

## REVIEW

Diversity in Rotavirus–Host Glycan Interactions:  
A “Sweet” SpectrumSasirekha Ramani,<sup>1</sup> Liya Hu,<sup>2</sup> B. V. Venkataram Prasad,<sup>2</sup> and Mary K. Estes<sup>1</sup><sup>1</sup>Department of Molecular Virology and Microbiology, <sup>2</sup>Verna and Marrs McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, Texas

## SUMMARY

Rotaviruses exploit host glycans as receptors for cell attachment. The discovery that human rotaviruses bind a spectrum of host glycans provides new insights into virus pathogenesis. Glycan expression is determined genetically and regulated developmentally, which may affect susceptibility to infection and vaccination.

Interaction with cellular glycans is a critical initial step in the pathogenesis of many infectious agents. Technological advances in glycobiology have expanded the repertoire of studies delineating host glycan–pathogen interactions. For rotavirus, the VP8\* domain of the outer capsid spike protein VP4 is known to interact with cellular glycans. Sialic acid was considered the key cellular attachment factor for rotaviruses for decades. Although this is true for many rotavirus strains causing infections in animals, glycan array screens show that many human rotavirus strains bind nonsialylated glycoconjugates, called *histo-blood group antigens*, in a strain-specific manner. The expression of histo-blood group antigens is determined genetically and is regulated developmentally. Variations in glycan binding between different rotavirus strains are biologically relevant and provide new insights into multiple aspects of virus pathogenesis such as interspecies transmission, host range restriction, and tissue tropism. The genetics of glycan expression may affect susceptibility to different rotavirus strains and vaccine viruses, and impact the efficacy of rotavirus vaccination in different populations. A multidisciplinary approach to understanding rotavirus–host glycan interactions provides molecular insights into the interaction between microbial pathogens and glycans, and opens up new avenues to translate findings from the bench to the human population. (*Cell Mol Gastroenterol Hepatol* 2016;2:263–273; <http://dx.doi.org/10.1016/j.jcmgh.2016.03.002>)

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The surfaces of cells are decorated heavily with glycans or glycoconjugates, with structures ranging from simple monosaccharides to complex sugars with many different branches, linkages, and orientations.<sup>1</sup> Interaction with host glycans is an essential and critical step in the

infectivity of most, if not all, microbial pathogens. Many pathogens exploit these glycans for initial cell recognition and attachment, and for enteric viruses such as rotaviruses and noroviruses, these interactions are frequently the first critical step for initiation of infections. Key fundamental, clinical, and epidemiologic questions on rotavirus disease have been answered through studies on the interactions of rotavirus with host glycans. The approaches exemplify multidisciplinary translational science and involve virologists, structural biologists, glycobiologists, physicians, and epidemiologists (Figure 1). For example, screens using transformative new technologies in glycobiology such as glycan arrays identified histo-blood group antigens (HBGAs) as new glycan partners for human rotaviruses. These interactions were confirmed by enzyme-linked immunosorbent assays (ELISA) using synthetic glycans, and the basis of these interactions was elucidated using x-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy. The biological relevance of these findings has been addressed using hemagglutination, saliva binding, and in vitro infectivity assays. Preliminary findings from cell lines now can be confirmed using physiologically relevant models of the human gut such as intestinal enteroids. Translational studies in people test if findings from the bench hold true at the bedside or at the population level. The findings from such team-science approaches not only have direct implications for our understanding of the biology of the virus, but also influence vaccine strategies, the development of therapeutics, and provide a new foundation for understanding other enteric infections. The diversity of glycans recognized by animal and human rotaviruses, insights gained into various aspects of viral pathogenesis such as interspecies transmission, host restriction, and tissue tropism, and the effect of genetic differences in glycan expression on susceptibility to infection and vaccination are reviewed here.

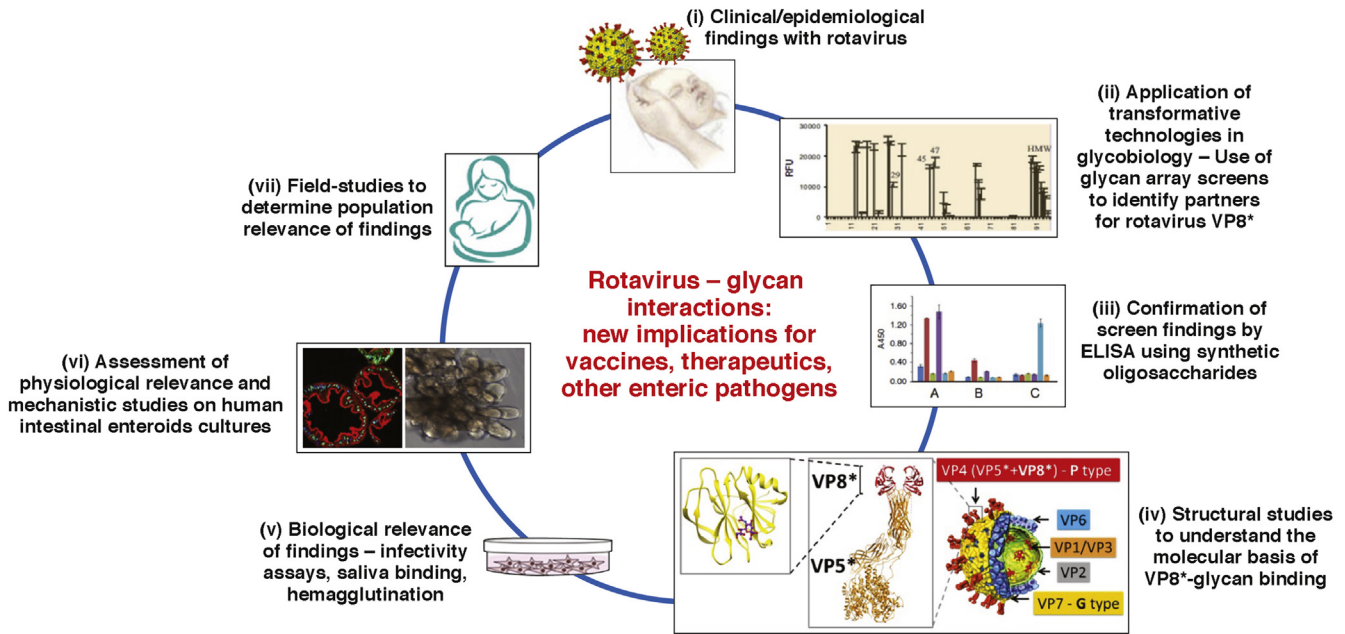
**Abbreviations used in this paper:** GlcNAc, N-acetylglucosamine; HBGA, histo-blood group antigen; HIE, human intestinal enteroid; LacNAc, N-acetyllactosamine; Le, Lewis; LNnT, lacto-N-neotetraose; LNT, lacto-N-tetraose; Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; NMR, nuclear magnetic resonance; RBC, red blood cell; Sia, sialic acid; VP, viral protein.

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**Figure 1. Schematic representation of multidisciplinary studies on rotavirus interaction with host glycans.** A combination of glycobiology, structural biology, basic virology, and field studies on infectious diseases have been used to understand rotavirus–host–glycan interactions. (i) Clinical and epidemiologic questions on rotavirus infections in children have been addressed through these approaches. (ii) A representative image of results from a glycan array is shown, with numeric order of glycans in the array listed on the x-axis and binding intensity in relative fluorescence units (RFU) on the y-axis. (iii) A representative image of enzyme-linked immunosorbent assay (ELISA) results showing the binding of VP8\* from 3 rotavirus strains A, B, and C to synthetic oligosaccharides. Each colored bar represents a synthetic oligosaccharide. Binding is measured by optical density value at 450 nm. (iv) A cut-away of a cryo-electron microscopic reconstruction of a rotavirus triple-layered particle. The core layer comprises VP2 and the intermediate layer is made of the protein VP6. The outer capsid is made of the glycoprotein VP7. Sixty spikes made of the protease-sensitive protein VP4 extend from the VP7 layer, and comprise 2 domains, VP5\* and VP8\* (inset). The crystal structure of rotavirus VP8\* in complex with a glycan is seen in the second inset. (v) The biological relevance of binding assays were confirmed through infectivity assays on transformed cell lines and through hemagglutination and saliva binding assays. (vi) HIEs provide a novel intestinal culture system to study rotaviruses. Confocal microscopy shows cross-section of an HIE stained with Ki-67 (green) for proliferating cells, actin (red) for highlighting the apical surface of the epithelial cells, and 4',6-diamidino-2-phenylindole (blue) for the nucleus (left panel). A multilobular, differentiated, 3-dimensional HIE is shown in the right panel. (vii) Field studies using samples from mother–infant pairs will determine the relevance of these findings at a population level.

## Rotavirus Structure, Classification, and Disease Burden

Rotaviruses are the leading cause of severe dehydrating gastroenteritis in children younger than 5 years of age. Globally, rotavirus infections result in approximately 453,000 deaths each year, accounting for approximately 5% of child deaths.<sup>2</sup> The majority of these deaths occur in developing countries in Asia and sub-Saharan Africa. Two live-attenuated oral vaccines against rotavirus (Rotarix, GlaxoSmithKline Biologicals, Belgium and RotaTeq, Merck & Co Inc, Whitehouse Station, NJ) were licensed for use in 2006, and as of October 2015, have been introduced into the national immunization programs of 79 countries worldwide.<sup>3</sup> Large clinical trials have shown that the vaccines are highly efficacious in developed countries,<sup>4,5</sup> and have significantly reduced the burden of rotavirus gastroenteritis in many high- and middle-income countries.<sup>6–12</sup> However, the vaccines remain less efficacious in developing countries, which have the greatest burden of disease.<sup>13–16</sup> A number of

factors contribute to the lower efficacy in these populations, including higher rates of malnutrition and tropical enteropathy, early infections, competing enteric infections at the time of vaccination, and interference by maternal antibodies.<sup>13</sup> Efforts are ongoing to improve the efficacy of existing vaccines and to develop more effective next-generation vaccines, and this is critical for decreasing the disease burden in highly affected populations.

Rotavirus is a member of the family *Reoviridae*. The viral genome consists of 11 segments of double-stranded RNA that code for 6 structural viral proteins (VP) and 6 nonstructural proteins.<sup>17</sup> The infectious virion is a triple-layered particle consisting of a core layer made of VP2, an intermediate layer made of VP6, and an outer shell made of glycoprotein VP7. Sixty protein spikes made of a protease-sensitive protein, VP4, extend from the VP7 shell (Figure 1, iv). Similar to the classification of influenza virus into H- and N- types based on the hemagglutinin and neuraminidase proteins, variability in the genes encoding

the 2 rotavirus outer capsid proteins (VP7 and VP4) form the basis of a dual-nomenclature system used to classify rotaviruses into G and P genotypes, respectively. To date, 27 G genotypes and 37 P genotypes (Figure 2, dendrogram) have been identified.<sup>18</sup> As many as 70 different combinations of G and P genotypes have been described in human infections, although a vast majority are caused by G genotypes G1, G2, G3, G4, and G9, in combination with P genotypes P[4], P[6], and P[8].

## Rotavirus Interacts With Cellular Glycans Through the VP8\* Domain of VP4

Rotavirus replicates primarily in differentiated epithelial cells at the tips of the small intestinal villi. In animal models of rotavirus infection, histopathologic changes range from no or slight lesions such as enterocyte vacuolization, to major changes such as villous blunting and crypt hyperplasia.<sup>17</sup> Pathogenesis is multifactorial. Rotavirus infection and a viral enterotoxin both alter intracellular calcium [ $\text{Ca}^{2+}$ ] signaling and stimulation of chloride [ $\text{Cl}^-$ ] secretion. Increased  $\text{Ca}^{2+}$  levels are thought to be central to stimulating  $\text{Cl}^-$  secretion.<sup>17</sup> Virus-mediated down-regulation of the expression of absorptive enzymes results in increased paracellular leakage through functional changes in the tight junctions between enterocytes.<sup>19,20</sup> Villous blunting also leads to decreased absorptive capacity. Furthermore, proliferation of crypt cells occurs during rotavirus infections, resulting in increased secretion. Secretion also is mediated by the activation of the enteric nervous system by the virus.<sup>17</sup> Recently, decreased sodium ( $\text{Na}^+$ ) absorption by inhibition of the ion transport protein sodium-hydrogen exchanger 3 (NHE3) activity also has been shown after rotavirus infection, suggesting another mechanism of diarrhea induction used by many bacterial pathogens.<sup>21</sup> In addition, rotavirus infection results in a decrease in brush-border enzymes, such as sucrase isomaltase, leading to an osmotic gradient that can contribute to diarrhea.<sup>22</sup> Villous ischemia and alterations in intestinal motility also have been reported in rotavirus infections,<sup>23</sup> with little or no evidence of inflammation.

The attachment of the virus to epithelial cells is a multistep process involving the interaction of viral proteins with cellular receptors such as cell surface glycans.<sup>24</sup> The spike protein VP4 is implicated in the initial attachment to host cell glycans. During rotavirus infection, proteolysis results in the cleavage of the spike protein VP4 (88 kilodaltons) into VP5\* (60 kilodaltons) and VP8\* (28 kilodaltons) fragments that remain noncovalently attached to the virion (Figure 1, iv, inset).<sup>17</sup> The binding to cellular glycans is mediated by the VP8\* domain of VP4, whereas VP5\* plays a role in host cell membrane penetration.<sup>25,26</sup>

NMR and x-ray crystallography studies of VP8\* from different rotavirus spike protein genotypes show that the protein exhibits a galectin-like fold with 2 twisted  $\beta$ -sheets separated by a shallow cleft (Figure 1, iv, inset).<sup>26–30</sup> Galectins are a family of proteins that bind  $\beta$ -galactoside-containing oligosaccharides. VP4 is proposed to be formed by the insertion of a host-derived, galectin-like carbohydrate

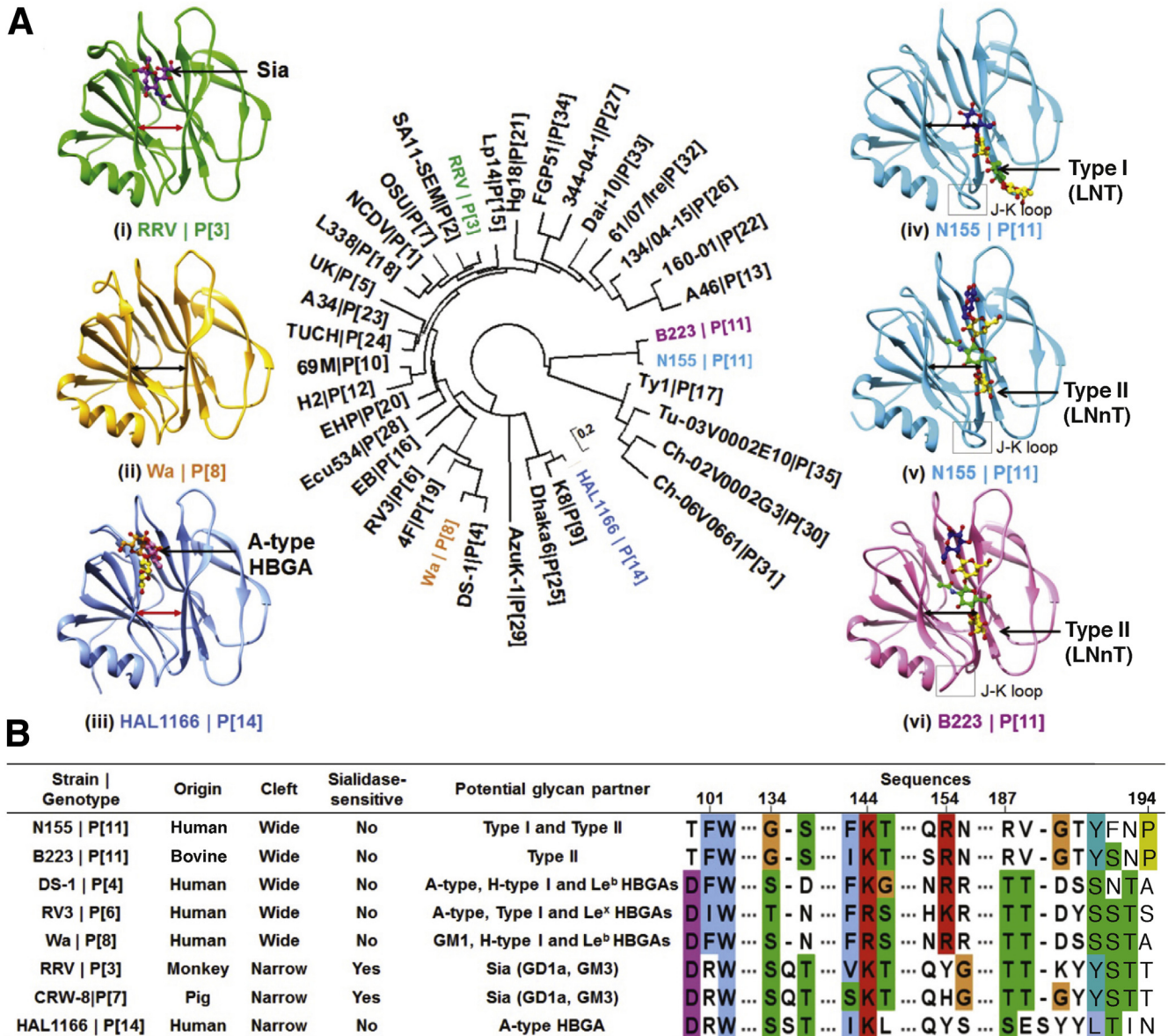
binding domain into a membrane interacting protein of the virus.<sup>26</sup> The insertion of a carbohydrate-binding domain in a membrane interaction protein has been described for other viral proteins such as the influenza hemagglutinin.<sup>31</sup> The crystal structures of VP8\* from 3 animal rotavirus strains, a rhesus rotavirus strain RRV, a porcine rotavirus strain CRW-8, and a bovine rotavirus strain B223, are determined and represent the genotypes P[3], P[7], and P[11], respectively.<sup>26,27,29</sup> The VP8\* structures of human rotaviruses Wa, DS1, HAL1166, and N155 representing the genotypes P[8], P[4], P[14], and P[11], respectively, also are known.<sup>27–30</sup> P[8] and P[4] are the most common VP4 genotypes described in children worldwide.<sup>32</sup> P[14] and P[11] rotaviruses are less common but both genotypes are highly interesting in that infections with these viruses appear to have resulted from interspecies transmission of animal rotaviruses into the human population.<sup>33–35</sup> P[11] infections in human beings are almost exclusive to neonates.<sup>36,37</sup> Although the galectin-like fold is conserved among all of these rotavirus VP8\* structures, there are differences in the width of the cleft between the 2  $\beta$ -sheets among some strains and this may play a role in the differences in their glycan binding profiles.<sup>38</sup>

## Sialic Acid as a Key Cellular Partner for Rotavirus VP8\*

For more than 30 years, sialic acid (Sia) was considered the key determinant of interactions for rotavirus VP8\*. This was based on early work showing that some rotavirus strains hemagglutinate red blood cells (RBCs) and that removal of terminal Sia residues by sialidase treatment of RBCs results in reduced virus binding.<sup>39–41</sup> Furthermore, the infectivity of some animal strains is sensitive to sialidase treatment, suggesting a role for Sia in virus attachment.<sup>42,43</sup> X-ray crystallography studies confirm this interaction. The crystal structure of the VP8\* of the sialidase-sensitive RRV strain in complex with the ligand shows Sia binding in an open-ended, shallow groove between the 2  $\beta$ -sheets (Figure 2A, i), distinct from the carbohydrate binding site in galectins.<sup>26</sup> The rim of the groove is formed by side chains of Y188 and S190 on one side and Y155 on the other side, and the floor of the groove is formed by R101, V144, K187, and Y189 side chains. Key Sia binding residues R101, Y189, and S190 are strongly conserved among other sialidase-sensitive rotavirus strains. A similar Sia binding groove is present in the VP8\* of a porcine P[7] rotavirus strain CRW-8 in complex with monosialodihexosylganglioside GM3.<sup>44</sup>

Although Sia binding is a common theme for the sialidase-sensitive animal rotavirus strains, there are variations in binding specificity to different members of the Sia family. The most common members of this family are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc), which differ by the presence of an additional hydroxyl group in Neu5Gc. RRV VP8\* shows greater affinity for Neu5Ac, and VP8\* from bovine strain NCDV, porcine strains OSU and CRW-8, and simian strain SA11 show greater specificity for Neu5Gc.<sup>45–47</sup> The binding





**Figure 2. Crystal structures of rotavirus VP8\*.** (A) Phylogenetic analysis of rotavirus VP4 types (*circular dendrogram*) was constructed by MEGA6 using the maximum likelihood method,<sup>75</sup> surrounded by the crystal structures of different VP8\*. The structures of VP8\* in complex with specific glycans are presented where known. (i) P[3] genotype, Rhesus rotavirus RRV in complex with Sia. (ii) P[8] genotype, human rotavirus Wa. Currently, there are no structures of P[8] VP8\* in complex with glycans. (iii) P[14] genotype, human rotavirus HAL1166 in complex with A-type HBGA in the same pocket as Sia binding in RRV. (iv) P[11] genotype, human neonatal rotavirus N155 in complex with type II tetrasaccharide LNT. The glycan binding occurs in a different site compared with RRV and HAL1166. (v) P[11] genotype, human neonatal rotavirus N155 in complex with type II tetrasaccharide LNnT. (vi) P[11] genotype, bovine rotavirus B223 in complex with type II tetrasaccharide LNnT. The difference in J-K loop (iv–vi, *black box*) between N155 and B223 contributes to the inability of B223 to bind type I glycans. (B) Structure-based sequence alignment of VP8\* from different rotavirus strains with residues colored with Clustal X shading using Jalview. Each residue in the alignment is assigned a color if the amino acid profile at that position meets a minimum criteria specific for that residue type.<sup>76</sup>

preference and affinity may be influenced by the amino acid residues at positions 187 and 157 of VP8\*, respectively.<sup>27,47</sup> The binding of VP8\* from many animal strains to Neu5Gc may be significant from an evolutionary perspective, given that Neu5Gc is expressed in mammalian tissues but not in normal human tissues. Binding to Neu5Gc

is not described for any human rotavirus strain and correlates with the evolutionary loss of Neu5Gc expression in human beings.<sup>48</sup>

The vast majority of strains causing human infections are insensitive to sialidase treatment.<sup>49</sup> For P genotypes that are detected in both human beings and animals, sialidase

sensitivity is VP4 genotype-specific and does not segregate by species of origin.<sup>50</sup> In the VP8\* of prevalent P genotypes (P[4], P[6], and P[8] genotypes), as well as that of some other sialidase-insensitive strains, the key Sia binding residue R101 is replaced by amino acids with hydrophobic side chains that would prevent the formation of hydrogen bonds with Sia (Figure 2B).<sup>26</sup> These results led to initial conclusions that Sia binding may not be an obligate requirement for all rotaviruses. However, NMR spectroscopy and molecular modeling of VP8\* from a sialidase-insensitive human rotavirus P[8] strain (Wa) show binding to gangliosides such as GM1 using internal Sia residues.<sup>51</sup> Infectivity assays on cells treated with sialidases showed a marginal increase in Wa titer, further suggesting a role for internal glycan residues as receptors for this virus strain. Of note, the cleft between the 2  $\beta$ -sheets in the VP8\* of the globally dominant human rotavirus P[4] and P[8] strains is considerably wider (Figure 2A, ii, and B) than the cleft in Sia binding animal rotavirus strains.<sup>38</sup> Although no crystal structures are available for these human genotypes in complex with glycans, NMR and modeling studies propose that a wider cleft would allow for binding of gangliosides such as GM1 using internal Sia residues.<sup>51</sup>

Taken together, these findings led to the paradigm that sialidase-sensitive strains with a narrow cleft recognize glycans such as GD1 with terminal Sia while the sialidase-insensitive strains possessing a wider cleft bind gangliosides such as GM1 that contain internal Sia residues.<sup>51</sup> The former group comprises predominantly animal rotavirus strains while the majority of human strains fall in the latter group. However, recent studies have shown that although recognition of sialoglycans is necessary for many animal rotaviruses, Sia binding may not be an obligatory requirement for all strains. Human rotavirus strains can bind nonsialylated glycans, showing genotype-dependent variations in glycan binding that may be critical for multiple aspects of virus pathogenesis.<sup>28,29,52–57</sup>

## VP8\* of Human Rotavirus Strains Bind Histo-Blood Group Antigens

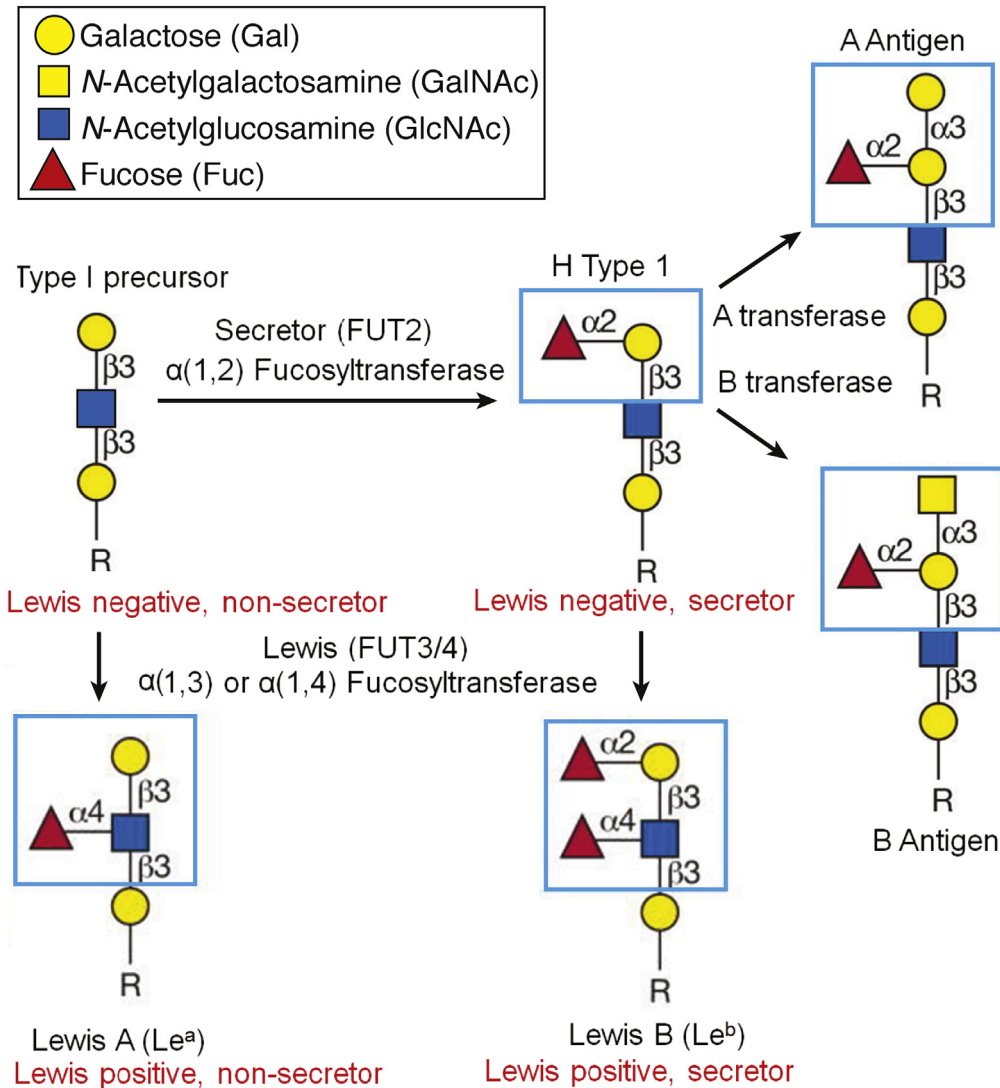
The discovery that the VP8\* of some human rotavirus strains bind HBGAs brought a paradigm shift in our understanding of rotavirus interactions with cellular glycans.<sup>28,29,52–57</sup> HBGAs are nonsialylated glycoconjugates expressed on the surface of RBCs and epithelial cells, and are present in mucosal secretions.<sup>58</sup> HBGAs are cellular receptors for a number of gastrointestinal pathogens such as noroviruses, *Helicobacter pylori*, and *Campylobacter jejuni*.<sup>59–61</sup> HBGA synthesis occurs by the sequential addition of monosaccharides to precursor disaccharides by genetically coded glycosyltransferase enzymes such as the A and B transferases (*ABH* genes), fucosyltransferase-2 (FUT2, *secretor* gene) and FUT-3/4 (*Lewis* gene). Thus, an individual's HBGA expression profile is genetically determined based on his/her ABH, Secretor, and Lewis genotypes. Precursor disaccharides contain the sugars galactose and N-acetylglucosamine (GlcNAc), linked by a  $\beta$ 1-3 or  $\beta$ 1-4 linkage. Based on the linkages, the glycans are categorized

as type I and type II, with type I sugars containing the  $\beta$ 1-3 linkage and type II containing the  $\beta$ 1-4 linkage, respectively. The biosynthesis of H-type I HBGA (Figure 3) involves the addition of a fucose residue in the  $\alpha$ 1,2 position to the terminal galactose of the type I precursor by the enzyme FUT2. The modification of H antigens by A and B transferases leads to the generation of the A or B antigens of the ABO system, respectively. Lewis antigens are synthesized by the addition of a fucose residue in the  $\alpha$ 1,3 or  $\alpha$ 1,4 position to the terminal GlcNAc of the precursor structures or H-type HBGA; addition to the precursor disaccharide results in the generation of Lewis-a ( $Le^a$ ), whereas the addition to the terminal GlcNAc of the H-type I HBGA leads to the generation of Lewis-b ( $Le^b$ ). Type II HBGA synthesis occurs on the type II precursor backbone, and similarly leads to the generation of H-type II, Lewis-x ( $Le^x$ ), and Lewis-y ( $Le^y$ ) antigens. Specific mutations in FUT2 and FUT3 genes render them nonfunctional and individuals with these mutations are referred to as secretor-negative and Lewis-negative, respectively. Major findings on rotavirus–HBGA interactions are discussed in the following subsections.

### Discovery of VP8\*-HBGA Interactions: P[14] VP8\* Binds A-Type HBGA

The first evidence for human rotavirus interaction with HBGA came through studies with a P[14] rotavirus strain HAL1166.<sup>28</sup> Human infections with P[14] viruses are thought to have resulted from interspecies transmission of P [14] strains from even-toed ungulates such as sheep and goats to human beings.<sup>34</sup> The x-ray crystallographic structure of the VP8\* of HAL1166 shows that the cleft in this strain is narrower than the VP8\* cleft in other human rotavirus strains such as P[4] and P[8], and is more similar to the narrow cleft seen in Sia binding animal strains. Despite this similarity, structural changes in the cleft region are not compatible with Sia binding. A high-throughput glycan array screen comprising 511 sialylated and nonsialylated glycans unexpectedly identified A-type HBGA as a partner for P[14] VP8\*. Specific binding to glycans with terminal residues characteristic of A-type HBGA was seen, although there was no binding to sialylated glycans with either internal or terminal Sia. Structural studies show that binding to A-type HBGA occurs in the same pocket where Sia binds RRV VP8\* (Figure 2A, i and iii). The binding of P [14] VP8\* to A-type HBGA is biologically relevant as seen by hemagglutination and infectivity assays. For example, the infectivity of HAL1166 is enhanced significantly in Chinese hamster ovary cells expressing A-type HBGA and infectivity is blocked by treatment with anti-A-type antibodies. P[14] infections in A–blood type individuals have been reported, but more epidemiologic data are needed to validate the clinical significance of this finding.

Binding to A-type HBGA also was seen for VP8\* from P [9] and P[25] rotaviruses, which are highly homologous to P [14] VP8\* in the glycan-binding domain.<sup>28,55</sup> Hemagglutination, saliva binding, and infectivity assays support the biological significance of this interaction while homology modeling and mutagenesis show that the carbohydrate



**Figure 3. Biosynthesis of type I HBGA.** The type I precursor contains the sugars Gal and GlcNAc linked by a  $\beta$ 1-3 linkage. The biosynthesis of H-type I HBGA involves the addition of a fucose residue in the  $\alpha$ 1,2 position to the terminal Gal of the type I precursor by the enzyme FUT2 (secretor gene). The modification of H antigens by A- and B-glycosyl transferases leads to the generation of the A or B antigens, respectively. Le<sup>a</sup> antigen is synthesized by the addition of a fucose residue in the  $\alpha$ 1,3 or  $\alpha$ 1,4 position to the terminal GlcNAc of the type I precursor by the enzyme FUT-3/4 (Lewis gene). The addition of a fucose residue in the  $\alpha$ 1,3 or  $\alpha$ 1,4 position by these enzymes to H-type HBGA leads to the generation of Le<sup>p</sup>. Persons who lack functional FUT2 cannot express fucose at the  $\alpha$ 1,2 position and are referred to as *nonsecretors*.

binding interface is shared with the Sia-binding animal rotaviruses through different amino acid residues.<sup>28,55</sup>

### Binding of Neonate-Specific P[11] VP8\* to Precursor Glycans: Basis of Age Restriction

Clinically and epidemiologically, rotavirus infections in newborns are distinct from infections in older children.<sup>62,63</sup> The majority of infections in neonates are asymptomatic, although association with severe gastrointestinal symptoms such as necrotizing enterocolitis and feed intolerance also have been described. Rotavirus infections in neonates are caused by remarkably stable, unusual virus strains that appear to be geographically restricted. Strains belonging to the P[11] genotype (in combination with either G9 or G10 VP7 genotype) are associated with high rates of neonatal infections in India.<sup>37,64</sup> Very few cases of G9P[11] or G10P[11] infections are reported beyond the neonatal period. The basis of the predilection of these viruses for newborns

was unclear until the discovery of HAL1166 VP8\* binding to A-type HBGA prompted studies to identify glycan receptors for neonatal P[11] viruses.<sup>54,56</sup>

Glycan screens for P[11] VP8\* using arrays of cellular and human milk glycans<sup>54,56,57</sup> show that the VP8\* of a neonatal P[11] virus strain N155 recognizes type I and type II precursor glycans (also referred to as lacto-N-biose and N-acetylglucosamine [LacNAc], respectively). The synthesis of precursors is constitutive in many cell types, and the addition of branches and terminal sugars is a developmentally regulated process that occurs in the postnatal period. The binding of P[11] VP8\* to precursor glycans may explain the predilection of these strains for newborns. With growth and development, the P[11] viruses are likely unable to bind modified glycans in the gut and therefore unable to infect children in the older age group. This is supported by biological relevance of P[11] VP8\* glycan binding: P[11] VP8\* binds saliva from infants but not adults,<sup>54</sup> hemagglutination occurs with cord blood RBCs, and infectivity of P[11] viruses



is increased in the presence of HBGAs.<sup>56</sup> Furthermore, the infectivity of a G9P[11] virus is reduced in the presence of poly-LacNAc, human milk, and infant saliva containing LacNAc, suggesting that soluble glycans in human milk can possibly act as decoy receptors for the P[11] VP8\* and prevent binding to intestinal LacNAc glycans.<sup>54</sup>

The crystal structures of N155 VP8\* in complex with a type I tetrasaccharide lacto-*N*-tetraose (LNT) and type II tetrasaccharide lacto-*N*-neotetraose (LNnT) show that there are additional glycan binding sites in rotavirus VP8\* (Figure 2A, iv and v).<sup>29</sup> Binding to LNT and LNnT in P[11] VP8\* occurs in a previously uncharacterized binding pocket, distinct from the Sia and A-type HBGA binding sites in animal and P[14] rotaviruses, respectively. The binding cleft in P[11] VP8\* is noticeably wider and is more similar to the cleft observed in the globally dominant P[4], P[6], and P[8] VP8\* despite sequence differences. Although the crystal structures of these common genotypes in complex with glycans are not yet described, it is tantalizing to speculate that the glycan-binding site in these strains with a wider cleft overlaps with the newly identified glycan-binding site discovered in P[11] VP8\*, with specific sequence changes that correspond to their glycan specificity. Galectin-3 is also known to bind LNT and LNnT, and the crystal structure of galectin-3 in complex with these glycans show that the ligand adopts a linear conformation similar to that observed in the P[11] VP8\*.<sup>65</sup> This leads to new questions on whether these glycans can engage both galectins and VP8\*, and whether galectins play a role in rotavirus entry into cells.

### *VP8\*–HBGA Interactions as a Basis for Interspecies Transmission*

The findings with P[14] and P[11] VP8\*s binding to HBGA suggest a role for VP8\*–HBGA interactions as a basis for interspecies transmission of rotavirus strains. The HAL1166 strain was isolated from a child in Finland<sup>66</sup>; however, this strain has its origins in even-toed ungulates.<sup>34</sup> The VP8\* of HAL1166 has a narrow cleft similar to Sia-binding animal rotaviruses, but A-type HBGA binds at the same location as Sia in the animal strains.<sup>28</sup> This difference in binding is brought about by subtle changes in the structural framework of P[14] VP8\*. Although the key Sia binding residue R101 is positionally conserved, there are differences in side-chain orientations, resulting in steric hindrance to Sia binding. An insertion in position 187 in HAL1166 allows for specific interaction with A-type HBGA (Figure 2B). Thus, comparisons of the complex structures of Sia binding animal rotavirus VP8\* and human P[14] VP8\* show how subtle sequence changes within the glycan-binding region results in the switch in specificity from Sia to A-type HBGA, which likely facilitates the interspecies transmission of this virus from ungulates to individuals with A-type HBGA expression.

The binding of the P[11] VP8\* to type I and type II precursor glycans is another striking example of interspecies transmission. The human neonatal P[11] viruses are naturally occurring bovine–human reassortant strains; these viruses have some genes of human rotavirus origin but others that have originated from bovine rotavirus

strains.<sup>33,35</sup> In particular, the spike protein VP4 is of bovine rotavirus origin. Phylogenetic analysis of the neonatal P[11] virus shows that the VP4 gene in these strains shows approximately 86% amino acid identity to P[11] viruses isolated from cattle.<sup>33,35</sup> A bovine G10P[11] strain B223 shows hemagglutination properties similar to the human neonatal P[11] virus, and infectivity is enhanced with expression of H-type HBGA in Chinese hamster ovary cells.<sup>54,56</sup> Despite these similarities, there are differences in the glycan binding profiles between the bovine and human P[11] VP8\* on a shotgun milk glycan array. Although the human neonatal P[11] VP8\* binds both type I and type II glycans, the bovine P[11] VP8\* only binds to type II glycans (Figure 2A, vi).<sup>57</sup> This difference is mediated by small changes in certain loop regions such as the J–K loop in the bovine VP8\*; the loop projects away in the bovine P[11] VP8\* compared with the human P[11] VP8\* and therefore cannot interact with the terminal sugars of type I glycans such as LNT (Figure 2A, iv–vi).<sup>29</sup> Although little is known about differences in glycan expression between the bovine and human gut, there is evidence to suggest that bovine milk contains an abundance of type II glycans whereas human milk contains type I and type II glycans.<sup>67</sup> Thus, the bovine strain may have achieved specificity for type II glycans in the bovine microenvironment, and through a few sequence changes, evolved to cross the species barrier by recognizing both type II and type I glycans present in human beings. This ability to bind a broader spectrum of glycans may be the basis for the zoonotic transmission of this virus from cattle to human beings.

### *Expanded Spectrum of Glycan Binding as the Basis for Global Prevalence of Some Strains*

P[14] and P[11] VP8\* provide extensive insights into the interaction of human rotaviruses with HBGA; however, much less is known about P[4], P[6], and P[8], which represent the 3 most prevalent VP4 genotypes worldwide. The crystal structures of P[4] and P[8] VP8\* show that these genotypes possess a wide cleft that correlates with an amino acid deletion at position 135, and a significant change from arginine to phenylalanine at position 101 (Figure 2B).<sup>29</sup> The structure of P[6] VP8\* remains to be solved, but this genotype also shows sequence changes associated with a wider cleft. Glycan binding assays using a panel of synthetic oligosaccharides show that P[4] VP8\* from strain DS1 and P[8] VP8\* from strain Wa bind Le<sup>b</sup> and H-type I HBGA, whereas VP8\* from a neonatal P[6] strain ST-3 binds H-type I (Figure 2B).<sup>53</sup> The binding to a type I terminal residue and an internal Le<sup>x</sup> determinant also is described for another P[6] neonatal strain RV3.<sup>57,68</sup> However, contradicting the enzyme-linked immunosorbent assay results, no interaction between Le<sup>b</sup> or H-type I HBGA with VP8\* from DS-1 or Wa was seen in saturation transfer difference NMR spectroscopy.<sup>52</sup> The saturation transfer difference NMR data suggest that P[4] VP8\* from DS1 and P[6] VP8\* from RV3 interact with A-type HBGA, leading to a conclusion that A-type HBGAs are receptors for human rotaviruses. It is hypothesized that strains show variability in the ways they

recognize A-type HBGA based on findings that P[14] and P[9] VP8\* interact with A-type HBGA predominantly through the N-acetylgalactosamine residue, whereas P[4] and P[8] interact using the fucose moiety. The discrepancy in results obtained from the binding studies and NMR using VP8\* from the same strains (DS-1, Wa, and ST3) remains to be resolved and validated through crystallography and epidemiologic studies.

## Genetic Basis of Strain Susceptibility and Vaccine Response

The expression of glycan partners for the prevalent rotavirus strains, H-type HBGA and Lewis antigens, are determined genetically; these findings stimulated several epidemiologic studies to determine if host genetics plays a role in susceptibility to specific rotavirus genotypes. Most studies focus on the genetic susceptibility to P[8] VP4 that binds H-type HBGA present only in secretor-positive individuals. The P[8] genotype not only represents one of the most prevalent VP4 types worldwide but is also a component of the Rotarix and RotaTeq vaccines. In a retrospective analysis of secretor status and rotavirus infection in 56 French children, a secretor-negative genotype was associated with resistance to symptomatic rotavirus infection.<sup>69</sup> Another study involving 85 rotavirus-positive children in Vietnam infected predominantly with a P[8] strain found all children were HBGA secretors or partial secretors.<sup>70</sup> A large study in the United States involving 1564 cases with gastroenteritis and 818 healthy controls found only 1 of 189 rotavirus-positive patients was a nonsecretor, compared with 23% in the control group. Overall, these data indicate that nonsecretors are protected from rotavirus infection.<sup>71</sup> However, it is possible that there are population-specific differences because contradictory results were seen in Tunisia, where, of 32 rotavirus-positive children, P[8] infections were detected in both secretors and nonsecretors.<sup>72</sup>

There is less clarity on the significance of Lewis status in susceptibility to rotavirus. Both secretor and Lewis antigen status are suggested to mediate susceptibility to rotavirus in a genotype-dependent manner in Burkina Faso and Nicaragua.<sup>73</sup> In these populations, P[8] infections were seen exclusively in Lewis- and secretor-positive children, but P[6] infections were seen in Lewis-negative children, independent of their secretor status. This correlates with the *in vitro* data on P[8] binding to H-type I and Le<sup>b</sup>, and with P[6] binding to H-type I and type I precursor in the glycan binding studies.<sup>53,57</sup> The comparatively higher prevalence of Lewis-negative phenotype in Africa may explain the higher prevalence of P[6] infections in this population. This raises important questions on the role of host genetic status in susceptibility to vaccine strains and protection from vaccination. Because Lewis-negative children are resistant to P[8] strains, they may be less susceptible to the vaccine strains but continue to be susceptible to infections with P[6] viruses. This provocative observation may explain the reduced vaccine efficacy in these populations. However, because this observation was based on a small number of children with

P[6] and P[8] infections in one region of Africa, the hypothesis remains to be validated in larger cohorts.

Advancements in *in vitro* culture techniques now allow us to mechanistically explore these findings alongside translational studies. For example, rotavirus infections in human intestinal enteroid (HIEs) models can assess secretor status-based differences in the growth of different rotavirus strains.<sup>74</sup> HIEs are a novel, genetically diverse, physiologically relevant model of human gut that show similar cellular and functional properties to the human gastrointestinal epithelium. Comparisons of the infectivity of P[8] strain Wa with the P[8] vaccine Rotarix indicated that the growth of Rotarix is attenuated in HIEs from different secretor-positive individuals when compared with Wa. Furthermore, Rotarix infection was attenuated consistently in an HIE from one secretor-negative individual. This novel *in vitro* model to study vaccine virus attenuation suggests that there is variability between individuals in susceptibility to rotavirus strains.

## Summary and Future Directions

The discovery of spike protein genotype-dependent variations in glycan binding for rotavirus strains has resulted in a new understanding of rotavirus host restriction, zoonotic transmission, and susceptibility to specific rotavirus strains. The role of host genetics in vaccine response, particularly in the context of the susceptibility of the population to different strains, is an important new consideration. Developmental regulation of glycan expression is another important consideration for age at the time of vaccination with specific strains. There is potential for the development of therapeutics through the use of soluble glycan decoys to prevent the binding of rotaviruses to cell surface glycans. These are exciting findings that open up new avenues for research on rotavirus interactions with host glycans, and can bridge the gap between the bench and bedside. Testing HIE cultures from individuals showing genetic differences in glycan expression will allow the exploration of the role of host genetics in susceptibility to rotaviruses. At this point, the clinical relevance of rotavirus-host glycan interactions is limited because there are few translational studies. Epidemiologic studies in many different populations may be required to fully understand the impact of these discoveries. An important lesson from the work on rotavirus-glycan interactions is that paradigms derived from laboratory or animal virus strains may not necessarily hold true for clinically relevant human virus strains and that applying transformative techniques from many different fields can lead to new lessons in the pathogenesis of gastrointestinal infections.

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- Conflicts of interest**  
The authors disclose no conflicts.