HISTO-BLOOD GROUP ANTIGEN AND HUMAN MILK OLIGOSACCHARIDES

Genetic Polymorphism and Risk of Infectious Diseases

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1. ABSTRACT

ABH and Lewis antigens are carbohydrates present on gut epithelial cells. These antigens provide diversity within the human population. Their biosynthesis largely is controlled by the enzyme products of alleles at the *ABO*, *FUT2* and *FUT3* loci. We have shown that Norwalk virus (NV) uses structures based on H type 1 as its primary receptor. Norwalk virus is the prototype of human caliciviruses, which collectively are responsible for the majority of gastroenteritis outbreaks in people of all ages. Individuals with two mutated *FUT2* alleles, and therefore devoid of H type 1 epitopes on their gut epithelial cells, are called nonsecretors and are resistant to infection by NV. This genetically controlled mechanism of resistance to NV also might be important in the protection of infants by human milk, yet in an inverse manner since, unlike milk from secretors, the milk from nonsecretor mothers does not inhibit attachment of recombinant NV particles to their primary receptor. This suggests that breastfeeding by a secretor mother should protect a secretor child from NV infection, whereas breastfeeding by a nonsecretor mother should not.

2. INTRODUCTION

ABO antigens were discovered a century ago on erythrocytes. Subsequently, similar molecules were identified in body fluids such as saliva and milk, as well as on various cell types. These molecules are carbohydrates that represent the terminal part of glycan chains of glycolipids or glycoproteins (Watkins 1999). They also may be present as free oligosaccharides in milk and have been found in many animal species, although their presence on red blood cells is restricted to humans and some anthropoid apes (Blancher & Socha 1997). ABO antigens also are expressed in relatively large quantities in the intestine and respiratory tracts, and they may play a role in the attachment of microorganisms (Marionneau et al. 2001). Furthermore, data support the fact that cell

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surface carbohydrates serve as initial receptors for numerous pathogens and toxins. For example, uropathogenic strains of *Escherichia coli* bind to the glycolipid Galβ3GalNAcβ3Galα4Galβ4Glcβ-Cer through type I or type II adhesins, strains of *Helicobacter pylori* equipped with the BabA adhesin bind to the Le^b antigen Fucα2Galβ3[Fucα4]GlcNAc, and some strains of *Staphylococcus saprophyticus* or *Pseudomonas aeruginosa* have been reported to attach to the histo-blood group A antigen GalNAcα3[Fucα2]Gal. Similarly, the cholera toxin (CT) and *Escherichia coli* labile toxin (LT) bind to the GM1 glycolipid Galβ3GalNAcβ4[NeuAcα2,3]Galβ4Glc-Cer, and Shiga and Shiga-like toxins from *E. coli* bind to the Gb_3 glycolipid Galα4Galβ4Glc-Cer. Toxin A from *Clostridium difficile* strains infecting rabbits bind to Galα3Galβ4GlcNAcβ3Galβ4GlcNAc. *Bacillus thuringiensis* toxin binds to glycans terminated with a β3-linked galactose residue (Moulds et al. 1996; Zopf & Roth 1996; Dai et al. 2000). *B. thuringiensis* toxin has been used in agriculture to control plant parasites such as caterpillars and beetles. However, this toxin also is lethal to the worm *Caenorhabditis elegans*. Most interestingly, a mutation that inactivates β3-galactosyltransferase from *C. elegans* was found to confer resistance to the toxin, indicating that the genetic polymorphism of expression of a glycan structure can confer protection against a pathogen (Griffitts et al. 2001).

Many viruses also are known to attach to glycans. This most often involves recognition of a sialic acid residue as in the case of influenza virus, rotavirus, coronavirus, or polyomavirus. Until recently, the only virus known to attach to a neutral glycan was parvovirus B19, which uses GalNAcβ3Galα4Galβ4Glc-Cer, the P histo-blood group antigen, as receptor (Brown et al. 1993).

The biosynthesis of ABO and related antigens proceeds from precursors by stepwise addition of monosaccharide units through the action of a set of glycosyltransferases. There are five types of precursors that have disaccharides sharing a galactose in β-linkage to the subjacent sugar unit: Type 1, **Galβ3**GlcNAcβ-R; Type 2, **Galβ4**GlcNAcβ-R; Type 3, Galβ3**GalNAcα**-R; Type 4, Galβ3**GalNAcβ**-R; and Type 5, Galβ4**Glcβ**-R. Type 1 and 2 precursors can be part of O- or N-glycoproteins, as well as of glycolipids of the lacto series (represented by the R letter). Type 3 is found exclusively on O-glycans, where it corresponds to core 1. Type 4, is only found on glycolipids of the globo and ganglio series, and Type 5 corresponds to the glycolipid lactosylceramide or to free lactose. In milk, all types of precursor structures can be present since this biologic fluid contains large amounts of glycoproteins with N-linked and O-linked glycans, glycolipids as well as free oligosaccharides. The major biosynthesis pathways of these antigens from the type 1 precursor is presented in Figure 1.

Addition onto precursors of a fucose in α1,2 linkage gives the H antigens. This step is catalyzed by an α1,2-fucosyltransferase. In humans, two α1,2-fucosyltransferases able to participate in the synthesis of these antigens are known. They are encoded by two distinct genes, *FUT1* and *FUT2*. There exists a third α1,2-fucosyltransferase gene called *Sec1* which is inactivated by insertion of a nucleotide leading to a frameshift in the coding sequence. *Sec1* is therefore a pseudogene. Fully or partially inactivating mutations of *FUT1* and *FUT2* define a genetic polymorphism at each of these two loci. Lack of a functional *FUT1* allele leads to the Bombay phenotype characterized by an absence of ABH antigens on erythrocytes. This phenotype is rare with a frequency in the 10^{-5} to 10^{-6} range. The lack of a functional *FUT2* allele is responsible for the nonsecretor phenotype, as opposed to the secretor phenotype, characterized by the absence of ABH antigens in

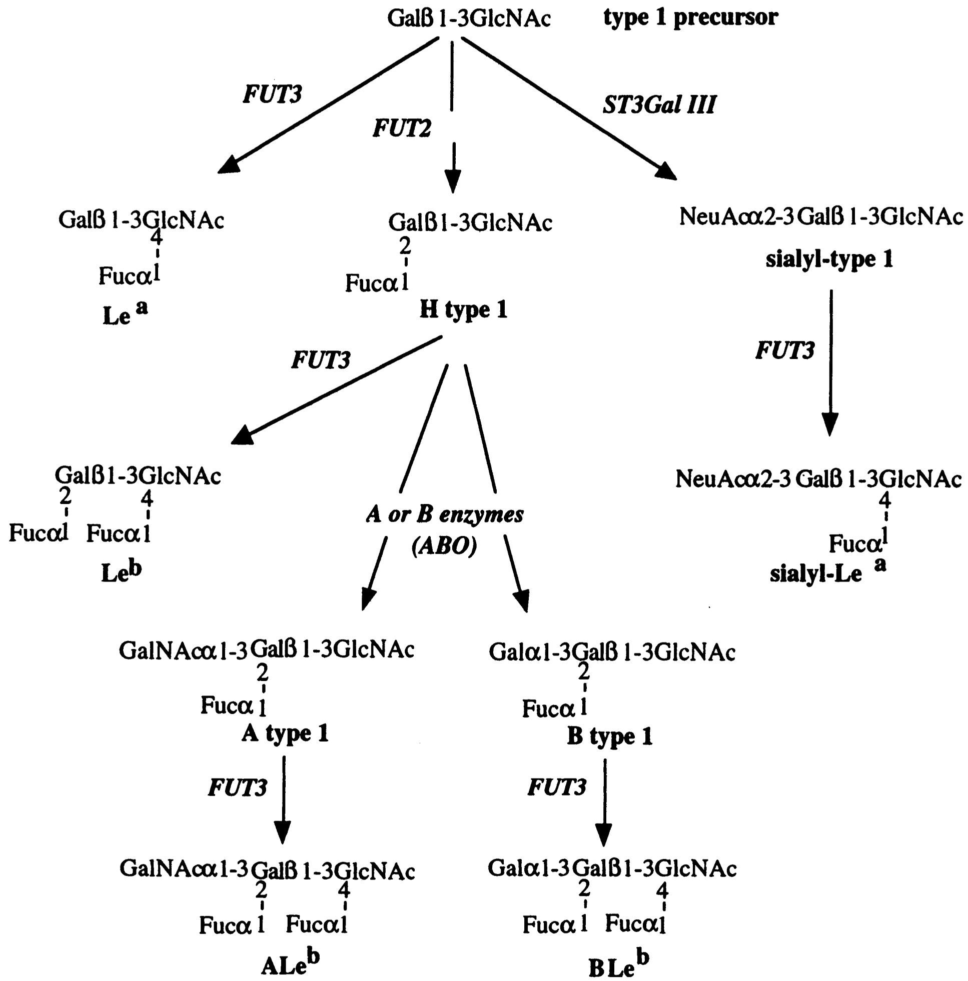

Figure 1. Major biosynthetic pathways of the ABH and Lewis antigens based on the type 1 precursor disaccharide. The name of the antigens are in bold, and the enzymes acting at each catalytic step are marked in italics.

saliva and on various epithelial cell types. Unlike the Bombay phenotype, nonsecretor phenotype is not rare since it is found in about 20% of Europeans and white Americans.

Once the H antigens are formed, biosynthesis can proceed by addition of either an *N*-acetylgalactosamine or a galactose in α1,3 linkage to give the A or B antigens, respectively. This step is catalysed by the A or B enzymes, which are encoded by different alleles at the *ABO* locus. A large series of alleles at this locus has been described: some alleles code for variants of A and B enzymes, others are inactivated by various mutations corresponding to O alleles (Hakomori 1999).

In the case of antigens derived from types 1 and 2 precursors, a fucose can be added in α1,4 or α1,3 of the *N*-acetylglucosamine residue of the precursor giving the Le^a or Le^x antigens, respectively. Addition of a fucose in the same position of the H types 1 and 2 antigens will give the Le^b and Le^y antigens, respectively. The A or BLe^b and A or BLe^y are obtained by addition of a fucose residue on the A/B type 1 or A/B type 2 antigens.

Six fucosyltransferases able to catalyze the addition of fucose in α1,3 or α1,4 position are known in humans. The FUT3 and FUT5 enzymes can use both type 1 and type 2 precursors as substrates, FUT3 having a marked preference for type 1 and FUT5 for type 2. The FUT4, FUT6, FUT7, and FUT9 enzymes catalyze addition of a fucose exclusively onto the type 2 precursor (Cailleau-Thomas et al. 2000). Like the *FUT1* and *FUT2* genes, polymorphisms have been described for *FUT3*, *FUT5*, *FUT6*, and more recently for *FUT7* (Bengston et al. 2001; Mollicone et al. 1994a, 1994b). Individuals lacking a functional *FUT3* allele are called "Lewis negative" and are characterized by the red cell phenotype Le^{a-b-}. They represent nearly 10% of the European population. People with a "Lewis positive" phenotype, either Le^{a-b+} or Le^{a+b-}, possess at least one functional *FUT3* allele and are, respectively, secretors or nonsecretors. The frequency of individuals deficient in FUT5, FUT6 or FUT7 enzymes is not well known as yet, but is low in the case of FUT6 (Mollicone et al. 1994a). The sialyl-Le^a and sialyl-Le^x antigens are obtained by addition of a sialic acid in α2,3 linkage followed by addition of a fucose in α1,4 or α1,3 linkage onto either the type 1 or type 2 precursors. Therefore, the genes involved in the biosynthesis of this group of carbohydrate structures can be either polymorphic, such as the *ABO*, *FUT2*, and *FUT3* genes, or nearly monomorphic such as the *FUT1*, *FUT4*, and *FUT7* genes. In epithelia, most of the biosynthesis is performed by the enzyme products of the polymorphic genes *ABO*, *FUT2*, and *FUT3*, which act in concert to generate a diversity of glycans at the population level. This diversity may be expected to be related with distinct individual susceptibility to a spectrum of pathogens.

3. HISTO-BLOOD GROUP ANTIGENS WITH AN α1,2-LINKED FUCOSE CONSTITUTE THE PRIMARY RECEPTOR FOR NORWALK VIRUS

The Caliciviridae constitute a family of small, positive strand RNA viruses that include animal and human pathogens. They are divided in four genera, two of them, the norovirus and sapovirus, causing acute gastroenteritis in humans (Thiel & König 1999). Epidemiologic studies indicate that noroviruses are responsible for the majority of outbreaks in developed countries, affecting people of all ages including very young children (Glass et al. 2001). Noroviruses are spread by the fecal-oral route from person to person or from contaminated water or food items.

Our group recently observed that another member of the calicivirus family, the lagovirus RHDV (rabbit haemorrhagic virus), binds to the histo-blood group antigen H

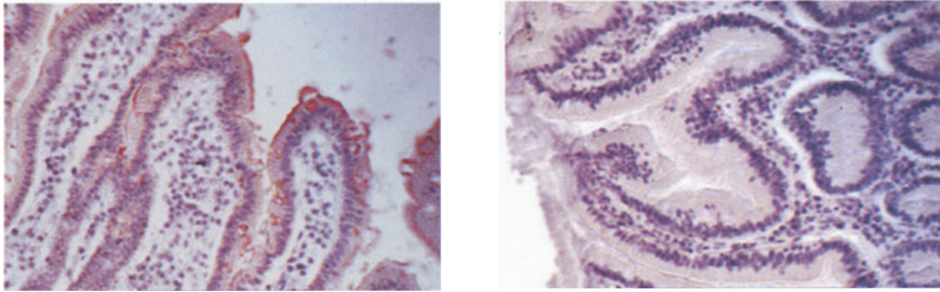

Figure 2. Binding of rNV VLPs to human duodenum tissue sections from a secretor and a nonsecretor individual. Sections were incubated with rNV VLPs, then with an anti-VLP followed by a peroxidase-labeled mouse anti-IgG detection system.

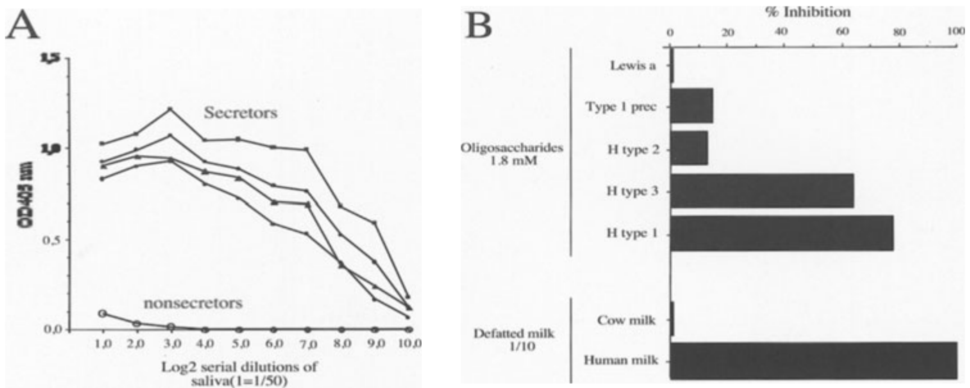

Figure 3. (A) Binding of rNV VLPs to serially diluted saliva samples from either secretors or nonsecretors coated to an EIA plate. (B) Inhibition of rNV VLPs binding to saliva from a secretor individual with synthetic oligosaccharides, cow milk or human milk from a secretor mother. Milk from nonsecretor mothers is not inhibitory (not shown).

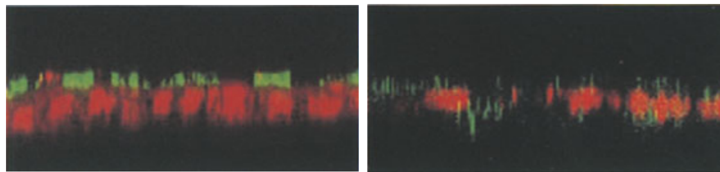

Figure 4. Confocal microscopy experiment showing internalization of rNV VLPs in Caco-2 cells. Caco-2 cells were maintained in culture on filters for 15 days postconfluency to obtain differentiation. The cells were then incubated with rNV VLPs at 4°C or 37°C. After incubation, cells were fixed and permeabilized. The localization of the rNV VLPs was visualized using an antibody against the capsid protein (green fluorescence) and the cell nuclei were stained with iodide propidium (red fluorescence).

type 2 (Fucα2Galβ4GlcNAc) present at the cell surface of the rabbit upper respiratory and digestive epithelial cells (Ruvoën-Clouet et al. 2000). This finding prompted us to determine if human caliciviruses could share this property of attachment to fucosylated glycans. For this aim, we used virus-like particles (VLPs) prepared from recombinant capsid protein of Norwalk virus (NV) by Jiang and colleagues (1992). NV is the prototype of the norovirus genera, discovered in 1972 by electron microscopy (Kapikian 2000). To study interaction of NV with digestive epithelial cells, we used an approach that we developed with RHDV where the VLPs are incubated with tissue sections and their attachment is detected with antibodies directed against the capsid protein by classical immunohistochemistry. Using this technique on human tissue sections from the stomach and duodenum, we observed a clear binding of rNV VLPs on the surface epithelial cells from all secretor individuals, but not from nonsecretors. This was the first indication that a functional *FUT2* allele, and therefore the presence of an α2-linked fucose residue, was necessary for attachment of rNV VLPs to the tissue sections (Fig. 2).

This finding was confirmed by treating tissue sections with a fucosidase that specifically cleaves α2-linked fucose residues, completely abolishing the binding of rNV VLPs. Since α2-fucosylated antigens also are present in the saliva under control of the *FUT2* genetic polymorphism, we developed an assay using boiled saliva samples coated on wells of microtiter plates. A strong binding of rNV VLPs to saliva samples from secretor individuals was observed. In contrast, no binding was obtained using saliva from nonsecretors (Fig. 3A). The binding of rNV VLPs to these saliva samples from secretors could be inhibited by synthetic oligosaccharides with an aliphatic tail. The best inhibition was obtained with the H type 1 trisaccharide, while the H type 3 trisaccharide also proved inhibitory. Other related oligosaccharides such as Le^a or the type 1 precursor were not inhibitory (Fig. 3B). Since then, others have shown that rNV VLPs bind to the H type 1, H type 3, and Le^b synthetic oligosaccharides coupled to polyacrylamide (Hutson et al. 2003; Harrington et al. 2002). These results indicate that these α2-fucosylated antigens are sufficient to mediate attachment of the recombinant virus particles. Using an anti-H type 1 specific antibody, we could inhibit binding of the rNV VLPs to duodenum tissue sections. An antibody specific to H type 3 and H type 4 antigens gave a partial inhibition whereas an anti-H type 2 specific antibody did not inhibit at all, showing that the H type 1 epitope of the digestive surface epithelial cells is recognized by the virus particles. In order to confirm the importance of the α2-linked fucose residue, CHO cells were transfected with a *FUT2* cDNA, allowing cell surface expression of H type 3 and H type 2 epitopes. This was sufficient to mediate attachment of rNV VLPs.

These data indicate that the α2-linked fucose residue under control of the *FUT2* gene is necessary and sufficient to mediate attachment of rNV VLPs. To further support the role of α2-linked fucose residues on the attachment of noroviruses to epithelial cells, we first performed confocal microscopy experiments in order to monitor the possible internalization of rNV VLPs following their attachment to cells expressing the α2-fucosylated ligand. At 4°C, rNV VLPs bound to the surface of either FUT2 transfected CHO cells or of differentiated Caco-2 cells. The latter cells originate from a human colon carcinoma and have the property to spontaneously differentiate in culture when maintained two weeks postconfluency. Additionally, these differentiated Caco-2 cells are the only cultivable cells known to express the H type 1 antigen. For this reason, Caco-2 cells are able to bind rNV VLPs. Upon incubation at 37°C, we could visualize internalization of the recombinant viral particles in both the transfected CHO cells and the differentiated Caco-2 cells (Fig. 4). This result provided the first indication that the

attachment of rNV VLPs to the α2-fucosylated antigens could be of functional relevance (Marionneau et al. 2002). However, it did not prove that native virions would behave similarly and that the carbohydrate recognition was absolutely required for infection.

At that stage we had the chance to collaborate with Christine Moe and her colleagues Ralph Baric and Lisa Lindensmith who had just performed a challenge study where 77 healthy volunteers had received an NV inoculum. Of these volunteers, 64% presented signs of infection. When their secretor status was determined, it appeared that all of these infected individuals were in the secretor group. None of the 22 nonsecretors presented signs of infection defined by clinical evidence of gastroenteritis, RT-PCR detectable virus particles in stool, or a 4-fold increase in anti-NV serum IgG (Lindesmith et al. 2003). This result clearly proved that the H type 1, or a closely related antigen requiring an active FUT2 enzyme for its synthesis, is not only sufficient to mediate attachment of the virus but also that it is necessary for infection. Therefore, it can be concluded that the α1,2 epitope constitutes the Norwalk virus primary receptor and that a genetically defined absence of this receptor confers full protection from NV infection.

Since the FUT2 enzyme collaborates with the *FUT3* and *ABO* gene products to synthesize A, B, H type 1, and Le^b antigens, it is quite possible that Norwalk virus will show different affinities for these carbohydrate structures and, therefore, that the polymorphisms at the *FUT3* and *ABO* loci will have some influence on the ability of the virus to infect people. As a first approach to test this possibility, we used the saliva binding assay and noticed an influence of the *ABO* polymorphism on rNV VLPs binding to saliva samples from secretor individuals. The strongest binding was obtained on saliva samples from blood group A individuals. A somewhat lower binding was obtained on saliva samples from blood group O individuals, and a much lower binding was detected on saliva samples from blood group B individuals (Fig. 5).

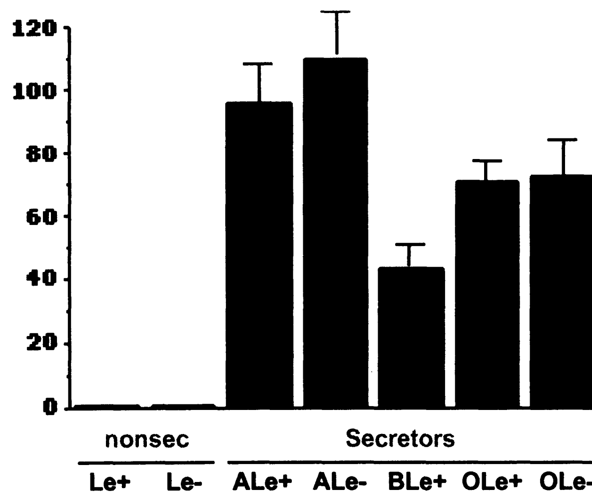

Figure 5. Influence of the ABO and secretor and Lewis phenotypes on the attachment of rNV VLPs particles to saliva samples. The binding of rNV VLPs to saliva samples, diluted 1/800, phenotyped for ABO, secretor and Lewis was determined by EIA. Values are normalized against a set of positive control saliva samples used on each plate.

Interestingly, a study published in 2002 reported that blood group B individuals had a lower risk of being infected by NV, indicating that besides the *FUT2* polymorphism, the *ABO* polymorphism can modulate the susceptibility to Norwalk virus (Hutson et al.). In contrast, no difference was observed between Lewis positive and Lewis negative saliva samples, that is, saliva specimens from individuals with at least a functional *FUT3* allele or with two mutated *FUT3* alleles, respectively. This result is not surprising since NV can attach equally well to H type 1 and to Le^b, which are the two major histo-blood group antigens present in saliva samples from the two subgroups. It is therefore unlikely that the *FUT3* polymorphism should influence infection by NV.

4. PROTECTION FROM NORWALK VIRUS INFECTION BY HUMAN MILK AND THE HISTO-BLOOD GROUP POLYMORPHISM—WHAT SHOULD WE EXPECT?

Human milk is well known to contain histo-blood group antigens either as free oligosaccharides, as glycolipids, and as glycoproteins. It is thus expected that some of these components could inhibit the attachment of NV to its cellular receptor and therefore participate in the protection of infants from NV-mediated gastroenteritis. When human milk samples were used in an inhibition assay of the binding of rNV VLPs to α2-fucosylated ligands, all samples from secretor mothers were strongly inhibitory whereas the samples from nonsecretor mothers were not, indicating that the *FUT2* polymorphism determines not only the attachment of the virus to its primary receptor but also controls the presence of potential protective molecules in human milk. We then tested the ability of the purified milk pentasaccharide Fucα2Galβ3GlcNAcβ3Galβ4Glc to inhibit rNV VLPs binding to immobilized H type 1 complexed to polyacrylamide. Despite the presence of the H type 1 trisaccharide as the terminal portion of this milk oligosaccharide, no inhibition could be obtained, even at a 10 mM concentration. This negative result tends to indicate that the specific inhibitory components from human milk are not the simple free oligosaccharides. Instead, they could be either the complex highly branched oligosaccharides or the multimeric glycans carried by proteins. For this reason we are now trying to characterize and purify the glycoproteins from the milk of secretor mothers that carry α2-fucosylated epitopes and potentially could inhibit the virus attachment to the infant's gut epithelial cells.

As shown in Figure 6, some children can be expected to be genetically resistant to NV since they are nonsecretors. Among the susceptible children, those who are breastfed by a secretor-positive mother could be protected by decoy receptors from the milk. Unfortunately, susceptible children whose mothers are nonsecretors would not be protected since the milk does not contain the appropriate decoy receptor. It is also important to remember that cow milk does not contain histo-blood group structures either as free oligosaccharides or on glycoproteins. Therefore, cow milk does not inhibit the binding of rNV VLPs to immobilized H type 1 (Fig. 3B). Thus, formula-fed infants do not benefit from the protection potentially conferred by the soluble histo-blood group antigens contained in human milk.

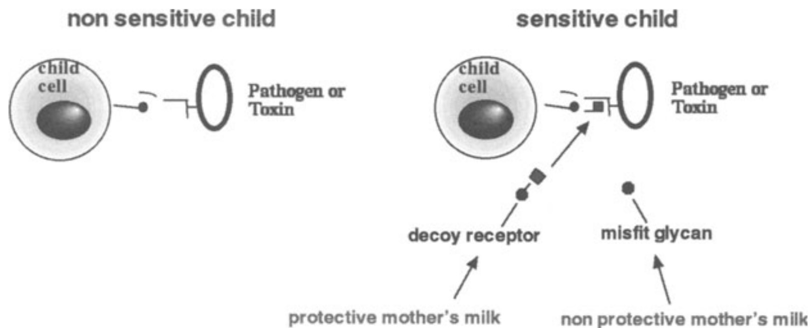

Figure 6. Hypothetical influence of the mother/infant combined histo-blood group polymorphism on the protection of infants from histo-blood group binding pathogens such as NV.

5. ACKNOWLEDGMENT

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