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Norwalk virus-like particles bind specifically to A, H and difucosylated Lewis but not to B histo-blood group active glycosphingolipids

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Abstract Noroviruses and norovirus virus-like particles (VLPs) exhibit strain specific patterns in their binding to ABH and Lewis histo-blood group antigens. In this study we demonstrate for the first time specific binding of Norwalk virus VLPs to type 1 and type 2 chain glycosphingolipids (GSLs) carrying ABH and Lewis antigens. *N*-succinimidyl-3-tributylstannyl benzoate (ATE) was precursor labeled with ^{125}I and then conjugated to VLPs. The ^{125}I-VLPs were used in GSL thin-layer chromatogram binding assays and displayed binding to H type 1, Lewis b, A type 1, A Lewis b GSLs but no binding to B type 1 or B Lewis b GSLs. For the type 2 chain GSLs the Norwalk VLPs bound to H type 2, Lewis y, A type 2 and A Lewis y. In addition, the VLPs bound to several complex GSLs from blood group O and A, but not from blood group B red blood cells.

Keywords Norovirus · Virus-like particle · Glycosphingolipid · ABH antigen · Secretor

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Abbreviations

ATE	*N*-succinimidyl-3-tributylstannyl benzoate
BSA	bovine serum albumin
CBA	chromatogram binding assay
Cer	ceramide
cpm	counts per minute
DMSO	dimethylsulfoxide
ELISA	enzyme-linked immunosorbent assay
Fuc	fucose
FUT	fucosyltransferase gene
G	genogroup
Gal	galactose
GalNAc	*N*-acetylgalactosamine
Glc	glucose
GlcNAc	*N*-acetylglucosamine
GSL	glycosphingolipid
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
Le	Lewis
NV	Norwalk virus
NMR	nuclear magnetic resonance
PAA	polyacrylamido
PBS	phosphate buffered saline
RBC	red blood cell
TLC	thin layer chromatography
TMB	3, 3', 5, 5'-tetramethylbenzidine
VLP	virus-like particle

Introduction

Norovirus infection is the major cause of viral epidemic gastroenteritis in adults and is an emerging problem worldwide. The strain specific binding of noroviruses to ABH blood-group carbohydrate antigens [1, 2] is a striking

example of viral glycan specificity [3]. Recent achievements regarding norovirus cultivation are promising [4], but at present sufficient amounts of live virus are not accessible for binding studies. However, the 58-kDa major capsid protein (VP1) has successfully been recombinantly expressed leading to the spontaneous assembly of 180 copies of VP1 to form ~30-nm sized virus-like particles (VLPs) that have the same morphological, antigenic and glycan binding properties compared to live virus [5–8]. The Norwalk virus (NV) was the first norovirus to be identified [9] and the crystal structure of the major capsid protein has been solved [10]. Recently, the crystal structures of the P-domain of the NV capsid protein in complex with the blood group A trisaccharide [11, 12] and H type 1 pentasaccharide were published [12]. The ABH histo-blood group antigens are typically displayed on glycosphingolipids, glycoproteins and mucins in mucosal tissues and secretions *e.g.* saliva, of secretors but not of non-secretor individuals (Fig 1) [13].

Genetic resistance [14] to NV infection for non-secretors is in accordance with NV VLP saliva binding experiments [15–19] and also with studies of NV VLP binding to epithelial cells of gastrointestinal tissue sections from secretors, but not from non-secretors [18]. In saliva binding studies NV VLPs bind saliva from secretors of blood group A and O but only to some of blood group B. The mixture of

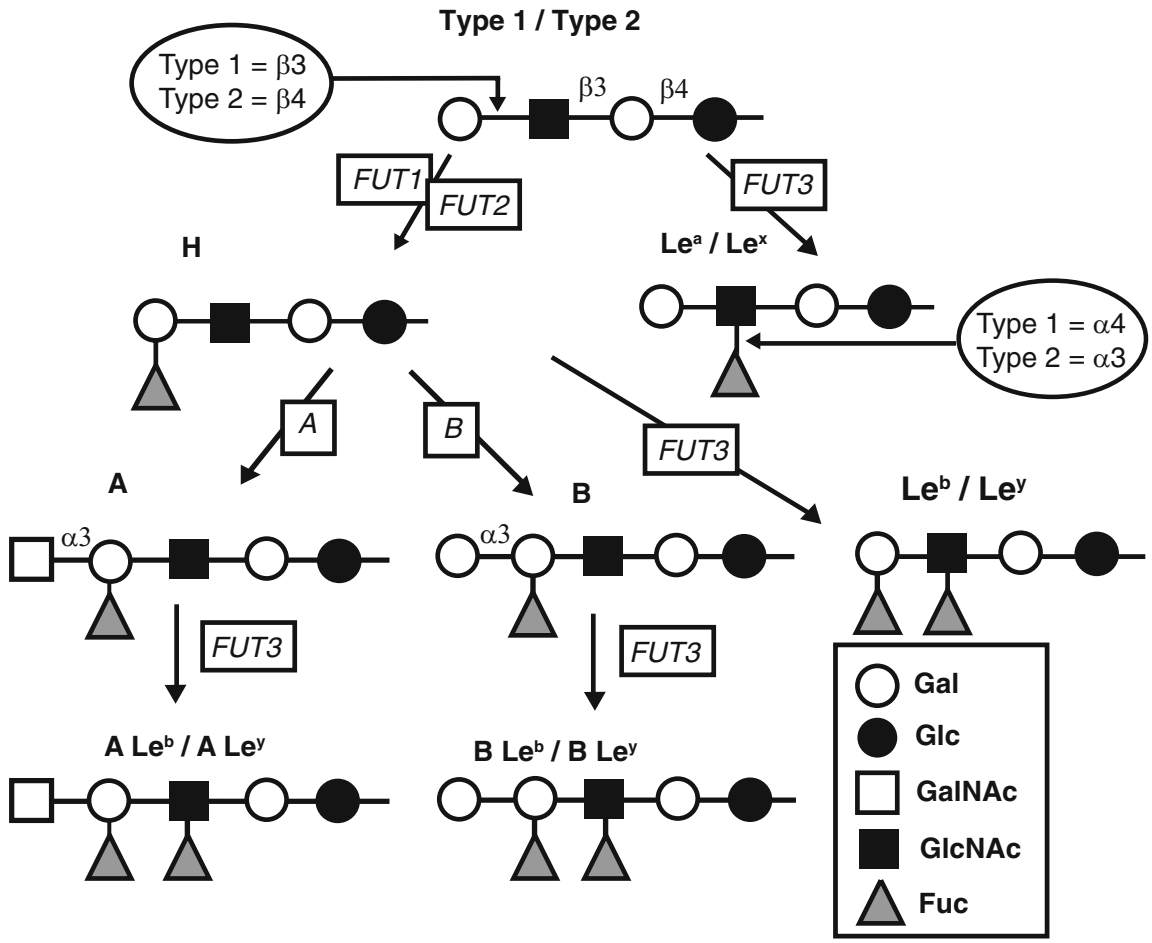

Fig. 1 Biosynthetic scheme and structures of type 1 (lactoserie) and type 2 (neolactoserie) histo-blood group GSLs. The α1,2-fucosyltransferases, encoded by *FUT1* or *FUT2,* add fucose (Fuc) onto the type 1 or type 2 chain precursors, Galβ3/4GlcNAcβ3-Galβ4Glcβ1Cer, to form the minimal blood group H epitope, Fucα2Galβ. The *FUT1* transferase is specific for the type 2 chain, whereas the *FUT2* transferase preferentially acts on the type 1 chain, but has activity also towards the type 2 chain. The H epitope may be further elongated by the A transferase to form the blood group A epitope, GalNAcα3(Fucα2)Galβ; or the B transferase to form the blood group B epitope, Galα3(Fucα2)Galβ. Non-secretors lack a functional *FUT2* transferase and thus do not synthesize the downstream ABH histo-blood group antigens. The α1,3/4-fucosyltransferase, encoded by the Lewis (*FUT3*) gene, may add a Fuc to the type 1 chain precursor to form Lewis a and a second Fuc to the type 1 chain ABH structures among secretors to form A Lewis b, B Lewis b and Lewis b, respectively. Besides the Lewis enzyme several additional α1,3-fucosyltransferases are responsible for the addition of Fuc to synthesize the Le^x, Le^y , ALe^y and BLe^y structures in various tissues [59]

glycan structures that are presented on the different saliva mucins is very complex [20] and the histo-blood group antigens present in saliva are a mixture of type 1 or type 2 structures [13]. It is thus difficult to physically isolate naturally occurring single epitopes that are responsible for norovirus binding to saliva mucins. Norovirus VLPs have also been shown to bind to multivalently displayed histo-blood group glycans on neoglycoproteins and on poly-acrylamide (PAA-) polymers [15, 16, 21, 22].

Besides glycoproteins, naturally occurring histo-blood group antigens can be found on glycosphingolipids (GSLs), which are particularly abundant on epithelial cells of the gastrointestinal tract [23–25]. GSLs are thus biologically relevant structures to study with respect to norovirus specificity, and may represent true cellular receptors for viral infection. GSLs have been shown to function as cellular receptors for some glycan-binding viruses [26–28]. In this study we determined, for the first time, the NV VLP binding specificities to GSLs using the thin-layer chromatogram binding assay (CBA) [29]. The NV VLPs were ^{125}I-labeled using a two-step labeling protocol involving initial ^{125}I-labeling of *N*-succinimidyl-3-(tributylstannyl) benzoate (ATE, activated tin-containing ester) [30] and subsequent conjugation to accessible lysine residues on the viral capsid. The ATE labeling protocol is commonly used for the purpose of radioimmunotherapy [31] and is here introduced for the purpose of VLP-radiolabeling.

Material and methods

Glycosphingolipids

The purified and structurally well characterized glycosphingolipids used in this study originate from human meconium samples pooled from individuals of the same ABO blood group or from meconium of single individuals of known ABO blood groups [25]. In addition, GSLs originating from pooled human red-blood cells of identical ABO blood groups and from single dog small intestines were purified and characterized. Total neutral and acidic GSLs were prepared essentially as described [32]. Individual GSLs were isolated by repeated silicic acid column chromatography either in stepwise elution (Lichroprep, Merck, Darmstadt, Germany) or in gradient elution (Iatrobeads 6RS-8060, Iatron Laboratories Inc., Tokyo Japan) of native non-acetylated and acetylated derivatives. The GSLs from RBCs and dog-small-intestines were purified by straight-phase HPLC in the final step. The identity and purity of reference GSLs used in this study were all analyzed as native structures by ^{1}H-NMR spectroscopy and compared to previous characterizations by ^{1}H-NMR and mass spectrometric analyses of permethylated and permethylated-reduced derivatives [33, 34].

Production of Norwalk VLPs

Norwalk strain VLPs (NV VLPs) were produced by use of a baculovirus-expression system. The construct was a kind gift of Dr. X. Jiang (Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA). Five days post-infection High-Five lysed cells and media were centrifuged at 4500 rpm for 15 min and the supernatant collected and centrifuged at 25.000 rpm, 3 h 30 min in SW28. The pellet was resuspended in distilled water and submitted to two rounds of purification on a sucrose gradient as previously described [5].

Radiolabeling of Norwalk VLPs

The intermediate labeling agent ATE, *N*-succinimidyl-3-(tributylstannyl) benzoate (5 nmol, Molecular Biosciences, USA) in methanol:acetic acid (99:1) was radioiodinated with 8.0 MBq Na^{125}I (Amersham) using *N*-bromosuccinimid (75 nmol) as the catalyst in a 1.3 ml reacti-vial (Cromacol, UK). The vial was vortexed for 20 min and the reaction was then quenched with $Na_2S_2O_5$ (3 nmol). A portion (100 kBq) of the labeling mixture was analyzed by HPLC to verify formation of the *N*-succinimidyl-3-^{125}I-benzoate product. The HPLC system (Waters 486) was used with a Kromasil C18 column, 10 μm, 250×4.6 mm (EKA Nobel AB, Sweden), isocratic elution with acetonitril:2 mM phosphoric acid (60:40 by volume) at 1 ml/min and an in-house built ionization chamber photon-detector. Standard ^{125}I activities had previously been injected onto the HPLC system to obtain the response factor for the photon detector. The labeling mixture was dried under a stream of nitrogen and VLPs (8 μg in 5 μl PBS) and phosphate buffer (10 μl, 0.2 M, pH 7.5) was added to the vial. The vial was vortexed for 2 h at room temperature and then placed at 4°C overnight. The ^{125}I-VLPs were desalted on a Sephadex PD-10 column (Amersham, Sweden) and an analytical amount of ^{125}I-VLPs were eluted on a second PD-10 column to verify that all ^{125}I was protein associated. For stability reasons BSA, final concentration 3 mg/ml, was added to the ^{125}I-VLPs. Radioactive measurements were done in a calibrated gamma counter (Wizard 1480, Wallace OY, Finland).

Thin-layer chromatography and VLP chromatogram binding assay

Pure GSLs (2–4 μg) or mixtures (10–20 μg) were applied to aluminum backed silica gel 60 HPTLC plates (Merck, Germany) using a Hamilton syringe and chromatographed with chloroform:methanol:water (60:35:8 by volume). Two sets of GSLs were run in parallel on the TLC-plate, and the dried plate was then cut in half. GSLs on one of the plates

were visualized by spraying with anisaldehyde:sulfuric acid:acetic acid (1:2:97 by volume) and heated (180°C) for one min [35]. The other plate was plasticized by dipping it for 60 s in P28 polyisobutylmethacrylate (0.5% in diethylether) and dried [29]. Plastic coated TLC-plates were blocked with buffer A (3% BSA, 0.05 % Tween-20) in PBS at room temperature for 2 h.

^{125}I-VLPs (2.5–3.5 ml, 1×10^6 cpm) in buffer A were added to the surface of plastic coated TLC-plates and incubated for 2 h at room temperature. The plates were washed 3×15 min under gentle agitation with buffer B (0.5% BSA, 0.05% Tween-20 in PBS) and then dried. ^{125}I-VLPs bound to GSLs were detected by autoradiography using Hyperfilm (Amersham, UK) at −20°C for 3–48 h. The H type 1 GSL was used as a positive control for NV VLP binding on all CBAs.

Antibody chromatogram binding assay

GSLs were applied on TLC plates, separated and plasticized as described above. The TLC plates were blocked with buffer A for 1.5 h and anti-A, (1:5,000) anti-B (1:10,000) or anti-H (1:500) monoclonal antibodies were applied and incubated at room temperature for 1.5 h. The monoclonal anti-H type 1 antibody was from a hybridoma cell line (825/7F45) and was a kind gift from Peter Påhlsson, Linköping University. The anti-H (Seraclone A46/B.B10) was from Biotest (Germany). The anti-blood group A (ABO1 clone 9113D10) and anti-B (ABO2 clone 9621A8) antibodies were from Diagast (Loos Cedex, France). The plates were then washed three times with PBS and goat alkaline phosphatase conjugated anti-mouse antibody (1:500, Sigma A0162) was added and incubated at room temperature for 1.5 h. The plates were washed three times with PBS and the immunostaining was developed with Sigma Fast BCIP/NBT.

ELISA

Saliva samples from a blood group AB Lewis positive secretor (ABLeSe) and a blood group O Lewis negative non-secretor (Olese) individual, genotyped by PCR with sequence specific primers (PCR-SSP) for *FUT2*, *FUT3* and *ABO* [36, 37], were used in the saliva ELISA. Boiled and centrifuged saliva samples (1 µg/ml, 100 µl/well) in carbonate buffer (50 mM, pH 9.6) were coated in flat-bottom MaxiSorp Microtiter wells (Nunc, Denmark) for 2 h at 37ºC and then overnight at 4ºC. The wells were washed three times with 0.05 % Tween 20 in PBS after each incubation step. The wells were blocked with 3% BSA in PBS at 37ºC for 1 h and VLPs (50 µl/well, 0.05 µg protein/ml) in buffer B was added to each well, and incubated at 37ºC for 1.5 h. Rabbit anti-Norwalk hyperimmune antisera (100 µl, 1:1000 in buffer B) was added to each well and incubated for 1.5 h at 37°C. HRP-conjugated goat anti-rabbit IgG (100 µl, 1:10,000 in buffer B) was added and incubated for 1.5 h at 37ºC. Color was developed with 100 µl TMB (Sigma) and the reaction stopped with H_2SO_4 (100 µl, 0.5 M) after 10 min. Absorbances were read at 450 nm (Labsystems iEMS Reader MF, Labsystems, Finland).

Solid phase VLP radio-binding assay

MaxiSorp tubes (Nunc, Denmark) were used for the solid phase radio-binding assay. The saliva coating and blocking were done as in the ELISA. ^{125}I-VLPs (5,000–20,000 cpm/tube) in buffer B were added and the tubes were incubated for 1.5 h at 37°C. The ^{125}I photons were measured in the gamma-counter to obtain the exact cpm added to each tube. The tubes were washed four times with 0.5 ml 0.05% Tween 20 in PBS and again measured in the gamma-counter.

NMR spectroscopy

All deuterated solvents were from Armar Chemicals, Switzerland. GSLs were deuterium exchanged in chloroform-d1:methanol-d4: D_2O (2:1:0.1 by volume) and analyzed with ^{1}H-NMR using a 600 MHz NMR spectrometer (Varian, USA) in DMSO-d6:D_2O (98:2) [38] at 30°C and 60°C and compared to published shifts and coupling constants for the anomeric protons, and shifts for fucose H-5 and H-6 protons for fucose-containing GSLs [25, 33, 34]. The chemical shifts were tabulated relative to tetramethylsilane by placing the residual DMSO protons at 2.50 ppm. The reference GSLs were found to be more than 95 % pure. Notably the Lewis b fraction contained almost 5 % Lewis y. Chemical shifts and coupling constants for the H1 anomeric protons coincided with published values [39–41].

Results

Radiolabeling of NV VLPs

The labeling agent *N*-succinimidyl-3-(tributylstannyl)benzoate (Bu-ATE) was radioiodinated with 8.0 MBq Na^{125}I. A small portion (100 kBq) of the resulting *N*-succinimidyl-3-^{125}I-benzoate was assayed by Radio-HPLC (Fig. 2a) to check for the purity and the incorporation of ^{125}I. The radiochemical yield for the labeling step was typically 95 %. The VLPs were conjugated in 0.1 M pH 7.5 phosphate buffer to ensure basic pH in the presence of residual acetic acid from the labeling mixture. Borate buffer pH 8 could not be used for the conjugation step since VLP binding was abolished after this treatment, which is in

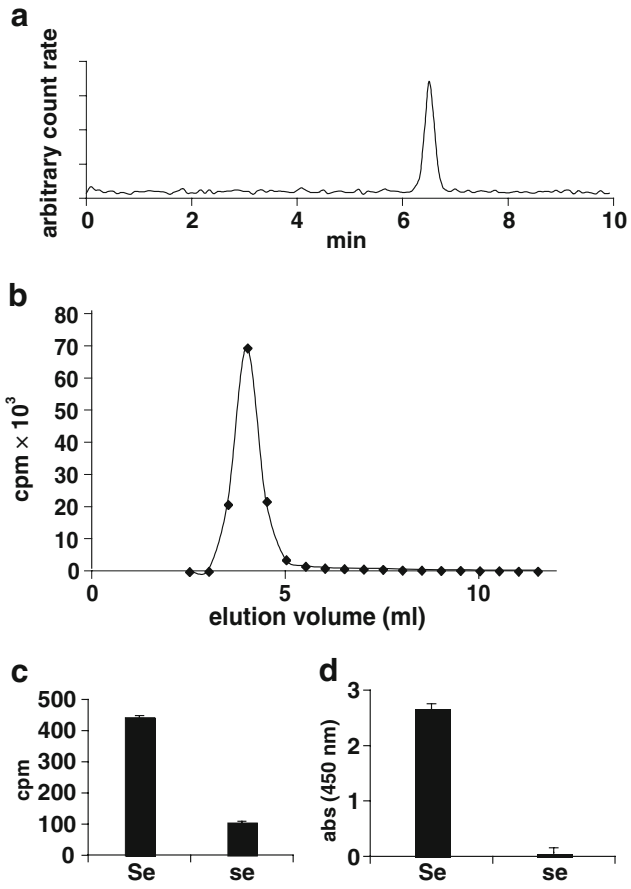

Fig. 2 **a** Analytical radio-HPLC of 100 kBq of 3-^{125}I-benzoic acid succinimidyl ester, which was used in the ^{125}I radiolabeling of NV VLPs. **b** Analytical amounts of ^{125}I-VLPs chromatographed on a PD-10 Sephadex column to verify that all ^{125}I is protein associated. **c** Solid phase binding of NV ^{125}I-VLPs and **d** ELISA of NV VLPs in their binding to saliva of a secretor (Se) and a non-secretor (se) individual

agreement with NV VLP instability at a pH above 8 [42]. After conjugation the VLPs were passed through a desalting column (Fig. 2b) and typically 8 μg of VLPs gave 1.1 MBq ^{125}I-VLPs, and the overall yield was thus about 10 %. This incorporation corresponds roughly to fifteen iodine atoms on each VLP based on the 180× 57 kDa weight for each particle, assuming that no VLPs were lost during the transfer of solutions and desalting steps. It was necessary to add 3 % BSA to the ^{125}I-VLPs for stability reasons and the ^{125}I-VLPs could be used and reused in radioassays with maintained binding specificity for more than one month after the labeling event. The radiolabeled VLP bound to saliva from a secretor individual, but not to saliva from a non-secretor in complete agreement with the ELISA for non-labeled NV VLPs (Fig. 2c and d). The radioiodination procedure thus did not impair or change the binding capability of the NV VLPs. The binding of ^{125}I-VLPs to saliva was used as a control to verify maintained secretor gene dependent saliva binding for batches of newly radioiodinated VLPs.

Chromatogram binding assay

GSLs were chromatographed on TLC-plates and radio-labeled NV VLPs were added on top of plasticized plates to detect binding to GSLs. (See Table 1 for the structures of the GSLs used in this study, and Fig. 1 for the biosynthetic relationships between the different type 1 and type 2 histo-blood group structures.) The H type 1 (lane 3, Fig. 3) and A type 1 (lane 2), but not the B type 1 (lane 4) GSLs were recognized by the NV VLPs. The type 1 precursor (lactotetraosylceramide, lane 5) or the Lewis a (lane 7) GSLs were not recognized. The Lewis b GSL (lane 6) showed a weaker VLP-binding, whereas A Lewis b (lane 1) was a good binder. The glycosphingolipid fraction in lane 8 originate from meconium of a single blood group B individual and contains B type 1 and B Lewis b as major fucosylated GSL components [33], and they were not recognized by the virus. The only VLP binding GSL of this fraction was migrating identically to H type 1 GSL. The presence of H type 1 GSL in this blood group B individual fraction has been well established [33] and was also verified by antibody binding (data not shown). The binding pattern found for the type 2 chain series of GSLs was similar to the NV VLP binding to the type 1 chain structures (Fig. 3) The NV VLPs thus bound the H type 2 (lane 11), A type 2 (lane 10) and A Lewis y (lane 9) but also to Lewis y GSL (lane 13). The VLPs did not bind Lewis x (lane 14) or globoside (lane 12). In addition to the CBAs presented here we compared the binding at ambient temperature with that at 4 °C and at 37 °C and found that the NV VLPs bound to H type 1, Lewis b, Lewis y, A Lewis b and A Lewis y equally well at 4 °C, at room temperature and at 37 °C (data not shown). Additional GSLs were tested in the CBA (Table 1), but no more ligands were found.

CBA of partially purified GSLs from red blood cells

Partially purified GSLs that exceed five sugar residues in size originating from pooled units of blood group A_1, A_2, O, and B red blood cells (RBCs), which predominantly display type 2 GSLs on their surfaces, were used in the CBA for binding to the NV VLPs (Fig. 4). The VLP had affinity to several long-chain GSLs from the A_1, A_2 and O RBCs, but had virtually no affinity to any of the GSLs present in the blood group B RBCs. The specific presence of A, H and B epitopes in these fractions were supported by the CBA with the use of anti-A, -B and -H antibodies (Fig. 4). The A_1 fraction did not contain any H structures and the NV VLP binding was thus dependent only on A structures. In the seven-sugar region a VLP binding band

Table 1 Structures of reference GSLs that were used in this study

Nr in Figs	GSL	Structure	NV VLP Binding
	Type 1 structures		
5	Lactotetra	Galβ3GlcNAcβ3Galβ4Glcβ1Cer	−
3	H type 1	Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+
7	Lewis a	Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	−
6	Lewis b	Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	+
2	A type 1	GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+
1	A Lewis b	GalNAcα3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	+
4	B type 1	Galα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glcβ1Cer	−
8[a]	B Lewis b	Galα3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	−
	Type 2 structures		
	Neolactotetra	Galβ4GlcNAcβ3Galβ4Glcβ1Cer	ND
11	H type 2	Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+
14	Lewis x	Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	−
13	Lewis y	Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	+
10	A type 2	GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+
9	A Lewis y	GalNAcα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	+
B[b]	B type 2	Galα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	−
	B Lewis y	Galα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	ND
	Other structures		
	Galactose	Galβ1Cer	−
12	Globoside (Globotetra)	GalNAcβ3Galα4Galβ4Glcβ1Cer	−
	Forssman	GalNAcα3GalNAcβ3Galα4Galβ4Glcβ1Cer	−
	Gangliotetra	Galβ3GalNAcβ4Galb4Glcβ1Cer	−

- , no VLP binding ;+, VLP binding; ND, not determined

[a] B Lewis b was a major GSL in this fraction from meconium of a blood group B individual

[b] B type 2 RBC GSL exceeding six sugars did not bind

was seen in the A_1 but not in the A_2 fraction. This GSL was most probably the Globo A GSL, GalNAcα3(Fucα2)Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer, typically found in A_1 but not in A_2 indivduals. The dominating presence of the blood-group ABH epitopes in the RBC GSL fractions were all verified with ^{1}H-NMR spectroscopy (data not shown), including the presence of Globo A in the A_1 fraction.

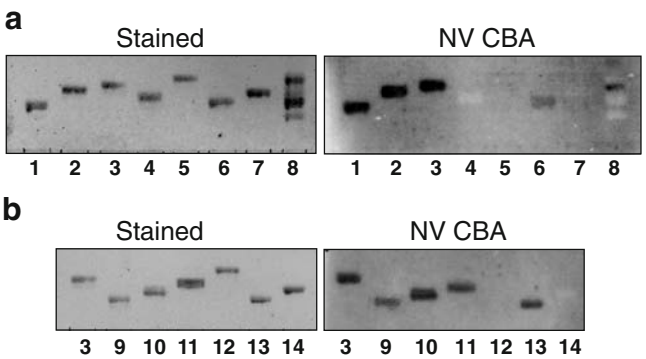

Fig. 3 ^{125}I-labeled NV VLP binding to type 1 (**Fig. 1a**) and type 2 (**Fig. 1b**) GSLs. The left plates are anisaldehyde stained to visualize the GSLs. The right plates show autoradiography with radiolabeled NV VLPs. **Lane 1**: A Lewis b; **2**: A type 1; **3**: H type 1; **4**: B type 1; **5**: lactotetraosylceramide; **6**: Lewis b; **7**: Lewis a; **8**: Partially purified meconium GSLs from a secretor positive blood group B individual; **9**: A Lewis y; **10**: A type 2; **11**: H type 2; **12**: globoside; **13**: Lewis y; **14**: Lewis x

Discussion

In this study the binding of norovirus VLPs to GSLs are demonstrated for the first time. GSLs are naturally occurring molecules that may represent true cellular receptors for noroviruses as they have been shown to be for other viruses [26–28]. We show here that Norwalk virus VLPs bind to reference GSLs with H and A histo-blood group epitopes on either the type 1 or the type 2 chains, but do not bind to the B type 1 or B type 2 GSLs. Also, we show for the first time the binding of NV VLPs to the A Lewis b and A Lewis y epitopes and the lack of binding to the B Lewis b epitope. An analogous B Lewis y structure [43] was not available for study. Neither the type 1 chain precursor (lactotetraosylceramide) nor the Lewis a GSL was recognized by the VLPs in the assay. The addition of Fucα4 onto H type 1 to form Lewis b or Fucα3 to H type 2 to form

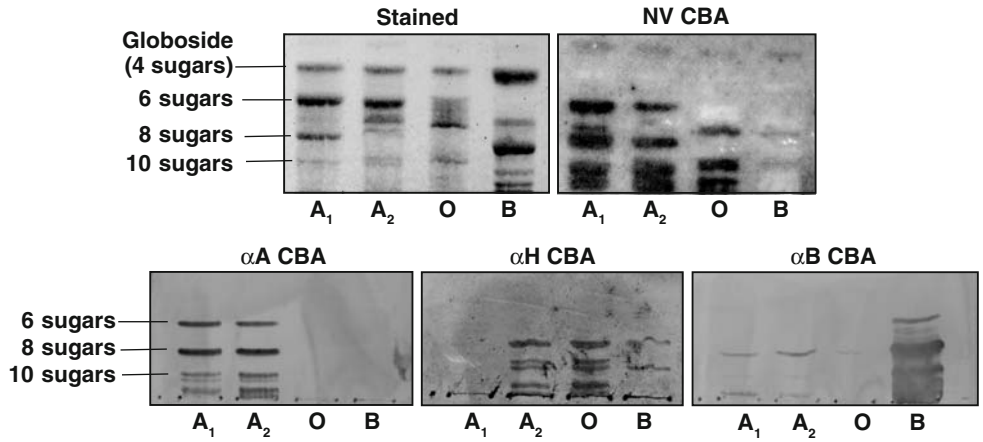

Fig. 4 ^{125}I-labeled NV VLP (top right) and anti histo-blood group A, H and B antibody binding (bottom) to partially purified GSLs from RBCs pooled from individuals of different blood groups; **A_1**, **A_2**, **O** and **B**. The top left plate was stained with anisaldehyde reagent to visualize the GSLs. The migration of GSLs with varying number of monosaccharides is indicated in the margin. The antibody binding is used as a reference to characterize the presence of A, B and H blood group epitopes in the A_1, A_2, O and B fractions. The TLC plates were chromatographed in chloroform:methanol:water (60:35:8, by vol.), cut into pieces, then overlaid with either VLP or antibodies and developed to give optimal contrast

Lewis y was tolerated by the NV VLPs. The presence of the terminal Fucα2Gal epitope was essential also for binding to the type 2 chain structures since the VLPs did not bind to the Lewis x GSL.

In conclusion, GSLs typically found in epithelial cells of secretors but not those found in non-secretors bound to the NV VLPs. The minimal binding epitope was the blood group H Fucα2Gal residue on either the type 1 or the type 2 chains. The NV GSL binding pattern was essentially in agreement with binding studies using PAA conjugates, which showed binding to H type 1 (trisaccharide), Lewis b (tetrasaccharide), H type 2 (trisaccharide), Lewis y (tetrasaccharide) and A (trisaccharide) conjugates [15, 21, 22]. An exception was however Lewis b, which was a good binder in the neoglycoconjugate studies but seemed to be less well recognized compared to the other binding GSLs in this study. Some of the binding may be due to Lewis y, since the ^{1}H-NMR analysis showed that the Lewis b fraction contained almost 5% Lewis y. Free Lewis b tetrasaccharide has previously been reported to be unable to inhibit VLP binding to tissue sections, whereas near-complete inhibition was seen with H type 1 trisaccharide [18]. However, Lewis b hexasaccharide-HSA conjugate was, in our hands, a better inhibitor than the H type 1 pentasaccharide-HSA for the blocking of NV VLP binding to secretor positive saliva [44].

NV VLPs have previously been shown to hemagglutinate H and A red blood cells in support of type 2 histo blood-group recognition, and in that study [21] NV VLPs were also demonstrated to bind H type 2 and Lewis y tetrasaccharides in the form of PAA/biotin conjugates linked to streptavidin-coated wells. The hemagglutination was run at 4°C and the PAA conjugate binding assay at 25°C [21]. In other studies, at 37°C, VLP binding to H type 2 as neoglycoconjugate or as free trisaccharide was not observed, whereas under the same conditions clear binding to H type 1 was reported [18, 19]. VLP from the closely related GI.1 r124 strain (98.7% identity to NV) was recently shown to recognize both H type 1, H type 2 and A (but not B) trisaccharide PAA conjugates in an ELISA run at 37°C [45]. However, using biotinylated conjugates none of the H (or B) structures and only A type 2, but not A type 1 pentasaccharide conjugates were binding the VLP in a surface plasmon resonance assay at 25°C. In the present study the binding of NV VLPs to both H type 1 and H type 2 GSLs was evident at 4°C, at room temperature and also at 37°C. Obviously, one method or temperature is seldom sufficient to get the whole picture of norovirus binding specificity to specific glycoconjugates.

From the binding of NV VLPs to the RBC GSLs we conclude that NV VLPs do not bind to any of the tested blood group B type 2 GSLs. A weak NV VLP interaction, most likely to an H type 2 GSL present in low amounts in the B fraction (Fig. 4), was however detected. The NV VLPs bound to GSLs originating from A_1, A_2 and O RBCs of sizes ranging from six up to about 20 sugars on the TLC plate. A sub-group difference between A_1 and A_2 individuals is due to different activities of the α1,3 GalNAc transferases in the conversion of the H type 4 (Globo H) GSL, Fucα2Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer into A type 4 (Globo A) GSL, GalNAcα3(Fucα2)Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer, found in A_1, but not in A_2 individuals [46, 47]. Our data support the NV VLP binding to the type 4 chain Globo A heptaglycosylceramide in A_1 individuals. However, much due to the complexity of these GSL fractions, VLP binding to the type 3 chain blood group A and H structures could not be conclusively

established. The very good binding of NV VLPs to complex, low abundant, GSLs in the RBC fractions of blood group A_1, A_2 and O individuals (Fig. 4) may indicate the presence of bivalent binding epitopes that can reach adjacent binding sites within one viral capsid. Branched GSLs carrying bivalent A epitopes have been reported to be present in GSLs both from blood group A [48] and blood group B RBCs [49].

Secretor positive blood group B individuals are partially protected against NV infection [17, 50] and NV VLPs bind to saliva only from some, but not all secretor positive blood group B individuals [17, 19, 45]. This may be explained by the results in this study since the VLP does not bind to blood group B active GSLs. Small amounts of the Fucα2Gal epitope present on saliva glycoproteins do, however, represent a potential binder and can explain binding to some saliva samples of blood group B individuals. The presence of H type 1 was demonstrated for the mixture of GSLs originating from meconium of a single blood group B individual (Fig. 3) and similarly H type 2 GSL was detected in the mixture of pooled blood group B RBCs (Fig. 4). The recently published crystal structures of the P-domain of the NV capsid protein in complex with H and A antigens suggest that the fucose is minimally involved in the binding of the A epitope [11, 12]. Our studies, however, suggest that a terminal GalNAc without the presence of a Fucα2 residue is not enough for binding the NV VLP. This is based on the observation that neither Forssman (terminal GalNAcα3) nor globoside (terminal GalNAcβ3) was a ligand for the NV VLP.

The radioiodination of NV VLPs via ATE was successful and did not interfere with the binding abilities when compared to the saliva ELISA (Fig. 2b and c). We achieved specific activities for the radiolabeled VLPs comparable to previous studies utilizing the Iodo-Gen radioiodination of viruses [51, 52]. The use of ATE is an established method to achieve the mild incorporation of radiohalogens at high yields in proteins. The bifunctional labeling agent is composed of an aryl derivative with an alkylstannyl group, which is a good leaving group in the electrophilic substitution reaction with halogens, and a succinimidyl ester moiety for the conjugation to accessible lysine residues on proteins. The standard procedure to radioiodinate proteins is to use e.g. the Iodo-Gen methodology for the radiohalogenation of tyrosine residues by electrophilic substitution in the presence of the Iodo-Gen oxidative conditions [53]. For precursor labeling methods, such as the one presented in this study, the oxidizing agent is quenched by the addition of $Na_2S_2O_5$ before the protein is added and the risk of oxidative damage to the protein is eliminated. Another advantage with the ATE method is that it is a one-pot procedure and the benzene extraction step used in the Bolton-Hunter methodology is not needed [54].

Outbreak and antibody titer studies have extended the correlation between secretor status and susceptibility to norovirus infection to include other strains of norovirus [8, 55–57]. It would be informative to use the methodology presented here for a variety of VLPs from other norovirus strains for the determination of their GSL binding specificities. NV VLP binding to histo-blood group GSLs suggests the possibility that GSLs may function as the true cellular receptors for norovirus infection, but further studies have to be done to prove this hypothesis. Studies have shown that NV VLPs were able to bind and be internalized in intestinal epithelial cells [18, 58]. The known glycan specificity of different norovirus strains determined by CBAs would add knowledge to our understanding of norovirus binding. The naturally occurring GSLs display more complex glycans in contrast to synthetic neoglycoconjugates, where the glycans often are shortened and composed merely of the minimal binding epitope. The CBA technique, as used in this report, may become an important tool to specifically map glycan binding of norovirus strains and also provide us with useful information for the design of sugar mimetics for the *in vivo* blocking of norovirus binding and infection.

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