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The carbohydrate moiety and high molecular weight carrier of histo-blood group antigens are both required for norovirus-receptor recognition

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Abstract Histo-blood group antigens (HBGAs) on human intestinal epithelium serve as receptors for noroviruses (NVs). These antigens also are expressed in milk and may act as decoy receptors to protect breast-fed infants and others against NV disease. In this study we demonstrated that human milk is highly variable in synthesis of HBGAs, which differs from that of saliva; a large quantity of small, soluble HBGAs are found in milk, but much less in saliva and are recognized by MAbs, but not by NVs. There is another group of HBGAs, of high MW, found in both milk and saliva, and recognized by both NVs and MAbs. These results suggest that the specificity of NVs and MAbs to HBGAs are different and the backbones in addition to the carbohydrate moiety are required for NV recognition. Further studies to define the structure and genetics of the high MW milk glycans are necessary.

Keywords Norovirus · Calicivirus · Human milk · Blood group antigens · Diarrhea · Gastroenteritis · Receptor

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Abbreviations

NVs	noroviruses
HBGAs	histo-blood group antigens
FPLC	fast protein liquid chromatography
MAb	monoclonal antibody
TFMS	trifluoromethanesulphonic acid
DTT	dithiothreitol
mAU	milliabsorbance unit

Introduction

Noroviruses (NVs), a major cause of acute gastroenteritis, belong to the calicivirus family, which includes more than 25 genetic clusters of NVs in five genogroups [1]. NVs are difficult to study for lack of a cell culture or animal model. The cloning of many NVs in the early 1990s greatly advanced NV research [2–5]. Expression of the major NV capsid protein in baculovirus [6–9] and other *in vitro* expression systems [10] results in self-forming virus-like particles (VLPs), which greatly facilitated the study of the immunology, diagnosis and host/pathogen interaction of NVs.

Human NVs have been found to recognize human histo-blood group antigens (HBGAs) as receptors [11–16]. HBGAs are the carbohydrate moieties of glycans found on cell membranes, such as red blood cells and mucosal surfaces, or of soluble glycans in body fluids, such as saliva, milk and intestinal contents [17]. Direct evidence for a role of HBGA receptors in infection by NVs has been obtained for the prototype Norwalk virus in human volunteer studies [18–20]. Direct evidence of such a role for infection by other NV strains remains limited.

Human milk contains multiple non-antibody factors that protect infants from diarrhea and other infectious diseases. Following the identification of HBGA receptors of NV in human saliva, we found that the high concentrations of HBGAs expressed in human milk can specifically block NV binding to their receptors [21]; milk HBGAs may act as decoy receptors to protect breast-fed infants from calicivirus infection [22, 23]. In this study, we resolve the milk and saliva glycans by fast protein liquid chromatography (FPLC), characterize the HBGA-containing glycans for interaction with NVs, and define the roles of the carbohydrate moieties and protein backbone of human HBGA in NV/host interaction.

Materials and methods

Preparation of milk and saliva samples A single milk sample for each of ten women at 1 to 4 weeks postpartum from the human milk bank at Cincinnati Children's Hospital Medical Center and ten saliva samples (five secretor and five non-secretor) from a saliva bank described in our previous studies [14] were selected for this study. Saliva samples were centrifuged at 5,000 rpm for 10 min to remove debris. The supernatant was boiled for 10 min and then centrifuged again at 10,000 rpm for 10 min. Milk samples were centrifuged at 10,000 rpm for 10 min and fat layer was removed. Aqueous phase of milk and supernatant of saliva were further passed through a 0.2 μm filter and subjected to fast protein liquid chromatography (FPLC). Milk and saliva samples and their FPLC fractions were stored in −80°C. Before testing for HBGAs and NV binding, each milk and saliva sample was boiled for 10 min to inactivate antibodies.

Fast protein liquid chromatography (FPLC) of milk and saliva samples Three to 5 ml of milk and saliva samples were fractionated by FPLC (Amersham Pharmacia Biosciences Corp, Piscataway NJ) on a Superdex 200 XK 16/60 (milk 1 to 10 and saliva 1 to 5) or Sephacryl S-500 XK 16/60 (saliva 6 to 10). Columns were equilibrated with phosphate buffered saline (PBS, 0.01 M, pH 7.4) and eluted at a flow rate of 1 ml/min. The eluate was collected as 1.2 mL fractions starting from 0.28 column bed volumes, and monitored at 280 nm. The columns were calibrated with HMW Gel Filtration Calibration Kit (cat. 17-0441-01, lot 303232) from Amersham Biosciences Corp.

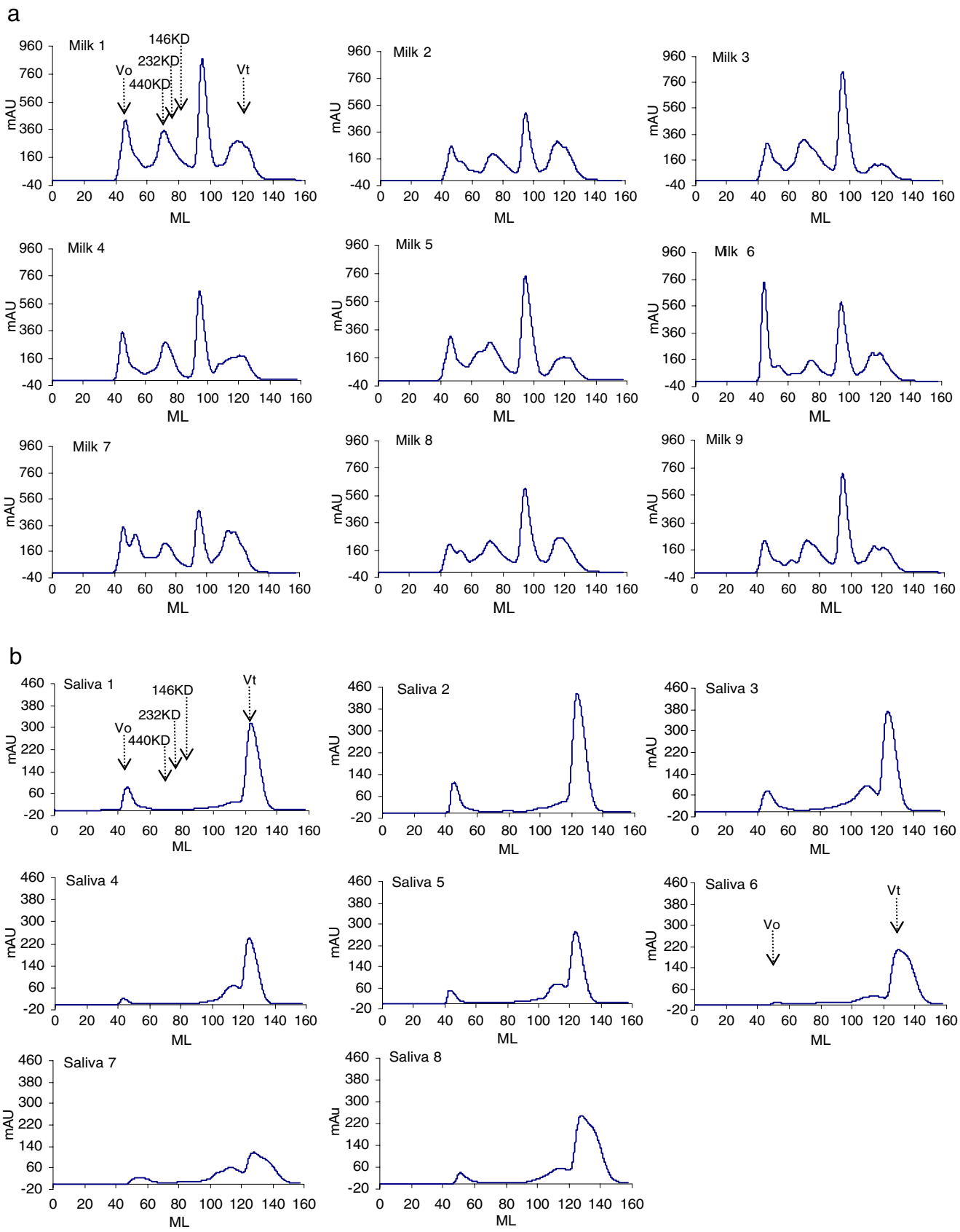
Detection of HBGA and mucin in milk and saliva samples HBGAs and mucin were detected in milk and saliva samples using the similar approach described previously [14, 24]. Briefly, FPLC fractions of milk or saliva samples were coated on microtiter plates (Dynex Immulon; Dynatech, Franklin, MA). After blocking with Blotto (nonfat bovine milk), specific monoclonal antibodies (MAbs) against the various HBGAs or mucins were incubated, and detected by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or IgM antibody binding. The binding was visualized by TMB (Kirkegard & Perry Laboratories, Gaithersburg, MD) and quantified by measuring the optical density (OD) at 450 nm in an EIA spectra reader (Tecan, Durham, NC). MAb anti-MUC1 (BC-2) (Signet Laboratories Inc., Dedham, MA) was used for detection of milk mucin and BAbCO 17B1 (Covance, the Development Service Company, Berkeley, CA) for detection of mucin in saliva.

Fig. 1 FPLC profiles of human milk and saliva with different histo-blood group antigen types. **a** Milk samples were centrifuged at 10,000×*g* for 10 min to remove cell debris and fat, 3 to 5 mL of clarified aqueous samples was applied to a Superdex 200 column (1.6×75 cm). **b** Saliva samples were centrifuged at 5,000 rpm for 5 min to remove pellets. The supernatant was boiled for 10 min and centrifuged at 10,000 rpm to remove any trace precipitates. Applied 3–5 mL of clarified sample to PBS (0.01 M, pH 7.4) equilibrated Superdex 200 (1.6×75 cm) column (saliva 1 to 5) or Sephacryl S500 column (saliva 6 to 10). Column was eluted with PBS at a flow rate of 1 ml/min and the elute fractions were monitored by UV adsorption at 280 nm. Fractions were collected at 1.2 mL each and started from 0.28 column bed volume (120 mL×0.28). *mAU* milliabsorbance unit

Binding of NV VLPs to FPLC fractions of milk and saliva samples Binding by recombinant VLPs from NV strain VA387 and VA207 was measured by EIA methods described previously [14, 21]. Strain VA387 (GII-4) was isolated in a nursing home outbreak and strain VA207 (GII-9) in a family outbreak of NV gastroenteritis in Virginia in the late 1990s. Briefly, FPLC fractions of milk and saliva samples were coated onto microtiter plates at a dilution of 1:15 for milk, 1:30 for saliva, or at specific dilutions indicated in the text, in PBS (pH 7.4). After blocking with 5% Blotto, NV VLPs were added. The bound VLPs were detected by hyperimmune guinea pig anti-VA387 or anti-VA207 antisera followed by a horseradish peroxidase (HRP)-conjugated goat anti-guinea pig IgG (ICN, Aurora, OH).

Measuring MAb blocking of NV binding to HBGAs For a blocking assay, the same principle and conditions described for the binding assay were used, except an incubation step with MAbs was added. Plates coated with FPLC fractions were incubated with MAbs specific to HBGAs described above at a dilution of 1:5 to 1:10. NV VLPs were added and captured VLPs were detected at 450 nm, as described above. The percentage of blocking was determined by comparing the optical density (OD) of wells with or without MAbs.

Chemical deglycosylation of HBGAs High MW fractions of milk and saliva samples resolved by FPLC were deglyco-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Milk 1
146KD
232KD
440KD
Vo
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Milk 2
Milk 3
Milk 4
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Milk 7
Milk 8
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mAU
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b
Saliva 1
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Saliva 4
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Saliva 6
Saliva 7
Saliva 8

sylated by trifluoromethanesulphonic acid (TFMS) (Por-Zyme Inc., San Leandro, CA). Briefly, 200 µl of high MW fractions of milk and saliva samples were lyophilized and placed in a dry-ice/ethanol bath and TFMS/Toluene mixture was slowly added via a metal needle. Samples were incubated at −20°C for 4 h with brief shaking, whereupon the reaction was quenched and neutralized. Deglycosylated samples were dialyzed and tested for binding activity using the above EIA binding assays.

Mucin specific MAb-affinity column IgG of anti-mucin MAb BAbCO 17B1 ascites was purified by Hitrap Protein G HP (Amersham Biosciences Corp, Piscataway NJ) and purified IgG was coupled to agarose by the AminoLink kit (Pierce, Rockford, IL). The coupled reaction slurry was packed into a column and uncoupled MAb IgG was washed away with binding buffer. The remaining active sites were blocked by a quenching solution followed by incubation with the Reductant Solution described in the kit.

Recombinant NV-affinity column The P domain of Norwalk virus capsid expressed in *E. coli*, of the same antigenicity and receptor binding activity as VLPs described previously [25, 26], was used to prepare a NV specific affinity column. Purified viral protein was immobilized on agarose beads by the AminoLink kit (Pierce) as described above. Milk components bound to the column were eluted with glycine buffer (pH 2.9) and the eluate was neutralized to pH 7.4 immediately and then concentrated on a 10,000 Da MWCO ultrafilter (Millipore, Bedford, MA).

Protease digestion of high MW peaks of milk and saliva The highest MW FPLC peaks of milk or saliva were pooled and digested by 0.5 mg/ml Pronase (Roche Applied Science, Indianapolis, IN) in 10 mM calcium chloride for 24 h at 37°C. After digestion, the sample was re-isolated by FPLC on a Superdex 200 column with PBS as the mobile phase, and the glycan characterized for HBGA by MAb EIA and for NV binding by VLP EIA.

Dithiothreitol (DTT) treatment of high MW peaks of milk and saliva FPLC fractions were reduced by incubating with 10 mM DTT at 37°C for 2 h, followed by FPLC resolution through a Superdex 200 column in PBS. All fractions were characterized for HBGAs by MAb EIA, and for NV binding by VLP EIAs.

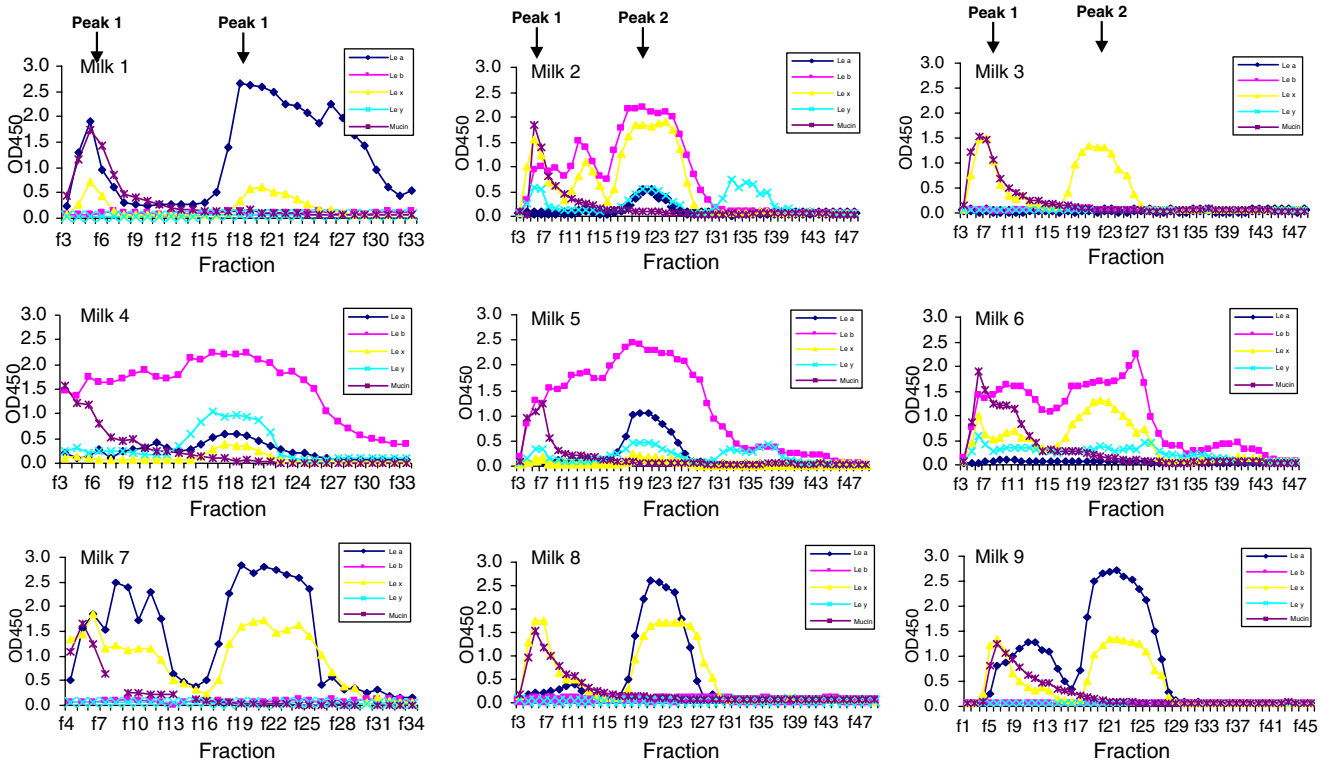

Fig. 2 Distribution of milk HBGAs and mucin in FPLC fractions. A. FPLC fractions of milk samples were diluted at 1:15 with PBS (0.01 M, pH 7.4) and coated to microtiter plate. After blocking with Blotto, MAbs specific to Le^a, Le^x, Le^b, Le^y and MUC1 were incubated and detected by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or IgM antibodies. The enzymatic reaction signals were detected at 450 nm and recorded as optical density (OD_{450}). Fractions 3 to 49 or fractions 3 to 33 were tested for the 4 HBGAs and MUC1 because fraction at either ends did not have any activity based on our preliminary results

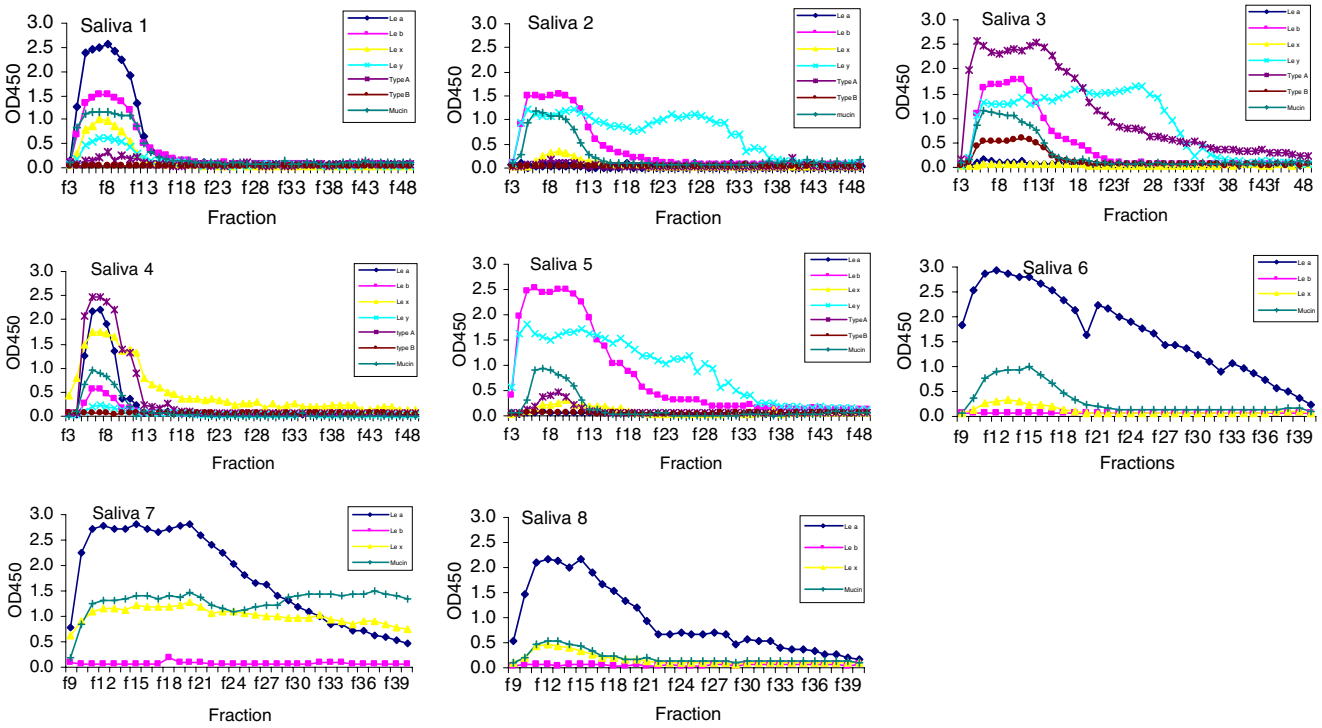

Fig. 3 Distribution of saliva HBGAs and mucin in FPLC fractions. **a** FPLC fractions of saliva samples were diluted at 1:15 with PBS (0.01 M, pH 7.4) and coated to microtiter plate. Same procedures as above were performed. MAbs specific to Le^a, Le^x, Le^b, Le^y and MUC5AC & 5B were used, anti-type A and -type B MAbs were also used. Fractions 3 to 49 or fractions 3 to 33 were tested for the four HBGAs and saliva mucin

Results

FPLC profiles of milk and saliva samples Under the FPLC conditions used in this study, the aqueous phase of 10 human milk samples from individuals of different HBGA types resolved into 4 major peaks, with minor individual variations (Fig. 1a). The first peak eluted with the expected void volume of Superdex 200 (exclusion limit 1.3×10^6 Da). Peaks 2 and 3 eluted where about 440 and <100 kDa would be expected, and peak 4 eluted at less than 5 kDa. Saliva samples 1 to 5 exhibited three primary peaks by FPLC through Superdex 200 (Fig. 1b), with the first peak in the void volume, the third peak less than 5 kDa and the second peak running slightly faster than the third peak. These patterns varied somewhat between saliva samples of some individuals (Fig. 1b). Peak 1 (the only peak that bound NV) of milk samples was estimated to be $1.3–2.0\times10^6$ Da using a Sephacryl S-500 column (exclusion limit 2.0×10^6 Da) (data not shown). Similar estimation of peak 1 of saliva samples was also obtained when the whole saliva samples (saliva 6–10), were analyzed using the Sephacryl S500 column, but the peaks were slightly wider.

HBGAs are expressed on a wide range of MW glycans The Le^a, Le^b, Le^x, and Le^y HBGAs were detected in the FPLC fractions of milk (Fig. 2) and saliva (Fig. 3). Types A and B antigens are not expressed in human milk [21]. Binding of these individual Lewis antigens by individual FPLC fractions displayed wide variations of intensities and/or sizes among individuals. Although the most Lewis HBGA binding was detected in the high MW fractions, especially coinciding with peak 1, significant quantities of HBGAs were also detected in fractions with smaller MW, with one prominent peak at approximately 200–440 kDa (Fig. 2). The salivary HBGA profiles of FPLC fractions were distinct from those of milk HBGA profiles. The peaks of HBGA on the low MW glycans found in milk was not observed or with a relatively lower signal in saliva compared with milk (Figs. 2 and 3). HBGA profiles varied among individuals for both milk and saliva samples (Figs. 2 and 3).

NVs recognize the high MW HBGAs only The above FPLC fractions of milk and saliva samples were tested for their ability to bind NVs. Binding by VLPs of VA387, a strain that binds A, B, and O (H) epitopes, and VA207, a strain that binds both the Lewis and secretor epitopes, were measured. Both strains mainly recognized the highest MW FPLC peak of both milks and saliva from donors with the corresponding HBGA types (Fig. 4). Binding of VLPs to the HBGAs of the high MW peaks from corresponding donors was confirmed by polyacrylamide gel electrophoresis, ultrafiltration, and sucrose gradient centrifugation (data not shown).

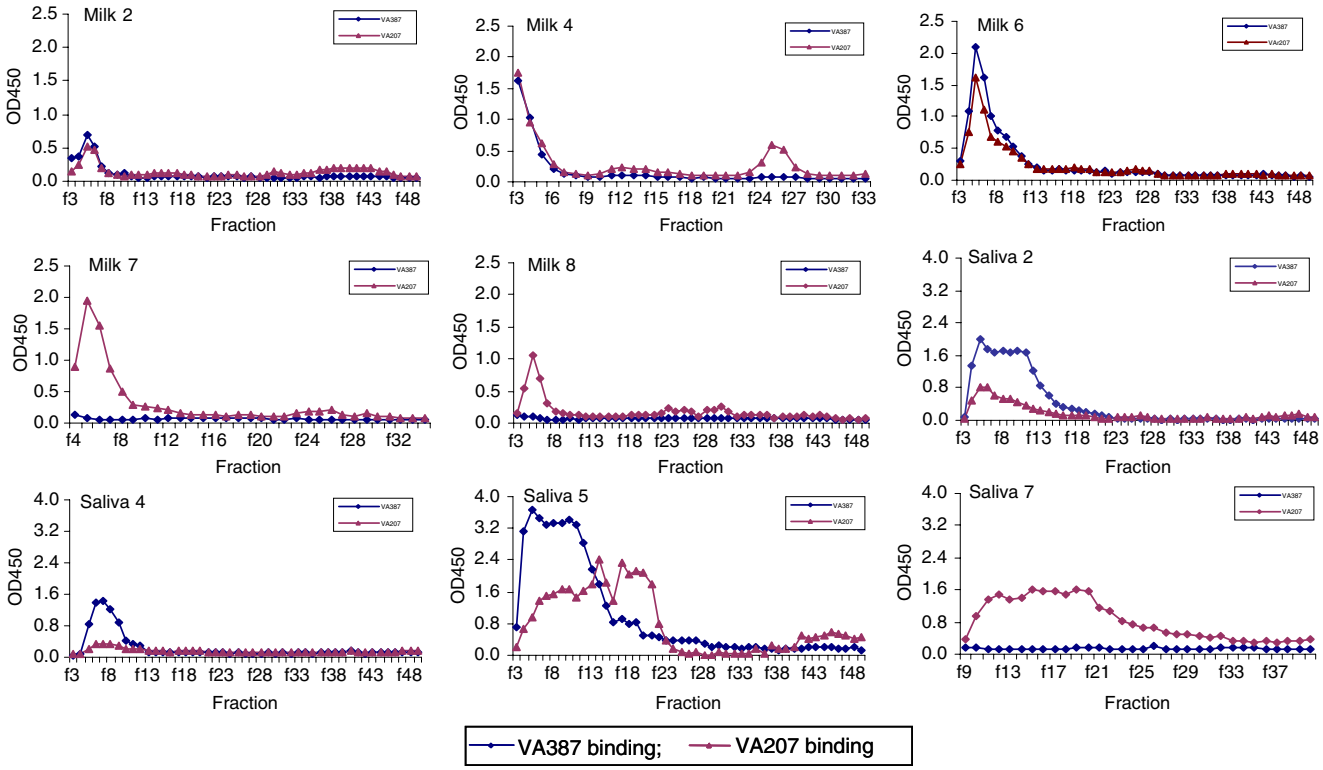

Fig. 4 Binding of VA387 and VA207 to milk and saliva HBGAs in FPLC fractions. FPLC fraction of five milk samples and five saliva samples were coated onto microtiter plates at a dilution of 1:15 (milk fractions) and 1:30 (saliva fractions) in 1× PBS (pH 7.4). The bound VA387 and VA207 VLPs were detected by hyperimmune sera. All five milk samples and saliva 2, 4 and 5 showed here were separated by Superdex 200, saliva 7 were separated by Sephacryl S500. All of the 82 collected FPLC fractions of milk 4 were tested. Only selected milk or saliva fractions were tested according to the HBGA activities

To demonstrate that HBGAs are essential to NV binding, the high MW FPLC peaks from a secretor (milk 4) and a non-secretor (milk 7) were tested for binding to NVs following blocking. Anti-Le^b and anti-Le^y MAbs blocked binding by both VA387 and VA207 to the secretor milk (Fig. 5a), consistent with the fact that these two antigens are major HBGA epitopes of secretors [13, 14]. Anti-Le^y MAb also blocked binding of VA207 to non-secretor milk. Since Le^y is not expressed in non-secretor individuals, the specific target of this MAb remains unknown. One possibility is that it may be cross-reactive with the Le^x antigen by the shared α1-3/4 fucose between Le^x and Le^y or by the common backbone of the antigens [13, 14]. Anti-Le^x MAb blocked binding of VA207 to the non-secretor but not to the secretor milk samples, consistent with the fact that Le^x plays the major role in NV binding with milk of non-secretors [13, 14]. Anti-Le^b MAb did not block binding of VA207 to the non-secretor (Fig. 5a), consistent with the fact that Le^b is not expressed in non-secretors [13, 14]. These results indicate that specific HBGA epitopes on the high MW molecules are the preferred binding sites of different NVs, confirming the importance of specific host HBGAs to NV binding [14, 27].

The importance of the carbohydrate HBGA moiety for NV recognition The endoglycosidase PNGase F failed to remove HBGAs from the high MW molecules of the milk and saliva samples, possibly due to steric hindrance by its dense glycosylation or its O-linked glycosylation because O-glycans are not affected by PNGase treatment. However, chemical deglycosylation with trifluoromethanesulphonic acid (TFMS) significantly abolished binding by both VA387 and VA207 to the high MW peaks of milk 4 obtained from a secretor, milk 7 from a non-secretor, and three saliva samples from secretors (Fig. 5b).

The importance of the glycan backbone for NV recognition The high MW peak of milk sample 7 from a non-secretor was treated with DTT, re-fractioned by FPLC, and the resulting fractions tested for their ability to bind MAbs. This treatment did not alter binding by MAbs to Le^x and mucin to the high MW glycan, but reduced the binding of Le^a in the high MW fractions and resulted in release of a new peak of Le^a binding of about 150 kDa (Fig. 6). Binding by VA207 to the high MW peak was not affected, and VA207 did not recognize the new, smaller Le^a peak. Treatment of a secretor saliva (Saliva 3, Le^b, Le^y, types A and B positive)

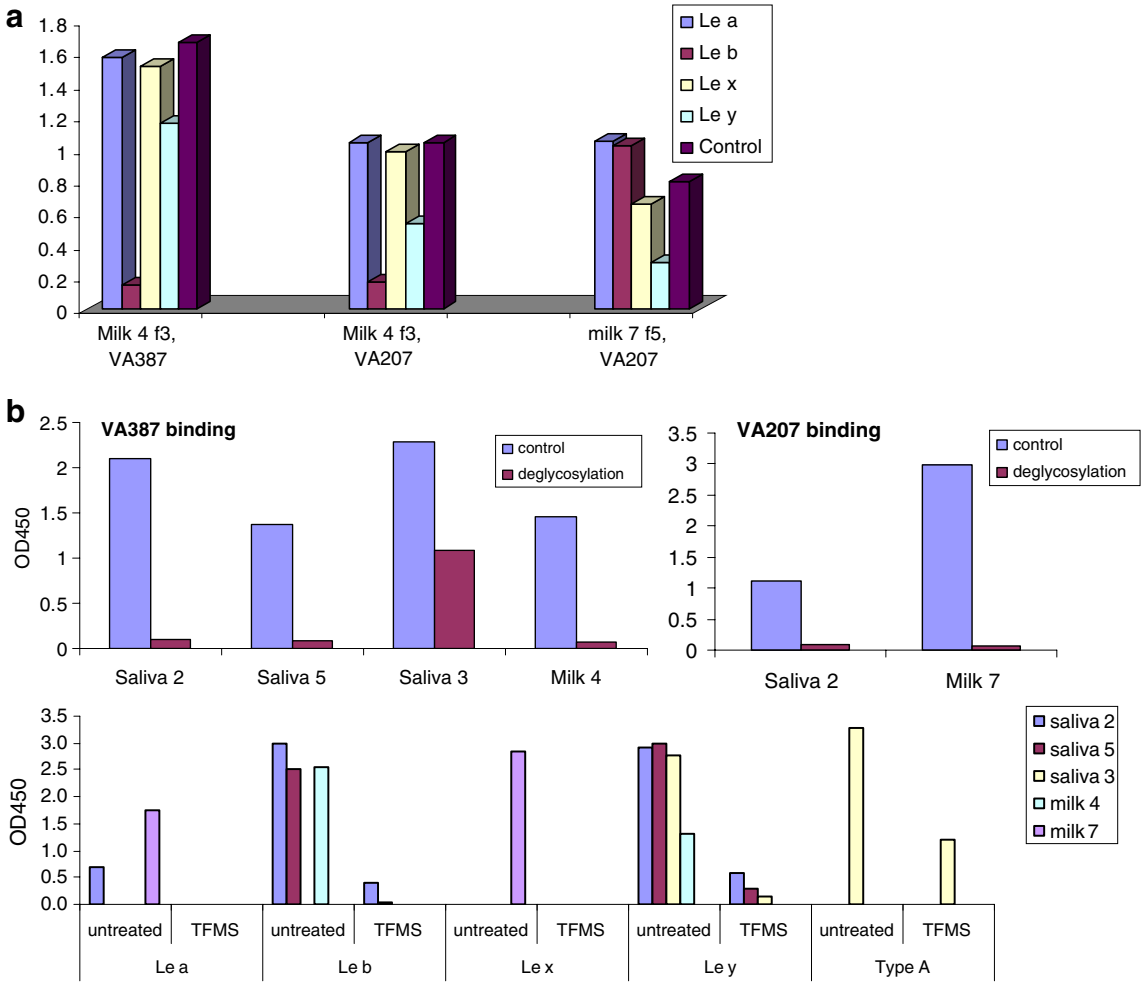

Fig. 5 Effects of MAbs and chemical deglycosylation on HBGAs binding to NVs. **a** MAbs specific to HBGAs blocked the binding of VA387 and VA207 to high-MW HBGAs in secretor and non-secretor milk samples. FPLC fraction 3 of milk 4 (secretor) and milk 7 (non-secretor) were coated to microtiter plates. After incubated with HBGA specific MAbs anti- to Le^a, Le^x, Le^b and Le^y, the binding of VA387 and VA207 VLPs were measured by NV specific antibodies. Wells without incubation with MAbs were included as negative controls. Specific blocking was determined by comparing the OD_{450} value in wells with MAbs to wells without MAbs. The non-secretor Milk 7 did not bind VA387 and it was only tested for blocking to VA207. **b**. Destruction of binding of HBGAs to NV by deglycosylation with TFMS. A pool of FPLC high-MW fractions (200 μl) of each of milk 4 and 7 and each of saliva 2, 3 and 5 were lyophilized and deglycosylated at low temperature by trifluoromethanesulphonic acid (TFMS). Undeglycosylated control fractions were brought to 600 μl, which was the final volume of the TFMS treated samples. Treated and untreated fractions were tested for their binding to NVs using the same procedure described above. HBGA signal reduction was also measured between treated and untreated fractions

with DTT similarly caused partial reduction of Le^b, Le^y, and B antigens at different levels, and VA387 binding to the high MW peak was not lost, apparently due to insensitivity of type A antigen to DTT reduction. A early report by Bara J. showed similar result that type A antigen is resistant to thiol reduction [28]. These results suggest that there is a high MW antigen in the initial high MW complex that is not released as lower molecular weight compounds by DTT treatment, and this specific molecule is responsible for NV recognition: the glycans released by DTT do not bind NVs.

Mucins and NV binding Mucins were detected in the high MW fractions of both milk and saliva using MAbs against milk and saliva mucins (Fig. 2). MAb BC-2 (anti-MUC1) detected mucins in only milk but not in saliva while MAb BAbCO 17B1 detected mucin in saliva but not in milk (dada not shown). MAb BAbCO 17B1 was generated against a partially purified mucin preparation derived from a rhesus monkey tracheobronchial epithelial secretion, it recognizes saliva MUC5AC and MUC5B ([29] and Reen Wu, personal communication). However, these MAbs did not block the high MW fractions binding to NVs. When a secretor milk sample was passed through a rNV-specific affinity column, the column-bound fraction was enriched with the Le^b and Le^y antigens, known ligands for Norwalk virus, but not milk MUC1 (Figs. 2 and 7a), indicating

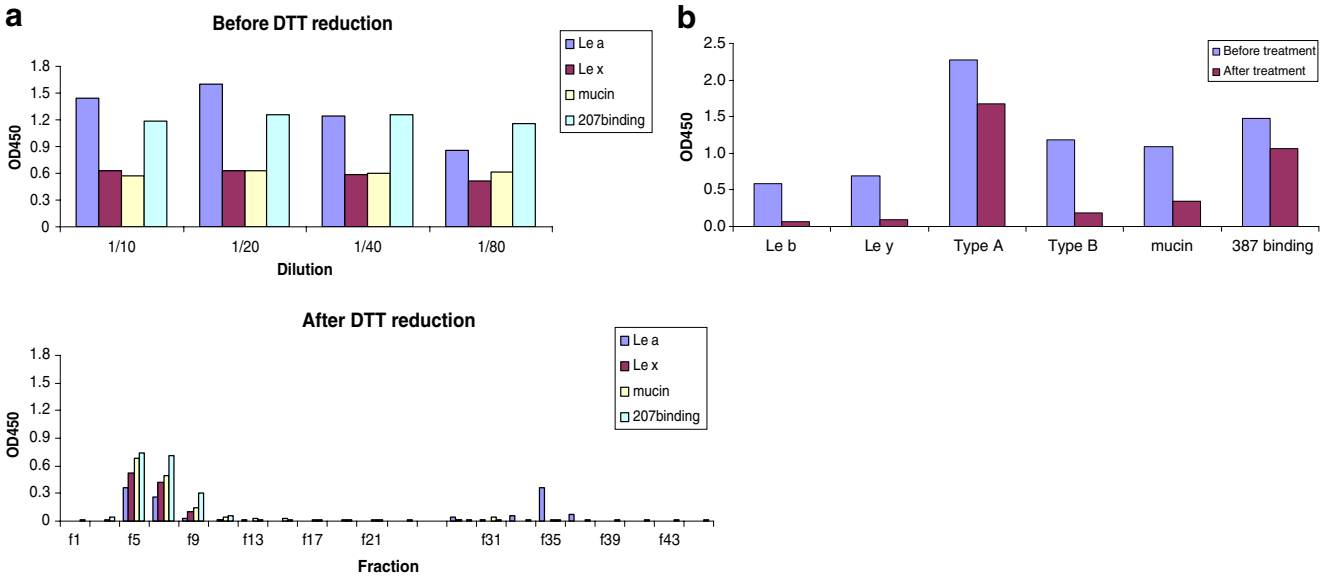

Fig. 6 Effects of DTT treatment on the high-MW HBGAs in milk and saliva. A pool of high-MW FPLC fractions of a non-secretor milk (milk 7) and of a secretor saliva (saliva 3) were treated with DTT at final concentration of 10 mM for 2 h. **a** The DTT reduced milk sample was re-fractioned by Superdex 200 and the distributions of HBGAs and VA207 binding signal were measured. Le^a, Le^x, MUC1 and VA207 binding of the high MW fractions were measured before re-fraction in different dilutions to compensate the dilution ratio due to re-fraction (top panel). Only Le^a was affected by the treatment as the signal of Le^a was shifted to a smaller size following the treatment (bottom panel). **b** DTT treatment significantly reduced the signals of antibodies binding to mucin and Le^b, Le^y and B antigens but less to type A antigen and VA387 binding

MUC1 was not involved in NV binding. Similar results also were obtained for saliva mucin when high MW peak of a saliva sample was analyzed by a mucin-MAb affinity column (Fig. 7b). Protease treatment of high MW fractions of a secretor saliva sample significantly reduced detection of mucin, but only slightly reduced the signals of type A antigen and binding activities to VA387 (Fig. 8).

Discussion

In this study we have characterized the HBGA glycans in human milk and saliva with respect to their interaction with human NVs. We found that the majority of milk HBGA that is reactive with NVs was in the high MW fractions of greater than 1.3×10^6 Da. This result is in contrast to a report of Ruvoen-Clouet *et al*., in which a smaller milk glycoprotein, BSSL (~130 kDa), was found to be the major determinants in blocking NV binding to their receptors [30]. In their study, affinity columns were used to enrich the BSSL and milk samples from one secretor and one non-secretor. In our study, we have studied milk and saliva samples from ten individuals for their ability to bind NVs representing two distinct receptor binding patterns. Although we did not observe major NV binding by BSSL, we did detect some minor binding to NV by smaller MW fractions from some milk samples. We also tested the samples under denaturing and reducing conditions and did not see release of BSSL-like proteins from the high MW fractions. Thus our data indicate that the high MW glycans are the major determinants for NV binding.

Mucin and mucin-like proteins were characterized with respect to their role in NV receptor binding. We found that MUC1, MUC5AC and MUC5B are not directly involved in NV receptor interaction despite being present in the same high MW glycan fractions that bind NVs. In reports by Ruvoen-Clouet *et al.* [30], both mucin 1 and mucin 4 were detected in the same regions of Western blots as the high MW glycans of human milk samples that react with Norwalk virus. However, in our studies using NV-specific affinity columns and quantitative measurement of mucins following denaturing and protease treatment of the milk samples, we found that these mucins co-migrated with NV-binding high MW molecules during FPLC but there was no indication that they were involved directly in NV-receptor binding. The hydrophobic tandem repeat of mucins confer a tendency to aggregate, and thus mucins isolated by ultrafiltration, electrophoresis, or gradient centrifugation [31–38] usually contain a mixture of different molecules. Because there are many species of mucins and mucin-associated proteins in human milk and saliva, and our study characterized only three of them, it remains early to make definitive conclusions on the role of mucins and associated proteins in NV/receptor binding.

We observed a wide distribution of different sizes of HBGAs in human milk but the majority of them are not

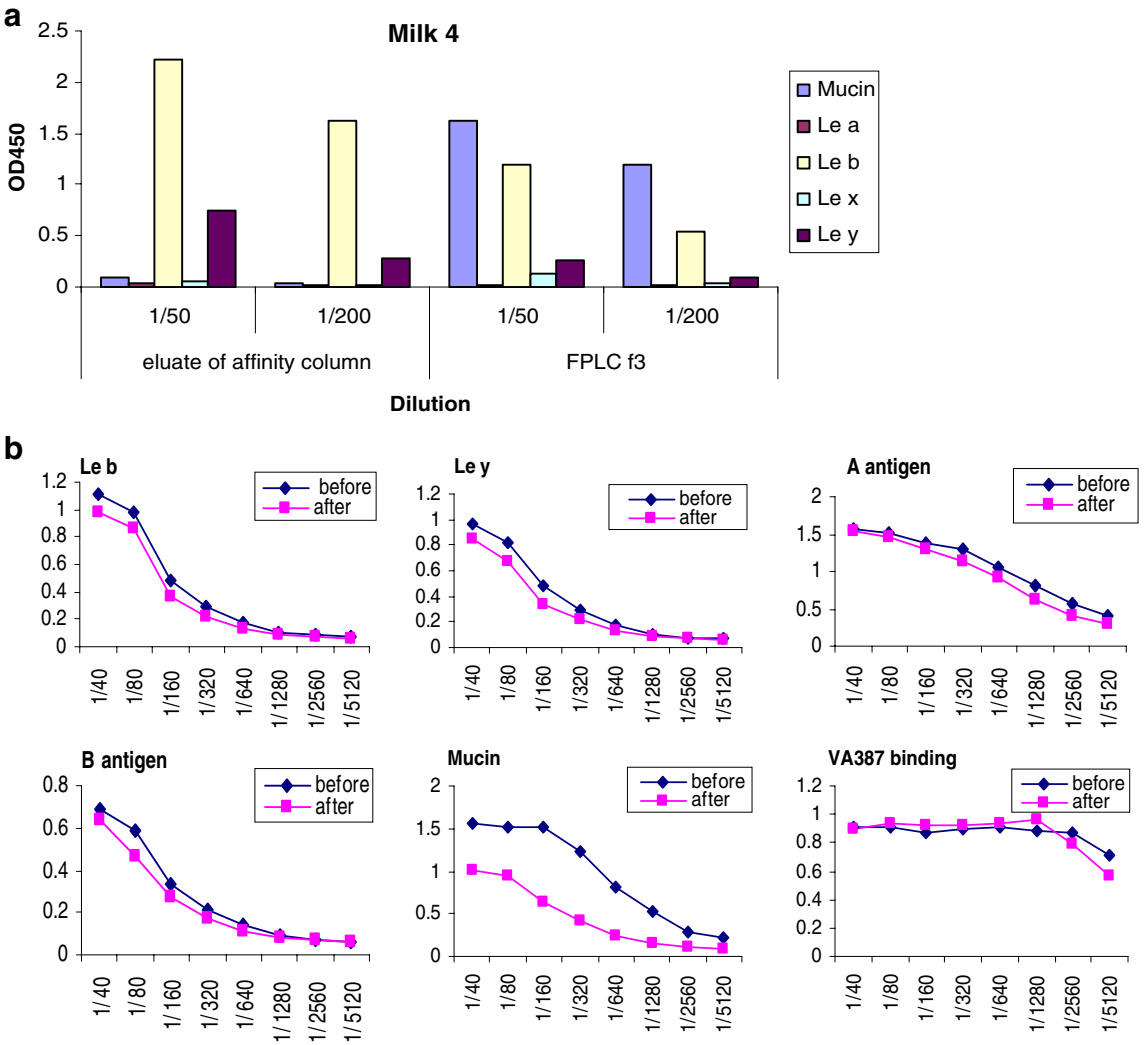

Fig. 7 MUC1, MUC5AC and MUC5 may not be involved in NV receptor binding. **a** HBGAs isolated by a NV capsid affinity column did not reveal signal of MUC1. Aqueous phase of a secretor milk sample (milk 4) was applied to a Norwalk virus affinity column. The Le^a, Le^x, Le^b, Le^y and mucin in the eluates (1:50 and 1:200) were measured by MAb-EIA. The NV affinity column enriched Le^b and Le^y but not MUC1, indicating MUC1 is not the carrier of Le^b and Le^y and is also not recognized by NVs. The dilutions were arbitrary and were not comparable. **b** A mucin specific MAb affinity column binds salivary mucin but not HBGAs. FPLC high-MW fraction 6 (f6) of a secretor saliva sample (saliva 3) was applied to a mucin specific MAb affinity column. The pass-through samples were tested for HBGA and mucin. The signal of mucin was significantly reduced compared with that before the column, while the signals of Le^b, Le^y, type A, and type B antigens were not significantly affected

recognized by NVs. This result explains some controversies on the NV receptor interaction found in our previous studies. For example, in our characterization of HBGAs in human milk for binding to NVs, we did not see clear correlation between the amounts of different HBGAs measured by MAbs and the binding activities of corresponding NV strains. Similarly, in a study on the influence of HBGA polymorphism on Norwalk virus attachment, Marionneau *et al.* [39] described a non-linear relationship between Norwalk binding and HBGA expression among individuals homozygous and heterozygous for the H gene and variable synthetic H trisaccharide neoglycoconjugates. They concluded that binding of Norwalk VLPs depends upon an optimal density of H epitopes on the backbone of approximately 20%, the presumed density ratio expressed in heterozygotes. In view of our finding of large amounts of non-NV reactive HBGAs in human milk and saliva, a reassessment of the experiments by measuring only the high MW fractions for NV binding may be warranted.

The finding of two species of HBGAs (recognizable and unrecognizable by NVs) in human milk and saliva raised a question about the biosynthesis of these HBGAs. The ABO, Lewis and secretor antigens are known to be synthesized from a common group of core disaccharides (types 1–5) that are linked to proteins or lipids in most glyconjugates; in milk, these antigens are also expressed as

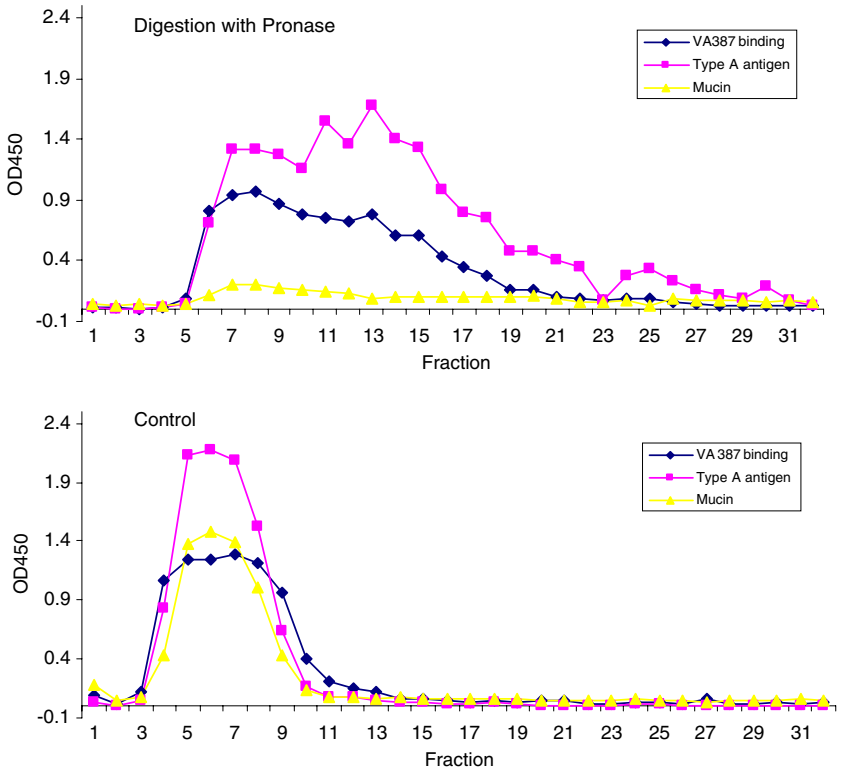

Fig. 8 Protease digestion reduced signal of mucin but not of HBGA or NV binding. A pool of FPLC fractions 4 to 7 of a secretor saliva (saliva 3) was split into two (1 mL each) and was applied to a second FPLC with or without a pronase digestion and the fractions were tested for type A antigen, mucin and VA387 binding. Compared to the undigested control, the pronase digestion almost completely abolished mucin signal, leaving the signal of the type A antigen and VA387 basically unchanged although the distributions of the type A antigen and VA387 binding became wider

free oligosaccharides bound to lactose. The carbohydrate moiety of different species of HBGAs should be the same, because they are synthesized by the same glycosyltransferases. The distribution of HBGAs in a broad range of MW in both milk and saliva resolved by FPLC and the differences in sensitivity of the high MW HBGAs to DTT reduction, protease digestion and deglycosylation indicated that the same carbohydrate epitopes may attach to different backbones and vise versa.

Our results demonstrated that unlike binding by MAbs, binding by NVs requires both specific carbohydrate moieties and a specific carrier or backbone to which it is attached. The carbohydrate moiety of HBGAs is the minimum requirement for ligand interaction, and is sufficient for recognition by the HBGA specific MAbs, but not by NVs, in other words, recognition of HBGA by NV is much stringent than that by HBGA specific MAbs. The requirement of backbones of HBGAs in NV recognition is also supported by our previous studies when HBGA oligosaccharides conjugated to different carriers [13]. Synthetic free oligosaccharides generated *in vitro*, or free oligosaccharides isolated from human milk had little affinity to NV relative to the natural receptors. Two factors may be involved including distance between HBGA epitope and functional domains of the backbone, and nature of the backbone. Although the backbone itself may not be recognized by NVs, it may merely function as a scaffold to ensure correct presentation of carbohydrate binding moiety for NV recognition.

Some limitations of this study should be noted. The human HBGA system is highly polymorphic and contains many gene families; we studied only the ABO, Lewis and secretor gene families. The precursors for synthesis of human HBGAs contain up to five types and we studied only types 1 and 2. Mucins appear to be a structural complex of three families with more than ten members, this study was limited to three major mucins. We measured the phenotypes of individuals using their milk and saliva samples, a genetic characterization of these individuals may be important for advancing understanding in future studies. FPLC resolution of milk and saliva samples has improved our understanding of NV glycan binding specificity; further resolution of these glycans may be more informative. Finally, we studied glycans expressed in samples in body secretions as surrogates for intestinal expression, which may not fully reflect the complexity or

specificity of human HBGA expression in the gastrointestinal tract; using models for gut expression might allow further testing of the conclusions of the studies reported herein. Thus, future studies will focus on determining the critical structures for binding by different strains of NVs, and on host genetics that underlie the expression of these NV receptors, and thus confer susceptibility to this pathogen. Determining the essential sequences of the backbone may allow rational design of high affinity antivirals against NVs based on the concept of receptor blocking.

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