβ1-3GlcNAcβ1 / 3
                      2
                      |
                    Fucα1
```

```
        Fucα1         Fucα1
          |             |
          4             3
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (55)
                                  6Galβ1-4Glc
               Galβ1-3GlcNAcβ1 / 3
                        4
                        |
                      Fucα1
```

```
      Fucα1           Fucα1
        |               |
        4               3
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (73)
                                  6Galβ1-4Glc
               Galβ1-3GlcNAcβ1 / 3
                        4
                        |
                      Fucα1
```

```
Fucα1                 Fucα1
  |                     |
  2                     3
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (55)
                                  6Galβ1-4Glc
               Galβ1-3GlcNAcβ1 / 3
                      2         4
                      |         |
                    Fucα1     Fucα1
```

```
Fucα1  Fucα1          Fucα1
  |      |              |
  2      4              3
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (55)
                                  6Galβ1-4Glc
               Galβ1-3GlcNAcβ1 / 3
                      2         4
                      |         |
                    Fucα1     Fucα1
```

```
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (75)
                                  6Galβ1-4Glc
               Galβ1-3GlcNAcβ1 / 3
                      3         4
                      |         |
                  Neu5Acα2    Fucα1
```

```
                      Fucα1
                        |
                        3
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (75)
                                  6Galβ1-4Glc
               Galβ1-3GlcNAcβ1 / 3
                      3         4
                      |         |
                  Neu5Acα2    Fucα1
```

```
    Fucα1
      |
      4
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (75)
                                  6Galβ1-4Glc
               Galβ1-3GlcNAcβ1 / 3
                      3         4
                      |         |
                  Neu5Acα2    Fucα1
```

```
Fucα1                 Fucα1
  |                     |
  2                     3
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1\                     (54)
                                  6Galβ1-4Glc
               Galβ1-3GlcNAcβ1 / 3
                      3         4
                      |         |
                  Neu5Acα2    Fucα1
```

By using this cultured cells, Crane *et al.* found that a crude mixture of human milk oligosaccharides showed a dose-dependent inhibition for the STa-induced cGMP production.[97] The inhibition activity was expressed by the fucosylated oligosaccharide fraction, but not by the non-fucosylated oligosaccha-

ride fraction, which were obtained by passing through an immobilized *Ulex europaeus* column. More recently, Morrow *et al.* reported that only the milk oligosaccharides, containing the Fucα1-2Gal group at their non-reducing termini, showed the inhibitory activity.[98]

Ruiz-Palacios *et al.* found that adhesion of *Campyrobacter jejuni* to the surface of intestinal epithelia is inhibited by the human milk oligosaccharides, which contain the Fucα1-2Galβ1-4GlcNAc group (H-2 antigen) at their non-reducing termini.[99] They further confirmed that adhesion of *Campyrobacter jejuni* to H-2 antigen expressed on the surface of the epithelial cells of human small intestine is essential for its infection, by indicating that the bacterium cannot bind to the surface of CHO cells, but binds well to the surface of CHO cells transfected with human α1,2-fucosyltransferase gene.

According to Le Pandu,[100] non-secretor individuals are resistant to Norwalk virus (NV) infection. This evidence indicated that H-1 antigenic determinant expressed on the surface of the epithelial cells of human intestine is working as the receptor to trigger NV infection. Actually, it was found that NV infection was inhibited by milk obtained from secretor mothers, which are known to contain a large amount of oligosaccharides containing the H-1 antigenic determinant, but not by milk from non-secretor mothers.

In 2005, Perret *et al.*[101] found that PA-IIL, a lectin purified from *Pseudomonas aeruginosa*, binds specifically to 3-FL and human milk oligosaccharides containing Leᵃ antigenic determinant, and estimated that Leᵃ and Leˣ antigenic determinants on the surface of mucous epithelial cells are the target to start the infection of *P. aeruginosa*.

F18-fimbriated *E. coli* infection to weaned piglet induces diarrhea and dropsy. Its infection starts by binding FedF, which is included in F18-fimbria, to the wall of small intestine of piglet. Quite recently, Coddens *et al.*[102] found that the adhesion of this bacteria to the microvilli of piglet small intestine is inhibited by LNF-I, but not by LNT. Based on this finding, they investigated the structures of glycolipids in the mucosa of piglet small intestine, which bind to FedF, and showed that glycolipids containing either H-1 antigenic determinant or blood type A or B antigenic determinant of Type I chain bind strongly to FedF. Because H-2 and blood type A or B determinants of Type II chain cannot bind to FedF, they concluded that H-1 is the basic structure of
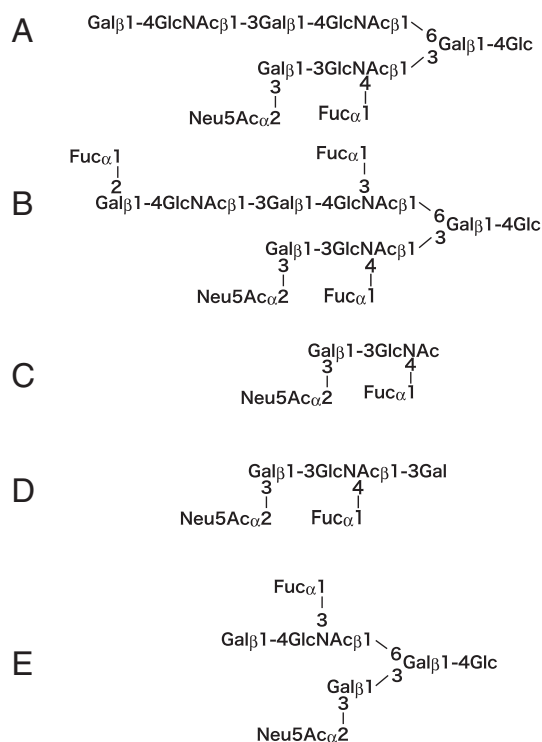

Fig. 4. Structures of milk oligosaccharides containing the sialyl-Leᵃ group (A–D), and three human milk oligosaccharides (C–E), which do not contain the known core oligosaccharides. Taken from the figure in Chang Gung Medical J. **26**, 620–636 (2003).

FedF ligand, and further addition of Gal or GalNAc at the C-3 position of the Gal residue of the ligand enhances the binding to FedF.

Future prospects

As shown by the data, so far introduced, studies of human milk oligosaccharides are expected to be very useful for the development of drugs, which are effective for protection of babies from harmful infections.

The key to lead to the success of this line of studies is how one will be able to develop an effective method to pick up minor but useful oligosaccharides from the mixture of a large number of different milk oligosaccharides.

Kitagawa *et al.* reported the structures of a series of human milk oligosaccharides, which were retained by an immobilized column of anti-CA19-9 antibody.[103] As shown by A–D in Fig. 4, they are oligosaccharides of various sizes containing the sialyl-Leᵃ determinant in common. Among them, two oligosaccharides, C and D, which occur as very minor components in the oligosaccharide fraction retained

by the column, have unusual features. They do not contain lactose, but contain either GlcNAc or Gal residue at their reducing termini.

Another unusual oligosaccharide (E in Fig. 4), which does not contain the core structures of milk oligosaccharides so far introduced, was isolated from human milk. However, this oligosaccharide also contains lactose at its reducing terminus. Therefore, the mechanism to produce oligosaccharides C and D, which lack lactose group, is totally unknown. They might be produced by an unknown degradation mechanism from larger milk oligosaccharides.

In any event, the report of Kitagawa *et al.* indicated that we would be able to pick up even a very minor oligosaccharide with particular ligand specificity, if a proper affinity chromatographic method is devised by isolating receptor proteins on the surface of bacteria and viruses. Perhaps, this can be performed in the near future by collaborating with the bacteriologists and virologists, who are investigating the virulent factors by cloning the surface lectins of bacteria and viruses.

The report of Perret *et al.*[101] can be considered as a pioneering work for this line of investigation.

For further development of such study, elucidation of the structures of all human milk oligosaccharides is also indispensable. As described already, 13 different core oligosaccharides were found for human milk oligosaccharides. Whether additional larger cores exist or not, and the rules in locations of additional fucoses and sialic acid residues remain to be elucidated. Accumulation of such results may be useful for elucidating the structural rules in the polylactosamine chains, which occur widely in the sugar chains of glycoproteins, glycolipids and proteoglycans.

Human milk contains more than three times of sialylated oligosaccharides of cow's milk. It is known that these sialylated oligosaccharides are absorbed from digestive tracts of babies, and used for the biosynthesis of glycoproteins and glycolipids in their brain.[104] It was also found that learning and memory are improved in piglets by administering sialic acid.[105] Development of this line of studies will afford another improvement for artificial nourishment of babies.

I would like to recommend readers of this review to read another comprehensive review of structures and functions of human milk oligosaccharides written recently by Urashima *et al.*[106]

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Profile

Akira Kobata was born in 1933, graduated from Faculty of Pharmaceutical Science, School of Medicine, the University of Tokyo in 1956, and received Ph.D. degree from the University in 1962. In 1967, he joined the Section of Biochemistry (Chief, Victor Ginsburg), Laboratory of Biochemical Pharmacology, National Institute of Arthritis and Metabolic Diseases in NIH as a visiting scientist. There, he was involved in elucidating the whole biosynthetic pathway of ABH and Lewis antigenic determinants, based on the finding that human milk can be classified into three groups by their oligosaccharide profiles. In 1971, he moved to Kobe University as the Professor of the First Department of Biochemistry, School of Medicine. While elucidating the structures of many novel oligosaccharides in human milk, he developed a series of new sensitive methods to investigate the structures of the sugar chains of glycoproteins. In 1982, he moved to the University of Tokyo as the Professor and Chairman of the Department of Biochemistry, a newly founded Department in the Institute of Medical Science, and expanded his research to the functions and pathologies of the N-linked sugar chains of glycoproteins. He received many Prizes including C. S. Hudson Award from American Chemical Society, and the 1992 Japan Academy Prize. He was a Fogarty Scholar-in-Residence in NIH from 1985 to 1987, Auckland Foundation Visiting Professor in New Zealand in 1988, and also served as the Director of the Institute of Medical Science from 1990 to 1992. Upon retiring from the University of Tokyo in 1993, he was appointed as the Director of Tokyo Metropolitan Institute of Gerontology (TMIG), and became a Professor Emeritus of the University of Tokyo. In this last carrier as a scientist, he developed a new glycobiology area in the field of aging research. From 2000, he has been the Director Emeritus of TMIG. Currently, he is the scientific advisor of Noguchi Institute, a non-profit institution established for the study of carbohydrate chemistry and biology.