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Evolutionary Glycomics: Characterization of Milk Oligosaccharides in Primates

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Abstract

Free oligosaccharides are abundant components of mammalian milk and have primary roles as prebiotic compounds, in immune defense, and in brain development. Mass spectrometry-based technique is applied to profile milk oligosaccharides from apes (chimpanzee, gorilla, and siamang), new world monkeys (golden lion tamarin and common marmoset), and an old world monkey (rhesus). The purpose of this study was to evaluate the patterns of primate milk oligosaccharide composition from a phylogenetic perspective in order to assess the extent to which the compositions of hMOs derives from ancestral, primate patterns as opposed to more recent evolutionary events. Milk oligosaccharides were quantitated by nanoflow liquid chromatography on chip-based devices. The relative abundances of fucosylated and sialylated milk oligosaccharides in primates were also determined. For a systematic and comprehensive study of evolutionary patterns of milk oligosaccharides, cluster analysis of primate milk was performed using the chromatographic profile. In general, the oligosaccharides in primate milk, including humans, are more complex and exhibit greater diversity compared to the ones in non-primate milk. A detailed comparison of the oligosaccharides across evolution revealed non-sequential developmental pattern, i.e. that primate milk oligosaccharides do not necessarily cluster according to the primate phylogeny. This report represents the first comprehensive and quantitative effort to profile and elucidate the structures of free milk oligosaccharides so that they can be related to glycan function in different primates.

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Keywords

Primates; milk oligosaccharides; evolution; mass spectrometry; glycomics

Introduction

Lactation is an ancient mammalian adaptation that serves to transfer critical resources from a mother to her offspring. Mammalian infants receive 100% of their nutrition via milk at the beginning of life; for many species, including primates, milk is the sole source of nutrition for an extended period of time. However, milk is a biologically complex fluid that contains many bioactive molecules that serve non nutritive functions as well. For example, colostrums, the first milk produced after parturition, contain high levels of immunoglobulin that transfer maternal immune function history to her offspring. Thus, milk performs immunological functions in addition to its nutritive function.¹

Energy is a fundamental necessity for life. The main energy sources in milk are lipids and simple carbohydrates. For many mammalian species, including primates, lactose is the predominant milk sugar, and the infant intestinal tract produces lactase in order to digest this important energy source. However, milk from many species also contains significant concentrations of free oligosaccharides that generally are not digestible by the infant and remain largely intact until they reach the large intestine. For example, human milk oligosaccharide concentrations range between 20–23 g/L for colostrums and 12–13 g/L for mature milk.^{2, 3} This represents approximately 15–20% of the gross energy provided by milk sugar, or about 7% of total milk gross energy. Thus, a significant fraction of milk energy is apparently not available, at least directly, to human infants. Evolutionary theory predicts that milk oligosaccharides perform significant adaptive functions, else they would not be in milk at such high concentrations. Over 200 different human milk oligosaccharides (hMOs) have been found,⁴ with at least 93 structures elucidated⁵ so far. Thus, hMOs are a very diverse group of molecules. Again, this argues that hMOs are performing significant adaptive functions of some kind, either in the infant, the mammary gland, or both.

HMOs have a lactose core at the reducing end and are further extended by the combined action of various glycosyl transferases. Milk from selected domestic animals including cows, goats, sheep and pig have also been recently analyzed.^{6–8} The free oligosaccharides from bovine and porcine milk are similar to each other and consist primarily of sialylated oligosaccharides.^{6–8} Generally, free oligosaccharides from milk of these domestic animals are smaller, consisting of fewer monosaccharide units than hMO. One distinct difference is the presence of the sialic acid variant N-glycolylneuraminic acid (NeuGc)-containing oligosaccharides in bovine, ovine and caprine milk contrasting to the complete absence found in human milk.^{6, 8} NeuGc is found at low levels in the free oligosaccharides of bovine milk and decreases further to trace amounts during lactation. hMOs are also highly fucosylated, while fucosylation in bovine is nearly absent and rare in porcine milk oligosaccharides.^{4, 6, 8} Thus, current evidence suggests that hMOs are more complex, varied, and in higher concentration than milk oligosaccharides of other species, suggesting an enhanced selective advantage to milk oligosaccharide content in human evolution. However, limited comparative data on non-human primate milk oligosaccharides has prevented determining whether the patterns of oligosaccharides might represent ancestral attributes among the primate order, as opposed to a derived condition in the human lineage.

There are currently over 200 primate species divided into two suborders: prosimians and anthropoids. The prosimians include lemurs, lorises, pottos, and galagos and were not considered in the present study. Anthropoids include monkeys (Old World and New World)

and hominoids (apes and humans). The primates considered in this analysis include two species of New World monkeys (golden lion tamarin, *Leontopithecus rosalia*; common marmoset, *Callithrix jacchus*), one species of Old World monkey (rhesus macaque, *Macaca mulatta*), and four species of hominoids (siamang, *Symphalangus syndactylus*; gorilla, *Gorilla gorilla*; common chimpanzee, *Pan troglodytes*, and human, *Homo sapiens*). Primate phylogeny and divergence dates derived from molecular investigation indicate that New World monkeys diverged from Old World Anthropoids ~46 MYA, Old World monkeys diverged from hominoids ~29 MYA, and among the hominoids, “lesser” apes (e.g. siamang) diverged from the “greater” apes ~21 MYA. The gorilla lineage diverged from the human-chimpanzee lineage ~9 MYA; chimpanzees and humans shared a common ancestor until ~6.6 MYA.

To improve our understanding of the role of free oligosaccharides in milk, we examined milk from the seven primate species listed above. The purpose of this study was to evaluate the patterns of primate milk oligosaccharide composition from a phylogenetic perspective in order to assess the extent to which the compositions of hMOs derives from ancestral, primate patterns as opposed to more recent evolutionary events. The results suggest a nearly independent evolution of glycosylation. Evolutionary glycomics is therefore the independent variation of glycan structures both from inherited traits and as a response to environmental pressures.

Experimental Section

Material

Nonporous graphitized carbon cartridges (150 mg of bed weight, 4 mL tube size) were obtained from Alltech (Deerfield, IL). Sodium borohydride (98%) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma-Aldrich (St. Louis, MO). Lacto-N-tetraose (LNT) and Lacto-N-neotetraose (LNnT) were obtained from Oxford Glycosystems (Oxford, UK). All reagents were of analytical or HPLC grade.

Milk Samples

Milk samples used in this study include hominoids (human, chimpanzee, gorilla, siamang), New World monkeys (golden lion tamarin, common marmoset), and an Old World monkey (rhesus macaque). The milk samples were all from mid lactation stage (4 month). Samples from each primate were limited to only one. However, four samples were obtained for the common marmoset allowing us to observe variability in multiple samples. For comparison, a single human sample that is representative of the other human samples was used for consistency.

The post-partum (around the 4th month) lactation milk samples from individual monkeys and apes were collected at the Centre International de Recherches Medicales de Franceville, Gabon, or the Yerkes Regional Primate Center and provided for this study By Dr F. Gillin, UC San Diego, and Smithsonian National Zoological Park. Milk samples were obtained then frozen at -80°C after collection. The human milk was collected from a mother (Gambia, Africa) at the fourth month of lactation. The gorilla and common marmoset samples were obtained from Smithsonian National Zoological Park (Washington D.C).

Sample preparation

Primate milk samples were totally thawed and 100 μL was centrifuged at 4,000 g in a *microcentrifuge* for 30 min at 4°C . After the top fat layer was removed, four volumes of chloroform/methanol (2:1 v/v) were added to the defatted milk samples. After centrifugation at 4,000 g for 30 min at 4°C , the upper layer was carefully transferred. The supernatant

(aqueous phase containing the milk oligosaccharide-rich fraction) was freeze-dried with a speed vacuum. Native milk OS were reduced to alditol forms by using 1.0 M sodium borohydride and incubated at 42° C overnight. Reduction was necessary because the HPLC separates the reducing-end anomers. Primate milk samples were purified by solid-phase extraction using a nonporous graphitized carbon cartridge (GCC-SPE) and eluted with 20% acetonitrile in water (v/v) prior to MS analysis.

MALDI and infrared multiphoton dissociation

A commercial MALDI mass spectrometer (Ionspec, Irvine, CA) with an external ion source was used to perform the analysis. The instrument is equipped with a 7.0- Tesla shielded superconducting magnet and a Nd:YAG laser operating at 355 nm. The sample spot was prepared by loading 0.5 μ L analyte, 0.3 μ L of salt: 0.1 M NaCl in 50% acetonitrile in water, followed by adding 0.5 μ L of 0.4 M matrix (2,5-dihydroxybenzoic acid (DHB)) in 50% acetonitrile in water. Samples were mixed on the probe before placing the probe into vacuum for drying and MS analysis.

A desired ion was readily selected in the analyzer with the use of an arbitrary-wave form generator and a frequency synthesizer. A continuous wave Parallax CO₂ laser (Waltham, MA) with 20 W maximum power and 10.6 μ m wavelength was installed at the rear of the magnet and was used to provide the photons for IRMPD. The laser beam diameter is 6 mm as specified by the manufacturer. The laser beam was expanded to 12 mm by means of a 2 $\times$ beam expander (Synrad, Mukilteo, WA) to ensure complete irradiation of the ion cloud through the course of the experiment. The laser was aligned and directed to the center of the ICR cell through a BaF₂ window (Bicron Corporation, Newbury, OH). Photon irradiation time was optimized to produce the greatest number and abundance of fragment ions. The laser was operated at an output of approximately 13 W.

HPLC-Chip/TOF MS Analysis

Milk OS fractions collected after GCC-SPE were analyzed using a microfluidic 6200 Series HPLC-Chip/TOF MS instrument (Agilent Technologies, Santa Clara). The microfluidic HPLC-Chip consists of an enrichment column and a LC separation column, both packed with porous graphitized carbon. The enrichment column has a volume of 40 nL and the LC column has the dimension of 75 $\times$ 50 μ m cross section with a length of 43 mm. The column terminates to a 2 mm spray tip.⁹ Separation was performed by a binary gradient A: 3% acetonitrile in 0.1% formic acid solution and B: 90% acetonitrile in 0.1% formic acid solution. The column was initially equilibrated and eluted with the flow rate at 0.3 μ L/min for nanopump and 4 μ L/min for capillary pump. The 65 min gradient was programmed as follows: 2.5 – 20 min, 0% – 16% B; 20 – 30 min, 16% – 44% B; 30 – 35 min, B increased to 100%; then 35 – 45 min, 100% B; and finally, 0% B for 20 min to equilibrate the chip column before the next sample injection.⁴ Each composition of milk oligosaccharide was identified with an in-house program “Glycan Finder.” Distinct compositions were identified based on accurate mass and retention times.

Relative quantitation of oligosaccharides in milk

The relative quantities of oligosaccharides in primate milk were calculated using peak intensities. Due to the prevalence of singly charged species ($z=1$), the absolute peak intensities can be directly related to the abundance of the molecule in the milk samples. To calculate the relative amounts of each oligosaccharide (mole%) the absolute peak intensities of each glycan was normalized by the sum of the absolute peak intensities of every oligosaccharide in the samples. The weighted absolute peak intensity of each was normalized by the sum of the values for all structures identified in a sample. The equation to calculate the relative quantity of each compound k (RQ_k) is as follows,

$$RQ_k(w/w) = \frac{(API_k/MW_k)}{\sum_{i=1}^n (API_i/MW_i)}$$

where API is the absolute peak intensity, and n indicates the number of oligosaccharides in the milk sample.

Hierarchical cluster analysis of oligosaccharide structures and composition

Hierarchical cluster analysis of primate milk was performed using the chromatographic profile. Milk oligosaccharides were integrated into a single table using the mass-to-charge (m/z) and retention times. The oligosaccharide compositions were assigned within a range of error < 10 ppm between theoretical and measured mass. When the compositions are the same and the retention times within a 10 second window the entry was deemed identical. The retention time was adjusted using LNT, LNFP I, and LNH as internal standards. For the clustering, HCE 3.5 has been used.¹⁰ The similarity in the cluster was measured by Euclidian distances, and cluster linkage was built by average linkage method. Taxonomy tree of primates used in these experiments were built by the NCBI Taxonomy browser.¹¹

Results

Milk samples from 10 primates including New World monkeys (golden lion tamarin; common marmoset (4 samples from 4 individual common marmosets)), one species of Old World monkey (rhesus), and four species of hominoids (siamang; gorilla; chimpanzee and human) were used in this study. Briefly, free oligosaccharides were extracted by Folch procedure, reduced by sodium borohydride, and enriched by solid phase extraction with a graphitized carbon cartridge. The reconstituted samples were analyzed by MALDI FTICR with high mass accuracy. Isomer separation and quantitation were performed on HPLC Chip/TOF MS. Structure identification was further performed by matching the retention time and accurate mass against the HMO structure library.^{12, 13}

Free Milk Oligosaccharide Profiling with LC/MS

HPLC-Chip/MS of the primate samples yielded chromatograms shown in Figure 1. Human milk has the highest ion abundances in the range of 4.7×10^5 counts, while gorilla has the lowest abundances with 2.5×10^4 counts, suggesting nearly an order of magnitude variation in the abundances. All three chromatograms showed a peak at m/z 710.28 as the most abundant. The peak could either be Lacto-N-tetraose (LNT) or Lacto-N-neotetraose (LNnT), which are isomers with similar retention times and differ only by the residue linkage Hex-HexNAc, namely LNT with $\beta(1-3)$ and LNnT with $\beta(1-4)$. Based on previous our study^{4, 8} and comparison of retention times of LNT, LNnT standards, the peak was identified as LNT. Another high intense peak in gorilla and common marmoset is m/z 1075.42 with the composition 4Hex:0Fuc:2HexNAc:0NeuAc, not so relatively intense in human milk. This peak was determined to be Lacto-N-neohexaose (LNnH) based on the comparison of HPLC retention times of oligosaccharide standards. This structure is also found in bovine and porcine milk in relatively high abundances.

Table S1-7 lists the components of each animal species as determined by Chip/TOF MS. Each list consists of total detected except for those where the number were over 100, in which case only the most abundant 100 oligosaccharides are listed. Included in the table are the retention times and the relative abundance of each compound, with the compounds listed according to their abundances. Table 1 lists the number of oligosaccharides observed where

every identifiable peak is listed. Human, chimpanzee, and common marmoset have the largest number of compounds. Because several of the structures are weakly abundant, difficult to structurally confirm and possibly not present in all animals in the species, we considered the oligosaccharides responsible for 90% of the abundances for comparison. When the 90% mole content is considered, the same trend is observed but the numbers are considerably smaller. For example, in humans 90% of the mole content is represented by only 50 oligosaccharides. This means that nearly half of the oligosaccharides have abundances of less than 10%. In other words, many of the primate milk oligosaccharides represent only minor fractions of the total oligosaccharides.

Primate milk is considerably more diverse than non-primate milk (e.g. bovine and porcine). The total number of oligosaccharides (from around 50 for gorilla to around 130 for chimpanzee) is significantly greater than the oligosaccharide species found in bovine (about 40) or porcine milk (about 30).^{6, 8} Figure 2 shows the number of oligosaccharides at various degree of polymerization (DP). These data also illustrate an interesting difference between human and the other primates. The median DP of the oligosaccharides in human milk is centered around 7–9, while for all the other primates oligosaccharides are centered around 5–6. Human milk thus contains generally larger oligosaccharides, consistent with a greater complexity than other primates.

Shown in Figure 3 are the numbers of oligosaccharides corresponding to the 90% mole fraction for the different species. Diversity is greater for human and chimpanzee in which 90% of the oligosaccharides constitute over 40 structures (Table 1). For common marmoset milk the value is 34 and for the remaining primate species the value is around 15. The amount of fucosylation similarly varies among the different primate species. For siamang and golden lion tamarin, the fucosylated oligosaccharides make up only approximately 1% by mole while for the rhesus they constitute nearly 50%. These results are consistent with a previous report on primate milk,¹⁴ which showed no fucose for siamang. However Table S6 lists six fucosylated compounds found in this study albeit with low abundances in siamang milk samples.

The net proportion of sialylation (NeuAc) at 60% (abundances) is highest in the milk of siamang. Such an extent of sialylation is more consistent with milk from bovine and much higher than human, which is typically between 10–30%. The sialylation found in chimpanzee and rhesus is more similar to values seen in human milk, whereas gorilla, common marmoset, and golden lion tamarin milk have slightly lower sialylated free oligosaccharides.

Among the primates, NeuGc containing oligosaccharides were found in chimpanzee, rhesus and gorilla, albeit in low abundances (1.1 ~ 9.4 %). The result is consistent with the findings for free oligosaccharides in bovine milk, where NeuGc is present more in bound milk glycans of glycoproteins. In addition, published results on various tissues from these nonhuman primate species show a relatively high NeuGc content (e.g. gorilla, chimpanzee and rhesus).¹⁴ Urashima et al found NeuGc only in 3'-NeuGc-sialyllactose in chimpanzee, bonobo, and orangutan, but not siamang.¹⁵ Sialyllactose is quantitatively a minor free oligosaccharide component in human (0.07 ~ 1.2 %) and in other primate milk (0.2 ~ 25 %) (Table S1-7), in contrast to mature bovine milk in which it is a major oligosaccharide component. To obtain the identity of sialyllactose in the previous study, the samples had to be significantly enriched because NMR is approximately six orders of magnitude less sensitive than MS; the present studies were performed on a few μ L of milk.

Another reason for the relative low abundant of NeuGc in the present study may be that the samples obtained were all mature milk at the fourth month of lactation and not colostrum,

which was sampled in the Urashima study.¹⁶ From the previous bovine milk study, we found that the free oligosaccharide NeuGc content decreased after delivery. In colostrum around 5% of the oligosaccharides contained NeuGc, which dropped to less than 1% in 4 month milk samples.⁷ Others have reported 3% NeuGc in total sialic acids (bound and free) in commercial bovine milk samples.¹⁴

Because of limitations in sample availability, intra-species variation was only assessed for the common marmoset. The spectra show biological variations however the dominant compounds are all the same with the greatest variations in the less abundance species (Figure S1). These results were further supported by the LC chromatogram (Figure S2), which also exhibit strong similarities among individuals. These results contrast with humans where distinct variations are observed with as few as five individuals.¹⁷ The four common marmoset monkeys were raised as a group and fed the same food and may actually belong to the same family. Human milk samples generally showed great diversity that may result from several factors including diet, environment, blood types, as well as age.¹⁷

Structural elucidation and variation between primate species

To annotate the structures of each oligosaccharide, a combination of standards developed from human milk and tandem MS techniques were used. Tandem MS is useful for obtaining sequence information and confirming saccharide composition and obtaining the basic connectivity as shown previously.⁴ For example, using a combination of HPLC and tandem MS techniques, the annotation of the most dominant peaks in common marmoset milk oligosaccharides are illustrated (Figure 4).

Table 2 lists the major oligosaccharides with structures commonly found in all primate milk including human milk, (“primate milk free oligosaccharide consensus structures”).¹² The relative intensities listed correspond to the abundances of each oligosaccharide related to the most abundant component. For example, the intensity of LNT in human milk is 100.0, which makes it the most abundant peak. Other peaks are normalized accordingly. For example, *m/z* 855.322 at 14.4 min representing Lacto-N-Fucopentaose (LNFP) III is 77.9 in relative intensity compared to 100.00 for LNT. The most abundant oligosaccharide in human milk is LNT, which is also the most abundant in chimpanzee, gorilla, and rhesus. For common marmoset, golden lion tamarin, and siamang, LNnH is generally the most abundant, with the exception of the siamang, LSTb (Gal β 1,3[NeuAc α 2,6]GlcNAc β 1,3Gal β 1,4Glc) is somewhat more abundant.

Cluster analysis shows the relationship between the milk oligosaccharides of the different primates (Figure 5). Two major groups were observed in terms of milk oligosaccharides; gorilla, rhesus, human and chimpanzee vs. common marmoset, golden lion tamarin and siamang. In milk oligosaccharides profiles, gorilla and rhesus are the two most similar in the group followed by human and chimpanzee. Among the second golden lion tamarin and common marmoset are the two most common followed by the siamang. This patterning of milk oligosaccharides only crudely parallels the phylogeny (Figure 5B) as the rhesus, an Old World monkey, clusters with the “great apes” and the siamang the “lesser” ape clusters with New World monkeys.

DISCUSSION

The large diversity in primate milk free oligosaccharides documented in these studies does not closely reflect the established primate species phylogeny. Evolution of the oligosaccharide structures apparently occurred independently of phylogenetic divergence and there appears to be ample parallel, and convergent evolution (e.g. highest fucosylation levels of free milk oligosaccharides in only distantly related humans and marmosets).

Different taxa may exhibit linkage-specific glycan repertoires,¹⁸ but within primates, the utilization of free oligosaccharides in milk appear only poorly correlated with phylogenetic similarity. This observation is consistent with the rapidity and apparent parallel nature of glycan evolution of ABO blood types in primates, where phylogeny only poorly predicts the existence of ABO polymorphisms in a given species.¹⁹

All primate species considered here have moderate degrees of sialylation; however two of the species have only trace fucosylation – more similar to bovine and porcine. Sialic acids may therefore be of more general and fundamental importance to the overall functions of free oligosaccharides and selective advantages afforded to infant mammals by their unusual presence in milk. Sialic acids are exploited by pathogens as receptors, and sialylated free milk oligosaccharides inhibit pathogen binding to epithelial cell surfaces.^{20, 21} Sialylation is also important in brain development.^{20, 21} Sialic acids increase the production of gangliosides, which are important components of membrane receptors and cell surfaces of the nervous system.²² Non-primate species such as bovine⁶ and porcine⁸ have high degrees of sialylation, with as much as 70 – 80% of the total oligosaccharide structures being sialylated. Among primates, the highest amount of sialylation was found in siamang milk, while for all the other species including human sialylation ranges between 10 and 20%; the human milk free oligosaccharide sialylation is consistent with previous values.⁴ Sialic acid may provide a distinct nutritional value as well. All mammals have the capacity to synthesize sialic acids and incorporate them into their neurological tissues; however, newborn infants are thought to incorporate exogenous sialic acids from their diet as well. As a result, both in part because of their immaturity, and in part the rapid growth and development of their brain the need for sialic acid may transiently exceed the infant's capacity to produce them.²³ The effect of sialic acid to influence cognitive brain development has recently been shown in model animals and humans. Porcine fed with bovine sialic acid supplements were reported to learn faster than those not fed with supplements²³ and breast fed human infants had higher ganglioside contents in the grey matter of their brains than formula fed infants.²⁴

Fucosylation in free oligosaccharides is apparently not as central to primate lactation considering its significant variation among the primates (Figure 3). Chimpanzee is closest to human with a fucosylation of nearly 50%. The human sample is highly fucosylated but is at the lower end of previous human samples that were between 50–80%.⁴ The other close human relative the gorilla is only 15% fucosylated, while siamang and golden lion tamarin show very low levels (<1%) of fucosylation. These values are more in line with bovine and porcine milk oligosaccharides. A more careful analysis of the supplement tables shows that multiple fucosylation is not common even among the other than hominids. In chimpanzee, 10 of the oligosaccharides contain at least two fucoses, while for gorilla seven are found. For common marmoset and golden lion tamarin only one in each list is difucosylated, and zero for siamang. The exception is rhesus with 12 difucosylated compounds.

Structural determination of milk oligosaccharides using the human structures as standard yielded the results in Table 2. The table allows the evaluation of the roles of specific structures as defined by previous studies in literature.^{13, 25} The epitope *Lewis a* is present in some of the common oligosaccharides such as LNFPII and DFpLNHII that both are presented in rhesus. No other *Lewis a* structures are found in other primates. The *Lewis x* epitope is found among milk oligosaccharides of all the primate species studied here. *Lewis x* is found in LNFP III (present in human, chimpanzee, gorilla, and rhesus) and in MFLNH III (human chimpanzee, gorilla, common marmoset and golden lion tamarin), DFLNH_a (Human and Gorilla), and MSMFLN_nH (all primates). Difucosylated free milk oligosaccharides could indicate the presence of *Lewis b* or *Lewis y* epitopes; however their structures could not be confirmed.

Oligosaccharides that contain Fuc(α 1–2) are found in secretors²⁶ because of the functional α 1–2 fucosyl transferase enzyme expressed in the epithelia. There are several known structures in humans that readily identify secretor status. However, we find only one structure with α 1–2 fucose (mass 1366.5, Table 2, gorilla) that is common to both human and primates. There are likely other secretor compounds in primates, but these may be unique structures that have not yet been elucidated.

The presence of NeuAc and NeuGc in different foods may influence the health properties of human diets. CMP-NeuAc, which is the substrate for sialyl transferases, is converted to CMP-NeuGc by active CMP-NeuAc hydroxylase.²⁷ NeuGc is then added to the nascent glycoconjugates using existing sialyl transferases. Humans are reported to synthesize only NeuAc.²⁷ The transforming enzyme Cytidine Monophosphate N-Acetylneuraminic acid hydroxylase (*CMAH*) was genetically inactivated approximately 3 MYA in the human ancestor.²⁸ Mammalian brains are exceptionally sialic acid rich but contain mostly NeuAc even in species that have abundant throughout other tissues in their bodies. The absence of NeuGc in free milk oligosaccharides as well as ganglioside-bound NeuGc in milk may be linked to the relative absence of NeuGc in the brain if some of the free sialylated oligosaccharides are incorporated into brain gangliosides.²³

The pattern of primate milk oligosaccharides detailed in the present study do not map directly to primate phylogeny, captivity, infant growth rate, geography, or ecology. What other selective forces may influence species profiles of milk oligosaccharides? In examining the possible ecological variables, one pattern is intriguing: notably sociality and group size. Marmosets, tamarins, and siamangs live in small social groups often comprised of a single breeding pair and their immature offspring.²⁹ Marmosets may have additional non-reproductive adult helpers in the social group, but group size is on average fewer than nine individuals.²⁹ In contrast rhesus, chimpanzees, and humans live in large social groups, all in turn associated with a conspicuous richness and diversity of helminthes protozoa, and viruses.^{30, 31} Group size in gorillas is variable, but is usually over ten individuals, comprised of one breeding male and multiple breeding females and their offspring.²⁹ Under these social conditions with strong and persistent pathogenic pressure, Darwinian selection is likely to favor immunological adaptations for the developing neonate and would include an important role for oligosaccharides with both highly selective prebiotic functions.^{32, 33} These same conditions would similarly favor the rapid co-evolution of a symbiotic microbiota, evidence in favor of which has recently been published.³⁴

Conclusion

We have performed the first comprehensive and quantitative study of free milk oligosaccharides by mass spectrometry regarding the glycan function and relationship between different primates. High resolution mass spectrometry and chip-based nano-LC/MS enabled us to interrogate free milk oligosaccharides. The relative abundances of fucosylated and sialylated milk oligosaccharides in primates were also determined. While several functions have been attributed to milk oligosaccharides, the best and most studied aspects involve infant-microbial interactions. Free oligosaccharides in milk are known to act as prebiotics and pathogen blocks. The variation in structures therefore is likely to correspond to selective pressures associated with the ongoing competition between commensal bacteria and pathogenic bacteria and the need to maintain a protective population of commensal bacteria. In this regard the degrees of fucosylation and sialylation are important as they play critical roles in bacterial binding.²⁰ From this perspective, the variations in sialylated and fucosylated oligosaccharides are noteworthy. Distinct pattern of evolutionary selection of free oligosaccharides by cluster analysis of primate milk and the independent emergence of

milk oligosaccharide repertoires in primates implies a role for this class of molecule in milk in specifically guiding prevalent microbe regimes associated with social group size.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. HPLC-MS chromatograms of (A) human (Gambia), (B) common marmoset, and (C) gorilla milk oligosaccharides in the positive mode. The numbers listed are the m/z of most abundant ions.

Figure 2.

Size distribution of glycans in the primate milk. Accumulated numbers of glycan in milk were plotted against the number of monosaccharide units (DP: degree of polymerization) in each glycan. Dot line indicates the median of DP distribution (DP=9).

Figure 3.

(A) Number of oligosaccharides that compose the 90% (% mol) of total milk oligosaccharides in the different primates. (B) The abundance of fucosylated glycans in milk oligosaccharide. Number in the key refers to the number of fucoses in the structure. Monofucosylated glycans therefore make up 40% of the total abundance for chimpanzee. (C) The abundance of sialylated glycans in milk oligosaccharide. Sialylated glycan contains only single NeuAc.

Figure 4. HPLC-Chip/TOF chromatogram of common marmoset milk oligosaccharides in the positive mode with possible structures.

Figure 5.

Quantitative comparison of glycans between primates (A) The hierarchical cluster analysis of quantitative glycan distribution between primates. The quantity of each glycan was calculated as a mole percentile among total glycans. The similarities between primates were measured by Pearson's product, and the linkage was built by Average linkage method. Glycans are listed by their molecular weight from the small to the large. The representative glycans are indicated with their composition. The shorthand compositions stand for the number of monosaccharide units in the order of hexose (Hex), fucose (Fuc), N-acetylhexosamine (HexNAc) and Sialic acid (NeuAc). The structural isomers with the same compositions are listed by their retention times. Two common glycans were found in all primates (arrow with names). (B) Phylogenetic tree of primates.

Table 1

Number of OS observed when all the OS intensities in the chromatograms are used (100%) or, conversely, fractions of the total intensity (70, 80, 90%). A large number of compounds are present in the milk of each species, however a relatively small number account for the majority of the total abundance.

mol%	Chimpanzee	Human	Gorilla	Siamang	Rhesus	Common Marmoset	Golden lion Tamarin
<i>a</i> 100%	100	116	52	69	69	100	66
<i>b</i> 90%	45	50	16	15	16	34	15
80%	27	30	10	9	11	17	9
70%	18	18	7	6	7	12	5

a Cutoff: Relative intensity 0.1

b Relative intensity ~2.0

Table 2

Possible structures of primates and human milk oligosaccharides with relative abundances (normalized to most abundant peak in the mixture) and retention times.

Mass (exp)	Composition	RT (min)	HMO	chimpanzee	Gorilla	Siamang	Rhesus	Common marmoset	Golden lion Tamarin		Lewis type
635.227	2001	23.0	0.4	36.0		65.2	9.8	0.8	14.7		
676.254	1011	22.8			8.3	7.3			0.3		
709.264	3010	14.8	100.0	100.0	100.0	48.9	100.0	45.7	14.7		
855.322	3110	11.4	4.6				0.2				A
855.322	3110	14.4	43.5	14.4	3.7		2.0				X
871.317	4010	15.7		8.9	13.9	25.3	9.9	9.1	32.8		
1000.359	3011	24.4	38.9						1.4		
1000.359	3011	24.9	16.4	3.6	11.1	100.0		0.5			
1000.359	3011	28.1	5.6	17.6				1.3	0.2		
1017.375	4110	13.5		16.2	0.3		8.1		0.7		
1074.396	4020	19.0	31.4	23.3	57.1	92.5	16.3	100.0	100.0		
1146.417	3111	23.1	1.8	13.5			5.4				
1162.412	4011	28.5		6.1	5.7	28.8	1.2	1.9	7.6		
1220.454	4120	15.7	4.0	21.6		0.2	27.9	8.3	0.9		
1220.454	4120	17.9	18.2	22.0	3.7			10.5	0.8		X
1308.470	4111	26.6					21.2				
1365.492	4021	25.7	1.5			9.3		23.2	4.4		
1365.492	4021	26.5	65.5	10.2	2.6	71.4	10.2	3.4	24.3		
1365.492	4021	27.7	3.5	8.1		10.2		3.8	0.8		
1366.512	4220	14.2					28.1	2.4			A
1366.512	4220	16.0	6.5		0.9						X
1439.528	5030	24.1			3.4	2.3	1.9	13.6	1.5		
1439.528	5030	25.0						7.1	2.5		
1511.550	4121	25.9	1.0	59.7	0.4	0.5	31.7	0.3	0.3		X
1804.661	6040	24.9			0.9	0.6		18.8	3.1		
1950.719	6140	24.6						5.2			

* ▲ Fucose; ● Glucose; ● Galactose; ■ HexNAc; ◆ NeuAc *monosaccharide composition 2Hex:1Fuc:0HexNAc:0NeuAc represented as 2100

*Fuc and NeuAc are in α-configuration. All the other residues are in the β-configuration

*These compounds were selected because their structures are known based primarily on human milk standards