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Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides

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Neutral oligosaccharides in human milk samples from approximately 50 women were analysed applying a recently developed high-pH anion-exchange chromatographic method. Three different oligosaccharide patterns could be detected in accordance with milk groups that had been already described. These oligosaccharide groups correspond to the Lewis blood types Le(a−b+), Le(a+b−) and Le(a−b−). In addition to these oligosaccharide patterns, a new carbohydrate pattern was detected in a milk sample from a Le(a−b−) individual. Here, only nonfucosylated oligosaccharides and compounds bearing *α*1,3 linked fucosyl residues were found, whereas structures with *α*1,2 and *α*1,4 fucosyl linkages were missing. This finding led to the hypothesis that there are four different oligosaccharide milk groups that fit well to the genetic basis of the Lewis blood group system.

***Keywords*: human milk, oligosaccharide pattern, Lewis blood group**

***Abbreviations*: HPAEC, high-pH anion-exchange chromatography; GPC, gel permeation chromatography; 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; LDFT, lactodifucotetraose; LNT, lacto-*N*-tetraose; LNnT, lacto-*N*-neotetraose; LNFP I–III, lacto-*N*-fucopentaoses I–III; LNDFH I–II, lacto-*N*-difucohexaoses I–II; LNH, lacto-*N*-hexaose; 2′-F-LNH, 2′-fucosyllacto-*N*-hexaose; 3′-F-LNH, 3′-fucosyllacto-*N*-hexaose; 2′,3′-DF-LNH, 2′,3′-difucosyllacto-*N*-hexaose**

Introduction

Carbohydrates from human milk comprise 7% lactose, approximately 1% neutral oligosaccharides and about 0.1% acidic oligosaccharides [1]. Roughly 90 different neutral and acidic oligosaccharides have so far been characterized [1–4]. The biological function of these compounds is not yet fully understood [5].

Analysis of milk oligosaccharides obtained from individual donors revealed that they can be classified into three groups according to the Lewis blood groups Le(a−b+), Le(a+b−) and Le(a−b−) [6]. There exists a close relationship between the structures of milk oligosaccharides and the Lewis blood group system [6]. The different Lewis blood types are genetically determined primarily by the action of a Lewis gene coding for an α1,3/4 fucosyltransferase and a secretor gene coding for α1,2 fucosyltransferase [7–9].

In human milk from individuals with blood type Le(a−b+), representing about 69% of the population [7], an oligosaccharide pattern corresponding to the first milk group was found. In these milk samples all common oligosaccharides with α1,2, α1,3 and α1,4 linked fucosyl residues occur [6]. The second milk group can be related to blood type Le(a+b−) which accounts for approximately 20% of the population [7]. The oligosaccharide pattern of the second milk group lacks compounds with fucosyl residues with α1,2 linkages. In milk samples from the remaining 10% of the population with blood type Le(a−b−), corresponding to the third milk group, oligosaccharides with α1,4 linked fucose residues were missing.

In the past few years high-pH anion-exchange chromatography (HPAEC) with pulsed amperometric detection has been established as a powerful tool in carbohydrate research [10]. HPAEC has also been applied to analysis of oligosaccharide standards from human milk [11, 12]. In this study we used a recently developed HPAEC method for the analysis of oligosaccharides from human milk [13]. We screened milk samples in order to confirm already known oligosaccharide patterns and in order to find a fourth milk pattern that can be deduced from the genetic basis of the Lewis blood group system.

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Methods

Collection of samples

Several hundred breast milk samples were taken from approximately 50 women living in Germany. About 5–10 ml aliquots were expressed manually into plastic containers. Milk samples were immediately frozen and stored at −20 °C until analysis.

Determination of Lewis blood groups

Lewis blood groups were determined on the day of blood sampling by a haemagglutination tube test. Haemagglutination was examined using corresponding erythrocyte suspensions (3–5% erythrocytes suspended in NaCl 0.9%) and monoclonal anti-Le^a and anti-Le^b antibodies (Immucor, Rödermark, Germany and BAG, Lich, Germany). Incubation was performed at room temperature for 15 min.

HPAEC analysis

HPAEC analysis of neutral oligosaccharides was performed as already described [13]. In short, human milk samples were heated for 30 min at 70 °C. One milliletre of human milk and 0.1 ml of a solution of the internal standard stachyose (400 mg per 100 ml) were mixed. Lipid and protein contents were reduced by centrifugation and ultrafiltration at 2000 × **g** and 6 °C using the Centrifree micropartition system (Amicon, Witten, Germany) equipped with 30 000 YMT membranes. The crude carbohydrate fraction (100 µl aliquots) was separated into acidic oligosaccharides, neutral oligosaccharides and lactose by gel permeation chromatography (GPC) in a 1.6 cm × 80 cm Toyopearl HW 40 (S) column (TosoHaas, Stuttgart, Germany). All components were eluted with 0.02% aqueous sodium azide (flow-rate of 1 ml min^{-1}) and monitored by refractive index detection. The HPAEC analyses were performed on a DX-300 Bio-LC-system (Dionex, Idstein, Germany) equipped with a pulsed electrochemical detector (PED 2, Dionex). Neutral oligosaccharides were separated on CarboPac PA-100 (Dionex) pellicular anion-exchange resins (4 × 250 mm) and CarboPac PA-100 guard columns (4 × 50 mm) at a flow-rate of 1 ml min^{-1} at room temperature using the following eluents: 0–20 min, 30 mM NaOH; 20–34 min, 30–100 mM NaOH; 34–48 min, 100 mM NaOH/0–28 mM NaOAc; 48–55 min, 100 mM NaOH/28–200 mM NaOAc; 55–60 min, 100 mM NaOH/200 mM NaOAc. Identification of the oligosaccharides from human milk separated by HPAEC was performed by comparison of their absolute retention times or their retention times relative to stachyose with that of oligosaccharide standards. These substances either were purchased from Oxford Glycosystems (Oxford, UK) or had been isolated from human milk as previously described [1, 14].

Results

Oligosaccharides in human milk samples from approximately 50 individual donors were analysed by HPAEC. Since abundant lactose in the crude oligosaccharide fraction prevented exact analysis of milk oligosaccharides by HPAEC, the major portion of lactose was removed by preparative GPC. This method also allowed a separation of neutral and acidic oligosaccharides. As neutral fucosylated oligosaccharides are much more abundant in human milk than acidic fucosylated compounds, the neutral oligosaccharide fraction was chosen for differentiating the oligosaccharides depending on Lewis blood groups.

When analysing milk samples from Le(a−b+) donors, reproducible patterns of neutral oligosaccharides were obtained. In Figure 1 the corresponding HPAEC profile including the internal standard stachyose is shown. The structures of fourteen neutral oligosaccharides that could be identified in milk samples from 40 women are listed in Table 1. The oligosaccharides present in this milk oligosaccharide group are indicated with a plus (first milk group). Besides the nonfucosylated structures LNT, LNnT and LNH a variety of fucosylated oligosaccharides with α1,2, α1,3 and α1,4 linkages were detected. Peak L represents residual lactose that was not completely separated from the oligosaccharide fraction during GPC separation.

Figure 2 shows the HPAEC profile of a neutral oligosaccharide fraction typical for Le(a+b−) donors. In milk samples from five donors nonfucosylated structures and fucosylated oligosaccharides with α1,3 and α1,4 linkages were found. However, the oligosaccharides 2′-FL, LDFT, LNFP I, LNDFH I, 2′-F-LNH and 2′,3′-DF-LNH were lacking (Table 1; second milk group). All these oligosaccharides have in common α1,2 linked fucose residues.

Figure 3 shows the HPAEC profile of the neutral oligosaccharides from a Le(a−b−) donor. In this milk

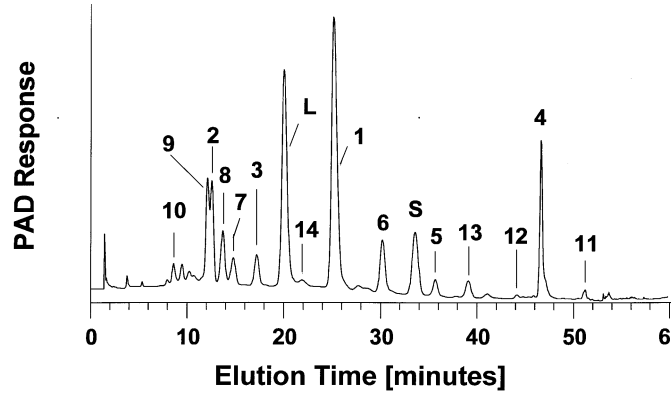

Figure 1. HPAEC profile of the neutral oligosaccharide fraction from a human milk sample typical for Le(a−b+) individuals. The neutral oligosaccharides that were identified are numbered according to Table 1. L, lactose; S, stachyose.

Table 1. Structures and patterns of human milk oligosaccharides as revealed by HPAEC

Peak	*Trivial name (abbreviated)*	*Structure*	*Milk groups*			
			First	*Second*	*Third*	*Fourth*
1	2′-FL	Fuc-*α*-(1 → 2)Gal-*β*-(1 → 4)-Glc	+	−	+	−
2	3-FL	Gal-*β*-(1 → 4)-Glc Fuc-*α*-(1 → 3)/	+	+	+	+
3	LDFT	Fuc-*α*-(1 → 2)Gal-*β*-(1 → 4)-Glc Fuc-*α*-(1 → 3)/	+	−	+	−
4	LNT	Gal-*β*-(1 → 3)-GlcNAc-*β*-(1 → 3)-Gal-*β*-(1 → 4)-Glc	+	+	+	+
5	LNnT	Gal-*β*-(1 → 4)-GlcNAc-*β*-(1 → 3)-Gal-*β*-(1 → 4)-Glc	+	+	+	+
6	LNFP I	Fuc-*α*-(1 → 2)-Gal-*β*-(1 → 3)-GlcNAc-*β*-(1 → 3)-Gal-*β*-(1 → 4)-Glc	+	−	+	−
7	LNFP II	Gal-*β*-(1 → 3)-GlcNAc-*β*-(1 → 3)-Gal-*β*-(1 → 4)-Glc Fuc-*α*-(1 → 4)/	+	+	−	−
8	LNFP III	Gal-*β*-(1 → 4)-GlcNAc-*β*-(1 → 3)-Gal-*β*-(1 → 4)-Glc Fuc-*α*-(1 → 3)/	+	+	+	+
9	LNDFH I	Fuc-*α*-(1 → 2)-Gal-*β*-(1 → 3)-GlcNAc-*β*-(1 → 3)-Gal-*β*-(1 → 4)-Glc Fuc-*α*-(1 → 4)/	+	−	−	−
10	LNDFH II	Gal-*β*-(1 → 3)-GlcNAc-*β*-(1 → 3)-Gal-*β*-(1 → 4)-Glc Fuc-*α*-(1 → 4)/ Fuc-*α*-(1 → 3)/	+	+	−	−
11	LNH	Gal-*β*-(1 → 4)-GlcNAc-*β*-(1 → 6)\ Gal-*β*-(1 → 4)-Glc Gal-*β*-(1 → 3)-GlcNAc-*β*-(1 → 3)/	+	+	+	+
12	2′-F-LNH	Gal-*β*-(1 → 4)-GlcNAc-*β*-(1 → 6)\ Gal-*β*-(1 → 4)-Glc Fuc-*α*-(1 → 2)-Gal-*β*-(1 → 3)-GlcNAc-*β*-(1 → 3)/	+	−	+	−
13	3′-F-LNH	Fuc-*α*-(1 → 3)\ Gal-*β*-(1 → 4)-GlcNAc-*β*-(1 → 6)\ Gal-*β*-(1 → 4)-Glc Gal-*β*-(1 → 3)-GlcNAc-*β*-(1 → 3)/	+	+	+	+
14	2′,3′-F-LNH	Fuc-*α*-(1 → 3)\ Gal-*β*-(1 → 4)-GlcNAc-*β*-(1 → 6)\ Gal-*β*-(1 → 4)-Glc Fuc-*α*-(1 → 2)-Gal-*β*-(1 → 3)-GlcNAc-*β*-(1 → 3)/	+	−	+	−

sample oligosaccharides with fucose residues in α1,2 and α1,3 linkages were detected. The carbohydrates LNFP II, LNDFH I, and LNDFH II with α1,4 fucosyl residues were missing. This oligosaccharide pattern as shown in Table 1 (third milk group) was detected in human milk from four individuals with blood type Le(a−b−).

However, one milk sample from a Le(a−b−) individual exhibited a different HPAEC profile (Figure 4). Besides nonfucosylated compounds only oligosaccharides bearing α1,3 linked fucosyl residues – that is 3-FL, LNFP III and 3′-F-LNH were found, whereas structures with α1,2 and α1,4 fucosyl linkages were missing (Table 1; fourth milk type).

Discussion

Analysis of human milk samples from approximately 50 women yielded only a few oligosaccharide patterns consisting of maximal 14 neutral oligosaccharides as revealed by HPAEC analysis. Further oligosaccharides characterized so far could not be identified with certainty by HPAEC because of low abundancy or insufficient resolution. However, a selection of 14 generally major compounds represents an adequate basis in order to differentiate human milk groups.

In human milk samples from Le(a−b+) individuals, all known major milk oligosaccharides with α1,2, α1,3, and α1,4 fucose residues were found. Two of these oligosaccharides,

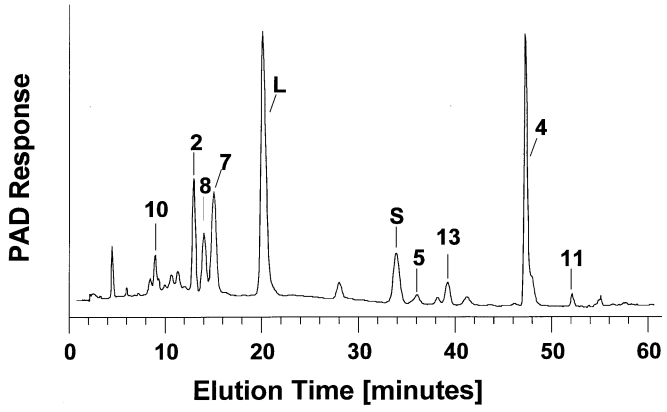

Figure 2. HPAEC profile of the neutral oligosaccharide fraction from a human milk sample typical for Le(a+b−) individuals. The neutral oligosaccharides that were identified are numbered according to Table 1. L, lactose; S, stachyose.

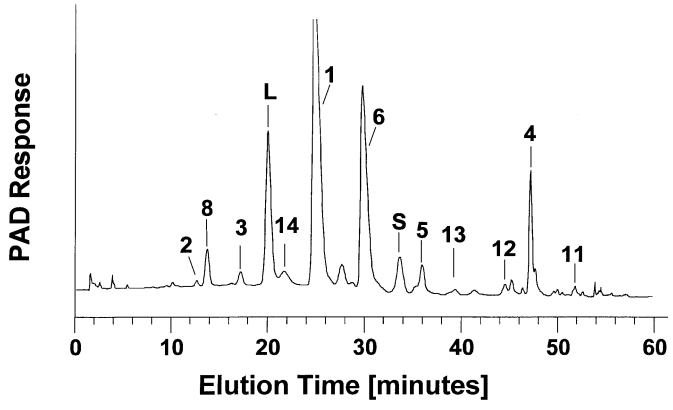

Figure 3. HPAEC profile of the neutral oligosaccharide fraction from a human milk sample typical for Le(a−b−) individuals. The neutral oligosaccharides that were identified are numbered according to Table 1. L, lactose; S, stachyose.

LNFP II and LNDH II, contain the carbohydrate sequence Galβ(1-3)[Fucα(1-4)]GlcNAc-R thus possessing Le^a activity [7]. The oligosaccharide LNDFH I found in this milk group with the carbohydrate motive Fucα(1-2)Galβ(1-3)[Fucα(1-4)]GlcNAc-R exhibits Le^b activity. In accordance with the results from Kobata *et al.* [6] and Viverge *et al.* [15] these milk samples can be attributed to the first human milk group that is characterized by active secretor and Lewis genes. In milk samples from Le(a+b−) donors oligosaccharides with α1,2 fucose linkages were absent. This oligosaccharide pattern can be explained by an active Lewis gene and an inactive secretor gene coding for an α1,2 fucosyltransferase. This oligosaccharide pattern fits perfectly to the second milk type described by Kobata [6]. Viverge

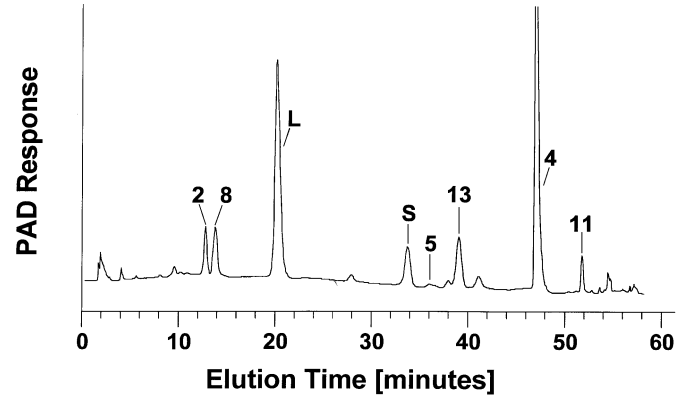

Figure 4. HPAEC profile of the neutral oligosaccharide fraction from human milk of a Le(a−b−) individual. The neutral oligosaccharides that were identified are numbered according to Table 1. L, lactose; S, stachyose.

et al. [15] found a similar oligosaccharide pattern with the exception of LNDFH II that was absent in human milk from Le(a+b−) donors. Lack of LNDFH II exhibiting Le^a activity contradicts an active Lewis gene. Milk samples from Le(a−b−) donors usually lacked oligosaccharides bearing α1,4 linked fucose residues. This pattern is the consequence of an active secretor gene and an inactive Lewis gene and corresponds to the third milk type as already described [6]. In all three milk groups oligosaccharides with α1,3 fucose residues were found. This finding can be explained by the occurrence of an additional fucosyltransferase in human milk with exclusively α1,3 fucosyltransferase activity [8, 16, 17].

The oligosaccharide pattern shown in Figure 4 has not been described so far. The absence of α1,2 as well as α1,4 fucosyl linkages should be the consequence of an inactive secretor as well as an inactive Lewis gene. From this finding we postulate that there exists a fourth human milk type. The expression of four different milk groups fits well to a logical matrix involving two genes and two fucosyltransferases respectively. In addition, four Lewis blood types involving the Lewis and the secretor gene have been described on erythrocytes [7]. The proposed correlation between the action of secretor gene, Lewis gene and of the Lewis phenotypes on erythrocytes on the one hand and the four oligosaccharide groups in human milk on the other hand is shown in Table 2. The third and fourth milk groups both are assigned to blood type Le(a−b−) since human milk samples from both groups lack Le^a and Le^b activities. In samples corresponding to the third milk group the oligosaccharides LNFP I, 2′-F-LNH and 2′,3′-LNH with the reducing terminus Fucα(1-2)Galβ(1-3)GlcNAc were found. This carbohydrate structure has been defined as Le^d antigen [7]. The Le^d antigen is also known as 'H type 1' antigen [18]. However, in the milk sample with a new carbohydrate pattern

Table 2. Correlation between Lewis blood groups and milk oligosaccharide groups

Genotypes		*Phenotypes*		
Secretor	*Lewis*	*Erythrocytes*		*Milk oligosaccharides*
		Lewis	*%*	
Se/−	Le/−	a−b+c−d−	69	First group
se/se	Le/−	a+b−c−d−	20	Second group
Se/−	le/le	a−b−c−d+	9	Third group
se/se	le/le	a−b−c−d+	1	Fourth group

oligosaccharides with Fucα(1-2) linkages and Le^d antigens respectively have not been found. Here, oligosaccharides with Galβ(1-3)GlcNAc residues were detected. This sugar chain has been defined as Le^c antigen [7]. As a consequence milk types three and four could be differentiated serologically if not only Le^a and Le^b activities but also Le^c and Le^d activities were analysed. The low frequency of blood group Le(a−b−c−d+) of 1% [17] should correspond to a low frequency of the fourth milk group. This could explain why this oligosaccharide pattern has not been described before. In contrast to our findings and to the results of Kobata [6], Viverge *et al.* [15] found oligosaccharide patterns in milk from Le(a−b−) individuals that do not correspond to the action of either an active or an inactive secretor gene.

HPAEC analysis of oligosaccharides from human milk has been demonstrated to be an appropriate method to reveal oligosaccharide patterns. Compared to previously applied methods such as GPC and paper chromatography [6, 14, 15], this technique is a fast and convenient method which allows the analysis of a large number of samples.

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