Chemical characterization of oligosaccharides in chimpanzee, bonobo, gorilla, orangutan, and siamang milk or colostrum

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Neutral and acidic oligosaccharides were isolated from the milk or colostrum of four great ape species (chimpanzee (Pan troglodytes), bonobo (Pan paniscus), gorilla (Gorilla gorilla), and orangutan (Pongo pygmaeus)) and one lesser ape species (siamang (Symphalangus syndactylus)) and one lesser ape species (siamang (Symphalangus syndactylus)), and their chemical structures were characterized by 1H-NMR spectroscopy. Oligosaccharides containing the type II unit (Gal(β1-4)GlcNAc) were found exclusively (gorilla and siamang) or predominately (chimpanzee, bonobo, and orangutan) over those containing the type I unit (Gal(β1-3)GlcNAc). In comparison, type I oligosaccharides predominate over type II oligosaccharides in human milk, whereas nonprimate milk almost always contains only type II oligosaccharides. The milk or colostrum of the great apes contained oligosaccharides bearing both N-glycolyneuraminic acid and N-acetylaceulaminic acid, whereas human milk contains only the latter. Great ape milk, like that of humans, contained fucosylated oligosaccharides whereas siamang milk did not. Since these analyses are based on a limited number of individuals, further research on additional samples of great and lesser ape milk is needed to confirm phylogenetic patterns.

Keywords: apes/bonobo/chimpanzee/orangutan/milk oligosaccharides/orangutan/siamang

Introduction

Human colostrum and mature milk contain 22–24 g/L and 12–13 g/L, respectively, of a variety of oligosaccharides (Newburg and Neubauer 1995; Kunz et al. 2000), which collectively represent the third largest solid component after lactose and lipids. To date the chemical structures of at least 93 human milk oligosaccharides have been characterized (Urashima et al. 2007); they are classified into 12 groups based on the chemical structures of their core units (Haeuw-Fievre et al. 1993). It is generally believed that human milk oligosaccharides act as soluble receptor analogs that inhibit the attachment of pathogenic bacteria, viruses, and bacterial toxins to the mucosa of the infant colon, and as prebiotics that stimulate the growth of beneficial bifidus bacterial flora in the infant colon; small amounts may be absorbed in the small intestine and exert immune modulating effects within the circulation (Urashima et al. 2007).

We have recently shown that the predominant oligosaccharides in human colostrum obtained during the first three days of lactation (see Table I for oligosaccharide abbreviations) are 2-FL, LNFP I, LNDFH I, and LNT (Asakuma et al. 2008). These four oligosaccharides are also predominant in transitional and mature human milk (Thurl et al. 1996; Coppa et al. 1999; Chaturvedi et al. 2001). It is noteworthy that LNFP I, LNDFH I, and LNT all contain the type I unit (Gal(β1-3)GlcNAc, lacto-N-biose I) within their structures, in contrast to the oligosaccharides of the milk or colostrum of a wide variety of nonhuman mammals, all of which have been shown to contain predominantly or, in most cases exclusively, the type II (Gal(β1-4)GlcNAc, N-acetyl lactosamine) unit (Urashima et al. 2007). These observations suggest that human milk oligosaccharides may be unique in that they contain predominantly the type I unit.

However, of the nonhuman species whose milk oligosaccharides have been studied in detail to date only one, the brown capuchin (Cebus apella), is a primate. Brown capuchin milk was shown to resemble that of other nonhuman mammals in that it contained type II but no type I oligosaccharides (Urashima, Kawai, et al. 1999). The apparent uniqueness of human milk oligosaccharides could merely be a consequence of the fact that the milks of the closest living relatives, the great apes, have not been examined. Warren et al. (2001) reported tentative identifications of several neutral oligosaccharides in the milks of chimpanzee, bonobo, gorilla, and orangutan by comparing the retention time of each peak with those of standard oligosaccharides using high-performance liquid chromatography (HPLC) after perbenzoylation. However, many of their identifications of milk oligosaccharides of nonprimate species, including tree kangaroo, do not correspond to oligosaccharide structures reported for the same or closely related species (Collins et al. 1981; Messer et al. 1982), indicating that HPLC peak retention by itself is not a sufficient criterion for characterization of the diverse oligosaccharides of mammalian milks. In the present paper, we have characterized the oligosaccharides of the milk/colostrum of the chimpanzee, bonobo, gorilla, and orangutan (great apes), as well as the siamang (lesser ape) using 1H-NMR spectroscopy. This method provides information on specific chemical bonds and is thus a more reliable method for the identification of milk oligosaccharides.

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Table I. Milk oligosaccharides characterized in this study

<table>
<thead>
<tr>
<th>Oligosaccharide (abbreviation)</th>
<th>Chemical structure</th>
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</thead>
<tbody>
<tr>
<td>2'-Fucosyllactose (2'-FL)</td>
<td>Fuc(α1-2)Gal(β1-4)Glc</td>
</tr>
<tr>
<td>3-Fucosyllactose (3-FL)</td>
<td>Gal(β1-4)Fuc(α1-3)Glc</td>
</tr>
<tr>
<td>6'-Galactosyllactose (6'-GL)</td>
<td>Gal(β1-6)Gal(β1-4)Glc</td>
</tr>
<tr>
<td>Lacto-N-tetraose (LNT)</td>
<td>Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc</td>
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<tr>
<td>Lacto-N-neotetraose (LNnT)</td>
<td>Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc</td>
</tr>
<tr>
<td>A-Tetrasaccharide (A-tetra)</td>
<td>GalNAc(α1-3)Fuc(α1-2)Gal(β1-4)Glc</td>
</tr>
<tr>
<td>B-Tetrasaccharide (B-tetra)</td>
<td>Gal(α1-3)Fuc(α1-2)Gal(β1-4)Glc</td>
</tr>
<tr>
<td>Lacto-N-fucopentaose I (LNFP I)</td>
<td>Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc</td>
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<tr>
<td>Lacto-N-fucopentaose III (LNFP III)</td>
<td>Gal(β1-4)Fuc(α1-3)GlcNAc(β1-3)Gal(β1-4)Glc</td>
</tr>
<tr>
<td>Lacto-N-neohexose (LNNH)</td>
<td>Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6)Gal(β1-4)Glc</td>
</tr>
<tr>
<td>Difuco-lacto-N-neohexose (DFLNNH)</td>
<td>Gal(β1-4)Fuc(α1-3)GlcNAc(β1-3){Gal(β1-4)}Fuc(α1-3)GlcNAc(β1-6)Gal(β1-4)Glc</td>
</tr>
<tr>
<td>3' Neu5Ac Lac (3' -NAC-SL)</td>
<td>Neu5Ac(α2-3)Gal(β1-4)Glc</td>
</tr>
<tr>
<td>3' Neu5GC Lac (3' -NGc-SL)</td>
<td>Neu5Gc(α2-3)Gal(β1-4)Glc</td>
</tr>
<tr>
<td>6' Neu5Ac Lac (6' -NAC-SL)</td>
<td>Neu5Ac(α2-6)Gal(β1-4)Glc</td>
</tr>
<tr>
<td>Sialyllacto-N-tetraose b (LSTb)</td>
<td>Gal(β1-3)Neu5Ac(α2-6)GlcNAc(β1-3)Gal(β1-4)Glc</td>
</tr>
<tr>
<td>Sialyllacto-N-tetraose c (LSTc)</td>
<td>Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc</td>
</tr>
<tr>
<td>Monosialyl-monofucosyl-LNNH (MSMFLNNH)</td>
<td>Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3){Gal(β1-4)}Fuc(α1-3)GlcNAc(β1-6)Gal(β1-4)Glc</td>
</tr>
<tr>
<td>Monosialyl-LNNH (MSLNNH)</td>
<td>Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3){Gal(β1-4)Glc(</td>
</tr>
</tbody>
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Material and methods

Samples and reference materials

Milk samples were collected at collaborating zoological parks and breeding colonies and shipped to the Nutrition Laboratory, Smithsonian National Zoological Park for storage and preparatory analysis. The samples were kept frozen at −20°C until thawed for analysis. The following samples were obtained.

(1) Chimpanzee (Hominidae: Pan troglodytes) milk was collected 451 and 550 days postpartum from lactating females (0430 and 095) at the Southwest Foundation for Biomedical Research, San Antonio, TX. The 2 mL and 3 mL milk samples were obtained on 25 May 2005 and 30 June 2005.

(2) Bonobo (Hominidae: Pan paniscus) milk was collected 54 days postpartum from a lactating female (ID3888, “Laura”) at the Milwaukee County Zoo, Milwaukee, WI. The 12 mL sample was obtained on 17 September 1995 via use of a breast pump (Lactina, Medela Inc., McHenry, IL) after the female voluntarily presented across a wire mesh barrier (as illustrated in Anonymous, 1959), using lactose monohydrate for preparation of standards.

(3) Gorilla (Hominidae: Gorilla gorilla) colostrum transitional milk was collected 3 days postpartum from a lactating female (“Dolly”) at the San Diego Wild Animal Park, Escondido, CA. The 10 mL sample was obtained on 23 June 1982 from the left breast during a routine medical exam.

(4) Gorilla (Hominidae: Gorilla gorilla) mature milk was collected 114 days postpartum from a 16-year-old lactating female (100016, “Samantha”) at the Philadelphia Zoo, Philadelphia, PA. The 15 mL sample was collected on 13 November 1984 after anesthesia with ketamine and atropine; oxytocin was not administered.

(5) Bornean orangutan (Hominidae: Pongo pygmaeus) colostrum was collected 2 days postpartum from a 34-year-old lactating female (“Maggie”) at the Toledo Zoo, Toledo, OH. Due to incorrect placement, the baby did not appear to suckle for the first 2 days of life. The female was immobilized on 14 September 1989 with ketamine, diazepam, and robinol, and after the baby was allowed to nurse, an ∼40 mL sample of colostrum was collected by manual expression from both breasts; oxytocin was not administered.

(6) Siamang (Hylobatidae: Symphalangus syndactylus) milk was collected 54 days postpartum from a lactating female (RZ-37) at the Riverbanks Zoo and Garden, Columbia, SC. The 12 mL sample was collected on 10 February 1998 from both breasts after the mother had been immobilized with ketamine and atropine for tuberculosis testing; oxytocin was not administered.

Oligosaccharide reference materials (see Table I for abbreviations). LNFP I, LNFP III, LNT, LNnT, 2'-FL, and 3'-FL, were purchased from Seikagaku Co. (Tokyo, Japan). 3'-NAC-SL and 6'-NAC-SL were from Sigma Co. (St. Louis, MO). A-Tetrasaccharide was obtained from Funakoshi Co. (Tokyo, Japan). B-Tetrasaccharide was isolated from Japanese black bear milk (Urashima, Sumiyoshi, et al. 1999), while 6'-NGc-SL and 3'-NGc-SL were isolated from ouve colostrum (Nakamura et al. 1998). Gal(β1-6)Gal(β1-4)Glc (6'-GL) was purified from caprine colostrum (Urashima et al. 1994).

Measurement of hexose content of whole milk and colostrum

Milk was thawed and assayed for total hexose content by the phenol–sulfuric acid method (Dubois et al. 1956; Marier and Boulet 1959), using lactose monohydrate for preparation of standards.

Preparation of the oligosaccharides from milk/colostrum

Subsamples for oligosaccharide analysis (chimpanzee milk, 5 mL; bonobo milk, 7 mL; gorilla colostrum, 4.6 mL; gorilla mature milk, 5.8 mL; Bornean orangutan colostrum, 8.6 mL; siamang milk, 14 mL) were thawed and extracted with four volumes of chloroform/methanol (2:1, v/v). After agitation, the emulsion was centrifuged at 5000 g for 5 min and the lower chloroform layer and the denatured protein were discarded. The methanol was evaporated from the upper layer, and the lyophilized residue was designated as the carbohydrate fraction.

The carbohydrate fraction from each sample was dissolved in 2 mL of water, and the solution passed through a Bio Gel
Fig. 1. Gel chromatograms of the carbohydrate fractions from (A) chimpanzee milk, (B) bonobo milk, (C) gorilla colostrum, and (D) gorilla mature milk. Elution from a Bio Gel P-2 column (2.6 × 100 cm) was done with distilled water at a flow rate of 15 mL/h, and fraction of 5.0 mL were collected. Each fraction was monitored by the phenol–H2SO4 method (at 490 nm, —) and the periodate–resorcinol method (at 630 nm, ...).

Fig. 2. Gel chromatograms of the carbohydrate fractions from (A) orangutan colostrum and (B) siamang milk. The gel chromatographies were done as in Figure 1.

Fractions C-1, B-1, GC-1, O-1, and S-1 from chimpanzee milk, bonobo milk, gorilla colostrum, orangutan colostrum, and siamang milk, respectively, were dissolved in 2 mL of 50 mM Tris–hydroxyaminomethane–HCl buffer (pH 8.7) and subjected to anion-exchange chromatography using a DEAE–Sephadex A-50 (Pharmacia Biotech, Piscataway, NJ) column (1.5 × 35 cm, void volume = 15 mL) equilibrated with the same buffer (chromatograms in Figure 3). The unadsorbed components were eluted with 250 mL of the buffer solution, after which elution was continued using a linear gradient of 0–0.5 M NaCl. Elution was done at a flow rate of 15 mL/h, and aliquots (50 μL) of each 5 mL fraction were analyzed for hexose. The peak fractions in the unadsorbed components were pooled and lyophilized. The components in each peak were analyzed by thin layer chromatography (TLC) on Silica Gel 60 (20 × 20; Merck, Darmstadt, Germany) with acetone/2-propanol/0.1 M lactic acid (2:2:1, v/v/v) as a developing solvent. The spots were detected by spraying with 5% sulfuric acid in 99.5% ethanol and heating above a flame. The components in C-2, C-3, C-4, C-5, and C-6 from chimpanzee milk; B-6, B-5, B-4, and B-3 from bonobo milk; GC-2 from gorilla colostrum, and GM-7, GM-6, and GM-5 from gorilla milk; O-7, O-6, O-5, O-4, O-3, and O-2 from orangutan colostrum; and S-3, S-4, and S-7 from siamang milk were characterized by 1H-NMR spectroscopy. The components in B-5 from bonobo milk were further separated into two peak fractions (B-5-1 and B-5-2) by a second gel filtration on Bio Gel P-2 and each was characterized by 1H-NMR spectroscopy. The components in GM-3 (Rf = 0.89 and 0.74, designated as GM-3-1 and GM-3-2) from gorilla milk were separated by preparative TLC in the same solvent system and further purified by passage through a Bio Gel P-2 column (2.0 × 35 cm). The component in GM-3-1 was characterized by 1H-NMR.
Fig. 3. Anion-exchange chromatogram of (A) O-1 separated from orangutan colostrum by gel chromatographies on Bio Gel P-2 (Figure 2). A DEAE–Sephadex A-50 column (1.5 × 35 cm) equilibrated with a 50 mM Tris–HCl buffer (pH 8.7) was used. Elution was done first with 250 mL of the same buffer, and then with a linear gradient of the same buffer containing NaCl from 0 to 0.5 M. The flow rate was 15 mL/h and fractions of 5 mL were collected. The fractions were monitored by the phenol–H$_2$SO$_4$ method. (B) Gel chromatogram on Bio Gel P-2 of the peak fraction, O-1, from the anion-exchange chromatogram shown in (A). The gel chromatography was performed on a Bio Gel P-2 column (3.0 × 35 cm).

Fig. 4. HPLC of the carbohydrate fractions (A) C-1, (B) B-1, and (C) GC-1 separated from chimpanzee milk, bonobo milk, and gorilla colostrum, respectively. HPLC was done using a Shimadzu LC-10ATVP pump on a TSK–gel Amido-80 column (4.6 × 250 mm, pore size 80 Å, particle size 5 μm). The mobile phase was 50% and 80% (v/v) acetonitrile (CH$_3$CN) in a 15 mM potassium phosphate buffer (pH 5.2). Elution was done using a linear gradient of acetonitrile, 80–50% at 60°C at a flow rate of 1 mL/min. The detection of peaks was done by UV absorption at 195 nm.

Results

The total hexose content of the samples prior to chromatographic separation was 7.4% for chimpanzee milk, 8.8% for bonobo milk, 9.0% for gorilla colostrum, 8.8% for gorilla mature milk, 4.8% for Bornean orangutan colostrum, and 8.2% for siamang milk.

As shown in the size-exclusion chromatograms in Figures 1 and 2, the carbohydrate fraction of each sample of milk or colostrum resolved into several peaks, each of which was designated as in Figures 1 and 2. Fractions, C-1, B-1, GC-1, O-1, and S-1, which reacted positively to periodate–resorcinol, had been subjected to anion-exchange chromatography on DEAE–Sephadex A-50 to remove peptides. During this chromatography, the unadsorbed fraction O-1 from orangutan colostrum separated into two peaks (Figure 3), which were assumed to contain neutral and monosialyl oligosaccharides.

$^1$H-NMR spectroscopy

The NMR spectra were recorded in D$_2$O (100.00 atom D%, Aldrich, Milwaukee, W) at 500 or 600 MHz for $^1$H-NMR with a JEOL-ECX-500 FT-NMR or Varian INOVA 600 spectrometer operated at 293.1 K. Chemical shifts are expressed in ppm downfield from internal 3-(trimethylsilyl)-1-propane sulfonic acid, sodium salt (TPS), but were actually measured by reference to internal acetone ($\delta = 2.225$).
Ape milk oligosaccharides

B-4. The 1H-NMR spectrum (supplementary Table S4) showed that B-4 contained two oligosaccharides, one major (B-4-1) and the other minor (B-4-2). As the major anomer shifts and characteristic resonances were essentially similar to those of authentic A-tetrasaccharide (see Urashima et al. 2000), the major oligosaccharide (B-4-1) was identified to be GalNac(\(\alpha\)1-3)\(\beta\)1-4)Glc and Neu5Ac\(\beta\)1-4)Glc. As the minor anomer shifts and characteristic resonances of H-4 of Gal and NAc of GlcNac were similar to those of authentic LNT, the minor oligosaccharide (B-4-2) was identified to be Gal(\(\beta\)1-3)GlcNac(\(\beta\)1-3)Gal(\(\beta\)1-4)Glc. The ratio of B-4-1 to B-4-2 was estimated by the signal intensities of both NAc shifts of \(\beta\)-(1-3)-linked GlcNac residues to be 1:1.

B-3. The 1H-NMR spectrum (supplementary Table S4) showed that this fraction also contained two oligosaccharides, one major (B-3-1) and one minor (B-3-2). As the major anomer...
shifts and characteristic resonances were similar to those of authentic LNFP III, B-3-1 was identified to be Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)Glc. As the minor anomeric shifts and characteristic resonances of H-4 of Gal, NAc of GlcNAc, and H-6 of Fuc were essentially similar to those of authentic LNFP I, B-3-2 was identified to be Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc. The ratio of B-3-1 to B-3-2 was estimated, by the signal intensities of both NAc shifts of β(1-3)-linked GlcNAc at δ 2.023 and 2.056, respectively, to be 4:1.

**Bonobo acidic milk oligosaccharides**

**B-1-1.** As the 1H-NMR spectrum (supplementary Table S5) had similar shifts of the anomers, the H-3 axial, equatorial, and NAc of Neu5Ac, and the H-3 of Gal to those of authentic 3′-NAc-SL, it is concluded that this fraction contained Neu5Ac(α2-3)Gal(β1-4)Glc as a major component (B-1-1-1). However, the spectrum had other shifts at δ 5.324 and 4.580, and the H-3 axial, equatorial, and a NAc shift of Neu5Ac at δ 1.820, 2.607, and 2.042, respectively. It has been shown that these resonances are observed, when 3′-NAc-SL is converted to its lactone (Nakamura et al. 2000). As these resonances corresponding to those of 3′-NAc-SL lactone, in which the COOH of nonreducing Neu5Ac is esterified to OH-4 of the neighboring β(1-4)-linked Gal residue, it was concluded that this fraction, designated B-1-1-2, contained Neu5Ac(α2-3)Gal(β1-4)Glc 1-4lactone as a minor component. The presence of 3′-NAc-SL, 1-4 lactone in bonobo milk might be the result of lactonization of S′-NAc-SL during purification of the acidic oligosaccharides.

**B-1-2.** As the 1H-NMR spectrum (supplementary Table S5) had similar shifts of the anomers, and the H-3 axial and NAc of Neu5Ac to those of authentic 6′-NAc-SL, it is concluded that this fraction contained Neu5Ac(α2-6)Gal(β1-4)Glc (B-1-2-1). However, there were another anomeric shift of Gal, and the H-3 axial, equatorial and NGc shifts of Neu5Gc (see supplementary Table S5), which were similar to those of 3′-NGc-SL. From these observations, it was concluded that this fraction contained Neu5Gc(α2-3)Gal(β1-4)Glc (B-1-2-2). In addition, there were small shifts at δ 5.331, 4.582, 2.603, and 1.837. It was concluded that these resonances arose from the lactonization of a sialyl residue to the neighboring Gal as in B-1-1-2, suggesting that this fraction, B-1-2-3, contained Neu5Gc(α2-3)Gal(β1-4)Glc 1-4 lactone.

**Gorilla neutral milk oligosaccharides**

**GM-7 and GM-6.** As the 1H-NMR spectra (supplementary Table S6) were completely identical with those of lactose and 2′-FL, respectively, these were identified to be Gal(β1-4)Glc and Fuc(α1-2)Gal(β1-4)Glc.

**GM-5 and GC-2.** As the 1H-NMR spectra (supplementary Table S6) of GM-5 and GC-2 were similar to the published data for B-tetrasaccharide (Urashima, Sumiyoshi, et al. 1999; Urashima et al. 2000), these oligosaccharides were identified to be Gal(α1-3)[Fuc(α1-2)]Gal(β1-4)Glc.

**GM-3-1.** The components in GM-3 had been further separated into two oligosaccharides, GM-3-1 and GM-3-2, by preparative TLC. The oligosaccharide in GM-3-1 was characterized by 1H-NMR, whereas that in GM-3-2 could not be characterized only with our NMR technique.

As the anomeric shifts and characteristic resonances (supplementary Table S6) were similar to those of authentic LNNt, the oligosaccharide in GM-3-1 was identified to be Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc.

**Gorilla acidic milk oligosaccharides**

**GC-1-1 and GC-1-2.** As the 1H-NMR spectra (supplementary Table S7) of GC-1-1 and GC-1-2 were essentially similar to those of 3′-NAc-SL and 3′-NGc-SL, respectively, these were identified to be Neu5Ac(α2-3)Gal(β1-4)Glc and Neu5Gc(α2-3)Gal(β1-4)Glc.

**Orangutan neutral milk oligosaccharides**

**O-7.** As the 1H-NMR spectrum (supplementary Table S8) of O-7 was completely identical with that of lactose, the saccharide in O-7 was characterized to be Gal(β1-4)Glc.

**O-6.** The 1H-NMR spectrum (supplementary Table S8) showed that O-6 contained two oligosaccharides, O-6-1 and O-6-2. As the spectrum of two oligosaccharides were essentially identical with those of 3′-FL and 6′-GL, O-6-1 and O-6-2 were characterized to be Gal(β1-4)[Fuc(α1-3)]Glc and Gal(β1-6)Gal(β1-4)Glc. The ratio of O-6-1 to O-6-2 was estimated by the intensities of both β-Glc signals to be 1:1.

**O-5.** The spectrum had the anomeric shifts of α-linked fucose, β-linked GlcNAc, and β-linked Gal at δ 5.106 and 5.098, 4.727, and 4.466, respectively, NAc of β-GlcNAc at δ 2.032, and H-6 of α-Fuc at δ 1.178 and 1.174. There were no anomeric shifts of reducing Glc, showing that the reducing end was not unsubstituted glucose in this saccharide. The details of its structure could not be obtained.

**O-4.** The 1H-NMR spectrum (supplementary Tables S8 and S9) showed that O-4 contained two oligosaccharides, one major (O-4-1) and the other minor (O-4-2). As the spectrum of two oligosaccharides were essentially identical with those of 3′-FL and 6′-GL, O-4-1 and O-4-2 were characterized to be Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc and Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc. The ratio of O-4-1 to O-4-2 was estimated by the signal intensities of NAc of both β(1-3)-linked GlcNAc residues to be 3:1.

**O-3.** As the 1H-NMR chemical shifts (supplementary Table S9) were essentially similar to those of LNFP III, the oligosaccharide in O-3 was characterized to be Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)Glc.

**O-2.** The 1H-NMR spectrum (supplementary Table S9) had the anomeric shifts of α-Glc, two of α(1-3)-linked Fuc, β(1-3)-linked GlcNAc, β-Glc, β(1-6)-linked GlcNAc, three of β(1-4)-linked Gal at δ 5.219, 5.126 and 5.105, 4.718, 4.665, 4.637, and 4.466, 4.452 and 4.426, respectively, H-4 of β(1-4)-linked Gal, which was substituted at OH-3 by β-linked GlcNAc at δ 4.142, NAc of β(1-6) and β(1-3)-linked Fuc at δ 2.053 and
in this study because the amounts obtained were too small. The saccharides in S-2, S-5, and S-6 could not be characterized.

Siamang neutral milk oligosaccharides

acterized to be Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc.

Siamang acidic oligosaccharides

S-1-1, S-1-2, and S-1-3. As the 1H-NMR chemical shifts (supplementary Table S13) of S-1-1, S-1-2, and S-1-3 were essentially similar to those of 3′-NAC-SL, 6′-NAC-SL, and LST c, respectively, these were identified to be Neu5Ac(α2-3)Gal(β1-4)Glc, Neu5Ac(α2-6)Gal(β1-4)Glc, and Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc.

S-1-4. The 1H-NMR spectrum (supplementary Table S13) of S-1-4 had the anomic shifts of α-Glc, β(1-3)-linked GlcNAc, β-Glc, β(1-6)-linked GlcNAc and three β(1-4)-linked Gal at δ 5.221, 4.726, 4.668, 4.647 and 4.640, and 4.472, 4.455 and 4.433, respectively. The spectrum had the characteristic H-3 axial, equatorial, and NAc of (α2-6) linked Neu5Ac at δ 1.724, 2.668, and 2.027, respectively, NAc of β(1-3) and β(1-6)-linked GlcNAc at δ 2.051 and 2.061, respectively, and H-4 of β(1-4)-linked Gal, which was substituted at OH-3 by β-GlcNAc, at δ 4.148. As this pattern was essentially similar to the published data (Gronberg et al. 1989) for MSLNnH, the oligosaccharide in S-1-4 was characterized to be the heptasaccharide Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc.

Discussion

The ratios of milk oligosaccharides to lactose in these milks were estimated from peak areas of the gel chromatograms shown in Figures 1 and 2, as follows: chimpanzee milk, 1.4; bonobo milk, 1.5; gorilla colostrum, 1.20; gorilla mature milk, 1.7; orangutan colostrum, 1.08; siamang milk, 1.3. This ratio in human milk obtained at 232 days of lactation was estimated from peak areas of the gel chromatogram of its carbohydrate fraction to be 1:2.7 (chromatogram not shown). We conclude, as had Warren et al. (2001), that the ratio of oligosaccharides to lactose in the milk or colostrum of apes is somewhat smaller than that in human milk, with the possible exception of the orangutan. It has been reported that in humans and other species, the ratio of milk oligosaccharides to lactose is usually higher in colostrum than in mature milk (Newburg and Neubauer 1995; Urashima et al. 2007), but in single samples of gorilla colostrum and milk we found that this ratio was lower in colostrum than in the mature milk. Future studies using a greater number of samples of gorilla colostrum and mature milk would be desirable in relation to this finding.

In the present study, milk oligosaccharides were characterized by 1H-NMR analysis of each oligosaccharide fraction. We believe that the analysis of reporter signals of 1H-NMR spectra for structural characterization is preferable to the methods used in previous studies of oligosaccharides in nonhuman milks that were based on the retention times of oligosaccharide peaks during HPLC or high-performance anion-exchange with pulsed amperometric detection (HPEAC-PAD), with or without derivatization, coupled with mass spectroscopy (Kunz et al. 1999; Warren et al. 2001). The latter methods do not provide sufficient structural detail about oligosaccharides, allowing imprecise or faulty conclusions about oligosaccharide identities, especially when dealing with taxa-containing milk...
oligosaccharides of quite different structure from those in human milk. For example, Warren et al. (2001) assigned HPLC peaks of various mammalian taxa to known human oligosaccharides, but more detailed studies indicate that some of these identities may be suspect. In particular, the identities of several oligosaccharides listed by Warren et al. (2001) for particular taxa, including 3-FL, LNT, and LNFP I, are inconsistent with prior or subsequent studies of the same or closely related taxa (dog, Bubb et al. (1999); bears, Urashima et al. (1997, 2000; Urashima, Sumiyoshi, et al. 1999); bottlenose dolphin, Uemura et al. (2005); kangaroos, Messer and Green (1979), Collins et al. (1981), Messer et al. (1982)).

Although great apes are closer to humans in a phylogenetic sense, and thus misidentifications of oligosaccharides are less likely, the data presented by Warren et al. (2001) on great ape milks need to be viewed with caution. They did not identify A- and B-tetrasaccharide in the milk of bonobo and gorilla, respectively, nor did they detect DFLNnH in orangutan milk. It is not clear how much of the discrepancy between Warren et al.’s findings (2001) and the current study is due to methodological differences, and how much might be due to variation among milk samples. For example, Warren et al. (2001) identified 2′-FL and LNFP I in orangutan milk; these sugars were not found by us in the colostrum of this species. In humans, 20% of donors do not have 2′-FL and LNFP I in their milk/colostrum (Kobata 2000), and it is possible that a similar phenomenon is found in orangutans. Minor oligosaccharides such as lacto difucotetraose (Fuc(α1-2)Gal(β1-4)[Fuc(α1-3)]Glc) or lacto-N-difucohexaose I (Fuc(α1-2)Gal(β1-3)(Fuc(α1-4))GlcNAc(β1-3)Gal(β1-4)Glc) might occur in our apes milk/colostrum, bearing in mind that the sensitivity for detection of oligosaccharides with NMR is smaller than with the methods used by Warren et al. (2001).

The major neutral and acidic milk oligosaccharides are compared among great apes and a siamang in Table II. Although we found differences among species, this study was performed using only one or two samples for each species; furthermore, differences of milk oligosaccharides have been found between individuals in human donors (Kobata 2000).

It has been shown that the type I saccharides significantly dominate over type II in human milk and colostrum (Thurl et al. 1996; Coppa et al. 1999; Chaturvedi et al. 2001; Asakuma et al. 2008). It is interesting to compare the milk oligosaccharides of humans to those of the closest living relatives, the chimpanzee (Pan troglodytes) and bonobo (Pan paniscus). The Pan lineage diverged from the human lineage about 4–7 million years ago, and the two Pan species diverged from each other about 1.3 million years ago (Hoboth et al. 2007; Caswell et al. 2008). In contrast to human milk oligosaccharides, type II saccharides dominate over type I in chimpanzee milk rather than the converse, as concluded by comparison of the ratio of LNFP I (type I) and LNFP III (type II) in both milks. Chinmapanzeemilk contained 2′-FL, 3-FL, LNT (type I), LNNt (type II), and LNFP III, while bonobo milk contained 2′-FL, 3-FL, A-tetrasaccharide, LNT, LNFP I, and LNFP III. This difference between chimpanzee and bonobo milks, namely the presence of A-tetrasaccharide in bonobo milk and its apparent absence from chimpanzee milk, warrants further study to determine whether this is a consistent species-specific trait. Thus, although chimpanzee and bonobo milks resemble human milk in containing both types of oligosaccharides, the ratios differ. The close similarity in the pattern of oligosaccharides between the two species of Pan is consistent with their recent divergence.

Among the great apes, gorillas diverged from the Pan–human lineage about 2 million years prior to the Pan–human split (i.e., 6–9 million years ago), while orangutan diverged considerably earlier, perhaps 17–18 million years ago (Hoboth et al. 2007). Gorilla milk or colostrum contained 2′-FL, B-tetrasaccharide, and LNNt (type II), but no LNT (type I) or other type I oligosaccharides, in contrast to human and Pan secretions. On the other hand, orangutan colostrum contained both types, including 3-FL, 6′-GL, LNT (type I), LNNt (type II), and LNFP III (type II). The ratio of LNT to LNNt was 3:1, indicating dominance of type I saccharide in the tetrasaccharide fraction, but the pentasaccharide fraction contained LNFP III (type II) but no LNFP I (type I). DFLNnH, a type II octasaccharide, was also found in orangutan colostrum. In the acidic oligosaccharide fraction, LST b, LST c, and MFMSLNNH were found as well as 3′-NAc-SL, 6′-NAc-SL, and 3′-Ngc-SL. The ratio of LST b (type I) to LST c (type II) was almost 1.0, as shown during HPLC of the acidic oligosaccharide fraction (see Figure 5), whereas MFMSLNNH (type II) was found as a major saccharide. Our observations suggest that orangutan colostrum contains predominantly type II oligosaccharides, but when compared with chimpanzee, bonobo, and gorilla milk, the ratio of type I to type II oligosaccharides in orangutan colostrum was higher. Further analyses using a greater number of milk samples per species need to be performed to solidify our understanding of phylogenetic patterns among the great apes.

In the milk of the siamang (lesser ape) only type II saccharides including LNNt, LNNH, LST c, and MSLNNH were found but not type I saccharides such as LNT, LNH, and LST b. It is also noteworthy that none of the siamang milk oligosaccharides were found to contain fucose or N-glycolyneuraminic acid, in contrast to the milk oligosaccharides of the great apes.

In summary, our results suggested that type I oligosaccharides are found in the milks of chimpanzee, bonobo, and orangutan, but in the samples we assayed these oligosaccharides did not predominate over type II oligosaccharides, in contrast to the situation in human milk/colostrum. Among mammals in which oligosaccharides have been studied in detail, milk and colostrum almost always contain only type II oligosaccharides, not type I. The only known nonprimate exceptions are the milks of the platypus, a monotreme (Amano et al. 1985) and the Asian elephant (Kunz et al. 1999), but we consider the latter case to be doubtful since Uemura et al. (2006) did not find type I oligosaccharides in Asian elephant milk. The presence of type I oligosaccharides in the milks or colostrum of the chimpanzee, bonobo, orangutan, and humans therefore appears to be a significant and unusual feature of the human–great ape lineage, notwithstanding the fact that type I oligosaccharides have yet to be found in gorilla milk. In this connection, it is noteworthy that type I, but not type II oligosaccharides have recently been suggested to promote the growth of beneficial bifidus flora in the infant colon (Kitaoka et al. 2005; Wada et al. 2008).

With respect to acidic oligosaccharides, chimpanzee milk contained 3′-Nac-SL (Neu5Ac(α2-3)Gal(β1-4)Glc) as well as 3′-Ngc-SL (Neu5Gc(α2-3)Gal(β1-4)Glc). In bonobo milk, 3′-Nac-SL and its 1-4 lactone, 3′-Ngc-SL and its 1-4 lactone, and 6′-Nac-SL (Neu5Ac(α2-6)Gal(β1-4)Glc) were found, whereas gorilla colostrum contained 3′-Nac-SL and 3′-Ngc-SL. On the other hand, the orangutan colostrum contained 3′-Ngc-SL as
Ape milk oligosaccharides

Table II. Comparison of oligosaccharides in the milk of primates

<table>
<thead>
<tr>
<th>Type of oligosaccharide</th>
<th>Chimpanzee</th>
<th>Bonobo</th>
<th>Gorilla</th>
<th>Orangutan</th>
<th>Siamang</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral tri</td>
<td>2′-FL</td>
<td>2′-FL</td>
<td>2′-FL</td>
<td>3′-FL</td>
<td>3′-FL</td>
</tr>
<tr>
<td></td>
<td>3-FL</td>
<td>3-FL</td>
<td>LNT</td>
<td>LNT</td>
<td>LNT</td>
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<tr>
<td>Neutral tetra</td>
<td>LNT</td>
<td>LNT</td>
<td>LNT</td>
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<td>LNT</td>
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<td>LNT</td>
<td>LNT</td>
<td>LNT</td>
<td>LNT</td>
<td>LNT</td>
</tr>
<tr>
<td>Acetic penta</td>
<td>LNFP I</td>
<td>LNFP I</td>
<td>LNFP III</td>
<td>LNFP III</td>
<td>LNFP III</td>
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<td></td>
<td>LNFP III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic hexa</td>
<td>LNFP III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic octa</td>
<td>LNFP III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acidic N-acetyl</td>
<td>3′-NaC-SL</td>
<td>3′-NaC-SL</td>
<td>3′-NaC-SL</td>
<td>3′-NaC-SL</td>
<td>3′-NaC-SL</td>
</tr>
<tr>
<td></td>
<td>3′-NaC1-4lactone</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Acidic N-glycoly</td>
<td>3′-Ngc-SL</td>
<td>3′-Ngc-SL</td>
<td>3′-Ngc-SL</td>
<td>3′-Ngc-SL</td>
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<td></td>
<td>3′-Ngc1-4lactone</td>
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</tr>
</tbody>
</table>

described above. It is noteworthy that milk oligosaccharides containing Neu5Gc, such as 3′-Ngc-SL, are found in the milk or colostrum of the great apes; glycoconjugates containing N-glycoly neuraminic acid are absent from the tissues or body fluids, including milk/colostrum, of healthy humans because of the absence of the enzyme that converts UDP-Neu5Ac to UDP-Neu5Gc (Brinkman-Van der Linden et al. 2000). Our results support the hypothesis that the loss of this enzyme occurred subsequent to the divergence of hominids from the apes (chimpan-bonobo) lineage (Schauer 2004). It is speculated that this loss occurred more exactly at ∼2.8 million years ago (Chou et al. 2002).

It is also noteworthy that 3′-SL but no 6′-SL was found in chimpanzee milk and gorilla colostrum, whereas both 6′- and 3′-SL were found in bonobo milk and orangutan colostrum. However, 3′-NaC-SL was more dominant than 6′-NaC-SL in the milk/colostrum of bonobo and orangutan. This situation is the reverse of that in human milk or colostrum (Asakuma et al. 2007).

Supplementary Data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest statement
None declared.

Abbreviations
2′-FL, 2′-fucosyllactose; 3′-FL, 3′-fucosyllactose; 3′-NaC-SL, 3′-N-acetylneuraminyllactose; 3′-Ngc-SL, 3′-N-glycolyneuraminyllactose; 3′-SL, 3′-sialyllactose; 6′-GL, 6′-galactosyllactose; 6′-NaC-SL, 6′-N-acetylneuraminyllactose; 6′-SL, 6′-sialyllactose; DFLNnH, difucosyl lacto-N-neohexaose; LNFPI, lacto-N-fucopentaose I; LNFP III, lacto-N-fucopentaose III; LNNH, lacto-N-neoehexaose; LNNt, lacto-N-neotetraose; LNPFI, lacto-N-difucohexaose I; LNT, lacto-N-tetraose; LSTb, sialyl lacto-N-tetraose b; LSTc, sialyl lacto-N-neotetraose c; MSLNnH, monosialyl lacto-N-neohexaose; MSMFLNnH, monosialyl monofucosyl lacto-N-neohexaose; NaC, N-acetyl; NAc-SL, N-acetylneuraminyllactose; Ngc, N-glycoly; Ngc-SL, N-glycolyneuraminyllactose; TLC, thin layer chromatography.

References


