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## Norwalk Virus Binds to Histo-Blood Group Antigens Present on Gastroduodenal Epithelial Cells of Secretor Individuals

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**Background & Aims:** Norwalk Virus (NV) is a member of the *Caliciviridae* family, which causes acute epidemic gastroenteritis in humans of all ages and its cellular receptors have not yet been characterized. Another calicivirus, Rabbit Hemorrhagic Disease Virus, attaches to H type 2 histo-blood group oligosaccharide present on rabbit epithelial cells. Our aim was to test if, by analogy, recombinant NV-like particles (rNV VLPs) use carbohydrates present on human gastroduodenal epithelial cells as ligands. **Methods:** Attachment of rNV VLPs was tested on tissue sections of the gastroduodenal junction and on saliva from individuals of known ABO, Lewis, and secretor phenotypes. It was also tested on human Caco-2 cells and on animal cell lines transfected with glycosyltransferases complementary DNA (cDNA). Competition experiments were performed with synthetic oligosaccharides and anticarbohydrate antibodies. Internalization was monitored by confocal microscopy. **Results:** Attachment of rNV VLPs to surface epithelial cells of the gastroduodenal junction as well as to saliva was detected, yet only from secretor donors. It was abolished by  $\alpha$ 1,2fucosidase treatment, and by competition with the H types 1 and 3 trisaccharides or with anti-H type 1 and anti-H types 3/4 antibodies. Transfection of CHO and TS/A cells with an  $\alpha$ 1,2fucosyltransferase cDNA allowed attachment of VLPs. These transfectants as well as differentiated Caco-2 cells expressing H type 1 structures internalized the bound particles. **Conclusions:** rNV VLPs use H type 1 and/or H types 3/4 as ligands on gastroduodenal epithelial cells of secretor individuals.

The *Caliciviridae* are a family of small, positive-strand RNA viruses that comprise animal and human pathogens.<sup>1</sup> It is subdivided into 4 genera and, among them, viruses from the 2 genera termed *Norwalk-like viruses* (NLVs) and *Sapporo-like viruses* cause acute gastroenteritis in humans.<sup>2</sup> Viral gastroenteritis is a highly prevalent illness that affects people of all ages, both in developed and developing countries.<sup>3</sup> Although rotaviruses are considered the most frequent etiologic agents

worldwide, recent epidemiologic studies indicate that caliciviruses are responsible for the large majority of outbreaks in developed countries, affecting young children as well as adults.<sup>4-8</sup> They are the etiologic agents of many sporadic cases previously considered to be of unknown cause and are spread by the fecal-oral route from person to person or from contaminated water and food items.<sup>9,10</sup>

Norwalk virus (NV), which is the prototype of the human caliciviruses (HuCVs), was discovered in fecal specimens in 1972 by immune-electron microscopy as a 27-nm particle.<sup>11</sup> Since then, other HuCVs have been found and genome sequencing revealed the existence of a large family with a wide range of variation. Among NLVs, at least 2 genogroups are defined, based on sequence comparison of both structural and nonstructural genes.<sup>12,13</sup> Their genomic RNA is about 7.5-kb long and predicted to encode 3 open reading frames. Open reading frame 2 encodes the major viral capsid protein, which has a molecular weight of 58 kilodaltons.<sup>14-16</sup> Until now, HuCVs have proven refractory to growth in cell culture, greatly impairing the study of their biologic properties. However, production of the capsid protein by insect cells infected with a recombinant baculovirus results in the spontaneous generation of virus-like particles (VLPs) that are morphologically and antigenically indistinguishable from authentic virions.<sup>17</sup> Such purified VLPs allow the generation of polyclonal and monoclonal anti-NV anti-

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**Abbreviations used in this paper:** BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; Gal, galactose; HuCV, human calicivirus; mAb, monoclonal antibody; NLV, Norwalk-like virus; NV, Norwalk virus; RACE-PCR, rapid amplification cDNA end polymerase chain reaction; RHDV, Rabbit Hemorrhagic Disease Virus; rNV VLPs, recombinant Norwalk Virus-like particles; UEA-I, agglutinin I from *Ulex europaeus*.

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bodies that can be used for diagnostic assays<sup>18–20</sup> and study of virus-cell interactions.<sup>21,22</sup>

Among many cell lines tested, only differentiated Caco-2 cells allowed significant attachment of recombinant NV (rNV) VLPs.<sup>21</sup> Caco-2 cells originate from a human colorectal carcinoma and have retained the ability to spontaneously differentiate into small intestine enterocytes in culture after reaching confluency. A monoclonal anti-rNV VLP was found to inhibit the interaction between differentiated Caco-2 cells and the VLPs.<sup>21</sup> Epitope mapping of this antibody revealed that it recognizes an epitope lying on the P2 domain of the capsid protein and structural analysis of the rNV capsids indicates that this domain forms a protrusion emanating from the capsid shell.<sup>23,24</sup> These results are consistent with the idea that the P2 domain is responsible for the attachment of NV particles to Caco-2 cells. However, the ligand at the cell surface to which they bind remains unknown.

We recently observed that another member of Caliciviridae, namely Rabbit Hemorrhagic Disease Virus (RHDV), attaches to rabbit epithelial cells through specific recognition of the H type 2 histo-blood group trisaccharide.<sup>25</sup> Histo-blood group antigens are complex carbohydrates present at the outermost part of N- or O-linked glycans of many glycoproteins or glycolipids. They may also be present as free oligosaccharides in biologic fluids such as milk. Their synthesis requires several glycosyltransferases acting on precursor oligosaccharides. Four main types of precursors are usually recognized that have in common a terminal galactose in  $\beta$  linkage to either an N-acetylglucosamine (type 1: galactose (Gal) $\beta$  N-acetylglucosamine  $\beta$ -R, and type 2 Gal $\beta$ 1–4 N-acetylglucosamine  $\beta$ -R) or an N-acetylgalactosamine (type 3: Gal $\beta$ 1–3 N-acetylgalactosamine  $\alpha$ -R, and type 4: Gal $\beta$ 1–3 N-acetylgalactosamine  $\beta$ -R). These precursors are converted into H antigenic structures by fucosylation in  $\alpha$ 1,2 linkage, a step catalyzed by an  $\alpha$ 1,2 fucosyltransferase for which 2 genes have been cloned and designated *FUT1* and *FUT2* in humans. The corresponding enzymes have previously been termed *H type* and *secretor type*, respectively. The H oligosaccharides can thereafter be substituted on the galactose by an N-acetylgalactosamine or a galactose in  $\alpha$ 1,3 linkage to give the A and B antigens, respectively, this step being catalyzed by the enzyme products of the A and B alleles at the *ABO* locus. Inactivating mutations of the *FUT1* and *FUT2* genes are known. *FUT1* inactivating mutations are very rare and responsible for the Bombay phenotype characterized by the lack of ABH antigens on erythrocytes and vascular endothelium. In contrast,

*FUT2* inactivating mutations are frequent and responsible for the nonsecretor phenotype found in about 20% of the European and North American populations and characterized by the absence of ABH antigens from saliva as well as from most epithelial cells of the respiratory, genitourinary, and digestive tracts. Additionally, a fucose residue can be added in  $\alpha$ 1,3 or  $\alpha$ 1,4 linkage on the type 1 or type 2 precursors by  $\alpha$ 1,3 or  $\alpha$ 1,4 fucosyltransferases. Six human genes encoding such glycosyltransferases have been cloned. Of these, *FUT3*, which presents a genetic polymorphism, is responsible in conjunction with *FUT2* for the various so-called Lewis phenotypes.

Sequence comparison of the capsid proteins from HuCVs indicates that the highest variability is in the P2 region. Owing to its localization within the capsid, this region is likely to be much exposed to immune recognition and therefore is expected to be under strong selective pressure for variation. Nevertheless, some residues are conserved between RHDV and NV in this region. Moreover, differentiated Caco-2 cells are known to carry carbohydrates of the histo-blood group family at their surface.<sup>26</sup> Because such antigens may additionally be present on human gastroduodenal epithelial cells depending on the individuals' ABO, Lewis, and secretor phenotype,<sup>27</sup> our aim in the present work was to test if, by analogy with RHDV, rNV VLPs bind to such carbohydrates and if this binding leads to internalization of the particles.

## Materials and Methods

### Reagents

Synthetic oligosaccharides corresponding to the H type 1, H type 2, H type 3, Lewis a, Lewis b, and type 1 precursor, in the form of sugar-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>, were gracious gifts from Dr. N. Bovin (Moscow, Russia). The  $\alpha$ 1,2-specific  $\alpha$ -fucosidase II from *Xantomonas manihotis* was obtained from Glyko (Upper Heyford, UK). The BG-4 anti-H type 1 specific monoclonal antibody (mAb) was purchased from Signet Pathology Systems (Dedham, MA). mAbs 19-OLE and 3-3A were from Dr. J. Bara (Paris, France). 19-OLE is an anti-H type 2 that shows a slight cross-reactivity with Lewis Y (unpublished results), and 3-3A is an anti-A that recognizes all types of A determinants: types 1, 2, 3, 4, and difucosylated types 1 and 2.<sup>28</sup> mAb MBr1, a kind gift from Dr. M. Colnaghi (Milan, Italy), is an anti-H types 3 and 4-specific antibody. The anti-NV capsid rabbit polyclonal antiserum was obtained by immunizing a rabbit with rNV VLPs as previously described.<sup>17</sup> mAb 9C3 was 1 of 12 mAbs isolated from mice immunized with 4 strains of NLVs including NV, MxV, HV, and GrV. This mAb reacts only with NV, based on enzyme immunoassays and blocking enzyme immunoassays. It recognizes nondenatured epitopes on recombinant NV capsid antigens based on Western blot analysis.<sup>20</sup> The agglutinin I

from *Ulex europaeus* (UEA-I) lectin, which recognizes H type 2 and Lewis Y oligosaccharides, either free, fluorescein isothiocyanate (FITC), or peroxidase conjugated, was obtained from Sigma (St. Louis, MO). Recombinant NV VLPs were produced by using a baculovirus-expression system and purified by rate-zonal centrifugation in a sucrose gradient as previously described.<sup>17</sup>

### Tissue Samples and Immunohistochemical Analysis

Strips of 1 × 10 cm including gastric and duodenal mucosa were resected from 13 kidney donors 5 minutes after death. These human samples were obtained before the law 88-1138 (December 20, 1988) concerning resection of human tissues after death for scientific investigations. Phenotypes of the tissue donors were: ALe(a+b-) 1 individual, BLe(a+b-) 1 individual, OLe(a+b-) 3 individuals, ALe(a-b-) 2 individuals, OLe(a-b-) 1 individual, ALe(a-b+) 2 individuals, BLe(a-b+) 1 individual, ABL(a-b+) 1 individual, OLe(a-b+) 1 individual. The 3 Le(a-b-) donors were secretors of H in the gastroduodenal junction. Tissue samples were fixed in 95% ethanol for 48 hours, coiled into Swiss rolls, and paraffin embedded. Sections (5 μm) were rehydrated in graded ethanol and washed in phosphate-buffered saline (PBS). Endogenous peroxidase was inhibited by using methanol/H<sub>2</sub>O<sub>2</sub> 0.3% for 20 minutes. Sections were then washed in PBS for 5 minutes and covered with PBS/bovine serum albumin (BSA) 1% for 20 minutes at room temperature in a humid atmosphere. After washing in PBS, sections were covered with either the primary antibodies or rNV VLPs diluted in PBS/BSA 1% and left at 4°C overnight. Antibodies were used at 1 μg/mL and VLPs at 5 μg/mL. Sections were then rinsed 3 times with PBS and incubated with biotinylated anti-mouse immunoglobulin (Ig)G (Vector Labs, Burlingame, CA) or rabbit anti-rNV VLPs diluted 1/1000, respectively, for 60 minutes at room temperature. After washing in PBS, the sections were covered with either peroxidase-conjugated avidin (Vector Labs) or with peroxidase-conjugated anti-rabbit IgG (Sigma) for 45 minutes, washed with PBS, and reactions were revealed with 3-amino-9-ethylcarbazol. Counterstaining was performed with Mayer's hemalun. Periodate treatment was performed immediately after the endogenous peroxidase quenching step by incubating sections with 10 mmol/L sodium periodate in 50 mmol/L, pH 5.0, sodium acetate buffer for 30 minutes at room temperature, followed by a 10-minute incubation with 1% glycine. Fucosidase treatment was performed on some sections by incubation at 37°C with 4 mU fucosidase II in 50 mmol/L, pH 5.0, sodium phosphate buffer for a total of 18 hours with a renewal after 6 hours. Controls were performed by using fucosidase, heat-inactivated by prior boiling in a water bath for 10 minutes. Inhibition of rNV VLPs attachment with synthetic oligosaccharides was performed by preincubating the VLPs with the oligosaccharides at a final concentration of 1.8 mmol/L in 1% BSA/PBS for 1 hour at room temperature. Thereafter, the VLPs/oligosaccharide mixtures were incubated overnight at 4°C on tissue-coded

sections as described earlier. To assay the ability of anticarbohydrate mAbs to compete with rNV VLPs attachment, coded tissue sections were first incubated with mAbs at 10 μg/mL for 1 hour at room temperature. rNV VLPs were then added and incubated as described earlier. Their binding was detected by using a rabbit anti-rNV VLPs antiserum at 1/1000 in 1% BSA/PBS, followed after washings by peroxidase-labeled anti-rabbit IgG (Sigma). Intensity of staining was semiquantitatively estimated by 3 persons; 2 of them did not know the code of sections.

### Binding of rNV VLPs to Saliva Samples

Saliva samples were boiled for 10 minutes immediately after collection and centrifuged for 5 minutes at 13,000g. The clear supernatant was collected and stored frozen until use. The secretor/nonsecretor phenotypes of the donors were determined by a classic inhibition of agglutination technique. Salivas from 4 unambiguous secretors and 4 unambiguous nonsecretors were chosen for further study. Their status was confirmed by genotyping of the individuals for the *FUT2* gene polymorphism in accordance with a published method.<sup>29</sup>

Attachment of rNV VLPs to saliva was tested by enzyme-linked immunosorbent assay. For this, NUNC (Raskilde, Denmark) immunoplates were coated with saliva samples serially diluted in 100 mmol/L, pH 9.6, carbonate/bicarbonate buffer by an overnight incubation at 37°C in a wet atmosphere. After a blocking step in 3% BSA/PBS for 30 minutes at 37°C, rNV VLPs at 1 μg/mL in 1% BSA/PBS were added and incubated for 1 hour at 37°C. Binding was detected by using the rabbit anti-rNV VLPs antiserum at 1/1000 in 1% BSA, followed by alkaline phosphatase-conjugated anti-rabbit IgG (Sigma), both reagents incubated for 1 hour at 37°C. Reactions were developed by using p-nitrophenyl phosphate (Sigma) as substrate and optical densities read at 405 nm. Between each step, plates were washed 5 times with PBS containing 0.05% Tween 20. The inhibitory potency of synthetic oligosaccharides was tested by preincubating the rNV VLPs with oligosaccharides at 1.8 mmol/L for 1 hour at 37°C before addition to the coated wells.

### Cell Lines and Transfections

The rat *FTB* complementary DNA (cDNA; Genbank accession number: AF131238) was excised from the pUC18 vector (Pharmacia-Amersham, Les Ulis, France) and inserted into the eukaryotic expression vector pBK-CMV (Stratagene, La Jolla, CA) as previously described.<sup>30</sup> This last vector was deleted from the lacZ promoter by digestion with SpeI and NheI to obtain higher expression of the inserted cDNA, as recommended by the supplier. The rat A enzyme cDNA was cloned by rapid amplification cDNA end polymerase chain reaction (RACE-PCR) into the pUC18 vector from a BDIX rat stomach cDNA library. Primers were deduced from the sequence of a rat exon homologous to the human A gene exon 6. This DNA fragment had been isolated after screening a rat genomic DNA library with a probe derived from the cDNA of the human A gene. The complete coding sequence of the rat A

gene (Genbank accession number: AF264018) was inserted into the pDR2 eukaryotic expression vector (Clontech, Palo Alto, CA) deleted of the sequences lying between the EcoRV and Cla I sites.

CHO cells and TS/A cells, Chinese hamster ovary carcinoma, and mouse mammary carcinoma cell lines, respectively, are devoid of  $\alpha$ 1,2-fucosyltransferase activity and therefore of ABH antigens. They were first transfected by using Lipofectamin with the *FTB* cDNA in the pBK-cytomegalovirus expression vector (Gibco, Paisley, UK) according to the manufacturer's instructions. Twenty-four hours later, fresh medium was added, and 48 hours later, selective medium containing 0.6 mg/mL G418 (Gibco) was added. Transfected cells expressing  $\alpha$ 1,2-linked fucose residues were selected by flow cytometry using FITC-labeled UEA-I, after cloning by limiting dilutions as previously described.<sup>30</sup> For each cell line, a strongly expressing clone was selected and transfected a second time by using the same procedure with the rat A enzyme cDNA in the pDR2 vector. Forty-eight hours later, cells were cultured in selective medium containing 0.6 mg/mL hygromycin. After cloning by limiting dilutions, strongly A antigen-expressing transfectants were selected. Control transfected cells were prepared by transfection with the empty vectors. These stable transfectants were cultured in RPMI 1640, 10% fetal-calf serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin (Gibco) supplemented with 0.25 mg/mL G418 and 0.2 mg/mL hygromycin in the case of double transfectants. Medium for CHO cells culture contained in addition free nucleotides (10  $\mu$ g/mL).

Caco-2 cells were cultured in Dulbecco's modified Eagle medium supplemented with 20% fetal-calf serum, 5% minimum essential medium, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. For confocal microscopy studies, cells were seeded at a density of 10,000 cells/cm<sup>2</sup> on either Transwell filters (NUNC) containing pores of 0.4  $\mu$ m or on glass slides. Apical and basal media were replaced each day from day 3. Differentiated cells were obtained late after confluency at 21 days.

All cells were maintained at 37°C in a 5% CO<sub>2</sub> humid atmosphere, passaged after dispersal with 0.025% trypsin in 0.02% ethylenediaminetetraacetic acid, and routinely checked for mycoplasma contamination by Hoescht 33258 (Sigma) labeling.

### Flow Cytometry Analysis

The binding of NV capsids to transfected cells was tested by incubating  $2 \times 10^5$  viable cells with rNV VLPs at 10  $\mu$ g/mL in PBS containing 0.1% gelatin for 1 hour at 4°C. After 3 washes with the same buffer, a second 45-minute incubation was performed with mAb 9C3 at 1  $\mu$ g/mL. After washings, a third incubation was performed with FITC-labeled anti-mouse immunoglobulins (Sigma) under the same conditions. The presence of histo-blood group antigens at the cell surface was detected by the same method by using primary antibodies (BG-4, 19-OLE, MBr1, 3-3A) at 1  $\mu$ g/mL and FITC-labeled anti-mouse immunoglobulins as secondary re-

agent. After final washings in the same buffer, fluorescence analysis was performed on a FACScan (Becton-Dickinson, Rungis, France) by using the CELLQuest program.

### Confocal Microscopy Analysis

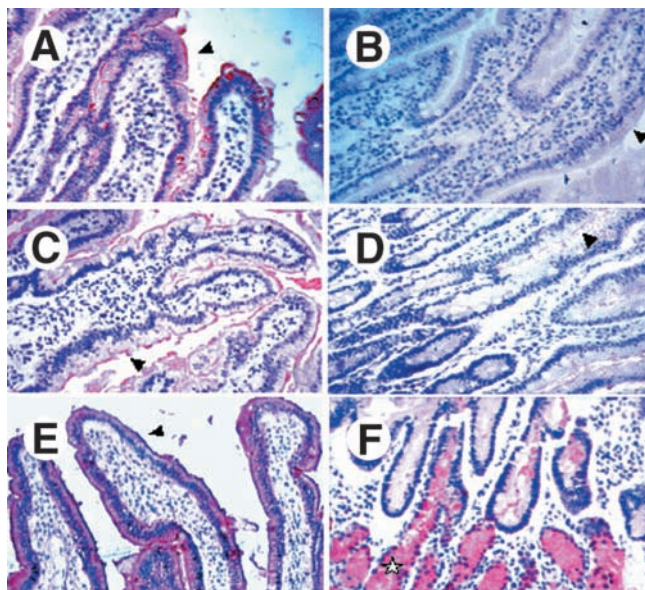
Caco-2 cells cultured on Transwell filters or transfected CHO cells cultured on glass coverslips were fixed with 2% paraformaldehyde and after washings with PBS and blocking with 1% BSA, they were incubated with rNV VLPs at 5  $\mu$ g/mL in 1% BSA/PBS for 1 hour at 4°C to monitor attachment to the cell surface. Alternatively, cells were incubated with rNV VLPs for 2 hours at 37°C to monitor internalization. At the end of the incubation period, cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% Triton X100/0.5% Tween 20. rNV VLPs were detected by incubating with mAb 9C3 diluted at 1/1000 from an ascitic fluid. After washings, preparations were incubated with biotinylated anti-mouse IgG (Vector) followed by FITC-labeled-streptavidin (Sigma) in the case of Caco-2 cells or with FITC-labeled anti-mouse IgG (Sigma) in the case of CHO cells. Propidium iodide at 2.5  $\mu$ g/mL was added for 1 minute before final washes. In some experiments, cells were treated with fucosidase II as described earlier for the treatment of tissue sections. Competition experiments were also performed by preincubating the rNV VLPs, for 1 hour at 37°C, with saliva from a secretor or a nonsecretor individual diluted 1/50 in 1% BSA/PBS. Fluorescence was observed by using a Leica TCS SP with a DM IRBE inverted microscope and a 63/1.4 objective (Rueil-Malmaison, France). Excitation was obtained with an Argon-Krypton laser, with line set at 488 nm for FITC and 568 nm for propidium iodide. A band-pass filter was used to recover FITC fluorescence and an LP 590 filter was used for propidium iodide. Both fluorochromes were excited and analyzed in 1 pass with no interference between the 2 channels. Image processing was performed with the Perfect Image software (Clara Vision, Orsay, France).

## Results

### rNV VLPs Attach to the Gastroduodenal Epithelial Cells of Secretor Individuals

The ability of rNV capsids to attach to normal human digestive cells was tested on tissue sections from the gastroduodenal junction of individuals with known ABO, secretor, and Lewis histo-blood group phenotypes. Recombinant NV VLPs bound to the apical surface of cells from the pyloric and duodenum epithelia of secretor individuals (Figure 1A), but not of nonsecretor individuals. In the duodenum, labeling was mainly located at the villi level and was weaker at the crypt level. No binding was detected in the pyloric or Brünner's glands. It was sensitive to periodate treatment (Figure 1B), suggesting involvement of a carbohydrate.

The association of the secretor status of the tissue donors with rNV VLP binding was clearly shown. Tissue



**Figure 1.** (A–D) Binding of rNV VLPs to duodenal tissue sections. Tissue sections from secretor individuals were incubated with VLPs and the binding was detected as described in the Materials and Methods section. Before incubation with VLPs, sections were treated with (B) 10 mmol/L periodate or with fucosidase II, either (C) heat inactivated or (D) not. rNV VLPs binding to the villi epithelial cell surface are indicated by arrowheads. No binding was detected on tissue sections from nonsecretors (not shown). No staining of Brunner’s glands was detectable, irrespective of the secretor status (not shown). Immunostaining of a duodenal section from a secretor individual by the (E) anti-H type 1 BG-4 and from (F) a nonsecretor individual by the anti-H type 2 19-OLE. (F) Staining of Brunner’s glands by the anti-H type 2 is shown by a star.

sections from all secretor individuals but none of 5 nonsecretors were stained (Table 1). No significant relationship could be found between the VLP attachment and the ABO or Lewis phenotypes. Because the secretor phenotype requires the presence of a fucose residue in  $\alpha 1,2$  linkage on glycoconjugates, tissue sections from secretors were treated with a fucosidase that specifically cleaves  $\alpha 1,2$ -linked fucose residues. As shown on Figure 1C and D, this treatment abolished the binding of rNV

**Table 1.** Distribution of the ABO, Lewis, and Secretor Phenotypes Among the Tissue Donors Whose Gastrointestinal Cells Showed Either Binding or No Binding of rNV VLPs

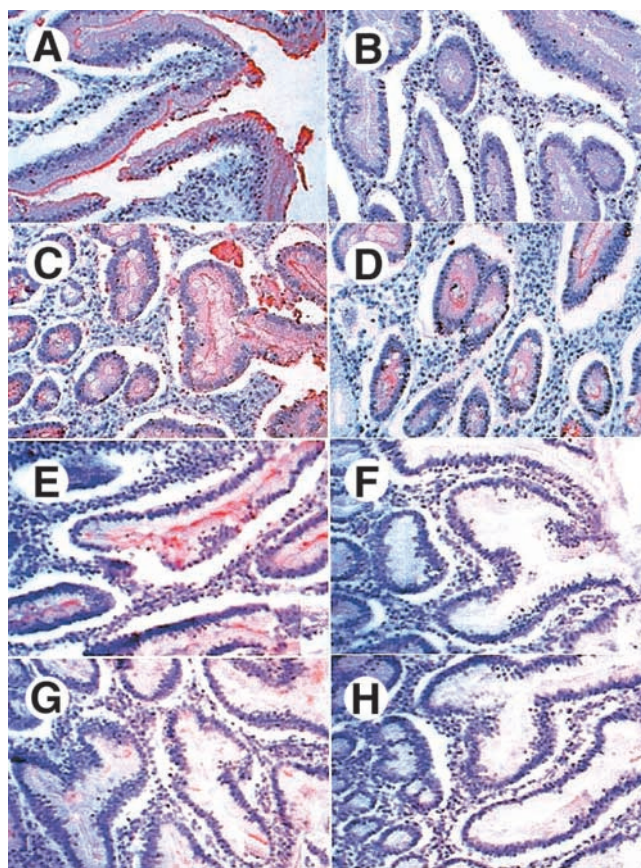
	ABO				Lewis		Secretor <sup>a</sup>	
	A	B	AB	O	Le+	Le-	sec	nonsec
Total number of cases	5	2	1	5	10	3	8	5
Cases with VLP binding	4	1	1	2	5	3	8	0
Cases with non-VLP binding	1	1	0	3	5	0	0	5

<sup>a</sup> Comparison of the secretor (sec) and nonsecretor (nonsec) groups by Fisher exact test:  $P = 0.0008$ .

VLPs, confirming requirement of a fucose residue in  $\alpha 1,2$  linkage.

### rNV VLPs Recognize the H Types 1 and 3 Antigens

Earlier studies have shown that the expression of ABH and Lewis antigens in the pyloric and duodenal surface epithelia is dependent on the secretor status, whereas that in the pyloric or Brunner’s glands is not. In addition, histo-blood group antigens based on type 1 precursor, such as Lewis a or Lewis b, are found exclusively at the level of the surface epithelia, whereas antigens based on type 2 precursor, such as Lewis Y and H type 2, are preferentially found at the glandular level.<sup>27</sup> By using a specific mAb, we now show that H type 1 epitopes are present at the level of the surface epithelia from secretor individuals (Figure 1E) irrespective of the

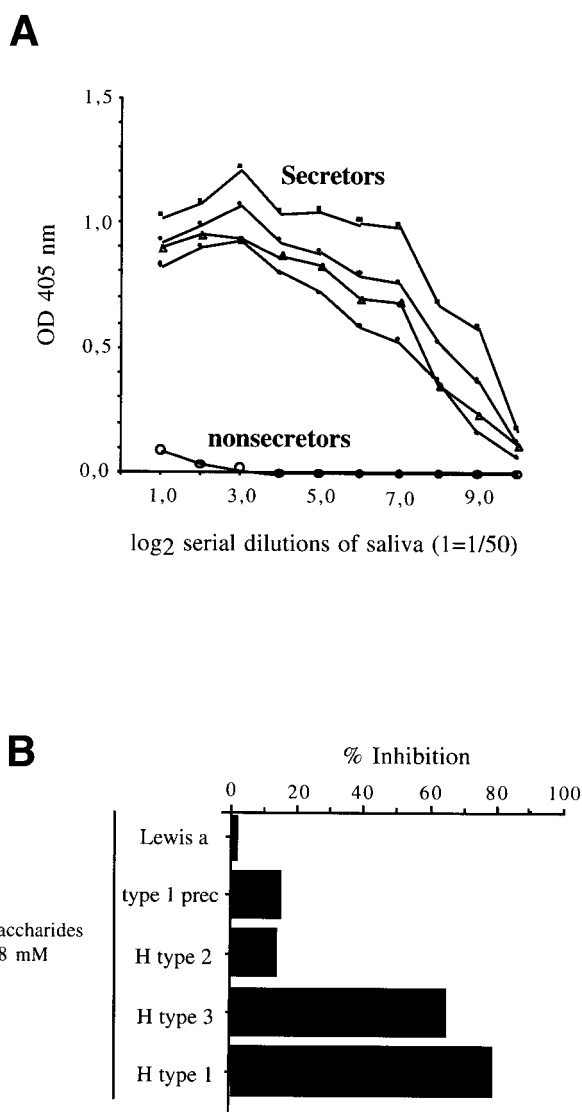


**Figure 2.** Inhibition of rNV VLPs binding to duodenal tissue sections from secretor individuals by (A–D) anti-H mAbs or by (E–H) synthetic oligosaccharides. Sections were first incubated with the anti-H mAbs and then with VLPs. Binding of the VLPs was detected as described in the Materials and Methods section. (A) No preincubated antibody, (B) preincubated anti-H type 1 BG-4, (C) preincubated anti-H type 2 19-OLE, and (D) preincubated anti-H types 3 and 4 MBr1. Inhibitions with oligosaccharides were performed by preincubating the VLPs with the following trisaccharides: (E) Lewis a, (F) H type 1, (G) H type 2, and (H) H type 3.

ABO and Lewis status and undetectable on digestive epithelial cells from nonsecretors (not shown). In contrast, H type 2 epitopes were readily detected both on the surface epithelia and the glands from secretors as well as on the glands from nonsecretors (Figure 1F). The expression of A and H types 3 and 4 epitopes in the normal gut has previously been reported.<sup>31,32</sup> They could not be detected at the cell surface, but were evidenced at the Golgi level. This was confirmed in the present study (data not shown). Thus, the binding of rNV VLPs to the gastroduodenal junction correlates with the presence of the H type 1 antigen and not with that of H type 2 or H types 3 and 4 antigens.

To confirm that rNV VLP attachment was mediated by recognition of a histo-blood group H-related structure and to define which one, competition experiments were performed with either type-specific anti-H mAbs or free oligosaccharides (Figure 2). Near-complete inhibition of rNV VLPs binding was obtained with the anti-H type 1 mAb as well as with the H type 1 trisaccharide (Figure 2B and F). Both the anti-H types 3 and 4 mAb and the H type 3 trisaccharide gave a partial inhibition (Figure 2D and H). Some degree of inhibition was also obtained with the anti-H type 2 and H type 2 trisaccharide (Figure 2C and G). In contrast, the Lewis a and Lewis b oligosaccharides did not inhibit (Figure 2E and data not shown). The H type 4 trisaccharide was not tested in these inhibition experiments. However, it is unlikely that rNV VLPs attach to the gut epithelial cells via H type 4 because the glycolipids carrying this antigenic determinant were not represented among glycolipids of the small intestine.<sup>33</sup>

ABH and Lewis antigens are present in saliva depending on the secretor status of the individuals. To get a more quantitative estimate of the qualitative results obtained on tissue sections, saliva samples from individuals phenotyped and genotyped as either secretors or nonsecretors were coated onto enzyme-linked immunosorbent assay plates and the ability of rNV VLPs to bind was assessed. In agreement with the histochemistry experiments, strong binding occurred on saliva from 4 of 4 secretors but not from 4 of 4 nonsecretors (Figure 3A). In addition, this binding was clearly inhibited by the H type 1 and H type 3 trisaccharides, whereas the closely related H type 2, type 1 precursor, or Lewis a oligosaccharides showed little or no inhibition (Figure 3B). These inhibitions required concentrations of free oligosaccharides about 4 orders of magnitude higher than the concentration of capsid protein. In addition, a preincubation step between the oligosaccharides and the rNV VLPs was required to obtain inhibition of the rNV VLPs



**Figure 3.** (A) Binding of rNV VLPs to serially diluted saliva samples from either secretor ( $n = 4$ ) or nonsecretor individuals ( $n = 4$ ) coated to an enzyme-linked immunosorbent assay plate. Three of the secretors were homozygotes, and 1 was heterozygote. Binding was detected by using the polyclonal rabbit anti-NV VLPs as described in the Materials and Methods section. The optical densities at 405 nm are plotted against the  $\log_2$  of reciprocal dilutions of saliva. (B) Inhibition of rNV VLPs binding to saliva from a secretor individual by synthetic oligosaccharides. VLPs were preincubated with oligosaccharides at a final concentration of 1.8 mmol/L before incubation on saliva coated at a dilution of 1/4000.

attachment to saliva. These observations suggest that the affinity of the free H type 1 or H type 3 trisaccharides for the recombinant capsid protein is quite low.

To determine if the presence of fucose residues in  $\alpha 1,2$  linkage on cellular glycoconjugates was sufficient to mediate rNV VLPs attachment, histo-blood group H determinants were expressed on 2 different cell lines by transfection of the rat *FTB*  $\alpha 1,2$ fucosyltransferase cDNA. *FTB* is the rat homologue of human *FUT2* and

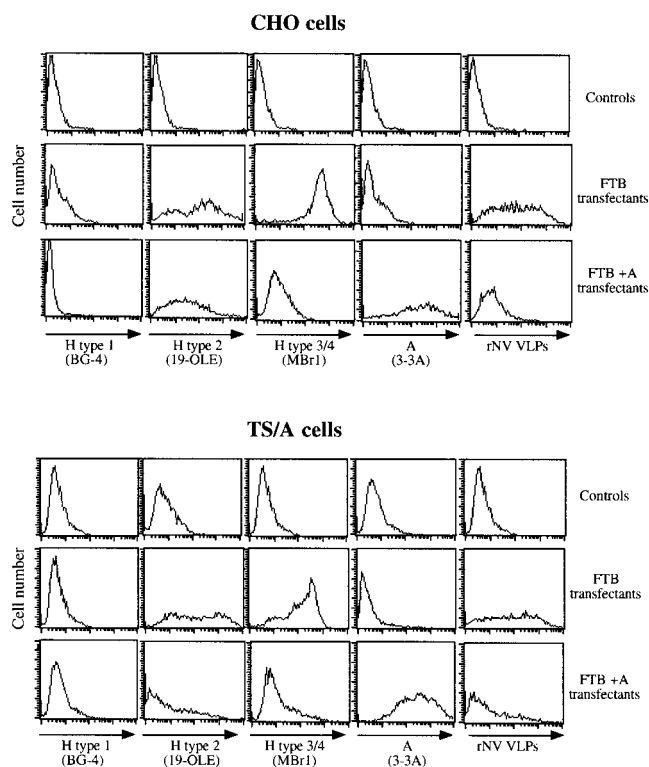
we recently showed that, like its human counterpart, it catalyzes the transfer of a fucose on either precursor, yet with a clear preference for types 1, 3, and 4 over type 2.<sup>34</sup> Neither parental CHO cells nor TS/A cells have detectable  $\alpha$ 1,2fucosyltransferase activity and, consequently, they are devoid of A, B, or H histo-blood group antigens. However, after transfection with the *FTB* cDNA, both cell lines express H types 2 and 3 and 4 determinants, but not H type 1 (Figure 4). The lack of H type 1 epitopes on these transfectants is likely caused by an absence of type 1 precursor, as in the case of CHO cells.<sup>35</sup> When assayed by flow cytometry, binding of rNV VLPs to these H antigen-expressing CHO and TS/A transfectants was readily detected. In contrast, no binding was evidenced on mock transfected cells (Figure 4). Addition of either an N-acetylgalactosamine or a galactose residue onto H structures generates A or B antigens, respectively, and this is known to hinder recognition by anti-H reagents.<sup>36</sup> To get further insight into the specificity of NV capsids, both CHO and TS/A cells were doubly

transfected with the *FTB* and rat A enzyme cDNAs. As expected, the double transfectants expressed the A antigen whereas their recognition by either the anti-H type 2 or the anti-H types 3 and 4 mAbs was severely diminished. The binding of rNV VLPs to these double transfectants also dropped, indicating that the presence of the A histo-blood group N-acetylgalactosamine hinders recognition of the subjacent H antigens by these viral capsids. In addition, this weak attachment of rNV VLPs to A-positive CHO and TS/A cells confirms specificity of the binding to strongly H-positive cells.

#### Attachment of rNV VLPs Through Recognition of 1,2Fucosylated Structures Is Followed by Internalization

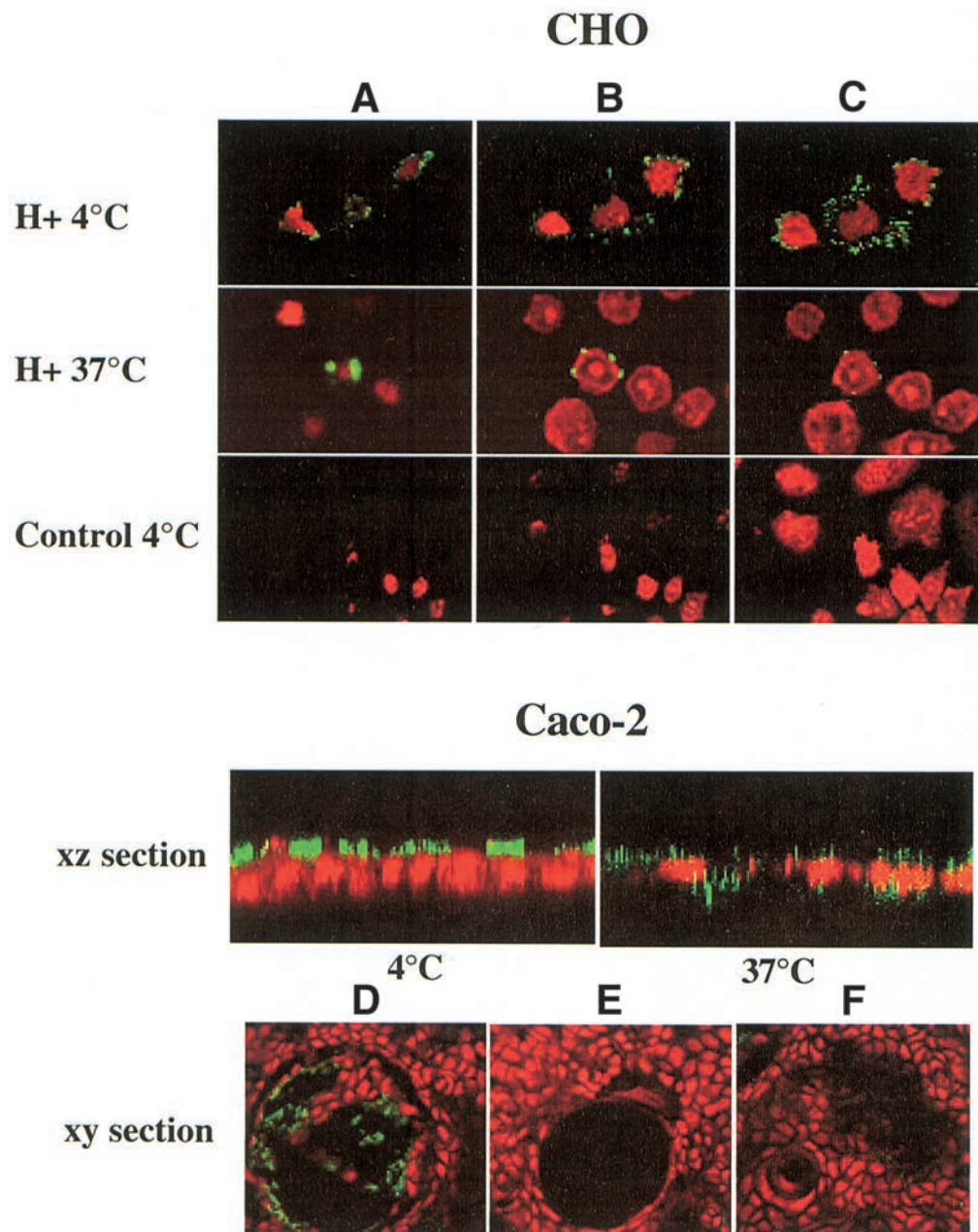
The ability of rNV VLPs attached to *FTB*-transfected CHO cells was monitored by confocal microscopy. Thus, *FTB*- or mock-transfected CHO cells were incubated with rNV VLPs at either 4°C or 37°C, and the presence of the recombinant capsids was observed by using the anti-NV capsid mAb 9C3. After incubation at 4°C, VLPs were clearly visible at the surface of *FTB* transfectants but not of control cells, in agreement with the results of the flow cytometry experiments (Figure 5). On incubation at 37°C, VLPs were still easily detectable on *FTB*-transfected cells, but they were no longer present at the cell surface. Instead, their detection required permeabilization and they were localized inside the cells around nuclei (Figure 5), indicating that internalization had occurred. The same results were obtained with the *FTB*-transfected TS/A cells (data not shown). When mock-transfected cells were incubated with rNV VLPs at 37°C, no signal was detected before or after permeabilization (data not shown).

It was shown previously by others that differentiated Caco-2 cells allow the binding and to some extent the internalization of VLPs from human NLVs.<sup>21,22</sup> Because this cell line represents a good in vitro model of normal differentiated human enterocytes, we tested their expression of H antigens in relationship with their binding of rNV VLPs. The alkaline phosphatase activity of Caco-2 cells was 4.8 nmol · min<sup>-1</sup> · mg<sup>-1</sup> at day 1 after confluency and progressively increased to reach 99.6 nmol · min<sup>-1</sup> · mg<sup>-1</sup> at day 15, indicating that the spontaneous differentiation process had taken place at day 15. Undifferentiated Caco-2 cells expressed H type 2 and moderate levels of H types 3 and 4 epitopes, but no detectable H type 1. At variance, differentiated Caco-2 cells strongly expressed all types of H epitopes. In agreement with previous reports, attachment of rNV VLPs was detectable at the surface of differentiated Caco-2 cells (Figure 5), but not of undifferentiated Caco-2 cells (data



**Figure 4.** Cytofluorimetric analysis of the expression of A and H histo-blood group antigens and of the rNV VLPs attachment to glycosyltransferases-transfected CHO and TS/A cells. Both cell types were transfected with empty vectors (controls), with the rat *FTB*  $\alpha$ 1,2fucosyltransferase cDNA (*FTB* transfectants), or with both the rat *FTB*  $\alpha$ 1,2fucosyltransferase and the rat A enzyme cDNAs (*FTB* + A transfectants). Cells were incubated with either the anti-H type 1 BG-4, the anti-H type 2 19 OLE, the anti-H types 3 and 4 MBR1, the anti-A 3-3A, or with rNV VLPs. The log of fluorescence intensities in arbitrary units is plotted against the cell number.





**Figure 5.** Confocal microscopy analysis of the attachment and internalization of rNV VLPs to transfected CHO cells and to differentiated Caco-2 cells. CHO cells transfected with the *FTB* cDNA and expressing H structures (H+) or mock transfected (CONTROL) were incubated at 4°C or 37°C for 2 hours with rNV VLPs. Before incubation at 4°C, cells were fixed to avoid any internalization. After incubation at 37°C, cells were permeabilized and fixed. VLPs were detected by using mAb 9C3 followed by FITC-conjugated secondary antibody (green). Nuclei were stained with propidium iodide (red). Confocal images were taken at the (A) top, (B) middle, and (C) bottom of cells. rNV VLPs were incubated at either 4°C or 37°C on differentiated Caco-2 cells grown on Transwell filters. Their presence was detected as described earlier for CHO cells. Confocal xz sections show the surface labeling after incubation at 4°C and the intracellular labeling after incubation at 37°C. Caco-2 cells grown on glass slides were incubated with rNV VLPs at 4°C and binding was detected as described earlier. Confocal xy sections were recorded. When grown on glass, differentiated Caco-2 cells form cysts as shown in D, E, and F. Before incubation on cells, VLPs were preincubated with either (D) a nonsecretor saliva or (E) a secretor saliva, or cells were treated with (F) fucosidase II. Images obtained in absence of preincubation with saliva or after treatment with inactivated fucosidase were similar to that shown in D.

not shown). The small amount of H types 3 and 4 detectable on undifferentiated Caco-2 cells is probably not sufficient to allow detectable attachment of rNV VLPs. A similar phenomenon was previously observed

with RHDV.<sup>25</sup> RHDV virions or recombinant particles bind to H type 2 present on adult rabbit epithelial cells. However, attachment could not be detected on young rabbit cells, which express much lower levels of H type

2 than adult cells. It is likely that VLPs require a minimal density of ligands to bind to cell surfaces. Thus, attachment of rNV VLPs to Caco-2 cells correlates with the presence of H type 1 or large amounts of H types 3 and 4 epitopes but not with that of H type 2. After 2 hours at 37°C, most of these VLPs were localized inside the cells, indicating that they have been internalized (Figure 5). The binding to differentiated Caco-2 cells was completely inhibited by preincubation with a saliva from a secretor individual but not from a nonsecretor individual (Figure 5D and E) and partly inhibited by pretreatment of the cells with fucosidase II (Figure 5F).

## Discussion

Carbohydrates represent 1 class of virus receptors among which heparin sulfates and sialic acids are the most frequently recognized. Viruses as diverse as influenza virus, some strains of rotaviruses, coronaviruses, and adenoviruses, as well as Sendai virus, are known to bind to their cellular targets via recognition of sialic acid residues.<sup>37-41</sup> So far, only parvovirus B19 has been shown to bind to a neutral oligosaccharide, namely the erythrocyte P antigen or globoside.<sup>42</sup> We have shown here that VLPs from NV attach to cells by recognition of the H type 1 or H types 3 and 4 histo-blood group oligosaccharides. This conclusion is based on the following observations: (1) attachment of rNV VLPs depends on the presence of a fucose in  $\alpha 1,2$  linkage; (2) it is inhibited by  $\alpha 1,2$  fucosidase treatment, by H types 1 and 3 trisaccharides, and by anti-H type 1 and anti-H types 3 and 4 mAbs; (3) tissue distribution of rNV VLPs binding sites correlates with that of H type 1 epitopes; and (4) transfection of an  $\alpha 1,2$  fucosyltransferase cDNA into animal cell lines devoid of such enzyme activity leads to expression of H types 2 and 3 and 4 epitopes at the cell surface and is sufficient to allow attachment of rNV VLPs.

The attachment of NV to cell surfaces through recognition of H-type oligosaccharides could be the primary step for viral infection. Alternatively, the carbohydrates could function as decoy receptors, in which case their recognition would impair entry of the virus particles into cells and lead to their elimination. To test these 2 hypotheses, we examined the internalization of rNV VLPs into Caco-2 and the transfected CHO and TS/A cells. Differentiated Caco-2 cells are the only intestinal cells known to express H type 1 in culture.<sup>26</sup> As previously described by others<sup>26</sup> and confirmed during the present work (data not shown), this carbohydrate structure is hardly detectable on undifferentiated Caco-2 cells and appears progressively during the course of differentiation. The results obtained with differentiated Caco-2

cells indicate that the interaction requires recognition of H types 1 and/or 3 by the rNV VLPs. After attachment, these particles are internalized. This is confirmed by the internalization that occurs after binding to *FTB*-transfected, but not -nontransfected, CHO cells. It is suggested that the expression of the carbohydrate ligand on the cell surface is required for internalization of the virus particles. This process is not species specific because CHO cells are derived from a Chinese hamster ovary carcinoma. Although these results tend to indicate that the fucosylated carbohydrate is not a decoy receptor, they do not conclude that it can be a functional receptor because cultivation of the virus has not been obtained as yet.

A previous study showed that rNV VLPs attach to differentiated Caco-2 cells, but only a small proportion, in the range of 5%, of attached particles were internalized.<sup>21</sup> A similar low rate of internalization by VLPs from a genogroup II NLV was reported.<sup>22</sup> The reason for these low rates of internalization is unclear. The differentiation state of the cells could be an important factor. Our cells were cultivated on Transwell chambers rather than on plastic culture wells. In addition, they were kept in culture for a longer period of time after confluency with frequent changes of medium. These different culture conditions could affect the differentiation of the cells. The confocal microscopy experiments that we performed were not quantitative and therefore do not allow us to draw conclusions as to the proportion of bound particles that were internalized. Observations were concentrated on areas where the cells presented a well-differentiated morphology. In these fields, after 2 hours at 37°C, most detectable VLPs were localized intracellularly and few cells with surface-bound VLPs remained visible. Observation of cells that were not clearly morphologically differentiated showed a weaker binding of VLPs and no clear internalization. Thus, partly differentiated Caco-2 cells may present sufficient levels of H epitopes to allow some attachment of NV particles but not to allow internalization. They may also lack additional molecules required for internalization that would only be expressed on fully differentiated cells.

Because the interaction between a virion and its cellular receptor is the initial event of the replication cycle, the failure of NV to grow in culture might be caused by the low levels or absence of specific receptor on the cell lines that have been tested for this aim. Our observation that strong expression of H histo-blood group antigens after transfection with an  $\alpha 1,2$  fucosyltransferase is required for internalization of rNV VLPs on animal cell lines, opens up the possibility to test the ability of

fucosyltransferase-transfected human cells to support the growth of NV. However, later events could also be limiting steps in the viral replication cycle.

Binding of nonreplicating VLPs to cells and tissue sections was studied in the present work. It is not clear at this point whether replicative NV would share the same properties. Yet, if this was the case, the fact that NV particles attach only to the gut of secretor individuals suggests that only these people can be infected by the virus. In contrast, nonsecretor individuals are not expected to be infected because they lack the histo-blood group H oligosaccharides on the digestive epithelial cells. Most interestingly, it has been observed in studies performed on volunteers challenged with NV that nearly 20% of the subjects did not develop clinical signs of the disease or antibodies to the virus.<sup>43</sup> This frequency matches that of the nonsecretors in the general European or North American populations, suggesting that the polymorphism at the *FUT2* locus could indeed be a major genetic determinant for sensitivity to NV. Among the remaining 80% volunteers, a significant proportion developed antibodies but no clinical signs, indicating that additional factors may be responsible for resistance to the infection. Our observation that the presence of the A histo-blood group antigenic motif hinders the binding site of rNV VLPs suggests that the polymorphisms at the ABO locus could also play a part in determining sensitivity to the virus. The availability of H epitopes at the digestive epithelial cell surface may depend on the interactions between the enzymes encoded by the *ABO*, Lewis (*FUT3*), and secretor (*FUT2*) genes. Our histochemical study did not reveal significant differences of rNV VLPs binding to digestive tissue from individuals of different ABO or Lewis types. However, this approach is not quantitative and does not allow us to rule out the possibility that A or B blood group individuals present less binding sites to the virus than do O individuals. Epidemiologic or volunteer studies are thus warranted to define the contribution of the *ABO*, *FUT2*, and *FUT3* genes to the sensitivity to NV.

NV is the prototype of the NLV group that presents a large diversity. It is not yet known if other strains of NLVs can also bind to H-type oligosaccharides. Viruses belonging to very distant families can use the same receptors, whereas highly homologous strains within the same family may use distinct receptors.<sup>44</sup> The ability to bind histo-blood group H structures is shared between the animal (RHDV) and human (NV) CVs despite little homology between the P2 subdomains of their capsid proteins. It is possible that this property is also shared among many HuCV strains, yet this remains to be

shown. If this was the case, it would be useful to design oligosaccharide mimics able to compete with the virus at the time of infection. Such molecules could be of major interest in preventing outbreaks. Finally, artificial virus vectors prepared by packaging DNA into recombinant VLPs proved efficient for gene transfer in in vitro experiments.<sup>45</sup> The exquisite property of NV capsids to recognize cells that express H types 1 or 3 antigens could thus be exploited in the context of gene therapy to specific target cells.

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