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JOURNAL OF VIROLOGY, Nov. 2008, p. 10756–10767 Vol. 82, No. 21
0022-538X/08/$08.00+0 doi:10.1128/JVI.00802-08

Noroviruses Distinguish between Type 1 and Type 2 Histo-Blood Group Antigens for Binding▽

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Received 15 April 2008/Accepted 6 August 2008

Norovirus (NoV) is a causative agent of acute gastroenteritis. NoV binds to histo-blood group antigens (HBGAs), namely, ABH antigens and Lewis (Le) antigens, in which type 1 and type 2 carbohydrate core structures constitute antigenically distinct variants. Norwalk virus, the prototype strain of norovirus, binds to the gastroduodenal junction, and this binding is correlated with the presence of H type 1 antigen but not with that of H type 2 antigen (S. Marionneau, N. Ruvoen, B. Le Moullac-Vaidye, M. Clement, A. Cailleau-Thomas, G. Ruiz-Palacois, P. Huang, X. Jiang, and J. Le Pendu, Gastroenterology 122:1967–1977, 2002). It has been unknown whether NoV distinguishes between the type 1 and type 2 chains of A and B antigens. In this study, we synthesized A type 1, A type 2, B type 1, and B type 2 pentasaccharides in vitro and examined the function of the core structures in the binding between NoV virus-like particles (VLPs) and HBGAs. The attachment of five genogroup I (GI) VLPs from 5 genotypes and 11 GII VLPs from 8 genotypes, GI/1, GI/2, GI/3, GI/4, GI/8, GII/1, GII/3, GII/4, GII/5, GII/6, GII/7, GII/12, and GII/14, to ABH and Le HBGAs was analyzed by enzyme-linked immunosorbent assay-based binding assays and Biacore analyses. GI/1, GI/2, GI/3, GI/4, GI/8, and GII/4 VLPs were more efficiently bound to A type 2 than A type 1, and GI/8 and GII/4 VLPs were more efficiently bound to B type 2 than B type 1, indicating that NoV VLPs distinguish between type 1 and type 2 carbohydrates. The dissociation of GII/4 VLPs from B type 1 was slower than that from B type 2 in the Biacore experiments; moreover, the binding to B type 1 was stronger than that to B type 2 in the ELISA experiments. These results indicated that the type 1 carbohydrates bind more tightly to NoV VLPs than the type 2 carbohydrates. This property may afford NoV tissue specificity. GII/4 is known to be a global epidemic genotype and binds to more HBGAs than other genotypes. This characteristic may be linked with the worldwide transmission of GII/4 strains. GI/2, GI/3, GI/4, GI/8, GII/4, and GII/7 VLPs bound to Le[a] expressed by nonsecretors, suggesting that NoV can infect individuals regardless of secretor phenotype. Overall, our results indicated that HBGAs are important factors in determining tissue specificity and the risk of transmission.

Norovirus (NoV), a member of the family *Caliciviridae* (1), is a major cause of water- and food-borne acute nonbacterial gastroenteritis and comprises many morphologically similar but antigenically diverse groups of viruses (8, 11, 22). Recent progress in genetic studies has enabled us to divide human NoV into at least three genogroups, genogroup I (GI), GII, and GIV, which contain at least 15, 18, and 1 genotype, respectively (20).

Histo-blood group antigens (HBGAs) are carbohydrates that contain structurally related saccharide moieties. H antigen (Fucα1-2Gal), i.e., O-type antigen, is generated by a fucose transfer to a galactose residue with an α1-2 linkage. A antigen [GalNAcα1-3(Fucα1-2)Gal] and B antigen [Galα1-3(Fucα1-2)Gal] of ABH HBGAs are generated by a transfer of GalNAc and Gal, respectively, to an H structure irrespective of the carbohydrate core structure. The core structures are classified into four major structures, type 1 (Galβ1-3GlcNAcβ), type 2 (Galβ1-4GlcNAcβ), type 3 (Galβ1-3GalNAcα), and type 4 (Galβ1-3GalNAcβ). The fucose transfer of ABH antigens in erythrocytes is catalyzed by FUT1, a member of the fucosyltransferase family, whereas it is catalyzed by a different fucosyltransferase, FUT2, in saliva and mucosal secretions (31). Individuals who have null *FUT2* alleles cannot synthesize ABH antigens in secretions and are called nonsecretors, although ABH antigens can be expressed in erythrocytes via FUT1 (21). *FUT2* alleles of Caucasian nonsecretors are completely inactivated by nonsense mutations, whereas those of Asian nonsecretors are incompletely inactivated by missense mutations (23, 41). Thus, Asian nonsecretors are incomplete nonsecretors and produce a small amount of ABH HBGAs in secretions. FUT2 is essential for the fucose transfer to the type 1 structure (Galβ1-3GlcNAc) required to generate the H type 1 structure (Fucα1-2Galβ1-3GlcNAc), a precursor structure for Lewis b (Le[b]) [Fucα1-2Galβ1-3(Fucα1-4)GlcNAc], in secretions. The FUT3 enzyme is required for the fucose transfer to type 1 or H type 1 to generate Lewis a (Le[a]) [Galβ1-3(Fucα1-4)GlcNAc] or Le[b], respectively.

Virus-like particles (VLPs) derived from the prototype strain of NoV, Norwalk virus (NV/68), bind to HBGAs in saliva from secretor individuals. They preferentially bind to H

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▽ Published ahead of print on 13 August 2008.

TABLE 1. Recombinant VLPs used in this study

Genogroup	Genotype	Strain	VLP	GenBank accession no.	Reference or source
GI	1	Hu/NV/GI/Aichi124-89/89/JP	r124	AB031013	Kobayashi et al., 2000 (22b)
	2	Hu/NV/GI/Funabashi258/96/JP	r258	AB078335	Tamura et al., 2000 (41a)
	3	Hu/NV/GI/Kashiwa645/99/JP	r645	BD011871	Kamata et al., 2005 (20a)
	4	Hu/NV/GI/Chiba407/87/JP	rCV	AB042808	Kobayashi et al., 2000 (22c)
	8	Hu/NV/GI/WUG1/00/JP	rW18	AB081723	Hansman et al., 2006 (11)
GII	1	Hu/NV/GII/Hawaii/71/US	rHV	U07611	Kindly provided by K. Green (NIH, Bethesda, MD)
	1	Hu/NV/GII/Noda485/00/JP	r485	DQ093065	Hansman et al., 2006 (11)
	3	Hu/NV/GII/Matsudo18/00/JP	r18-3	DQ093062	Hansman et al., 2006 (11)
	3	Hu/NV/GII/Kashiwa336/00/JP	r336	DQ093063	Hansman et al., 2006 (11)
	4	Hu/NV/GII/Narita104/97/JP	r104	AB078336	Kitamoto et al., 2002 (22a)
	5	Hu/NV/GII/Ichikawa754/98/JP	r754	BD011877	Kamata et al., 2005 (20a)
	6	Hu/NV/GII/Ueno7k/94/JP	r7k	AB078337	Tamura et al., 2000 (41a)
	6	Hu/NV/GII/Sanbu445/00/JP	r445	DQ093064	Hansman et al., 2006 (11)
	7	Hu/NV/GII/Osaka10-25/99/JP	r10-25	BD011881	Kamata et al., 2005 (20a)
	12	Hu/NV/GII/Chitta/Aichi76-96/96/JP	r76	AB032758	Kobayashi et al., 2000 (22d)
	14	Hu/NV/GII/Kashiwa47/97/JP	r47	AB078334	Kitamoto et al., 2002 (22a)

type 1 and Le^b synthetic carbohydrates (12, 14, 15, 24, 26). Although NV/68 VLPs bind to type A antigens in saliva and synthetic type A carbohydrates, they bind to neither type B synthetic carbohydrates nor the majority of type B antigens in saliva (12, 14, 15, 24). From previous volunteer challenge studies, there is strong evidence that this carbohydrate binding is essential for NV/68 infection (16, 24). Nonsecretors were not infected after the challenge with NV/68. Furthermore, type O secretors are more likely to be infected with NV/68; conversely, type B secretors are less likely to be infected with NV/68. However, other NoV VLPs display different ABH and Lewis carbohydrate-binding profiles (12–15): indeed, epidemiological studies have shown that some NoV strains could infect individuals with other ABH or secretor phenotypes (34).

Type 1 core structures are widely expressed in endodermally derived tissues, such as lining epithelia and glandular epithelia (32). On the other hand, type 2 core structures are found mainly in ecto- or mesodermally derived tissues, including skin and erythrocytes (7, 10, 32). In the human gastroduodenal junction, type 1 structures are found exclusively at the level of the surface epithelia whereas type 2 structures are preferentially found at the glandular level (27). Immunohistochemical analysis showed that the binding of recombinant NV/68 (rNV/68) to the gastroduodenal junction correlated with the presence of H type 1 antigen but not with that of H type 2 antigen (26). In a human GII/4 infection experiment, pigs that expressed either A or H antigen on the intestinal mucosa had significantly higher rates of diarrhea and seroconversion in response to the strain, and fecal shedding of virus was also significantly higher (5). Therefore, in pigs and in humans, the expression of HBGAs may lead to increased susceptibility to NoV infection. The rNV/68 VLP has been reported to bind synthetic H carbohydrates in the following order of strength: H type 1 trisaccharides, H type 2 trisaccharides, and H disaccharides (17). Meanwhile Harrington et al. reported that this strain had higher binding activity for H type 1 trisaccharides than for H type 3 trisaccharides (12). Although these reports suggest that rNV/68 VLP may recognize the linkages and components in the core structures of the HBGAs, this has not yet been proven. Moreover, it has not been known whether NoV VLPs distinguish between the type 1 and type 2 chains of A and B antigens.

To investigate the binding properties of NoV strains for HBGAs and to determine whether NoV distinguishes between type 1 and type 2 chains of HBGAs, the attachment of 5 GI VLPs from 5 genotypes and 11 GII VLPs from 8 genotypes to ABH and Le^aLe^b HBGAs was analyzed by enzyme-linked immunosorbent assay (ELISA)-based binding assays and Biacore analysis.

MATERIALS AND METHODS

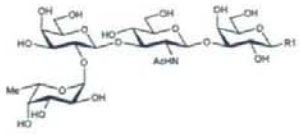
Recombinant VLPs. The recombinant VLPs used in this study are shown in Table 1 (11, 20a, 22a, 22b, 22c, 22d, 41a). VLPs were prepared by infecting subconfluent Tn5 insect cells with the recombinant baculoviruses as described previously (11). Briefly, the culture medium was harvested at 6 days after infection, centrifuged at 1,000 × *g* for 10 min to remove the cell debris, and further centrifuged at 10,000 × *g* for 30 min to remove the baculoviruses. The VLPs in the supernatant were concentrated by centrifugation at 100,000 × *g* for 2 h at 4°C in an SW28 rotor (Beckman Instruments, Inc., Palo Alto, CA). The pellet was resuspended in a solution containing CsCl (1.9 g/4.5 ml) and centrifuged at 120,000 × *g* for 20 h at 10°C in an SW50.1 rotor (Beckman). Peak fractions containing the VLPs were pooled, diluted with phosphate-buffered saline (PBS) (pH 7.5), and centrifuged at 200,000 × *g* for 2 h at 4°C in an SW50.1 rotor. The purified VLPs were examined by electron microscopy, and it was confirmed that the preparations form particles similar to native NoV and do not contain VP2 aggregates, as described previously (11). The VLPs were also examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the protein standard. Tn5 cells, an insect cell line from *Trichoplusia ni* (Invitrogen, San Diego, CA), were grown at 27°C with Ex-CELL 400 medium (JRH Biosciences, Lenexa, KS). The phylogenetic analysis of the 16 NoV strains, which classified them into 13 clusters, was described previously (11).

Saliva samples and blood group antigens. Saliva samples were collected from 29 healthy Japanese donors: specifically, 13 (45%) males (average age, 41 years old; range, 29 to 59 years old) and 16 (55%) females (average age, 36 years old; range, 23 to 62 years old). The saliva samples were boiled for 10 min immediately after collection and centrifuged for 5 min at 13,000 × *g*. The clear supernatant was collected and stored at −30°C until use.

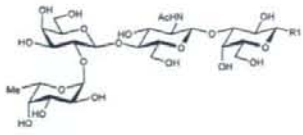
The samples were assayed for the presence of H, A, and B antigens by hemagglutination inhibition. Either 100 μl of anti-H lecithin (Gamma Biologicals, Inc., Houston, TX), 50 μl of anti-A antibody (Gamma Biologicals, Inc.), or anti-B antibody (Gamma Biologicals, Inc.) was mixed with an equal volume of

A

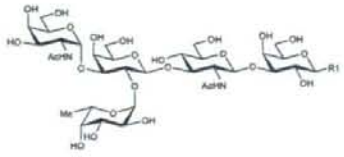
H type 1: Fucα1-2Galβ1-3GlcNAcβ1-3Galβ-R2 (Tetrasaccharide)



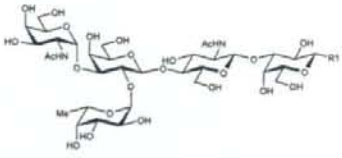
H type 2: Fucα1-2Galβ1-4GlcNAcβ1-3Galβ-R2 (Tetrasaccharide)



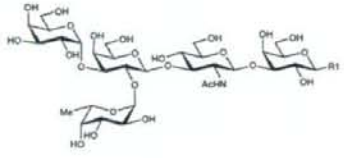
A type 1: GalNAcα1-3(Fucα1-2)Galβ1-3GlcNAcβ1-3Galβ-R2 (Pentasaccharide)



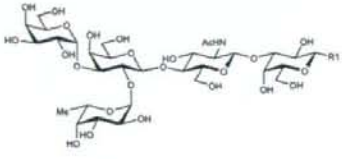
A type 2: GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ-R2 (Pentasaccharide)



B type 1: Galα1-3(Fucα1-2)Galβ1-3GlcNAcβ1-3Galβ-R2 (Pentasaccharide)



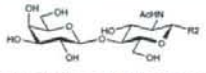
B type 2: Galα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ-R2 (Pentasaccharide)



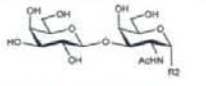
B

type1: Galβ1-3GlcNAcβ-R1 (Disaccharide)

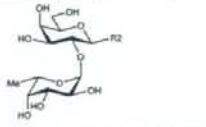
type2: Galβ1-4GlcNAcβ-R1 (Disaccharide)



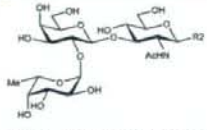
type3: Galβ1-3GalNAcα-R1 (Disaccharide)



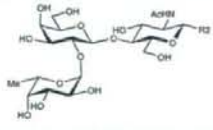
di-H: Fucα1-2Galβ-R1 (Disaccharide)



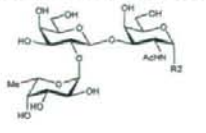
tri-H type 1: Fucα1-2Galβ1-3GlcNAcβ-R1 (Trisaccharide)



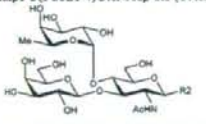
tri-H type 2: Fucα1-2Galβ1-4GlcNAcβ-R1 (Trisaccharide)



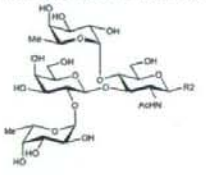
tri-H type 3: Fucα1-2Galβ1-3GalNAcα-R1 (Trisaccharide)



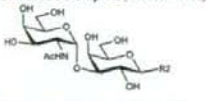
Le^a: Galβ1-3(Fucα1-4)GlcNAcβ-R1 (Trisaccharide)



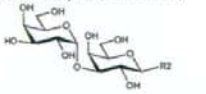
Le^b: Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ-R1 (Tetrasaccharide)



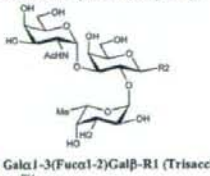
di-A: GalNAcα1-3Galβ-R1(Disaccharide)



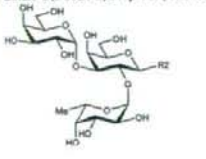
di-B: Galα1-3Galβ-R1 (Disaccharide)



tri-A: GalNAcα1-3(Fucα1-2)Galβ-R1 (Trisaccharide)



tri-B: Galα1-3(Fucα1-2)Galβ-R1 (Trisaccharide)



each undiluted saliva sample and incubated for 10 or 20 min at 26°C. Then, 50 μl of a 3 to 4% suspension of indicator O (Gamma Biologicals, Inc.), A1 (Gamma Biologicals, Inc.), or B (Gamma Biologicals, Inc.) was added. The mixture was left at 26°C for 5 min and centrifuged at 125 × *g* for 1 min. The amount of HBGAs was measured in a semiquantitative manner using serially diluted saliva (40). Serial twofold dilutions of the samples were prepared (1- to 256-fold dilution) and assayed by hemagglutination inhibition assay as described above. Informed consent was obtained from all donors in 2003 before their participation according to ethical code 28 of the National Institute of Infectious Diseases, Japan.

Enzymatic preparation of monovalent carbohydrate-biotin reagents. For the preparation of the GlcNAcβ1-3Galβ1–biotin derivative, a reaction mixture containing 25 mM HEPES buffer (pH 7.0), 10 mM $MnCl_2$, biotin-labeled galactose, an appropriate concentration of UDP-GlcNAc, and purified β1,3-*N*-acetylglucosaminyltransferase 2 (β3GnT2) (38) was used. Biotin-labeled galactose, UDP-GlcNAc, and β3GnT2 were the acceptor, donor substrate, and enzyme, respectively. The substrates and enzyme were added to a reaction mixture containing 25 mM HEPES buffer and 10 mM $MnCl_2$ and incubated at 37°C for 36 h. The enzyme was removed with an Ultrafree-MC column (Millipore, Bedford, MA), and the product was purified using reversed-phase high-performance liquid chromatography. For the preparation of the galactosylated derivatives Galβ1-3GlcNAcβ1-3Galβ1–biotin and Galβ1-4GlcNAcβ1-3Galβ1–biotin, a reaction mixture containing GlcNAcβ1-3Galβ1–biotin and UDP-Gal was used. β1,3-Galactosyltransferase-5 (18) and β1,4-galactosyltransferase-1 (28) were added to the solution to synthesize type 1 and type 2 structures, respectively. After incubation at 37°C for 20 h, the removal of the enzyme and the purification of the products were performed as described above. For the preparation of the fucosylated derivatives Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1–biotin and Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1–biotin (H types 1 and 2, respectively, in Fig. 1A), a reaction mixture containing GDP-Fuc, FUT2, and the galactosylated derivatives as the acceptor substrate was used. After incubation at 37°C for 24 h, the products were purified as described above. For the preparation of the derivatives with the A-antigen structure, GalNAcα1-3(Fucα1-2)Galβ1-3GlcNAcβ1-3Galβ1–biotin and GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ1–biotin (A types 1 and 2, respectively, in Fig. 1A), a reaction mixture containing UDP-GalNAc, the A enzyme, and the fucosylated derivatives as the acceptor substrate was used. After incubation at 37°C for 15 h, the product was purified. For the preparation of derivatives with B-antigen structure, Galα1-3(Fucα1-2)Galβ1-3GlcNAcβ1-3Galβ1–biotin and Galα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ1–biotin (B types 1 and 2, respectively, in Fig. 1A), a reaction mixture containing UDP-Gal, the B enzyme, and the fucosylated derivatives as the acceptor substrate was used. After incubation at 37°C for 15 h, the products were purified. The monovalent carbohydrate-biotin reagents were identified by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (Reflex IV; Bruker-Daltonik GmbH) as previously described (19).

ELISA-based binding assay. Two ELISA-based assays were used to detect and quantify NoV VLP attachment to HBGAs. In the first assay, a 96-well microplate (Thermo Labsystems, Franklin, MA) was coated with 100 μl of serially twofold-diluted saliva with coating buffer (50 mM carbonate-bicarbonate buffer, pH 9.6) (Sigma, St. Louis, MO) and incubated overnight at 37°C in a wet atmosphere. The wells were washed three times with 300 μl of PBS containing 0.05% Tween 20 (PBS-T) and then blocked with 200 μl of PBS containing 5% skim milk (SM/PBS) for 1 h at room temperature. After the well was washed three times with PBS-T, the VLPs (1 μg/ml) in 100 μl of 1% SM/PBS-T were added and incubated for 1 h at 37°C. The coating buffer was used for the negative control. The plates were washed six times with PBS-T, and 100 μl of the rabbit anti-recombinant NoV VLP antiserum (1:2,000) in 1% SM/PBS-T was added and incubated for 1 h at 37°C. After the well was washed six times with PBS-T, 100 μl of horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Zymed Laboratories Inc., San Francisco, CA) in 1% SM/PBS-T was added and incubated for 1 h at 37°C. The plates were washed six times with PBS-T, 100 μl of *O*-phenylenediamine (Sigma) was added as a substrate, and incubation proceeded at room temperature. After 30 min, 50 μl of 4 N H_2SO_4 was added to stop the reaction and the optical density at 492 nm was measured. A convalescent-phase serum from a patient infected with the GI/2 258 strain and r258 were used for the internal standard.

In the second assay, multivalent carbohydrate-biotin reagents conjugated to polyacrylamide (CHO-PAA-biotin; Glycotech, Rockville, MD) (Fig. 1B) were rehydrated to 1 mg/ml with 0.3 M sodium phosphate buffer and diluted to 2.5 μg/ml with Tris-buffered saline. The carbohydrates (100 μl per well) were added to streptavidin-precoated plates (Thermo Electron Corporation, Vantaa, Finland) and incubated for 2 h at 37°C. The plates were then blocked with 300 μl of 5% SM/PBS overnight at 4°C. VLPs (1 μg/ml) in a 100-μl volume of 5% SM/PBS were added and incubated for 4 h at 37°C. The plates were washed six times with PBS-T, and 100 μl of the rabbit anti-recombinant NoV VLP antiserum (1:2,000) in 5% SM/PBS was added and incubated for 2 h at 37°C. After the well was washed six times with PBS-T, 100 μl of horseradish peroxidase-conjugated anti-rabbit immunoglobulin G in 5% SM/PBS was added and incubated for 1 h at 37°C. The plates were washed six times with PBS-T, and binding was detected using *O*-phenylenediamine. To measure dose-dependent binding of VLPs to the monovalent carbohydrate-biotin reagents (Fig. 1B), the reagents were rehydrated with 0.3 M sodium phosphate buffer and diluted to 1 pmol/μl with Tris-buffered saline. Serial twofold dilutions of the regents were prepared (1.0 to 0.016 pmol/μl) and used to coat streptavidin-precoated plates. The binding of the VLPs was detected by polyclonal rabbit anti-VLPs.

SPR assay. The interaction between the VLPs and monovalent carbohydrate-biotin reagents (Fig. 1A) was examined by surface plasmon resonance (SPR) assay at 25°C with a Biacore 2000 instrument. The running buffer used in this assay was 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$ containing 0.005% p20. A research-grade streptavidin-coated sensor chip (Biacore AB, Uppsala, Sweden) was pretreated with three injections of 1 M NaCl–50 mM NaOH at the flow rate of 5 μl/min/injection. The monovalent carbohydrate-biotin reagents (Fig. 1A) were rehydrated to 1 mg/ml with 0.3 M sodium phosphate buffer and diluted to 10-fmol/μl with the same buffer. The biotinylated oligosaccharides were captured on the chip. A 10-fmol/μl solution of each biotinylated oligosaccharide was then injected at 5 μl/min until an amount corresponding to 80 resonance units (RU) for H type 1 and H type 2 or 100 RU for A type 1, A type 2, B type 1, and B type 2 was captured on each independent surface of the sensor chip. A signal of 100 RU corresponds approximately to a surface concentration change of 0.1 ng/mm^2. The carbohydrate-free surface of a sensor chip was used as a negative control. The VLPs (100 μg/ml) in the running buffer were injected at a flow rate of 20 μl/min for 120 s over all surfaces of the sensor chip to monitor the associations between VLPs and oligosaccharides. After injection, the VLPs were replaced with the running buffer to monitor their dissociations.

RESULTS

Saliva phenotypes. HBGAs and secretor status are known to be associated with susceptibility to NoV infection, and these characteristics are represented in saliva (9). To determine the phenotypes of saliva samples, a hemagglutination inhibition assay was performed as described in Materials and Methods. According to the presence or absence of H, A, and B antigens, the 29 saliva samples were divided into 5 groups (Table 2), in which 1 specimen, no. 26, had an unexpected phenotype, H negative and A positive (Table 2). Therefore, this phenotype was further examined by an ELISA-based binding assay using r124, a VLP derived from a GI/1 strain which has 98% amino acid identity with the prototype Norwalk virus (NV/68) in the P2 domain. Two amino acid differences, at residues 370 and 376, are known not to be related to HBGA binding (3, 4, 42). The abilities of recombinant NV/68 to bind to H and A antigens have been well documented (12, 14–16, 24, 26). GII/1

FIG. 1. Diagram of carbohydrate structures used in this study. Monovalent carbohydrate-biotin reagents (A) were synthesized and used in the experiments shown in Fig. 5 and 6, and multivalent carbohydrate-biotin reagents (B) were used in the experiments shown in Fig. 4. Glc, glucose; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Lac, lactose; GalNAc, *N*-acetylgalactosamine; R1, biotin; R2, polyacrylamide with biotin.

TABLE 2. Detection of soluble ABH antigens in saliva by hemagglutination inhibition assay

Donor no.	Grouping	Presence of HBGA		
		H	A	B
1–5, 25, 27–29	H/A	+	+	−
6–13	H/A/B	+	+	+
14–19	H/B	+	−	+
20–24	H	+	−	−
26	A	−	+	−

rHV was used as a negative control for the binding assay (12). As depicted in Fig. 2, the binding of r124 occurred in a dose-dependent manner and high optical density values were observed up to 1:6,400 saliva dilutions (Fig. 2A, B, and D). The binding of r124 to the saliva containing B antigens was low, probably due to the inefficient binding ability of the terminal galactose of the type B epitope, as described previously (Fig. 2C) (12, 14, 15, 24). Although the saliva included A antigens and they were recognized by NV/68, the binding of r124 to the saliva from donor no. 26 was weak (Fig. 2E). In addition, four saliva samples containing H and A antigens had weak binding ability (Fig. 2A, no. 25 and 27 to 29). None of the saliva bound to rHV, as described previously (Fig. 2F) (12, 15).

Identification of incomplete nonsecretors. To further characterize these low binding abilities, the amounts of HBGAs in saliva samples were measured by semiquantitative hemagglutination inhibition assay. The phenotypes according to the amounts of HBGAs are summarized in Table 3. The saliva was grouped into five categories, characterized by A, AB, B, and O secretor status and nonsecretor status. These results were consistent with the results shown in Table 2. The phenotype of secretors was characterized by high secretion of H, A, or B antigens in saliva, as observed for donors 1 to 24. Each group of secretors was characterized by antigen content: H and A antigens for phenotype A; H, A, and B antigens for the AB phenotype; H and B antigens for phenotype B; and H antigen for phenotype O. The phenotype of the nonsecretors was characterized by low secretion of ABH antigens in saliva as observed for donors 25 to 29. These nonsecretors differ from those observed in Europe, because Japanese nonsecretors secrete a small amount of either H, A, or B antigen or both (23, 41). Therefore, they are described as incomplete nonsecretors. Measurement of the HBGAs enabled us to clarify the phenotypic difference between donors 1 to 5 and donors 25 and 27 to 29.

Binding of GI NoV VLPs to HBGAs. The binding between GI VLPs and saliva was analyzed by using an ELISA-based binding assay. The saliva dilution of 1:1,600 was used because the strength of the binding to r124 was observed clearly at this dilution (Fig. 2). GI/1 r124 bound more efficiently to saliva from type O, A, and AB secretors than to that from type B secretors and incomplete nonsecretors (Fig. 3A), indicating that GI/1 r124 recognizes the H and A antigens. These results were consistent with the observation shown in Fig. 2. Three GI VLPs, GI/2 r258, GI/3 r645, and GI/4 rCV, had the same binding profiles and high binding activities for type O, A, and AB secretors and incomplete nonsecretors (Fig. 3B, C, and D). These results indicated that these three GI strains recognize the Le^a antigen as well as the H and A antigens, because the saliva of the nonsecretors is characterized by high secretion of

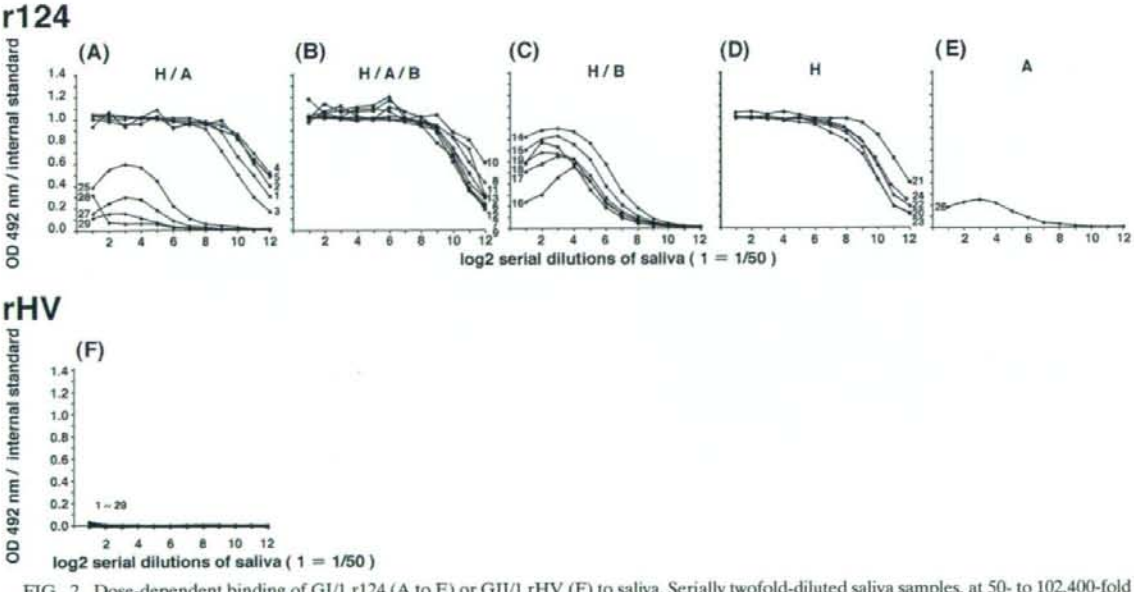


FIG. 2. Dose-dependent binding of GI/1 r124 (A to E) or GII/1 rHV (F) to saliva. Serially twofold-diluted saliva samples, at 50- to 102,400-fold dilution, were used to coat the microplates. Convalescent-phase serum from a patient infected with the GI/2 258 strain and r258 were used for the internal standard. Coating buffer was used for the blank. The binding of the VLPs was detected by using polyclonal rabbit anti-VLPs as described in Materials and Methods.

TABLE 3. Semiquantitation of soluble ABH

Secretion status of donor	HBGA secretion phenotype	Donor no.	Titer of HBGA		
			H	A	B
Secretor	A	1	32	128	0
		2	32	64	0
		3	64	64	0
		4	128	>256	0
		5	32	128	0
	AB	6	4	128	8
		7	16	128	8
		8	8	>256	1
		9	4	128	8
		10	16	>256	32
		11	8	>256	8
		12	64	128	64
		13	64	128	>256
	B	14	16	0	128
		15	8	0	>256
		16	16	0	>256
		17	8	0	>256
		18	16	0	128
		19	16	0	>256
	O	20	>256	0	0
		21	>256	0	0
		22	128	0	0
		23	>256	0	0
		24	>256	0	0
Incomplete nonsecretor		25	16	16	0
		26	0	1	0
		27	4	8	0
		28	4	1	0
		29	4	1	0

Le^a antigen in saliva (40). GI/8 rW18 bound efficiently to all saliva samples regardless of secretor phenotype or blood type, indicating that GI/8 rW18 recognized the H, A, B, and Le^a antigens (Fig. 3E). To further examine which HBGAs are involved in GI NoV attachment, a carbohydrate-VLP binding assay was performed with seven multivalent carbohydrate-biotin reagents: H type 1, H type 2, H type 3, tri-A, tri-B, Le^a, and Le^b. Basically, no discrepancy was found between the carbohydrate- and saliva-VLP binding assays, although the sensitivity of the former was higher than that of the latter (Fig. 4A to E; Table 4). Moreover, the following observations were obtained from the carbohydrate-VLP binding assay: (i) the results provide direct evidence that GI/2, GI/3, GI/4, and GI/8 VLPs recognized the Le^a carbohydrate (Fig. 4B, C, D, and E); (ii) GI/1 and GI/2 VLPs bound to H type 3 (Fig. 4A and B); and (iii) GI/1, GI/4, and GI/8 VLPs recognized the Le^b carbohydrate (Fig. 4D and E). We concluded that the binding properties of GI NoV VLPs for HBGAs were variable. However, four of five genotypes, GI/2, GI/3, GI/4, and GI/8, had high abilities to bind to the Le^a antigen. This was a unique characteristic of GI which was not seen with GII, as mentioned below.

Binding of GII NoV VLPs to HBGAs. Similarly, a binding assay was performed with 11 GII VLPs. Two GII/1 VLPs (rHV and r485) and one GII/14 VLP (r47) did not bind to any of the saliva samples (Fig. 3F, G, and P), indicating that these three GII strains did not recognize the HBGAs in saliva. Two GII/3 VLPs (r18-3 and r336) and one GII/5 VLP (r754) bound to saliva samples from type A, AB, and B secretors, whereas they bound neither to type O secretors nor to incomplete nonsecretors (Fig. 3H, I, and K). The results indicate that these strains recognized the A and B antigens. GII/4 r104 and GII/7 r10-25 bound to all saliva samples (Fig. 3J and N), indicating that these two GII strains recognized the H, A, B, and Le^a antigens. Two GII/6 VLPs, r7K and r445, had high binding ability for all blood-type secretors (Fig. 3L and M), indicating that these GII/6 strains recognize the H, A, or B antigen. GII/12 r76 bound to saliva from type AB and B secretors, although the binding levels were extremely low (Fig. 3O), indicating that r76 attached weakly to the B antigen. The saliva-VLP binding assay clearly showed that the strains of the same genetic clusters, rHV and r485 in GII/1, r18-3 and 336 in GII/3, and r7K and r445 in GII/6, have the same carbohydrate-binding patterns. To address which HBGAs are involved in the GII NoV attachment, a carbohydrate-VLP binding assay was performed as described above. The sensitivity was higher in the saliva- than the carbohydrate-VLP binding assay, and no discrepancy was found between the two binding assays, as was also the case with the GI assays (Fig. 4F to P and Table 4). Five other observations from the carbohydrate-VLP binding assay were made: (i) GII/3, GII/4, GII/6, and GII/7 VLPs bound to H type 3 (Fig. 4J, L, M, and N); (ii) GII/7 VLPs recognized the Le^a carbohydrate (Fig. 4N); (iii) r104 hardly recognized the Le^a carbohydrate (Fig. 4J), although the strain bound to incomplete nonsecretors strongly (Fig. 3J); (iv) r7K had binding activities for the Le^a carbohydrate (Fig. 4L) (it had been hard to predict the r7K binding to the Le^a antigen based on the saliva-VLP binding assay due to low binding activity for incomplete nonsecretors [Fig. 3L]); and (v) GII/4, GII/6, and GII/7 VLPs recognized the Le^b carbohydrate (Fig. 4J, L, and N). We concluded that the binding properties of GII NoV VLPs for HBGAs were variable. However, there was no strain which strongly bound to the Le^a antigen, although the strains of GII/6 and GII/7 had weak binding abilities for this antigen.

Both α1,2-fucosyl residue and carbohydrate core structures are needed for binding to HBGAs. To investigate the effect of the terminal saccharide residue(s), the binding activities between VLPs and synthetic type 1, 2, and 3 carbohydrates and synthetic H disaccharides that do not include the core structures were examined. As depicted in Fig. 4, none of the VLPs bound to synthetic type 1, 2, and 3 disaccharides, although GI/1, GI/2, GI/3, GII/3, GII/4, GII/6, and GII/7 VLPs had binding activities for H type 1, 2, or 3 (Fig. 4A to C, H to J, and L to N). These results suggested that the terminal α1,2-fucosyl residue on those H trisaccharides is one of the determinants responsible for the NoV binding. Moreover, NoV VLPs recognized the components in the core structures, because (i) seven VLP genotypes, including GI/2, GI/3, GII/3, GII/6, and GII/7 VLPs, unequally bound H type 1, 2, and 3 trisaccharides (Fig. 4B, C, H, I, and L to N); (ii) the binding abilities of those VLPs for H disaccharides were undetectable (Fig. 4B, C, H, I, and L to N); and (iii) the binding abilities of GI/1 r124 and GII/4 r104 for H disaccharides were lower than those for the H type 1, 2, and 3 trisaccharides (Fig. 4A and J). Next, we addressed whether the VLPs bound to synthetic A and B disaccharides that lack the α1,2-fucosyl residue. As shown in Fig. 4A to E, J, K, L, and N, those strains did not bind to the synthetic A and

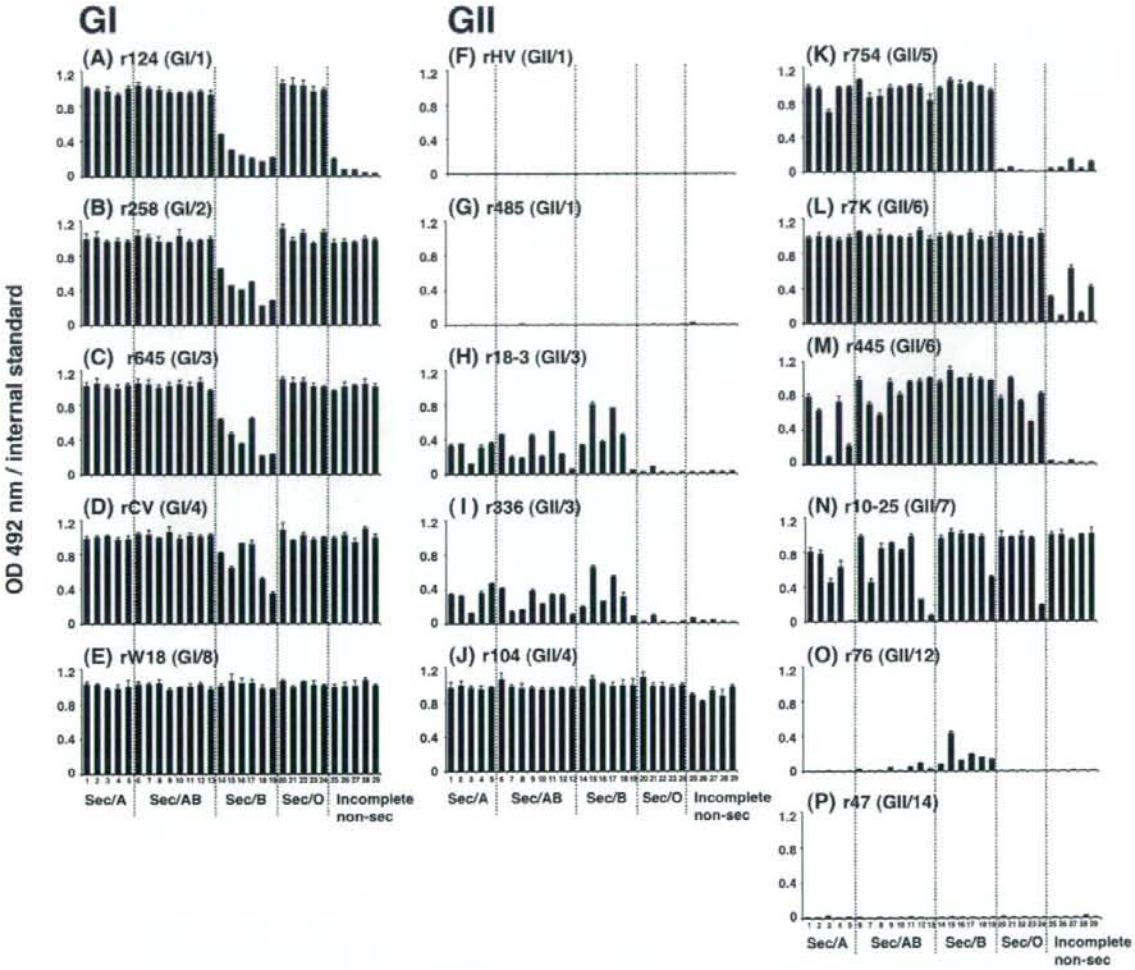


FIG. 3. Binding between VLPs and saliva samples. The saliva samples were tested at a dilution of 1:1,600. The binding of the VLPs was detected by polyclonal rabbit anti-VLPs as described in Materials and Methods. The experiments were performed in triplicate and reproduced at least twice. Each data point represents the mean value (with error bar).

B disaccharides, with the exception of GI/2 r258. Therefore, besides the r258 strain, the α1,2-fucosyl residue is one of the determinants responsible for NoV binding, not only to H antigens but also to A and B antigens. We concluded that both the α1,2-fucosyl residue and core structures are needed for the binding of VLPs to the HBGAs.

NoV VLPs distinguish between type 1 and type 2 carbohydrates. Six monovalent carbohydrate-biotin reagents, H type 1, H type 2, A type 1, A type 2, B type 1, and B type 2 (Fig. 1A), were used in SPR Biacore experiments to determine whether NoV VLPs distinguish between type 1 and type 2 chains. These oligosaccharides were prepared using human recombinant glycosyltransferases. Unlike the organic synthesis of oligosaccharides, the reaction using glycosyltransferases is quite clear and efficient, and the monovalent carbohydrate-biotin reagents were identified by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (Reflex IV; Bruker-Daltonik GmbH) (data not shown). Six strains, including GI/1, GI/2, GI/3, GI/4, GI/8, and GII/4 strains, bound to the monovalent carbohydrate-biotin reagents (Fig. 5), whereas 10 strains, including GII/1, GII/3, GII/4, GII/5, GII/6, GII/7, GII/12, and GII/14 strains, did not (data not shown). Basically, no discrepancy was found between the results of the ELISA-based binding assay with multivalent biotinylated reagents and those of the SPR Biacore experiments with monovalent biotinylated reagents, although the sensitivity of the former was higher than that of the latter (Fig. 4 and 5). GI/1, GI/2, GI/3, and GI/4 VLPs bound to A, but not to B, carbohydrates in the Biacore experiments, whereas GI/8 and GII/4 VLPs bound to both A and B carbohydrates. These results were consistent with the

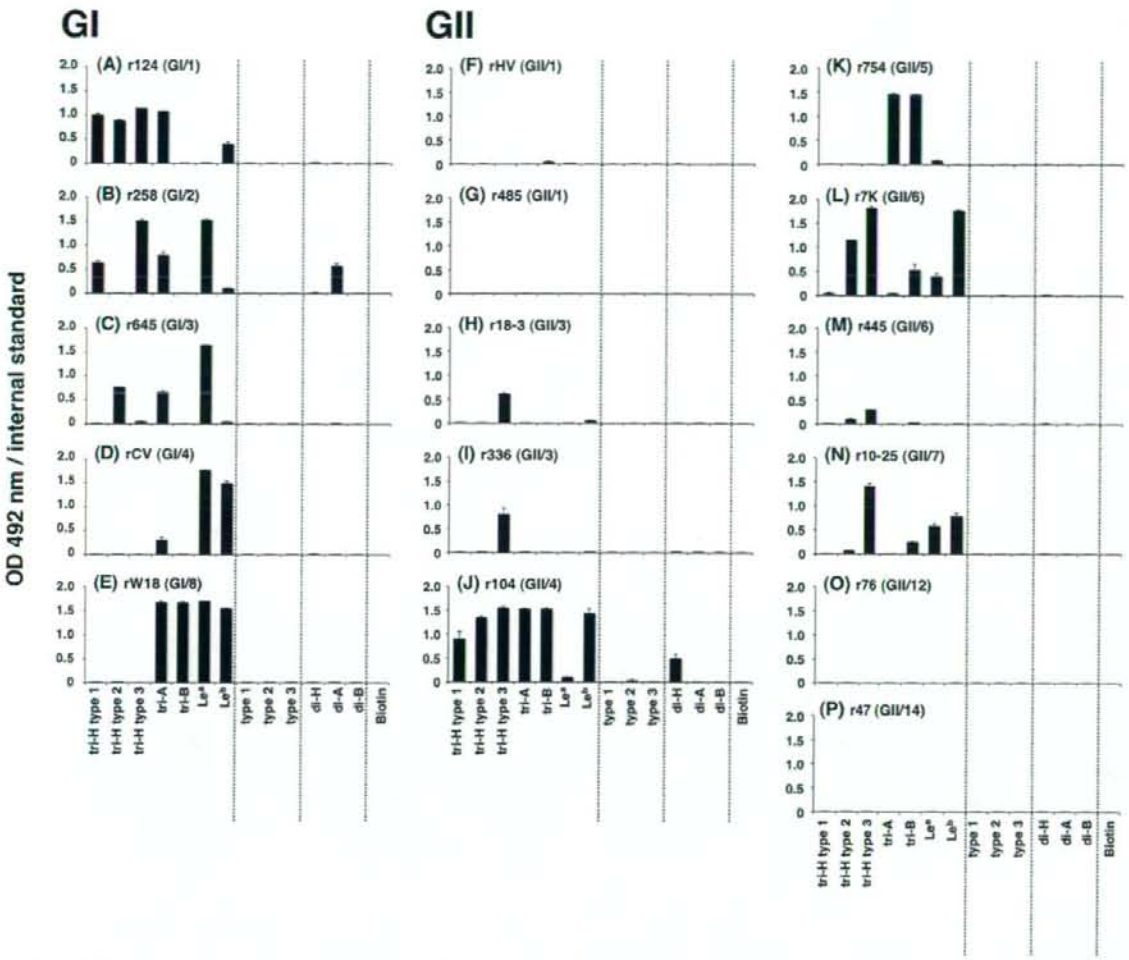


FIG. 4. Binding between VLPs and synthetic histo-blood group carbohydrates. The multivalent carbohydrate-biotin reagents conjugated to polyacrylamide were tested at a concentration of 2.5 μg/ml. H type 1 trisaccharides and r124 were used for the internal standard. Tris-buffered saline was used for the blank. The binding of the VLPs was detected by polyclonal rabbit anti-VLPs as described in Materials and Methods. The experiments were performed in duplicate and reproduced at least twice. Each data point represents the mean value (with error bar).

observation shown with the ELISA-based binding assay. Moreover, the following three observations were obtained from the Biacore experiments: (i) GI/3 and GII/4 VLPs were more efficiently bound to H type 2 than to H type 1 tetrasaccharides (Fig. 5C and F); (ii) five GI VLPs and r104 were more efficiently bound to A type 2 than to A type 1 pentasaccharides (Fig. 5G to L); and (iii) GI/8 and GII/4 VLPs were more efficiently bound to B type 2 than to B type 1 pentasaccharides (Fig. 5Q and R). These results indicate that NoV VLPs are able to distinguish between type 1 and type 2 carbohydrates. Moreover, type 1 carbohydrates are likely to bind more tightly to NoV VLPs than do the type 2 carbohydrates, because the dissociation of GII/4 r104 was slower in B type 1 than B type 2 (Fig. 5R). To further characterize this strong binding ability of type 1 carbohydrates, the binding between r104 and the six monovalent carbohydrate-biotin reagents was examined using an ELISA-based binding assay. The bindings of GII/4 VLPs were stronger in B type 1 pentasaccharides than in B type 2 pentasaccharides (Fig. 6). The Biacore assay allowed us to visualize each binding step in the 120-s reaction time, whereas ELISA allowed us to visualize only the last step in a total reaction time of about 7.5 h. Therefore, these results indicated that NoV VLPs bind more tightly to type 1 carbohydrates than to type 2 carbohydrates.

DISCUSSION

To date, the interaction between carbohydrates and 16 NoV VLPs, including 5 GI and 11 GII VLPs, has been described, with the conclusion that the carbohydrate-binding properties

TABLE 4. HBGA recognition predicted by carbohydrate- and saliva-VLP binding assay

Genogroup	Genotype	VLP	Binding assay result									
			Saliva-VLP					Carbohydrate-VLP[b]				
			H	A	B	Le[a]	Le[b]	H type 1/2/3	Tri-A	Tri-B	Le[a]	Le[b]
GI	1	r124	+	+	−	ND[a]	ND	+	+	−	−	+
	2	r258	+	+	−	ND	ND	+	+	−	+	−
	3	r645	+	+	−	ND	ND	+	+	−	+	−
	4	rCV	+	+	−	ND	ND	−	+	−	+	+
	8	rW18	+	+	+	ND	ND	−	+	+	+	+
GII	1	r485	−	−	−	ND	ND	−	−	−	−	−
	1	rHV	−	−	−	ND	ND	−	−	−	−	−
	3	r18-3	−	+	+	ND	ND	+	−	−	−	−
	3	r336	−	+	+	ND	ND	+	−	−	−	−
	4	r104	+	+	+	ND	ND	+	+	+	−	+
	5	r754	−	+	+	ND	ND	−	+	+	−	−
	6	r7k	+	+	+	ND	ND	+	−	+	+	+
	6	r445	+	+	+	ND	ND	+	−	−	−	−
	7	r10-25	+	+	+	ND	ND	+	−	+	+	+
	12	r76	−	−	+	ND	ND	−	−	−	−	−
	14	r47	−	−	−	ND	ND	−	−	−	−	−

[a] ND, not clarified by the saliva-VLP binding assay.
[b] Relative optical densities (optical density at 492 nm/internal standard) greater than 0.178 were considered positive in the carbohydrate-VLP binding assay.

of NoV VLPs vary (12–15, 39). Our experiments with 16 VLPs from 5 GI genotypes and 8 GII genotypes have confirmed and extended these findings: not only the outmost sugar residues but also the type 1 and 2 core structures are important for VLP recognition.

In this study, we investigated the function of the core structures in the binding between NoV and H/A/B antigens. The GI/1, GI/2, GI/3, GI/4, GI/8, GII/3, GII/4, GII/6, and GII/7 VLPs were able to distinguish between type 1 and type 2 carbohydrates (Fig. 4 and 5). Moreover, the type 1 carbohydrates bound more tightly to NoV VLPs than did the type 2 carbohydrates, as indicated in the following results: (i) the dissociation of GII/4 VLPs from B type 1 pentasaccharides was slower than that from B type 2 pentasaccharides in the Biacore experiments (Fig. 5), and (ii) the binding of GII/4 VLPs to B type 1 pentasaccharides was stronger than that to B type 2 pentasaccharides in the ELISA experiments (Fig. 6). Avian and equine influenza viruses are known to preferentially bind to the terminal sialic acid α2-3Gal (SAα2-3Gal) linkage, while human influenza viruses preferentially bind to the SAα2-6Gal linkage (6, 35, 36), affording a major impact on the host specificity in the infection of influenza viruses. A similar relationship may exist between NoV carbohydrate recognition and its tissue specificity, because the binding of rNV/68 to the gastroduodenal junction has been reported to correlate with the presence of the H type 1 antigen but not that of the H type 2 antigen (26).

The importance of the terminal α1,2-fucosyl residue in the binding between NoV and H/A/B antigens has been analyzed in detail with the GI/1 NV/68 and GII/4 VA387 strains (3, 12, 26). In this study, we confirmed these findings with GI/1 and GII/4 strains. Moreover, we revealed that other genotypes which bind to H, A, and/or B antigen also require the α1,2-fucosyl residue for the binding. Although the GI/1, GI/2, GI/3, GII/3, GII/4, GII/6, and GII/7 VLPs bound to H type 1, type 2, and/or type 3 carbohydrates (Fig. 4A to C, H to J, and L to N), none of these VLPs bound to type 1, type 2, and type 3 carbohydrates (Fig. 4A to C, H to J, and L to N), suggesting that the terminal α1,2-fucosyl residue on those H trisaccharides may be the determinant responsible for the binding between NoV and the H antigen. Moreover, as shown in Fig. 4A to E, J to L, and N, GI/1, GI/2, GI/3, GI/4, GI/8, GII/4, GII/5, GII/6, and GII/7 VLPs, which bound to A and/or B trisaccharides, did not bind to A and B disaccharides, with the exception of GI/2 VLPs. Therefore, besides the GI/2 strain, the α1,2-fucosyl residue is the determinant for NoV binding not only to H antigens but also to A and B antigens. We had predicted that NoV would require additional terminal sugars, such as α1,2-fucose, for HBGA recognition. Unexpectedly, however, we found that GI/2 r258 recognized synthetic A disaccharide, which does not include fucose (Fig. 4B). Therefore, both core structures and additional terminal sugars may contribute to the virus-carbohydrate interaction.

In a previous work, the strain specificities of NoV-HBGA binding were reported (15). Those authors concluded that NoV-HBGA binding patterns could be classified into two groups, an A/B-binding group and a Lewis-binding group, and that there was no correlation between the binding patterns and the genogroup. Our results were consistent with their results when GI/1, GI/2, GII/1, GII/4, and GII/5 VLPs were used. However, GI/3 and GII/3 VLPs gave different results. This may be due to the difference in the amino acid residues at the putative carbohydrate-binding sites. The amino acid residues 267N, 291R, 292G, 293D, 300N, 322D, 327D, 328W, 329H, 331N, 333T, 334Q, 335F, 339S, 341T, 364I, 368N, 373L, 374S, 375W, 377S, and 430A (NV/68 numbering) on the P2 domain were predicted to be important for HBGA binding (3, 4, 42). There were no differences in residues between our GI/1, GI/2, GII/1, GII/4, and GII/5 strains and their corresponding strains, whereas some different residues were found in the GI/3 and

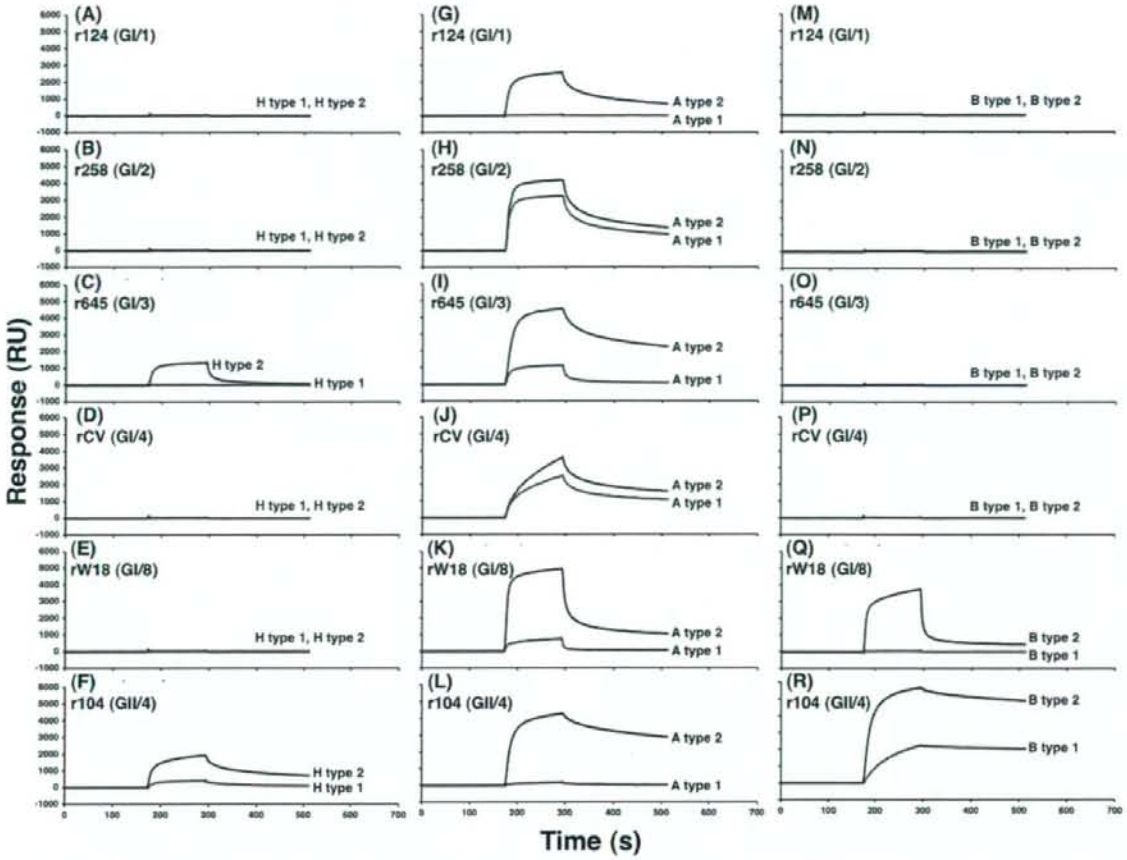


FIG. 5. Interaction between NoV and synthetic histo-blood group carbohydrates. The monovalent carbohydrate-biotin reagents were captured on a streptavidin-coated sensor chip as described in Materials and Methods. Sensorgrams show the binding of the VLPs to immobilized carbohydrates, H type 1 and type 2 (A to F), A type 1 and type 2 (G to L), or B type 1 and type 2 (M to R). At 180 s, 40 μl of the VLP was injected at a flow rate of 20 μl/min and was replaced by the running buffer at 300 s. The binding curves of 180 to 300 s showed the association, whereas those of 300 to 500 s showed the dissociation. The binding curves between VLPs and two different carbohydrates were compared by overlaying the sensorgrams obtained on each surface. The experiments were reproduced at least twice. The *y* axis indicates the resonance signal as shown in resonance units (RU).

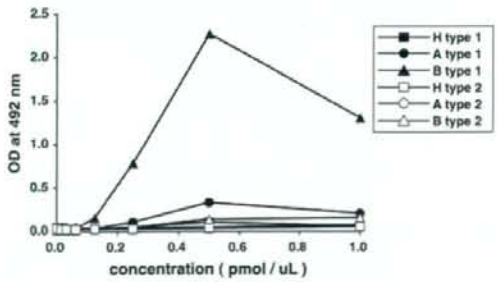


FIG. 6. Dose-dependent binding of GII/4 r104 to the monovalent carbohydrate-biotin reagents. Serially twofold-diluted carbohydrate-biotin reagents, at 1.0 to 0.016 pmol/μl, were used to coat streptavidin-precoated plates. Tris-buffered saline was used for the blank. The binding of the VLPs was detected by polyclonal rabbit anti-VLPs as described in Materials and Methods. The optical densities at 492 nm are plotted against the dilutions.

GII/3 strains. The finding that a single amino acid change in the P domain resulted in a change in the pattern of HBGA binding (42) could explain the inconsistency of the results.

Differences in the reactivities between saliva samples and synthetic carbohydrates may be due to structural differences between the synthetic products and authentic antigens, which are thought to be present on mucin or mucin-like molecules (15, 39). In our experiment, the sensitivity of the saliva-VLP binding assay was better than that of the carbohydrate-VLP binding assay. On the other hand, the carbohydrate-VLP binding assay demonstrated H type 3, Le^a, and Le^b antigen recognition by NoV, which could not be detected with the saliva-VLP binding assay. This is why we have performed the binding assay with two ELISA methods.

In Biacore assays, we used 11 GII strains; however, only one strain (GII/4 genotype) revealed binding (Fig. 5). The remaining 10 GII strains did not bind to either A or B pentasaccharides (Fig.

5). GII/4 VLPs may recognize complicated carbohydrate structures as the authentic antigens and pentasaccharides, as shown in Fig. 3 and 5, whereas the remaining 10 GII viruses may only recognize simple structures, as shown in Fig. 4.

In conclusion, it is obvious that HBGAs are important factors in determining tissue specificity, although it is still unclear whether the HBGAs function as the primary receptor or enhance NoV infectivity and/or attachment to a common cellular receptor. GII/4 is known to be a global epidemic genotype (25, 29, 30) and to bind more HBGAs than other strains (15) (confirmed in this study). This characteristic may be linked with the worldwide transmission of GII/4 strains. Lewis HBGAs are also carbohydrate antigens expressed in the epithelial cells of gastrointestinal tracts (2, 33, 37). Interestingly, some strains of GI and GII bound to Le^a expressed by nonsecretors. This means that NoV can infect individuals regardless of secretor phenotype. We are going to synthesize various Lewis-type glycans to further characterize NoV-Lewis HBGA binding abilities. In this study, the linkages and carbohydrate core structures appeared to be important in NoV-carbohydrate interaction. Since NoV forms many antigenically diverse groups, identification of the common NoV binding epitopes on host cells, if any, should be useful for the development of possible antiviral agents.

ACKNOWLEDGMENTS

We thank Katsuo Natori, Tsutomu Kageyama (National Institute of Infectious Diseases, Tokyo, Japan), and Kunio Kamata (Denka-Seiken Co., Niigata, Japan) for their valuable assistance.

This work was supported in part by a grant for Research on Food Safety from the Ministry of Health, Labor and Welfare of Japan and by the R&D Project of the Industrial Science and Technology Frontier Program, supported by the New Energy and Industrial Technology Development Organization.

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Microbiol Immunol 2009; 53: 53–57
doi:10.1111/j.1348-0421.2008.00087.x

MINI-REVIEW

Non-human primate surrogate model of hepatitis C virus infection

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ABSTRACT

More than 170 million people worldwide are chronically infected by HCV, which is the causative agent of chronic hepatitis C, cirrhosis, and finally liver cancer. Although animal models of viral hepatitis are a prerequisite for the evaluation of antiviral and vaccine efficacy, the restricted host range of HCV has hampered the development of a suitable small animal model of HCV infection. Use of the chimpanzee, the only animal known to be susceptible to HCV infection, is limited by ethical and financial restrictions. In this regard GBV-B, being closely related to HCV, appears to be a promising non-human surrogate model for the study of HCV infection. This review describes the characteristic of GBV-B infection of New World monkeys, and discusses current issues concerning the GBV-B model and its future directions.

Key words GBV-B, HCV, hepatitis C, monkey.

INTRODUCTION

Since HCV was identified as a major causative agent for non-A, non-B hepatitis in 1989 by Choo *et al.* (1), it has become evident that HCV is disseminated worldwide and is carried by an estimated more than 170 million people (2). In most advanced nations, the prevalence of HCV infection is roughly 1–2% and further dissemination is suppressed. By contrast, among developing countries the number of HCV-infected patients is still increasing due to iatrogenic exposure, including blood transfusion from unscreened donors and reuse or inappropriate sterilization of contaminated medical equipment, and injecting drug use (3). After HCV exposure, about 70% of individuals who exhibit acute infection progress to chronic liver disease, and many of these patients develop hepatic cirrhosis and hepatocellular carcinoma (2). Currently, the only treatment available for patients with chronic HCV infections is combination therapy with pegylated interferon and ribavirin. As the standard therapy is effective in only approximately 50% of patients with chronic HCV hepatitis, the other half of affected patients are still threatened by poor prognosis (4). It is therefore urgent to develop more effective therapeutics for HCV infection. At the same time, prophylactic vaccines are indispensable for prevention of further spread of HCV in developing countries, including reduction of the risk to health care workers of occupational transmission.

ANIMAL MODELS OF HCV INFECTION: RODENTS AND CHIMPANZEES

Research in infectious diseases will never progress without animal models. Because conventional small animals are not susceptible to HCV infection due to its limited host range, development of an effective prophylactic vaccine, as well as unveiling of the molecular mechanism of viral pathogenesis, has been hampered. Nonetheless, decades

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Received 11 August 2008; revised 12 September 2008; accepted 17 September 2008

List of Abbreviations: ALT, alanine aminotransferase; CTL, cytotoxic T lymphocytes; GBV-B, GB virus-B; GE, genome equivalents; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; SIV, simian immunodeficiency viruses; SIVmac, SIV derived from rhesus macaques.

of struggle have resulted in a number of animal models for HCV infection and hepatitis C.

Recently, a number of rodent models have been developed (for a review of this topic see reference 5). Rodent models, which permit HCV replication and involve transplantation of human hepatocytes, include immunotorelated fetal rat (transplantation of human hepatoma cell line Huh7 into newborn rats after prior injection of the same cells into pregnant females) (6) and immunodeficient mice such as trimera mice (7, 8) and uPA mice (9–11). These rodent models are highly useful for evaluating the efficacy of antiviral drugs and neutralizing monoclonal antibodies. In addition, a number of HCV transgenic mice have been developed. These enable direct characterization of the effects of expression of HCV genes on liver injury (5). These small animal models do not require costly facility for primates as mentioned later. While having a number of merits as mentioned above, these rodent models still have some limitations. For example the former models are not suitable for investigation of the pathogenesis of hepatitis C and the development of effective vaccine strategies, while in the latter models the proteins of interest are usually over-expressed as compared with natural HCV infection, and the integration site of the transgene may have an influence on the outcome of the study.

The chimpanzee model is the most straightforward since this animal can be experimentally infected with HCV. One third of HCV-inoculated chimpanzees develop chronic infection, while infection resolves in the remainder after an acute phase lasting 2–3 months, indicating that the chronicity rate in chimpanzees is somewhat lower than in humans (12). The chimpanzee model has been considered the primary choice for studying the relationship between the virus and host anti-viral immune responses, as well as for evaluating immunopathogenesis and the efficacy of prophylactic vaccination. However, irrespective of its benefits, many obstacles need to be overcome in order to use this model. For example in many countries it is illegal to employ the chimpanzee as an experimental animal, primarily due to ethical, (and secondly to financial), reasons. In fact, in 2004 the Dutch government decided to stop all research with chimpanzees at the biomedical primate research center in Rijswijk, Netherlands. As this was the only primate center in Europe where chimpanzees were used for biomedical research, this decision made chimpanzees unavailable as experimental animals in Europe. It is still possible to employ chimpanzees for biomedical research in some other countries, including the USA. However, the National Center for Research Resources of the National Institute of Health in the USA has recently decided not to continue to breed chimpanzees for research (13). It is estimated that the existing chimpanzees in the National Center for Research Resources will die within 30 years.

AIDS-related research has been one of the major purposes for using chimpanzees. However, due to the reasons mentioned above, as well as the endangered status of chimpanzees, nowadays AIDS scientists mainly make use of macaque monkeys infected with SIV as a non-human primate surrogate AIDS model (14, 15). The macaque/SIV model is useful since SIV is highly related to HIV-1, and induces AIDS-like diseases that are comparable to those of humans infected with HIV-1. Taking this into consideration, an alternative surrogate model which employs New World monkeys infected with GBV-B may be promising for future HCV/hepatitis C research.

GBV-B AS A NON-HUMAN PRIMATE SURROGATE MODEL OF HCV INFECTION

Among viruses so far known, GBV-B is the most closely related to HCV. However, due to a lack of epidemiological information as discussed below, GBV-B has been tentatively classified in the Hepacivirus genus of the Flavivirus family. Originally, Deinhardt *et al.* (16) found that some tamarins (genus *Saguinus*) developed hepatitis after inoculation with an inoculum obtained from a surgeon with the initials GB who had contracted hepatitis. After 11 passages in tamarins they obtained serum including GB agent(s), and were then able to achieve molecular cloning of GBV-A and GBV-B as flavivirus-like genomes (17) and to demonstrate GBV-B as an agent which could cause hepatitis in tamarins (18). Although it was unclear whether GBV-B originated from the GB inoculum or the tamarins themselves, later animal studies demonstrated that GBV-B is infectious for tamarins but not chimpanzees (19) and reciprocally that HCV is infectious for chimpanzees but not tamarins (20). These findings led to the retrospective conclusion that at least one of the tamarins employed for the *in vivo* passage study was persistently infected with GBV-B, and therefore GBV-B is probably a virus that originated in tamarins (20). However, GBV-B has not so far been isolated from additional tamarins, probably due to limited epidemiological analyses. Thus the natural host(s) and prevalence of GBV-B are yet to be determined.

CHARACTERISTICS OF GBV-B INFECTION OF NEW WORLD MONKEYS

Previous data have shown that a number of New World monkeys (parvorder *Platyrrhini*) including tamarins, the common marmoset (*Callithrix jacchus*) and the owl monkey (*Aotus trivirgatus*) are susceptible to GBV-B infection, as summarized in Table 1, although in tamarins peak concentrations of viruses in plasma are higher (10^7–10^{10} GE/ml) than in other monkeys (10^5–10^8 GE/ml) (21–29). In general, in any monkey species viremia

Table 1 Summary of characteristics of acute GBV-B infection in monkeys

Monkeys permissive of experimental infection	Tamarins (Genus *Saguinus*) Common marmoset (*Callithrix jacchus*) Owl monkey (*Aotus trivirgatus*)
Appearance of viremia	1–2 weeks post infection
Peak levels of viremia	Tamarins; 10^7–10^{10} GE/ml Marmoset and owl monkey; 10^5–10^8 GE/ml
Peak ALT levels	Approximately 200–500 IU/ml
Duration of viremia	2–3 months
Timing of seroconversion	A couple of weeks before clearance of viremia

persists for 2–3 months and is followed by clearance. GBV-B-infected monkeys with viremia usually develop self-resolving subacute hepatitis, as indicated by increases in the concentrations of serum enzymes such as ALT, gamma-glutamyltranspeptidase, and isocitrate dehydrogenase. Pathologically, degeneration and apoptosis of hepatocytes, as well as disruption and dilation of sinusoids, have been observed in the livers of GBV-B-infected tamarins with higher viremia and ALT activity (29). It is possible that GBV-B-specific CTL may cause the liver damage. However, a recent study reported that CTL are induced at a late stage of subacute GBV-B infection, and are inversely correlated with reduction in viremia (30). Since liver damage is usually found very early (1–2 weeks) after infection, when specific CTL are not observed, it is likely that viral replication in the hepatocytes leads directly to the early onset of cytopathic effects, while lower numbers of CTL may also contribute to cytotoxicity.

The clearance of viremia in the acute phase of GBV-B infection should require an effective antiviral immune response. In particular, in both GBV-B and HCV intrahepatic CTL appear to play a major role in viral clearance (30, 31). In addition, secondary GBV-B infection after clearance of the primary viremia induces a strong T cell response, leading to virtual absence of viremia, indicating that efficient memory is a key to protection from chronic viral infection (30, 32). In pre-immune chimpanzees antibody-mediated depletion of either CD4 or CD8 T lymphocytes affects their ability to control viral replication, resulting in prolonged viremia, demonstrating essential roles for both CD4 and CD8 memory in protection from viral persistence (33, 34).

On the other hand, the significance of humoral immunity in controlling GBV-B replication is still unclear. It is reasonable to assume that neutralizing antibodies also play important roles in the clearance of subacute viremia and protection from viral persistence. In the case of HCV, in one well characterized single-source outbreak of hepatitis C, viral clearance was associated with rapid induction of neutralizing antibodies in the early phase of infection, while chronic HCV infection was characterized by absent or low-titer neutralizing antibodies in this phase. Patients with resolution of infection were shown to exhibit broader cross-neutralizing activity of antibodies in the early phase of infection (35). In one chronic HCV patient who was followed up for 30 years, it has also been shown that HCV continuously escaped the host's immune system by repeated mutational changes, resulting in loss of recognition of the HCV envelope glycoproteins by antibodies (36). The fact that the sequences of envelope glycoprotein and specificity of neutralizing antibody change over time suggests that neutralizing antibodies exert selective pressure on HCV evolution. Thus, although neutralizing antibodies (and/or CTL) are not necessarily capable of controlling chronic viral infection, frequent escape from the antibodies needs so called fitness cost, resulting in the partial suppression of viral loads. Indeed, HCV-infected patients with primary antibody deficiencies have accelerated rates of disease progression (37).

Although features of the subacute phase of GBV-B infection are similar to that of HCV, a major defect of GBV-B infection as a surrogate model for HCV is that it is difficult to chronically infect monkeys. While as many as 70% of humans with HCV infection become chronically infected, only approximately a third of chimpanzees do so (2, 12). By contrast, only a few cases regarding chronic GBV-B infection have been reported so far. The best example was a case of a tamarin persistently infected with GBV-B (24); the monkey exhibited acute mild hepatitis with viremia (peak level; $\sim 10^9$ GE/ml), which reduced to a set point level (less than 10^4 GE/ml) at 16 weeks post infection, followed by a gradual increase in viremia which reached $>10^7$ GE/ml at 112 weeks post infection, along with a significant ALT increase. However, the viremia suddenly declined thereafter and became undetectable, in association with a reduction in antibody titer, and subsequent *in vivo* passage of virus obtained from the tamarin failed to reproduce persistent infection in other tamarins (24). In addition, immunosuppression of a GBV-B-infected tamarin by FK506 treatment, or infection of GBV-B with deletion of poly(U) tract in the 3′ UTR, reportedly resulted in relatively long-term persistent infection of GBV-B for up to 46 and 90 weeks, respectively (23, 27). These results indicate that GBV-B may have the potential for establishing chronic infection.

Furthermore, our recent study has demonstrated that among four common marmosets infected with GBV-B derived from a molecular clone pGBB (21), two developed long-term chronic infection for up to three years, with recurrent viremia in which plasma viral RNA levels fluctuated between undetectable and 10^5 GE/ml, which is equivalent to the case of chimpanzees chronically infected with

HCV (Iwasaki *et al.*, manuscript in preparation). Notably, the induction of antiviral antibody response as measured by anti-Core and -NS3 antibodies was delayed in both cases, followed by a gradual increase, and then sustained high antibody titers. This was in contrast with an abrupt and transient increase at the end of periods of subacute viremia in marmosets and tamarins with viral clearance. Whether a delayed antibody response is associated with persistent GBV-B infection remains to be determined.

Taken together, these findings indicate the similarity between HCV and GBV-B in regard to their ability to induce chronic infection, and also shed light on the further potential of GBV-B as a surrogate model for HCV.

FUTURE PROSPECT OF GBV-B SURROGATE MODEL

Although many questions are still to be addressed, accumulating evidence from extensive studies to date has greatly advanced the usefulness of the GBV-B as a surrogate model for HCV. The GBV-B model may be applicable for evaluating the feasibility and safety of anti-HCV vaccines employing novel viral vectors and gene therapy which creates RNA interference. For example, in a recent pilot study we showed that systemic administration of cationic liposome-encapsulated small interfering RNA to marmosets resulted in efficient regulation of GBV-B replication, indicating the usefulness of the surrogate model for proving the feasibility of RNA interference technology for future clinical application (38). This GBV-B model will also be helpful in identifying the virological and immunological factors which determine whether the outcome is acute resolving or chronic infection. While the GBV-B model appears to be valuable, development of an HCV/GBV-B chimeric virus would greatly expand the utility of the surrogate model, since it would enable us to directly evaluate antiviral vaccines and chemicals for HCV as a preclinical study. Rijnbrand *et al.* have reported that a chimeric GBV-B with 5′ untranslated region from HCV is infectious and causes hepatitis in tamarins (39). As recently demonstrated by Chevalier *et al.* (40), this will be a good model for evaluating the potential of small interfering RNA specific to HCV genome for future clinical application.

In regard to this, we may refer to an elegant precedent in the case of the macaques AIDS model. SIVmac is well known to efficiently infect, and result in the development of AIDS in macaques. Furthermore HIV-1, of which only 7% of the entire genome is derived from SIVmac, has been demonstrated to overcome the host range of authentic HIV-1, and to acquire the ability to productively infect macaque cells (41, 42). Instead of endangered chimpanzees, tamarins/marmosets which can be chronically infected with an HCV/GBV-B chimera (hopefully capable of inducing chronic hepatitis) should be the next generation of a promising non-human primate surrogate model for HCV infection, one which is similar to the macaques AIDS model. Whatever animals are used for pre-clinical study, it is important to keep in mind that results obtained from monkey models using either GBV-B or HCV/GBV-B chimera (as well as SIV or HIV/SIV chimera) may not necessarily be applicable to humans, because of potential differences in the molecular structure and/or mechanism by which antivirals and/or viral and host proteins function. Further characterization and understanding of the molecular biology and immunology of virus-host interactions will help in developing novel antiviral strategies.

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Cancer Immunol Immunother
DOI 10.1007/s00262-008-0652-9

REVIEW

The extrinsic RNA-sensing pathway for adjuvant immunotherapy of cancer

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Received: 6 December 2008 / Accepted: 30 December 2008

Abstract Infection with RNA viruses presents a typical pattern of virus products, double-stranded RNA (dsRNA), and induces the maturation of antigen-presenting dendritic cell (mDC). There are several dsRNA sensors that are diVerentially distributed on the cell membrane and in the cytoplasm and are variably expressed depending on the cell type. Among these sensors, TLR3 links to the adaptor TICAM-1 (TRIF), which is characterized by its unique multipronged signaling cascades for cytokine/chemokine production, apoptosis and autophagy in both immune and tumor cells. In the context of mDC maturation, various cellular events are further induced in response to dsRNA; these include cross-priming followed by CD8+ CTL induction, NK activation and proliferation of CD4+ T cells including Th1, Th2, Treg and Th17 cells. In this review, we focus on the potential role of dsRNA in modulating the inXammatory milieu around mDCs and tumor-associated antigens to drive speciWc cellular eVectors against the tumor.

Keywords Immunotherapy for cancer · RNA adjuvant · Toll-like receptor · TICAM-1 (TRIF) · Dendritic cells · Cellular eVectors

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Published online: 31 January 2009

Introduction

Tumor progression often occurs during inXammation because cell growth is an event that is closely connected to both extrinsic and intrinsic inXammatory stimulation [1]. Many biological mediators such as cytokines and chemokines are involved in immune cell recruitment, which accelerates tumor development in an inXammatory milieu [2]. Immune-related cells are incorporated into the tumor matrix and evoke complicated immune responses against the tumor through cell–cell interactions. Ultimately, the antigen (Ag)-presenting cells (APC) mature as a result of the inXammatory stimuli and tumor-associated antigens (TAAs) and Xow out to the regional lymph nodes where TAAs are presented to lymphocytes [3]. However, tumor remission does not occur frequently despite TAA presentation by APC [3, 4]. In contrast, most other infections facilitate myeloid dendritic cell (mDC) maturation [5] and provoke a robust immune response that contributes to pathogen eradication. If PRRs fail to be activated due to the lack of appropriate microbial patterns in APC of cancer patients even in the presence of TAAs, no eVectors are generated for tumor targeting, thereby neither immune edition nor surveillance occurring against tumor.

Double-stranded (ds) RNA is a product of virus replication. A variety of RNA and DNA viruses generate replication-mediated dsRNA, polyU/UC or stem-loop structures [6], which serve as ligands for pattern-recognition receptors (PRRs). TLR3 [7], TLR22 [8], RIG-I/MDA5 [9], PKR [10], NALP3 [11, 12] and Dicer in the RNAi system [13] along with as yet unidentiWed receptors are believed to serve as PRRs for dsRNA sensing (Fig. 1a). These PRRs induce intracellular signaling cascades that regulate cell growth, diVerentiation, apoptosis and immune activation [6, 14]. Ultimately, dsRNA and its synthetic analog polyI:C

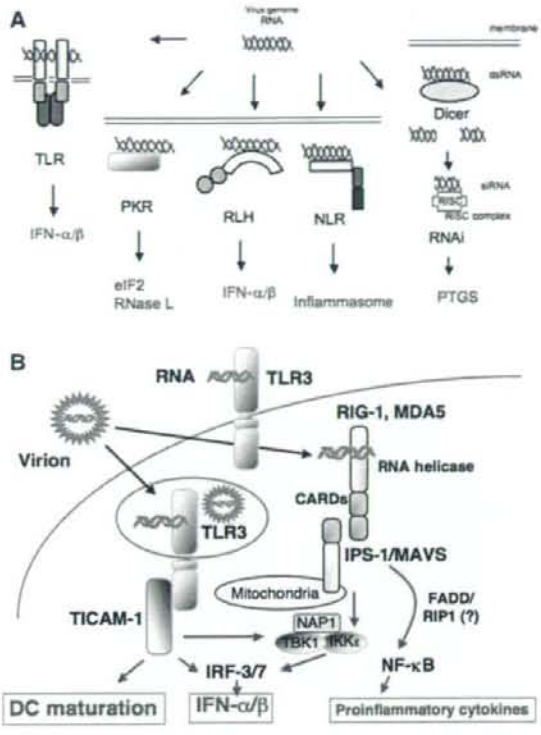

Fig. 1 dsRNA-sensing systems. **a** Double-stranded (ds)RNA are primarily generated during virus replication. Major dsRNA sensors in human cells are indicated. Dicer and RNA-recognizing helicases work in invertebrates as antiviral receptors, but in humans no evidence of these receptors for antiviral action has been proposed. How dsRNA selects a variety of RNA pattern sensors remains largely unknown. PTGS, post-transcriptional gene silencing. **b** TLR3 is mainly localized in the endosome of limited cell types, while RLH (RIG-I and MDA5) are ubiquitously distributed in the cytoplasm. Adaptor molecules, TICAM-1 and MAVS, are localized in the cytoplasm. Upon stimulation, TLR3 recruits TICAM-1 near the endosomal membrane, while MAVS recruits RLH on the mitochondrial membrane. The known outputs of TLR3 and RLH are indicated by red

exert a wide range of biological activities and can elicit immune responses. Since dsRNA-sensing PRRs are distributed across a variety of host cells in diVerent combinations [6, 15], systemic inXammation occurs in various modes depending on the receptors and cell types involved in viral infection, virus vaccine inoculation or dsRNA administration for RNA therapy. An inXammatory environment promotes tumor growth and priming of dendritic cells. Many sterile and infectious RNAs induce inXammation.

The signaling pathways of PRRs are linked via adaptor proteins (Fig. 1b). The intra-cytoplasmic RNA sensors, RIG-I and MDA5, interact with MAVS (Cardif/IPS-1/VISA) on the outer membrane of mitochondria [16], and TLR3 resides in the endosome and interacts with TICAM-1 (TRIF) [17]. The signal selection systems of other dsRNA sensors are relatively less deWned. Typically, stimulation of the TICAM-1 and MAVS pathways induces type I interferons (IFN) [18]. This is a reXection of the fact that the signaling cascades of both pathways converge upon the complex of the virus-activated kinase (VAK), i.e., NAP1/SINTBAD-IKKe/TBK1 [18, 19] (Fig. 2a). Other cellular responses, autophagy [20], proliferation [21] and apoptosis [22], are induced in cells stimulated with dsRNA (Fig. 2a). Study of the molecular mechanism of these responses is currently underway.

In mDCs, a variety of cellular eVectors are driven in response to dsRNA. CD4 Th1, CD8 CTL, NK cells, regulatory T cells (Treg), and Th17 cells are activated/proliferated through dsRNA-stimulated mDCs [15]. Some inXammatory cytokines and chemokines, as well as IFN-inducible gene products are also up-regulated in mDCs. These eVectors appear to be independently induced in a situation-dependent manner. However, the molecular mechanisms whereby these variable eVectors are diVerentially induced by mDCs are unknown. We have determined that the TICAM-1 pathway in mDCs is involved in inducing all these eVector cell types (Fig. 2b).

In this review, we focus on the TICAM-1 pathway in which cellular eVectors are induced by mDCs. We also discuss the involvement of the TICAM-1 pathway in cancer progression and the therapeutic potential of TICAM-1 in antitumor immunotherapy.

TLR3 agonists in cancer immunotherapy

PolyI:C is a representative agonist for human and mouse TLR3 [23]. This compound is believed to be an analog of viral double-stranded RNA (dsRNA) and is a strong inducer of type I IFN in both humans and mice [24]. Initially, polyI:C was regarded as a PKR activator [25]. Later, it was determined that this compound is not only a TLR3 agonist, but also a stimulator of the cytoplasmic RNA sensor, MDA5 [26]. PolyI:C also activates RIG-I [26], but other viral RNA patterns, 5•-triphosphate [27, 28] and polyU/UC [29] may be natural ligands for RIG-I. Earlier, it was reported that polyI:C, which is capable of activating various PRRs, causes endotoxin-like cytokine stormsl; therefore, this compound was deemed to be too toxic for application in clinical therapy [30].

mDCs mature into APCs that drive cellular eVectors (Fig. 3). TLR3 resides in the endosome of mDCs [17], senses dsRNA in the endosome, and relays signals to the TICAM-1 pathway, thereby leading to maturation of mDCs [6]. Thus, endosomal stimulation of TLR3 by ligands links to activation of mDCs (Fig. 1b). Certain dsRNA derivatives preferentially activate TLR3 rather than RLH receptors