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ORIGINAL CONTRIBUTION

Silvia Rudloff
Christina Stefan
Gottfried Pohlentz
Clemens Kunz

Detection of ligands for selectins in the oligosaccharide fraction of human milk

Summary *Background* Human milk contains a large variety of oligosaccharides which show structural similarities with ligands for selectins, a family of cell adhesion molecules which are involved in many cell-cell interactions. *Aim of the study* Due to their structural similarity with selectin ligands, human milk oligosaccharides were labelled with phosphatidyl ethanolamine to be able to investigate specific effects of antibodies against carbohydrate epitopes. *Methods* Various monoclonal antibodies against physiological selectin ligands were used to determine whether epitopes within human milk oligosaccharides are recognized. Oligosaccharides were isolated from human milk, transferred into neoglycolipids and separated using high performance thin layer chromatography prior to incubation with monoclonal antibodies for the selectin ligands sialyl-Lewis a (sLe a), sialyl-Lewis x (sLe x), Lewis x (Le x) and Lewis y (Le y) after Western blotting. Fast atom bombardment-mass spectrometry was then used to identify antibody-binding compounds. *Results* In the immunoassays all ligand epitopes except for Le y were detected in the oligosaccharide fraction of human milk. Anti-sLe a showed the most distinct reaction with N-acetylneuraminic acid containing neoglycolipids of which two were identified as neuraminyl-fucosyl-lacto-N-hexaose and neuraminyl-lacto-N-tetraose. Such oligosaccharides as well as similar structures are present in relatively high concentrations in human milk. *Conclusions* The presence of sialyl-Lewis ligands on milk oligosaccharides together with their abundancy in human milk may suggest that they could be selectin ligands and they may be part of inflammatory processes.

Key words Human milk – selectin ligands – oligosaccharides – neoglycolipids

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Dr. S. Rudloff (✉)
Justus Liebig University
Institute of Nutritional Sciences
Wilhelmstrasse 20
35392 Giessen, Germany
Tel.: +49-641/99-39041
Fax: +49-641/99-39049
E-Mail:
silvia.rudloff@ernaehrung.uni-giessen.de

S. Rudloff · C. Stefan · C. Kunz
Research Institute of Child Nutrition
Heinstück 11
44228 Dortmund, Germany

G. Pohlentz
Institute for Physiological Chemistry
University of Bonn
Nussallee 11
53115 Bonn, Germany

Introduction

Selectins, a group of cell adhesion molecules, are expressed on the cell surface of leukocytes, lymphocytes as well as on endothelial cells and are involved in the cell adhesion cascade leading to the extravasation of blood cells into the tissue during inflammatory processes [1–3]. Through the binding of their lectin-like domains to carbohydrate ligands situated on the membrane of blood or endothelial cells [4, 5], leukocytes are removed from the blood stream and start rolling along the vessel wall until they are firmly attached, a process which is mediated by other types of cell adhesion molecules.

Human milk is an unusual biological fluid that contains a large variety of free oligosaccharides in quantities of 5–10 g/l [6, 7]. Among these carbohydrates there are various structures that carry epitopes similar to the ligands for selectins [8, 9]. However, it is unclear whether milk oligosaccharides are able to exert immunomodulating functions by blocking cell adhesion and thus preventing overshooting immune reactions.

Table 1 Monoclonal antibodies (from Seigagaku, Tokyo, Japan) against epitope ligands (Lewis antigens)

Antibody	Clone	Ig class
anti-sialyl Lewis a	2D3	mouse IgM kappa
anti-sialyl Lewis x	KM–93	mouse IgM
anti-Lewis x	73–30	mouse IgG_3 kappa
anti-Lewis y	H18A	mouse IgG_3 kappa

In this study, we used an overlay technique to test whether the monoclonal antibodies, shown in Table 1 (see Methods section), directed against physiological selectin ligands also recognize epitopes within milk oligosaccharides. The main focus was on the detection of epitopes for Lewis x (Galβ1-4[Fucα1-3]GlcNAc-R), Lewis y (Fucα1-2Galβ1-4[Fucα1-3]GlcNAc-R), sialyl-Lewis x (NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAc-R) and sialyl-Lewis y (NeuAcα2-3 [Fucα1-2]Galβ1-4[Fucα1-3]GlcNAc-R).

Material and methods

Milk sampling

Milk from 6 women in the third week of lactation was collected by completely emptying the milk from the breast using electric pumps into sterile glass bottles. Aliquots were stored at –20 °C until further analysis.

Isolation and characterization of milk oligosaccharides

The isolation of oligosaccharides from human milk was performed as previously described [28, 29]. Briefly, fat and protein were removed from the milk samples by centrifugation and subsequent precipitation using ethanol. The resulting soluble phase was subjected to gel filtration chromatography (Sephadex G25, Pharmacia, Uppsala, Sweden) to remove lactose and proteins. Complex carbohydrates remaining in solution were then separated into neutral and acidic (containing N-acetylneuraminic acid, NeuAc) oligosaccharides using anion exchange chromatography (FPLC-Resource Q, Pharmacia, Uppsala, Sweden). The composition of these oligosaccharide fractions was characterized by silica-high performance thin layer chromatography (HPTLC) and high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [29]. HPAEC-PAD was performed on a Carbo Pac™ PA1 column (Dionex, Sunnyvale, CA, USA) with a gradient of buffer 1 (100 mMol NaOH) and buffer 2 (100 mMol NaOH, 250 mMol Na-acetate). 25 µl of each milk carbohydrate solution was injected.

Synthesis of neoglycolipids

For the further use of oligosaccharides in overlay techniques to identify ligand epitopes, they were converted into water-insoluble components by coupling to dipalmitoyl-phosphatidyl-ethanolamine (PE; Sigma, St. Louis, MO, U. S. A.) in a reductive aminoreaction according to methods reported by Feizi et al. and Pohlentz and Egge [14, 30]. The course of the reaction was followed by HPTLC and the plates were sprayed with a "Stains All" reagent consisting of 2.5% molybdatatophosphoric acid, 1% cerium sulfate in 6% sulfuric acid.

Antibody reactions with neoglycolipids after HPTLC

After the separation of the neoglycolipids using HPTLC, the plates were coated with Plexigum P28 (polyisobutylacrylate; Röhm & Haas, Darmstadt, Germany) by dipping them into a solution of 5 g polyisobutylacrylate, 95 g chloroform and 5 g ethanol diluted with n-hexane (plexigum stock solution: n-hexane, 1: 10) for 90 s. The plates were allowed to dry and were then incubated in a blocking solution which consisted of 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS; 0.01 M Na_2HPO_4, 0.01 M KH_2PO_4, 0.15 M NaCl, pH 7.4). Thereafter, the plates were incubated with monoclonal antibodies (Table 1; 1–4 µg/ml PBS containing 1% BSA) for 6 h at room temperature. Unbound antibodies were removed by washing the plates with PBS. Then, a secondary antibody conjugated with alkaline phosphatase (anti-mouse IgM (Caltag, San Francisco, CA, U. S. A.) or anti-mouse Ig (Boehringer, Mannheim, Germany) was diluted (antibody solution: PBS containing 3% polyethylene glycol, 1: 1000) and incubated with the HPTLC plates. After 1 h at room temperature the plates were developed in the substrate for alkaline phosphatase (5-bromo-4-chloro-3-indolyl-phosphate and 4-nitrobluetetrazolium chloride) until positive reacting bands were visible.

Identification of neoglycolipids by fast atom bombardment mass spectrometry (FAB-MS)

Neoglycolipids were characterized by FAB-MS according to Pohlentz et al. (1992) [31]. Briefly, FAB-MS analyses were carried out with a VG Analytical ZAB-HF reverse geometry mass spectrometer fitted with an Ion-Tech atom gun (VG, Manchester, UK). For atom bombardment xenon was used and the applied acceleration voltage was 7 kV. Thioglycerol (tgl) was used as the matrix. The sample bands were scraped from the HPTLC plates and the obtained silica gel was directly mixed with the matrix on the probe tip. Positive ions (FAB(+)) were detected and the spectra were run in a mass range from

100 to 3000 atom mass units (amu) with a scan rate of 15 s per decade. The spectra were recorded and evaluated on a SAM II/68 K computer (KWS, Ettlingen, Germany) using the DP10 program of AMD (Harpstedt, Germany).

Results

Isolation and characterization of oligosaccharides

In Fig. 1, the various steps of the isolation procedure for human milk oligosaccharides are shown after HPTLC. It can be seen that lactose as well as monosaccharides were successfully removed from the sample using chromatographic methods.

HPAEC-PAD and HPTLC analysis revealed considerable differences within the fraction of neutral oligosaccharides of some milk donors (Fig. 2). HPAEC-PAD of samples I, II, III and IV showed a similar oligosaccharide

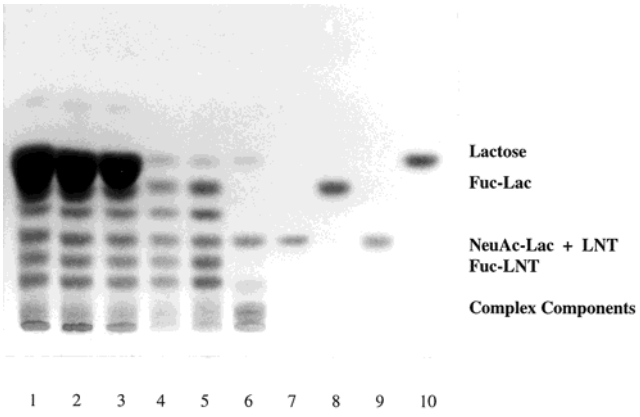

Fig. 1 HPTLC of oligosaccharides prepared from whole milk. 1: untreated human milk; 2: defatted human milk; 3: defatted and deproteinized human milk; 4: total oligosaccharide fraction after Seph G25 gel filtration; 5: neutral oligosaccharide fraction after FPLC anion exchange; 6: acidic oligosaccharide fraction after FPLC anion exchange; 7: standard α2-6-NeuAc-Lac; 8: standard α1-2-Fuc-Lac; 9: standard Lacto-N-neo-tetraose; 10: standard lactose.

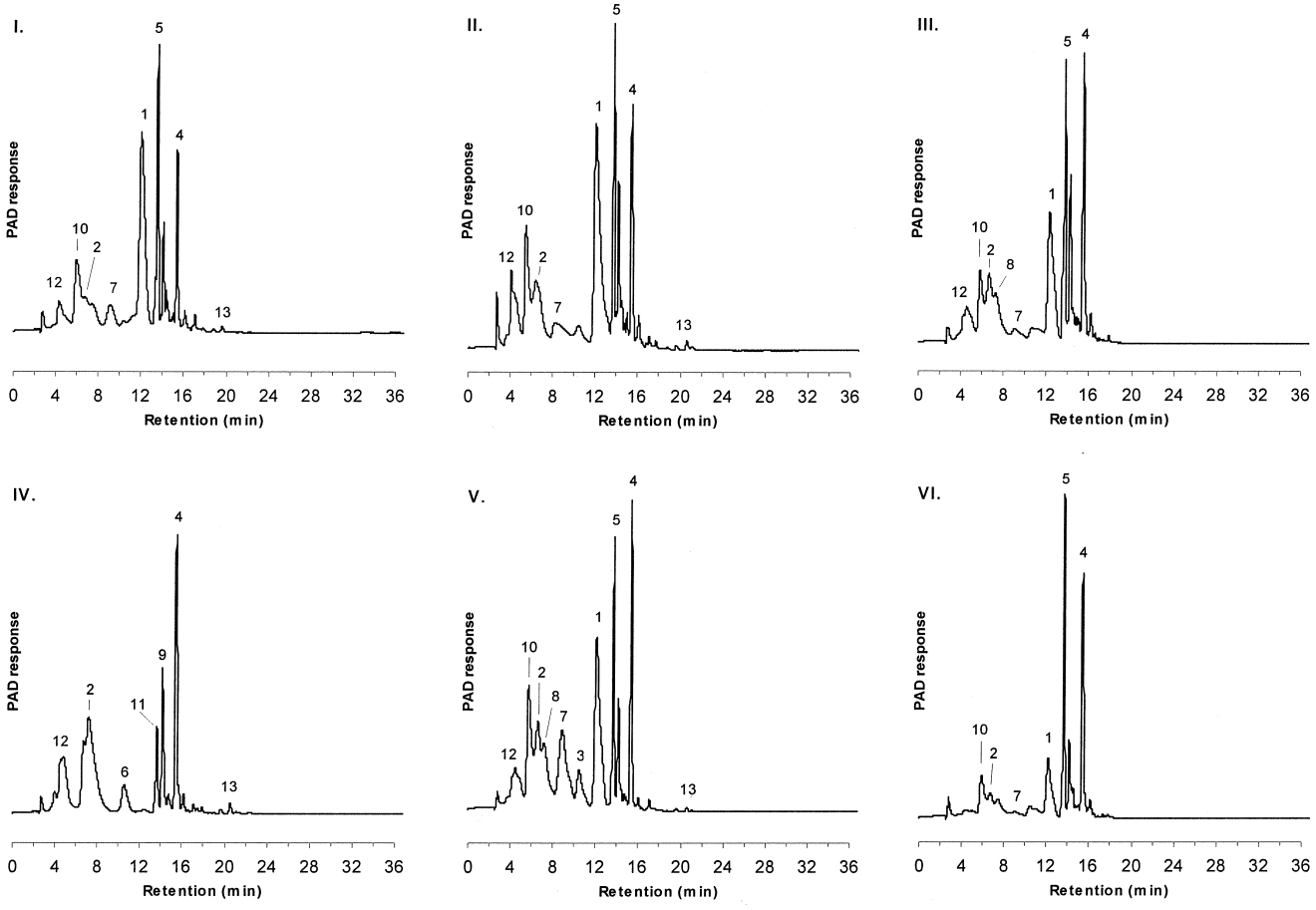

Fig. 2 HPAEC-PAD of neutral oligosaccharides after FPLC-Resource Q anion exchange from milk of 6 lactating mothers (samples I–VI).
1: α1-2-Fuc-Lac; 2: α1-3-Fuc-Lac; 3: Fuc_2-Lac; 4: LNT; 5: LNFP I; 6: LNFP II; 7: LNFP III; 8: LNDFH I; 9: α1-3-Fuc-LNH; 10: LNDFH II; 11: Fuc-LNOctaose; 12: di- and trifucosylated hexaoses and more complex components; 13: acidic oligosaccharides.
Samples I, II, III and V are from milk with Le (a-b+)/secretor activity; sample IV is from milk with Le (a+b-)/nonsecretor activity; sample VI is from milk with Le (a-b-)/secretor activity.

pattern with lacto-N-tetraose (LNT), lacto-N-fucopentaose I (LNFP I) and α1-2-fucosyl-lactose (α1-2-Fuc-Lac) as the main components. In samples I and II, the ratio of LNFP I to LNT was larger than in samples III and V. Furthermore, sample V contained greater amounts of difucosylated compounds, e. g., difucosyl-lactose (Fuc_2-Lac) and lacto-N-difucohexaose I and II (LNDFH I and II) (Fig. 2). These observations were confirmed by HPTLC (Fig. 3).

The HPAEC-PAD profile of sample IV differed significantly from all other samples (Fig. 2). LNT was the major component, whereas LNFP I, α1-2-Fuc-Lac as well as Fuc_2-Lac were not detectable.

These observations allowed a classification into the different Lewis blood types and the secretor status of the milk donors which are shown in Fig. 2 for each individual.

In contrast to the fraction of neutral oligosaccharides, the pattern of acidic components was remarkably similar in the milk samples. They mainly consisted of α2-3-sialyl-lactose (α2-3-NeuAc-Lac), α2-6-NeuAc-Lac, NeuAc-Fuc-LNT and NeuAc-LNT (LST) a, b and c (Fig. 4).

Neoglycolipids from human milk oligosaccharides

To analyze the binding specificities of antibodies against carbohydrate ligands, we used an HPTLC-overlay technique that was developed for glycosphingolipids. Thus, oligosaccharides were changed into neoglycolipids by coupling them to PE, which were then subjected to HPTLC. The separation of these lipid-coupled oligosaccharides is shown in figure 5 a and b. Some of these compounds, e. g., Fuc-Lac-PE, Fuc_2-Lac-PE, LNT-PE, NeuAc-Lac-PE and NeuAc-LNT-PE, were identified by FAB-MS analysis.

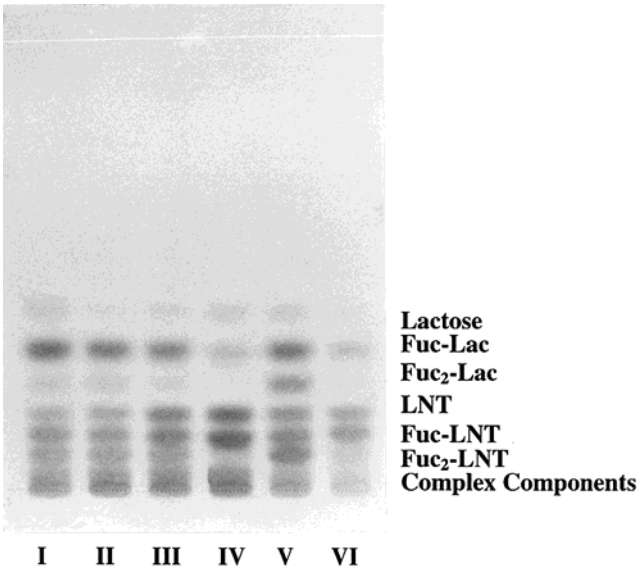

Fig. 3 HPTLC of the neutral oligosaccharide fractions from milk of 6 lactating mothers (samples I–VI). The plate was sprayed with orcinol to visualize carbohydrates.

Sialyl-Lewis a epitopes

After using a monoclonal antibody against the ligand epitope sialyl-Lewis a (sLe a) in the HPTLC overlay with neoglycolipids (NeoGL) derived from acidic oligosaccharides, one major band could be detected in all samples (band 1; Fig. 6 b) whereas two further bands were stained weakly or were hardly detectable (bands 2 and 3; Fig. 6 a). Bands 1 and 3 were identified by FAB-MS as NeuAc-LNT-PE ($M+H^+$: m/z 1674, $M+Na^+$: m/z 1696) and NeuAc-Fuc-LNH-PE ($M+H^+$: m/z 2185, $M+Na^+$: m/z 2207), respectively. Band 2 could not be characterized, probably due to the very low amount.

The overlay of the NeoGL from neutral oligosaccharides with anti-sLe a revealed two distinct bands in samples I and IV that could not be identified yet (Fig. 6 a).

Lewis x epitopes

A positive reaction with monoclonal anti-Lewis x (anti-Le x) was observed with two bands derived from the neutral oligosaccharide fraction from samples III and V, and also with band 1 of sample VI that has been identified as NeuAc-Fuc-LNH-PE ($M+H^+$: m/z 2185, $M+Na^+$: m/z 2207)(Fig. 7 a).

NeoGL prepared from acidic oligosaccharides reacted with anti-Le x as well (Fig. 7 b). Band 1 shown in figure 7 b could be identified as NeuAc-Lac-PE ($M+H^+$: m/z 1309, $M+Na^+$: m/z 1331) by FAB-MS. A further band (band 2) was visible in the samples I – V but could not be detected by staining for carbohydrates with orcinol.

Sialyl-Lewis x epitopes

Fig. 8 shows the HPTLC-overlay after using anti-sialyl Lewis x (anti-sLe x) antibodies and displays striking similarities with the results shown in Fig. 7 b. However, the band (band 1) which was identified as NeuAc-Lac-PE reacted much weaker and band 2 much stronger than with anti-Le x antibodies.

Lewis y epitopes

In contrast to the results described above, no distinct positive reaction was visible when using anti-Lewis y (anti-Le y) antibodies on neoglycolipids after HPTLC separation (data not shown).

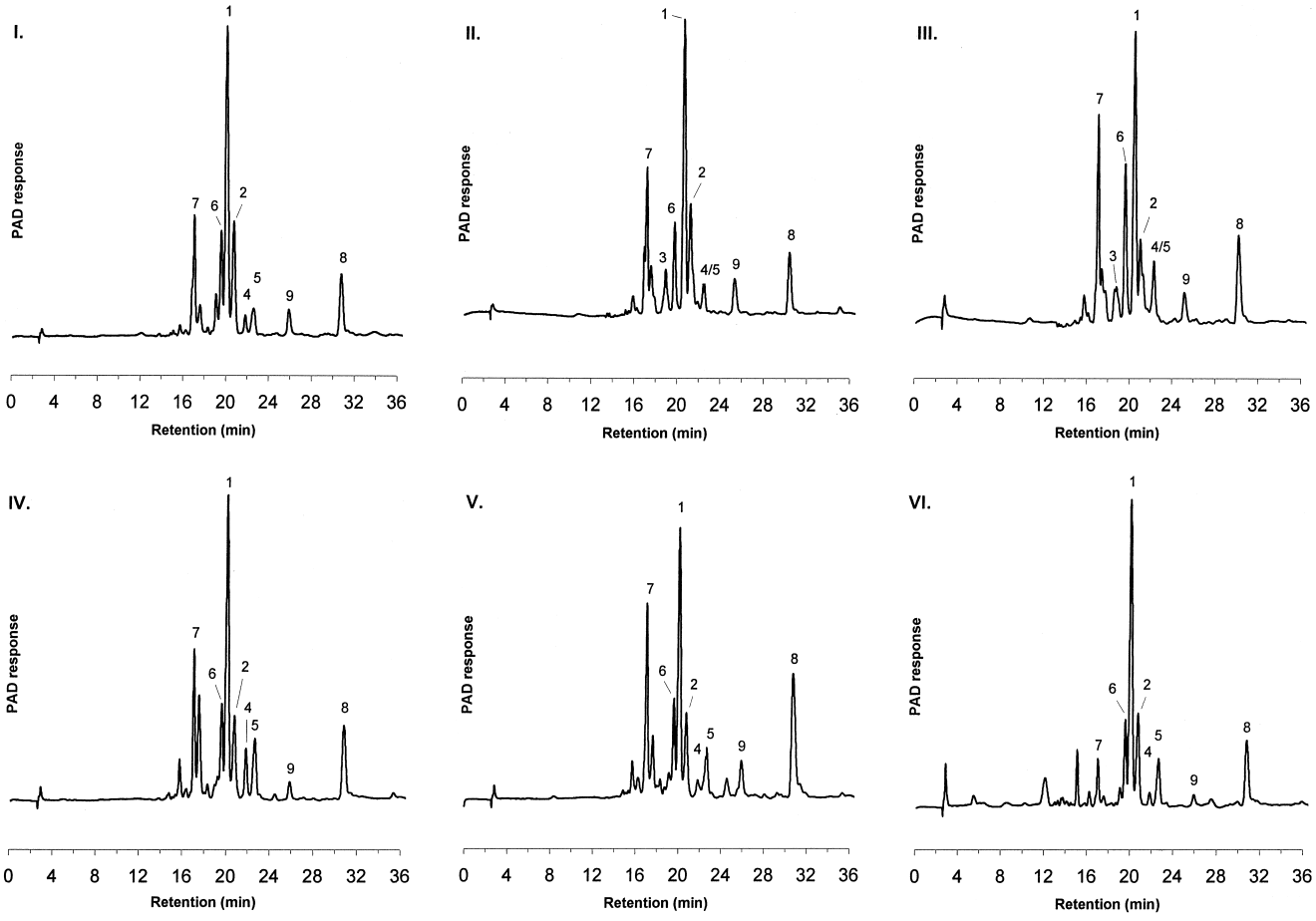

Fig. 4 HPAEC-PAD of the acidic oligosaccharide fractions after FPLC-Resource Q anion exchange from milk of 6 lactating mothers (samples I–VI). 1: α2-6-NeuAc-Lac; 2: α2-3-NeuAc-Lac; 3: α2-3-NeuAc-α1-3-Fuc Lac; 4: LST a; 5: LST b; 6: LST c; 7: NeuAc-Fuc-LNT; 8: $NeuAc_2$-LNT; 9: $NeuAc_2$-Fuc-LNH.
Samples I, II, III and V are from milk with Le (a-b+)/secretor activity; sample IV is from milk with Le (a+b-)/nonsecretor activity; sample VI is from milk with Le (a-b-)/secretor activity.

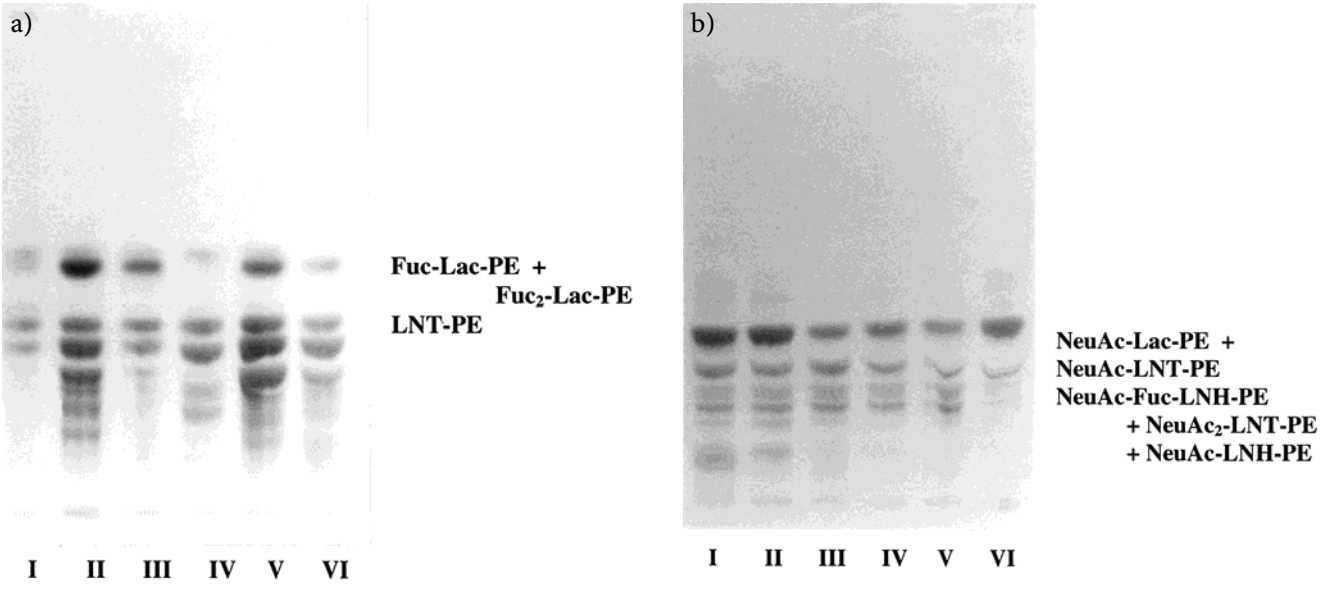

Fig. 5 HPTLC of neutral (**a**) and acidic (**b**) neoglycolipids (samples I–VI). Plates were sprayed with orcinol.

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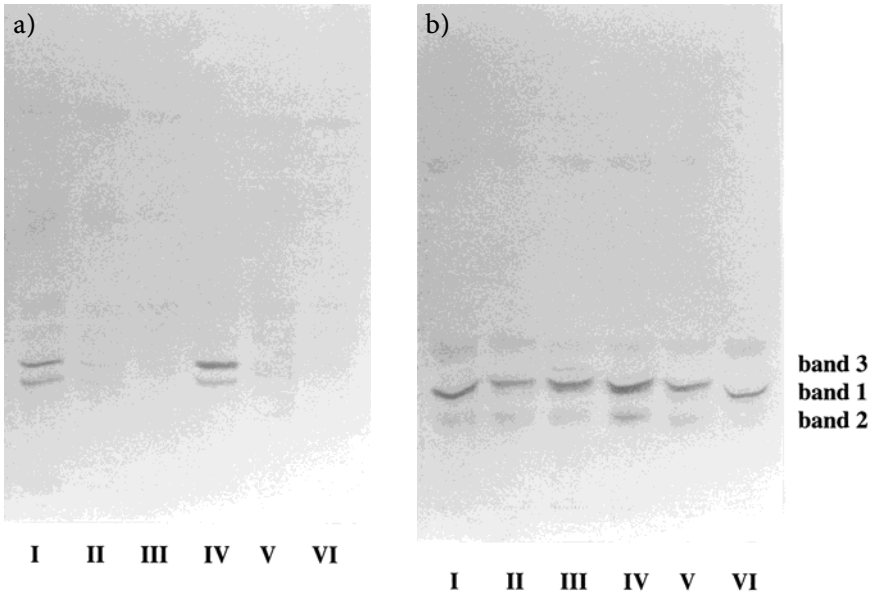

Fig. 6 HPTLC overlay of neutral (**a**) and (**b**) acidic neoglycolipids using anti-sLe a (samples I–VI).

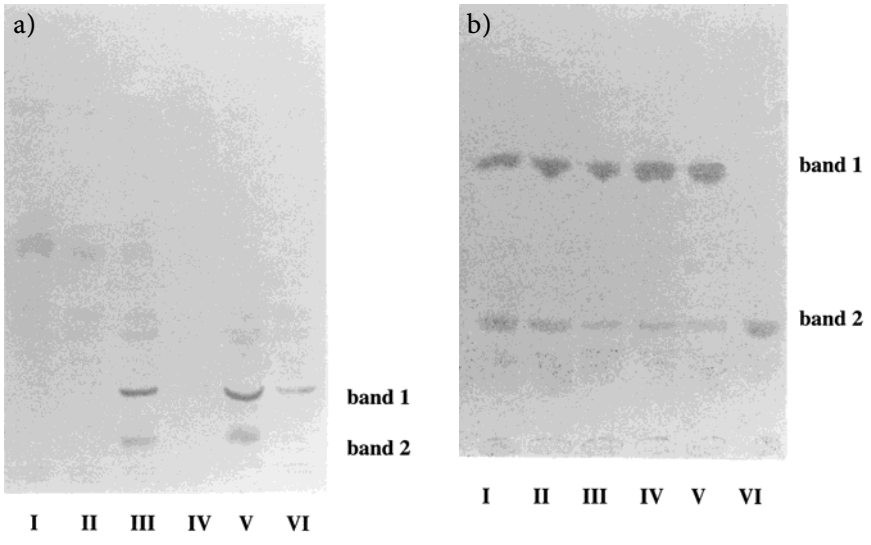

Fig. 7 HPTLC overlay of neutral (**a**) and acidic (**b**) neoglycolipids using anti-Le x (samples I–VI).

Discussion

In the present study, we investigated whether oligosaccharides from human milk contain epitopes for ligands of selectins as a group of cell adhesion molecules mediating initial steps leading to leukocyte extravasation in inflammatory processes [1–3, 10].

In order to use monoclonal antibodies on carbohydrate epitopes, free oligosaccharides such as those from human milk have to be changed into glycoconjugates. During the synthesis of neoglycoproteins multiple glycosylations might occur whereas neoglycolipids usually contain only one carbohydrate chain and can thus be identified much more easily [11, 12]. In addition, since many of the natural selectin ligands are glycoproteins where the protein core might also interfere with the binding to ligands, the use of neoglycolipids has the advantage that a possible protein effect can be excluded.

In contrast to ELISA techniques, HPTLC overlays open the possibility of differentiating between the binding specificities of single component within a mixture of compounds.

Using anti-sLe a antibodies on acidic neoglycolipids, a positive reaction was found with NeuAc-LNT-PE, a nonfucosylated precursor of sLe a and with NeuAc-Fuc-LNH-PE which contained the sLe-a epitope (Fig. 6 b). This means that the monoclonal antibody used in this study not only recognized the complete sLe a epitope, but also similar structures without fucose residues.

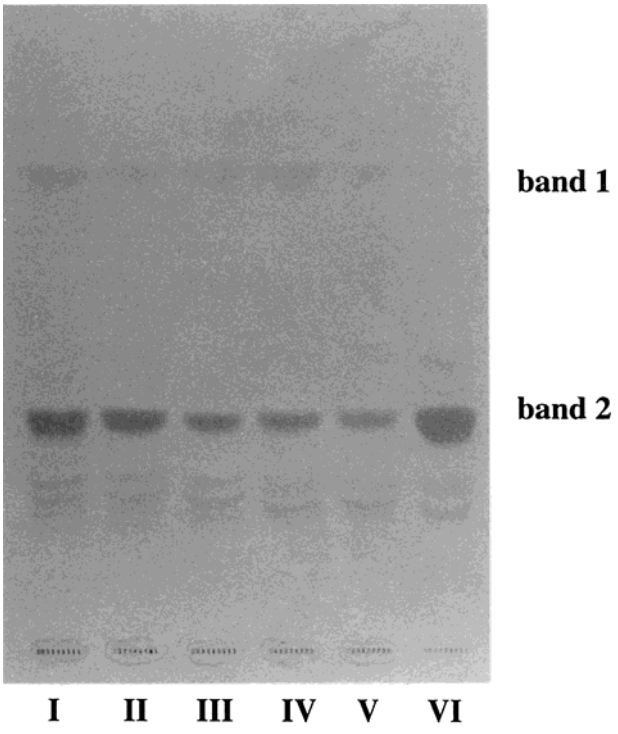

Fig. 8 HPTLC-overlay of acidic neoglycolipids using anti-sLe a (samples I–VI).

Within the neutral fraction of neoglycolipids Anti-sLe a antibody also leads to a positive reaction (Fig. 6 a). This may be attributed to an incomplete chromatographic separation of neutral and acidic oligosaccharides due to their complexity as many oligosaccharides are both fucosylated as well as sialylated.

All three types of selectins (E-, P- and L-selectin) bind sLe a as well as sLe x [13]. Sialyl-Le a was even reported to have the highest binding affinity for E-selectin [14–16]. It is unlikely that sLe a precursors without fucose are able to bind selectins since it has been shown that α1-3- or α1-4-bound fucose is necessary for an efficient binding [4, 17, 18]. However, Lundblad and coworkers (1993) hypothesized that milk oligosaccharides may be modified within the intestinal cells of infants and then re-enter the intestinal lumen as a result of their studies in term and preterm infants [19]. If fucosylation occur by these means, then, non-fucosylated precursors may also be of importance as selectin ligands.

NeuAc-Fuc-LNH-PE was identified among the components reacting with anti-Le x antibodies. This oligosaccharide also binds to anti-sLe a although the epitope structure of Le x and sLe a is quite different. An explanation for this may be that NeuAc-Fuc-LNH-PE exists in various isomer forms and 4 of these have already been identified in human milk [8, 9]. One of these derivatives contains the Le x epitope, whereas another contains both the Le x and the sLe a epitopes.

Furthermore, anti-Le x also reacted with a component within the acidic fraction of neoglycolipids which was not visible with the carbohydrate stain orcinol (compare Figs. 5 b and 7 b). A plausible explanation is the very high sensitivity of the immunostaining method compared to orcinol staining.

The relatively weak reaction of neoglycolipids with anti-sLe x antibodies might be explained by the very small amount of sLe x structures in human milk. In contrast, Le x epitopes are present in the highest quantities. Although the inhibitory effect of sLe x and sLe a in most in vitro tests was found to be higher, Larsen et al. (1990) reported that Le x structures also blocked cell adhesion, especially the P-selectin dependent adhesion of activated platelets to leukocytes [20]. Since some oligosaccharides in human milk contain various Le epitopes within one molecule [21], their combined action may result in an increased affinity for selectins.

The prerequisites for selectin ligands to mediate effective binding between leukocytes and endothelial cells are still being discussed. Structural analysis of natural ligands on leukocytes such as PSGL-1 or GlyCAM-1 suggests that the linkage of the glycan chain as well as additional sulfate groups may play a role in the binding affinity [22, 23]. However, other reports indicate that the binding of selectin ligands is completely independent of the polypeptide chain which may simply function as a ligand carrier [24]. In vitro inhibition studies have shown that oligosaccharide molecules are effective in preventing cell adhesion [14, 25] which would suggest that the epitopes in oligosaccharides from human milk could exert similar functions. It has recently been shown in "in vitro" experiments that human milk oligosaccharides are not digested [26, 27], and therefore, may still carry epitopes for selectins. Furthermore, recent results confirm that some lactose-derived oligosaccharides in infants survive the gastrointestinal passage and the absorptive processes and are excreted via urine [7]. Therefore, some carbohydrate epitopes from HM might circulate in the infant's blood for a period of time before they are excreted. Hence, the prevention of inflammatory bowel diseases or their chronic manifestation in breast fed infants could be possible by carbohydrate interaction with mucosal leukocytes.

Further studies are necessary to investigate whether these oligosaccharide structures also act as immune modulators *in vivo*. An important tool for such investigations might be the application of in vivo labelled ^{13}C-oligosaccharides in addition to *in vitro* tests with synthetic components [7].

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