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MINIREVIEW

Role of sialic acids in rotavirus infection

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Abstract Rotaviruses are the leading cause of childhood diarrhea. The entry of rotaviruses into the host cell is a complex process that includes several interactions of the outer layer proteins of the virus with different cell surface molecules. The fact that neuraminidase treatment of the cells, or preincubation of the virus with sialic acid-containing compounds decrease the infectivity of some rotavirus strains, suggested that these viruses interact with sialic acid on the cell surface. The infectivity of some other rotavirus strains is not affected by neuraminidase treatment of the cells, and therefore they are considered neuraminidase-resistant. However, the current evidence suggests that even these neuraminidase-resistant strains might interact with sialic acids located in context different from that of the sialic acids used by the neuraminidase-sensitive strains. This review summarizes our current knowledge of the rotavirus-sialic acid interaction, its structural basis, the specificity with which distinct rotavirus isolates interact with sialic acid-containing compounds, and also the potential use of these compounds as therapeutic agents.

Keywords Rotavirus · Sialic acid · Ganglioside · Glycolipid · Glycoconjugate

Introduction

Diarrhea is among the most common illnesses of infants and young children throughout the world, with an estimated number of episodes of about 1.4 billion occurring each year in children under 5 years of age [1]. It was originally believed that bacteria were the most important cause of diarrhea, and it was not until the late nineteen forties that a virus was associated, for the first time, with diarrhea in infant mice [2]; this virus was later identified as rotavirus, strain EDIM [3]. Also, although these findings were not seen as significant at the time, the experimental production of diarrhea by virus particles in bacteria-free fecal filtrates from calves changed that opinion [4]. Soon afterwards, viral particles were observed in biopsies from duodenal mucosa of children with severe non-bacterial diarrhea [5]. These particles have since come to be recognized as rotavirus, the leading etiological agent of severe diarrheal disease in infants worldwide, being responsible for an estimated 500,000 deaths each year [1], mainly in developing countries. In developed countries they are an important cause of childhood morbidity, accounting only in United States for 55,000 hospitalizations and 500,000 physician visits, that cost in excess of US $1 billion annually [6]. It is estimated that by the age of 5 nearly every child will have an episode of rotavirus gastroenteritis, 1 in 5 will visit a clinic, and 1 in 65 will be hospitalized [1]. Rotaviruses also infect other mammalian and avian species, resulting in important economical losses due to diarrhea in calf, pig, sheep, and poultry rearing.

Rotaviruses are large non-enveloped viruses, consisting of three concentric layers of protein which surround the viral genome composed of 11 segments of double-stranded RNA (dsRNA). The innermost layer is formed by the VP2 protein, which encloses the viral genome and two virus encoded enzymes (VP1, the viral RNA polymerase, and VP3, a guanylyltransferase), forming the core of the virus. The middle layer is formed by the most abundant protein of the virus, VP6, which sits on top of the VP2 layer to form

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double-layered particles (DLP), which are non-infectious. The outermost layer, characteristic of the complete, infectious triple-layered particle (TLP), is composed of two proteins, VP7 and VP4. VP7 is a calcium-binding glycoprotein that forms the smooth surface of the virion, while VP4 forms the spikes that extend from the surface of the viral particle [7]. Both of these proteins have essential functions in the virus life cycle, including receptor binding and cell penetration [8] and, thus, represent important targets of neutralizing antibodies. Rotavirus infectivity is increased by, and most probably depends on, trypsin treatment of the virus. This proteolytic treatment, which results in the specific cleavage of VP4 (776 amino acids) to polypeptides VP8 (amino acids 1–231) and VP5 (amino acids 248-776) [9–11], is not needed for the virus to attach to the cell surface [12], but for the virion to penetrate into the cells' interior [13]. The mechanism through which trypsin enhances virus penetration is not yet understood.

The initial step in a viral infection consists in binding of the virus to the surface of the host cell, followed by penetration of the virus particle into the cytoplasm. These events depend on the recognition of specific receptors on the cell surface by the virus; consequently, receptors are important determinants of viral tropism and pathogenesis. *In vivo* rotaviruses have a specific cell tropism, infecting primarily the mature enterocytes of the villi of the small intestine, suggesting that these cells contain specific receptor for the virus. However, recent reports of extra-intestinal spread of rotavirus during infection, indicate a wider host tissue range than previously thought [14]. *In vitro*, rotaviruses bind to a wide variety of cell lines, however only some of these, including cell lines from renal or intestinal origin and some transformed cell lines of breast, stomach, bone, or lung origin, are efficiently infected [15]. This observation suggests that the initial binding of rotavirus to the cell surface is promiscuous, and that the subsequent interaction with specific post-attachment receptors is responsible for the entry of the virus into the cell.

Soon after their discovery, it was found that rotaviruses were able to agglutinate human type O erythrocytes [16], and in a short period of time this observation was extended to several other rotavirus strains of animal and human origin [17–20]. However, despite the original reports of hemagglutination by human rotavirus strains [19,20], these observations were not confirmed, and further studies showed that human rotavirus isolates were only able to agglutinate fixed, one-day old chicken erythrocytes, and that this activity was abolished by trypsin treatment of the viruses [21]. At present, human rotavirus isolates are considered in general, as non-hemagglutinating. The agglutination of erythrocytes by animal rotavirus strains is associated with infectious TLP [18], and is independent of the trypsin treatment of the virus [22]. Several lines of evidence have shown that the interaction of rotaviruses with red blood cells is mediated, as in the case of many other viruses, including influenza A virus, reovirus type 3, various coronaviruses and Sendai virus, among others, by N-acetylneuraminic acid (sialic acid, Sia) containing compounds. Treatment of erythrocytes with sialidases and/or periodate oxidation prevents their agglutination by rotaviruses, as does preincubation of the virus with the highly sialylated protein glycophorin A [21-24].

The hemagglutination activity of some rotavirus strains led to the idea that Sias were also involved in the interaction of the virus with the cell surface and, indeed, it was found that the cell binding and infectivity of some animal rotavirus strains required the presence of Sia on the cell surface, since these events were greatly diminished by treatment of cells with neuraminidases (NA) from different origins, including those isolated from *Arthrobacter ureafaciens* and *Vibrio cholerae* [24,25]. As expected, the rotavirus isolates having hemagglutination activity were those whose infectivity was decreased by NA treatment of the cells. These types of rotavirus strains were initially called Sia-dependent, however, more recently it has been recognized that a more appropriate term is NA-sensitive, since there are Sia moieties in oligosaccharide structures, like those present in ganglioside GM1, that are either less sensitive or not sensitive at all to treatment with neuraminidases [26], and which could play a role in rotavirus cell attachment. Although it was initially considered that most rotavirus strains of animal, non human origin were NA-sensitive, it was recently shown that the infectivity of many strains of animal origin, as well as that of most rotavirus strains isolated from humans is not affected by treatment of the cells with NA, and they are therefore classified as NA-resistant [27–29]. However, this does not mean that these strains do not need Sia for cell attachment. As will be discussed later, the importance of Sias in the infectivity of the NA-resistant rotavirus isolates is still to be defined. The ability of different rotavirus strains to infect cells treated with NA, and their hemagglutination activity, are summarized in Table 1. It is known that the initial interaction of NA-sensitive rotaviruses with Sias is generally followed by specific interactions with several other cell surface molecules, including integrins $\alpha 2\beta 1$, $\alpha v\beta 3$, $\alpha x\beta 2$, and the heat shock cognate protein hsc70. The role of these post binding interactions in rotavirus cell infection has been reviewed recently [8], and will not be discussed here any further.

The aim of this review is to summarize the current knowledge of rotavirus—sialic acid interactions, with emphasis on the latest structural characterization of this interaction, and on the description of the specificity of rotavirus isolates to bind different Sia—containing compounds. The use of Sia-containing molecules as potential therapeutic agents to control rotavirus infection is also discussed.

Table 1 Summary of hemagglutination activity (HA) and sensitivity to neuraminidase (NA) of different rotavirus strains

Virus strain	Origin	*G* serotype	*P* serotype[a]	HA	NA	Reference
NCDV,C486 RF, BRV033	bovine	6	6[1]	+	+	[28–31]
SA11	simian	3	5B[2]	+	+	[18,22,28]
HCR3	human	3	5A[3]	NT	+	[28]
Ro1845	human	3	5A[3]	+	NT	[32]
RRV	simian	3	5A[3]	+	+	[33,24]
C U-1, K9	canine	3	5A[3]	+	+	[28,33,34]
FRV64,	feline	3	5A[3]	+	+	[26,34]
Cat97	feline	3	5A[3]	+	+	[28,34]
GRV	caprine	3	[3]	+	NT	[35]
DS-1, S2, KUN	human	2	1B[4]	–	–	[21,28,26,34]
L26	human	12	1B[4]	NT	–	[28]
UK, WC3, B-641	bovine	6	7[5]	–	–	[28,31]
678	bovine	8	7[5]	NT	–	[28]
McN13	human	2	2A[6]	NT	–	[28]
ST3	human	4	2A[6]	NT	–	[27,38]
Gottfried	porcine	4	2B[6]	+	–	[27,61]
C95	porcine	1	9[7]	NT	+	[28]
CRW8, A131 A138, A411	porcine	3	9[7]	NT	+	[28]
SB-1A	porcine	4	9[7]	+	+	[28,37]
OSU, C134, TFR-41, EE	porcine	5	9[7]	+	+	[28,37,38]
H1	equine	5	9[7]	+	+	[28,38]
YM, A253	porcine	11	9[7]	+	+	[28,39]
Wa, KU, D, M37, 1076	human	1	1A[8]	–	–	[28,29]
MO, YO, Ito, Nemoto	human	3	1A[8]	–	–	[21,28,34]
VA70, Hochi, Hosokawa	human	4	1A[8]	NT	–	[28]
W161	human	9	1A[8]	NT	–	[28]
K8	human	1	3[9]	NT[b]	–	[28]
O264, AU-1	human	3	3[9]	–	–	[28,34]
Cat2	feline	3	3[9]	–	–	[28,34]
69M	human	8	4[10]	NT	–	[28]
B223	bovine	10	8[11]	NT	–	[28]
I321	human	10	8[11]	NT	–	[28]
H2, FI14	equine	3	4[12]	–	–	[28,34,40]
FI-23, FR5	equine	14	4[12]	NT	–	[28]
A46	porcine	5	13[13]	NT	–	[28]
Ala, C-11, BAP-2, R-2	lapine	3	11[14]	–	–	[28,35]
PA169	human	6	11[14]	NT	–	[27]
HAL1166	human	8	11[14]	NT	–	[28]
Mc35	human	10	11[14]	NT	–	[28]
Lp14	ovine	10	[15]	NT	–	[28]
EDIM, EW, EC, EB	murine	3	10[16]	–	+/–[c]	[28]
Ch-1	chicken	7	[17]	NT	+	[41]
TY-1, TY-3	turkey	7	[17]	NT	+	[41]
PO-13	pidgeon	7	[17]	NT	+	[41]
L338	equine	13	12[18]	+	+/–	[28]
4F	porcine	3	[19]	NT	–	[28]
EHP	murine	3	[20]	–	+/–	[29,36]

[a] *P* serotype, when known, precedes *P* genotype in brackets [].
[b] NT–not tested.
[c] Partial inhibition of the infectivity.

Characterization of the rotavirus protein that interacts with sialic acids

In 1983, Greenberg *et al.* [42] isolated a panel of monoclonal antibodies directed to the surface proteins VP4 and VP7 of the simian rotavirus strain RRV. These antibodies were neutralizing, and some of them, directed to either VP4 or VP7, had the ability to inhibit the virus hemagglutination activity. To determine the protein responsible for this activity, Kalica and coworkers [43] generated a set of reassortant viruses (gene reassortment is a property of viruses with segmented genomes that has been very useful to identify the function of the viral proteins) by coinfecting a cell with the HA positive (HA+) simian strain RRV, and the HA negative (HA−) bovine strain UK. They found that the HA activity segregated with rotavirus gene 4, later shown to code for the spike protein VP4. The ability of some monoclonal antibodies directed to VP7 to inhibit the hemagglutination activity of the virus was considered the result from steric hindrance of these antibodies preventing the interaction of VP4 with red blood cells. A more direct proof showing that VP4 was responsible for the HA activity of the virus was the finding that a recombinant rotavirus VP4 protein, produced in insect cells, was able to agglutinate erythrocytes [44].

As previously mentioned, the VP4 protein is cleaved by trypsin to produce two subunits, VP8 and VP5, both of which remain associated to the virus particle (Fig. 1). In 1991, two groups used similar approaches to identify which of the two subunits of VP4 was responsible for the HA activity of the virus. Lizano *et al.* [45] expressed in bacteria the amino—terminal half of VP4 (containing VP8) as fusion protein with 98 amino acids of the MS2 phage polymerase. Alternatively, Fiore *et al.* [46] produced the VP8 protein in insect cells using a recombinant baculovirus. In both cases, the expressed proteins were shown to contain HA activity, narrowing the functional HA domain to this VP4 subunit. To further characterize the region containing the Sia binding site, recombinant baculoviruses that direct the expression of chimeric VP4 proteins between the HA+ porcine strain YM, and the HA- human strain KU, were constructed. The chimeric proteins expressed in insect cells were tested for their ability to hemagglutinate. Using this approach it was found that the region between amino acids 93 and 208 of VP8 contained the domain necessary to agglutinate red blood cells [39].

More recently, to narrow down the amino acid residues involved in the binding of Sia, alanine mutagenesis of selected amino acids of a hemagglutinating recombinant rotavirus RRV VP8 protein expressed in bacteria, was performed [47]. Of 15 mutants analyzed, only three (with mutations in tyrosines 155 and 188, and in serine 190) led to loss of the Sia dependent HA activity of the protein without affecting its overall conformation, suggesting these amino acids played an important role in the interaction with Sia. The fact that these three amino acid residues were predicted to map to loops separating β-sheets also supported their involvement in Sia binding [47].

Our understanding of the VP8-Sia interaction was further clarified by the nuclear magnetic resonance spectroscopy of the HA domain of rotavirus RRV complexed with sialosides [48]. The protease resistant VP8 core (consisting of amino acids 46–231) was shown to bind α-anomeric Sia with a K_d of 1.2 mM, and not to require additional carbohydrate moieties [48]. In addition, VP8 was shown not to distinguish $3'$ from $6'$ sialyllactose and to have approximately tenfold higher affinity for N-acetylneuraminic acid (NeuAc) than for N-glycolylneuraminic acid (NeuGc).

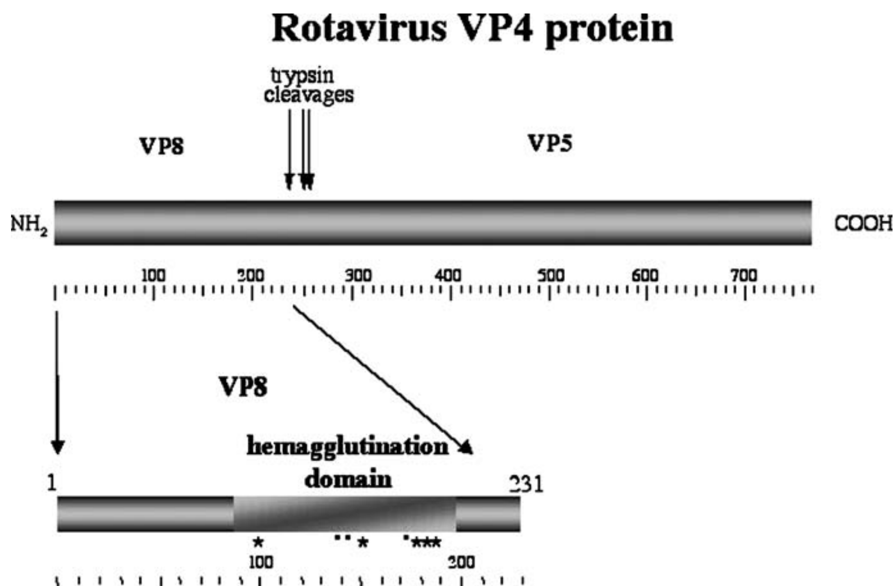

Fig. 1 Structural features of the VP8 protein. The VP4 protein of rhesus rotavirus strain RRV is 776 amino acids long, and is cleaved by trypsin at arginines 231, 241, and 247, to produce two final products, VP8 (amino acids 1–231) and VP5 (amino acids 248–776). The trypsin cleavage sites are represented by arrows. The VP8 hemagglutination domain (amino acids 93 to 208) is shown. Critical amino acid residues for binding sialic acids are marked by asterisks (amino acids 101, 155, and 188–190). Amino acids 144, 146 and 187, which also make contacts with sialic acid, are marked by dots.

Table 2 Sequence alignment of the VP4 regions containing the amino acids involved in binding to sialic acids

Rotavirus strain	Phenotype	101 ↓	144 ↓ 155 ↓	188 190 ↓↓↓
RRV	HA +	...NNTD**R**WLAT...	...FID**V**VKSTQNGSYSQ**Y**GPLQSTPKL...	...ETPNVTTK--**YYS**TTNYDSVN...
SA11	HA +	...NNTD**R**WLAT...	...FID**V**VKTTANGSIGQ**Y**GSLLSSPKL...	...QTPNARTG--**HYS**TTNYDSVN...
OSU	HA +	...NNTD**R**WLAT...	...FID**V**STTTPTGSYTQ**H**GPLFSTPKL...	...TTPNATTG--**YYS**ATNYDTVN...
YM	HA +	...NNTD**R**WLAT...	...FID**V**SKTTLTGNYTQ**H**GPLFSTPKL...	...TTPNATTG--**YYS**TTNYDTVN...
H1	HA +	...NNTD**R**WLAT...	...FID**V**AKTTLTGNYTQ**H**GPLFSTPKL...	...TTPNATTG--**YYS**TTNYDTVN...
L338	HA +	...NNTN**R**WLAI...	...FIV**V**SKQTLDGAYAQ**Y**GPLLSATKL...	...ETPNATTA--**YYS**TTNYDTVN...
FRV64	HA +	...NNTD**R**WLAT...	...FVD**V**SKTTQNGSYSQ**Y**GPLLSTPKL...	...QTPNATTG--**YYS**STNYDSVN...
Ro1845	HA +	...NNTD**R**WLAT...	...FVD**V**SKTTQNGSYSQ**Y**GPLLSTPKL...	...QTPNATTG--**YYS**ATNYDSVN...
Cat97	HA +	...NNTD**R**WLAT...	...FVD**V**SKTTQNGSYSQ**Y**GPLLSTPKL...	...QTPNATTG--**YYS**ATNYDSVN...
K8	HA −	...NTTD**R**WFAC...	...FIL**F**IKLTPYGYTYTQ**Y**STLSTPHKL...	...ATPNASES--**YYL**TINNDNSN...
RV5	HA −	...NNND**F**WTAV...	...FFE**M**FKGSSQGDFSN**R**RTLTSNNRL...	...ETPRATTD--**SSN**TADLNNIS...
KU	HA −	...NNSD**F**WTAV...	...FLE**M**FRGSSQNEFYN**R**RTLTSDTKL...	...ETPRATTD--**SSN**TANLNDIS...
Wa	HA −	...NNSD**F**WTAV...	...FLE**M**FRSSSQNEFYN**R**RTLTSDTRF...	...ETPRATTD--**SSS**TANLNNIS...
1076	HA −	...NKTD**I**WVAL...	...FFE**M**FRSSVSAEFQH**K**RTLTSDTKL...	...ETPHATTD--**YSS**TSNLSEVE...
UK	HA −	...DNAG**R**WLSV...	...FVE**M**VKTAVDGDYAE**W**GTLLSDTKL...	...ETPNATTK--**GYF**ITNYASAE...
EW	HA −	...NTVN**R**WIAI...	...FID**F**MKTPTTGSYVR**Y**NILLSSTKL...	...ETPTAGQA--**YYS**SF--NIFN...
H2	HA −	...NSIN**R**WLAT...	...FMN**L**IKTTLSGNFTL**Y**STLLSEPKL...	...ETPNATTT--**GYV**TSNYDSLT...
69M	HA −	...NNTN**R**WLAT...	...FID**L**MKTTSSGTYTQ**H**SPLLSEPKL...	...ETPNAITN--**GYP**TTNYDSVN...
MDR13	HA −	...NNTS**I**WLAT...	...FID**F**GRSSQNDSYTN**Y**GTLLSENKL...	...DTPNAVPYEW**GYT**TNNYDEIV...
B223	HA −	...DNST**F**WMFT...	...FVD**Y**IKTSSTQAYGS**R**NYLNTAHRL...	...ADTQGDLRVG**TYS**NPVPNAV-...

The HA-positive (HA +) and HA-negative (HA −) phenotype of the rotavirus strains is indicated. The amino acids which are thought to be involved in binding to Sia in HA-positive strains, and the corresponding amino acids in HA-negative strains, are shown in bold.

Finally, the X-ray crystallography of the VP8 core of the HA+ strain RRV bound to 2-*O*-methyl-α-D-*N*-acetyl neuraminic acid, directly identified the amino acid residues involved in this interaction [49]. The VP8 domain that interacts with Sia was shown to have the β-sandwich fold of galectins, a family of sugar—binding proteins. The Sia moiety binds VP8 within a shallow groove between two β-sheets, with tyrosine 188 and serine 190 side chains forming one rim of the groove, and the aromatic ring of tyrosine 155 forming the opposite rim, while the floor of the groove is formed by arginine 101, valine 144, lysine 187 and tyrosine 189. The critical residues involved in the interaction with Sia are highly conserved among rotavirus hemagglutinating strains, with arginine 101, tyrosine 189, and serine 190 being completely conserved, and tyrosines 155 and 188 accepting a conservative change to histidine (Table 2). On the other hand, the amino acids at those positions in the VP8 sequence of the non-hemagglutinating strains are more variable (Table 2). The 3-D structure of the VP8 core shows that it is capable of interacting with a variety of oligosaccharide linkages proximal to the Sia moiety. This may allow VP8 to bind internal Sia linked to distal sugar residues in glycolipid moieties [49].

The VP8 sequences of the non-hemagglutinating strains suggest that they may bind modified sialosides, or sialosides that are presented in a different context. As it will be discussed latter, the non-hemagglutinating bovine strain UK binds Sia-containing gangliosides, but with a specificity different from that of the hemagglutinating strains SA11 and NCDV [50]. In a similar way, the murine rotavirus strain EHP was described as NA-resistant when tested in the monkey kidney cell line MA104 [28], while it behaved as NA-sensitive when tested in the human colon carcinoma cell line CaCo-2 [36]. Furthermore, previous studies had shown that a mutation of lysine 187 to arginine (K187R) in the simian strain RRV was associated with the loss of the virus sensitivity to NA treatment of cells [51]. In this regard, it is important to point out that the isolated RRV rotavirus mutants whose infectivity became resistant to the treatment of cells with NA (nar3 and gpr8), were still capable of binding to Sia, as demonstrated by their HA activity and their ability to bind to glycophorin A in Sia-dependent manner [51]. By X-ray crystallography, the VP8 core of a K187R mutant was shown to bind Sia, but with approximately one quarter of the affinity of the wild type protein [52].

Glycoconjugate binding specificities of rotaviruses

The susceptibility of the infectivity of some rotaviruses to the treatment of cells with neuraminidase, and the ability of Sia-containing glycoproteins to inhibit rotavirus infectivity, both *in vitro* and *in vivo* [23,25], led to the effort to identify the Sia-containing cellular molecule(s) involved in the initial binding of the virus to the cell surface. The interaction between rotaviruses and glycolipids was investigated by characterizing the binding of radiolabeled virus to different glycolipids separated on high-performance thin-layer chromatography (HPTLC) plates. Following this approach, Willoughby *et al.* [53] demonstrated that the NA-sensitive simian rotavirus strains SA11 and RRV, and the bovine strain NCDV, as well as the NA-resistant human strains Wa and DS-1, specifically interacted with asialo-GM1 (GA1), and this binding was shown to be inhibited by pre-incubation of the virus with serotype–specific hyperimmune sera. The binding was shown to be carbohydrate specific, as suggested by its susceptibility to periodate oxidation. Surprisingly, the inclusion of a Sia residue on an internal galactose (in ganglioside GM1) prevented the binding of rotavirus in this assay. In a later study, the binding of the simian strain SA11 to glycoconjugates was also determined by TLC; it was found that this virus bound preferentially to O-linked sialylglycoproteins, with measurable binding at <10 pmol of Sia, while N-linked glycoproteins required 140 pmol of Sia to support the binding of the virus [54]. When other substrates were tested, gangliosides with terminal Sia (GM3 and GT1b) did not support the binding at 140 pmol of terminal Sia. Experiments with polyvalent N-acetyllactosamine cores, having either α2-3 or α2–6 Sia linkages, showed that SA11 virus binds to both sialylated neoglycoproteins with equal avidity, in contrast to the preference for NeuAcα2-3Galβ1-4GlcNAc-PE, when presented in monovalent form. These results indicated that SA11 binding to sialylated ligands was dependent primarily on valency. In a similar study by Srnka *et al.* [55], the binding of simian strain SA11 to glycolipids isolated from adult mouse small intestine was investigated after separation on TLC. Neutral glycolipids GA1, GA2, and pentaosylceramides with terminal N-acetylgalactosamine, were shown to bind rotavirus, as did acidic lipid cholesterol 3-sulfate and two other compounds with lactosyl ceramide cores. Interestingly, no Sia-containing glycolipids tested, bound rotavirus.

The findings discussed above conflicted with the known sensitivity of the infectivity of rotaviruses SA11, RRV, and NCDV to the NA treatment of cells [21,24]. To clarify these discrepancies, the glycosphingolipid binding specificities of two NA-sensitive rotavirus strains (simian SA11 and bovine NCDV), and one NA-resistant rotavirus strain (bovine UK) were thoroughly investigated using a TLC binding assay, and a large panel of glycosphingolipids [50]. It was found that both DLPs and TLPs from strains SA11 and NCDV bound to several nonacid glycosphingolipids, although the strongest interaction was with GA1. As mentioned previously, DLPs lack the outer capsid proteins and are therefore non-infectious. Given this result, Delorme *et al.* [50] concluded that the interactions of purified rotavirus DLPs and TLPs with nonacid glycosphingolipids (GA1, GA2, lactosylceramide with phytosphingosine, lactotetraosylceramide, neolactotetraosylceramide, and hydroxy fatty acids) were not specific. Nevertheless, in that study it was found that TLPs but not DLPs of all strains tested bound to gangliosides. Interestingly, they found that the ganglioside binding specificities of the NA-sensitive and NA-resistant strains differed. While both NA-sensitive and NA-resistant strains bound to sialylneolactotetraosylceramide, GM2, and GD1a gangliosides, only the NA-resistant strain UK bound to NeuAc-GM3, and GM1. Similarly, the NA-sensitive strains (SA11 and NCDV) were the only to bind *N*-glycolylneuraminic (NeuGc)-GM3. Therefore, NA-sensitive strains bound to external Sia residues in gangliosides, while the NA-resistant strain UK recognized gangliosides with internal Sia, which are resistant to neuraminidase treatment. The minimal structural element required for binding of the NA-sensitive strains was found to be the terminal sequence NeuGc- or NeuAc-α2-3Galβ of gangliosides. A number of exceptions to this general observation suggested the contribution of some additional carbohydrate moieties to the ganglioside molecule. One of these exceptions was the lack of binding of TLPs from the NA-sensitive strains SA11 and NCDV to NeuAc-GM3, while they strongly bound to NeuGc-GM3. The binding of TLPs from these strains to NeuGc-GM3, and their lack of binding to NeuAc-GM3 is in contrast to the observed higher affinity of the RRV VP8 core for NeuAc as compared to NeuGc [49] by NMR. This differential preference for NeuAc or NeuGc can be explained by variations in the amino acids present in the Sia-binding site of the strains characterized. The strains reported to bind NeuGc (OSU, SA11, and NCDV) [50,56] have a glycine at position 187, while strain RRV has a lysine residue in this position, which may sterically hinder the binding to NeuGc [49]. The difference between the previously reported binding specificity of simian strain SA11 [53,55], and the results presented in the work by Delorme *et al*. [50] might be explained by the different methods used to purify the viral particles. While in earlier works the virus was purified using metrizamide gradients, which did not have a good resolution to separate DLPs from TLPs [25], latter works used cesium chloride gradients, which allowed a better separation of DLPs and TLPs. It is possible that the preparations used by Willoughby *et al.* and Srnka *et al.* [53,55] contained both TLPs and DLPs that could account for the observed interaction of SA11 with GA1 and GA2.

A different approach was used to investigate the interaction of the NA-sensitive simian SA11 strain with the monkey

kidney cell line LLC-MK2 [57]. In that study, the infection of rotavirus SA11 was inhibited by preincubating the virus with bovine brain gangliosides, and purified GM1 was shown to have best inhibitory activity. Interestingly, it was also found that the infection of SA11 in NA-treated LLC-MK2 cells was restored by the addition of exogenous GM1 to the cells. These results are in contrast with a report by Guo *et al.* [26], which describes GM1 as not being important for the infectivity of NA-sensitive feline rotavirus strain FRV64, while being involved in the infection of NA-resistant human rotavirus strains KUN and MO.

Another NA-sensitive strain that has been used to study the interaction with gangliosides is the porcine isolate OSU [56,58]. Initially, it was found that a family of monosialogangliosides isolated from piglets had the capacity to specifically block the binding of rotavirus OSU to the surface of MA104 cells [58], and it was found that this activity was resistant to treatment with trypsin or heat, and sensitive to periodate oxidation and digestion with NA or ceramide glycanase. In a subsequent, more detailed study, two gangliosides (NeuGc- and NeuAc-GM3) were found to inhibit the viral binding of OSU to the cell surface [56]. NeuGc-GM3 was approximately two to three times more effective than NeuAc-GM3 in blocking virus binding to MA104 cells. NeuGc-GM3 was also more effective in inhibiting virus infectivity as compared to NeuAc-GM3. These results are in agreement with the previously mentioned binding specificities of NA-sensitive and resistant rotavirus strains to GM3 gangliosides [50]. The ability of gangliosides isolated from Italian buffalo milk to bind to simian rotavirus strain RRV and to its neuraminidase resistant mutant nar3 has also been analyzed [59]. While RRV virus interacted only with lipophilic GM3, its variant nar3 also bound to hydrophilic GD3 species, in addition to lipophilic GM3.

Biochemical nature of the receptors for rotavirus

To determine the biochemical nature of the cellular receptor for rotaviruses, two studies followed a similar approach [60,61]; MA104 cells were treated with specific inhibitors of glycosylation prior to infection, and their effect on viral infectivity was determined. Both studies found that treatment of cells with tunicamycin, a drug that blocks the N-glycosylation pathway by preventing transfer between UDP-GlcNAc and dolichol-1-phosphate [62], inhibited the infectivity of several rotavirus strains (RRV, and its NA-resistant mutant nar3, SA11, NCDV, CRW8, and Wa). Another inhibitor of N-linked glycosylation used was 1-deoxymannojirimycin, which blocks the glycan chain processing at a later step, and as a result, although the proteins are partially glycosylated, they do not contain N-linked neuraminic acid and galactose [63]. Treatment of MA104 cells with this inhibitor resulted in 15 to 30% reduction of viral infectivity [61]. On the other hand, inhibition of O-linked glycosylation by benzylGalNAc did not have a signifficative effect on the infectivity of the rotavirus strains tested [60,61]. In addition, a synthetic analog of ceramide, PDMP, was used to inhibit the biosynthesis of the glycosphingolipid precursor glucosylceramide [60]. This treatment resulted in the inhibition of rotavirus infectivity by about 60 to 80%, showing again the importance of gangliosides for rotavirus infection.

An alternative approach to characterize the biochemical nature of the rotavirus receptor was based on the ability of the non ionic detergent octyl glucoside (OG) to extract molecules from the cell surface without affecting the cells viability. Cells treated in this way were found to be 40–70% refractory to infection by rotavirus, depending on the viral strain tested [60,61]. The effect of this detergent was reversible, since the cells became fully susceptible to rotavirus infection after about 3 to 8 h. The only exception was the porcine strain CRW8, which was able to infect the cells just one hour after detergent extraction, and inhibition of protein synthesis with cycloheximide during the recuperation period had no effect on the reestablishment of the susceptibility of the cells to CRW8, while inhibition of protein synthesis during the recovery period rendered cells non susceptible to infection by rotavirus strains NCDV and Wa [61]. Taken together, these observations suggest that the NA-sensitive strain CRW8 uses a cellular receptor different from that used by strains NCDV and Wa [61].

Inhibition of rotavirus infectivity by Sia-containing compounds

To prevent the infection by rotavirus, several attenuated live rotavirus vaccines have been developed and subjected to extensive clinical trials, and some of them will soon be available for routine immunization [64]. However, in the particular case of immunocompromised hosts, alternative therapeutic approaches would be required. Given that Sia plays an important role during the initial interactions of rotavirus with its host cell, it has been proposed that Sia-containing compounds could be used as therapeutic agents to treat the diarrhea caused by rotavirus. The compounds that have been shown to inhibit rotavirus infection *in vivo* or *in vitro* are listed in Table 3. Various sialylated glycoproteins have been shown to have the ability to inhibit cell infection if preincubated with the virus at concentrations of 10–100 μg/ml [25] before cell infection. The capacity of these glycoproteins to inhibit the replication of rotavirus in the intestine of mice was a promising observation. In later studies, mucins of different origins were shown to inhibit rotavirus replication and to prevent experimental gastroenteritis [65–68]. The inhibition

Table 3 List of sialic acid containing compounds capable of inhibiting rotavirus infection *in vivo* or *in vitro*

Component	Rotavirus strain	NA[a]	Tested in	ID50[b]	Reference
ovomucin glycoprotein	SA11	+	MA104	$<10\,\mu$g/ml	[25]
ovalbumin glycoprotein	SA11	+	MA104	$<10\,\mu$g/ml	[25]
fetuin	SA11	+	MA104	$<100\,\mu$g/ml	[25]
bovine submaxilar mucin	SA11	+	MA104	100–1000 μg/ml	[25]
ovomucin glycoprotein	EDIM	+/−	mice	ND[c]	[25]
ovalbumin glycoprotein	EDIM	+/−	mice	ND	[25]
bovine salivary mucin	SA11	+	MA104	ND	[67]
GM1	SA11	+	LLC-MK2	8 μg/ml	[57]
GD1a	SA11	+	LLC-MK2	200 μg/ml	[57]
GT1b	SA11	+	LLC-MK2	200 μg/ml	[57]
human milk mucin	SA11	+	MA104, mice	ND	[68]
murine intestinal mucin	RRV	+	MA104	0.12 μg/ml	[65]
	UK	−	MA104	1.86 μg/ml	[65]
	EW	+/−	MA104	0.93 μg/ml	[65]
	Wa	−	MA104	1.86 μg/ml	[65]
human intestinal mucin	RRV	+	MA104	1 μg/ml	[67]
	Wa	−	MA104	2 μg/ml	[67]
rat intestinal mucin	RRV	+	MA104	1 μg/ml	[67]
	Wa	−	MA104	2 μg/ml	[67]
NeuGcGM3	OSU	+	MA104	3.97 μM	[56]
NeuAcGM3	OSU	+	MA104	9.84 μM	[56]
Collectins	NCDV	+	MA104	10 μg/ml	[69]
	B223	−	MA104	10μg/ml	[69]
	SA11	+	MA104	10 μg/ml	[69]
lactoferin	SA11	+	HT-29	50–60 μg/ml	[70]
lactadherin	Wa	−	Caco-2	20 μg/ml	[71]
macromolecular whey proteins	Wa	−	Caco-2	20 μg/ml	[71]
GM1	KUN, MO	−	MA104	7.5 μg/ml	[26]
α-linked sialosides	NCDV	+	MA104	0.35–1.5 mM	[72]
sialylphospholipid	SA11	+	MA104	4.35 mM	[73]
sialylphospholipid	MO	−	MA104	1.61 mM	[73]
sialylmimetics	NCDV	+	MA104	6.25 mM	[74]
sialylmimetics	Wa	−	MA104	6.25 mM	[74]
sulfated sialyl lipid	Wa	−	MA104	2.1 μg/ml	[75]
	DS-1	−	MA104	4.7 μg/ml	[75]
	S-2	−	MA104	1.7 μg/ml	[75]
	MO	−	MA104	2.2 μg/ml	[75]
	Hosokawa	−	MA104	1.7 μg/ml	[75]
sulfated sialyl lipid	MO	−	mice	10 μg[d]	[75]

[a]NA = sensitivity of rotavirus strains to treatment with neuraminidase as shown in Table 1.
[b]ID50 = dose necessary to obtain 50% inhibition of infectivity.
[c]ND = not determined.
[d]dose applied to mice three times per day.

activity was abolished by treatment of mucins with NA, or by digestion with trifluoromethanesulfonic acid, suggesting that Sia present in these molecules was directly involved in the inhibition of rotavirus infectivity.

On the other hand, breast-feeding has been recognized as an important factor in protection against enteric infections, however, the results that support its role in preventing the diarrhea caused by rotavirus have been variable. The degree of protection observed after breast-feeding does not completely correlate with the levels of maternal anti-rotavirus antibodies, suggesting that other non immunoglobulin factors might play a role in protection. Milk and colostrum contain more than 100 different oligosaccharides [76] that might play an important role in this process. In fact, several active components of the milk, which have the ability to inhibit rotavirus infection, have been described. Among these, human milk mucin was shown to be able to inhibit rotavirus replication and to prevent experimental gastroenteritis [68]. This protection was associated with an acidic fraction of the milk; a 46 kDa glycoprotein isolated from this fraction was found to be able to specifically bind to rotaviruses, and both, the protection of infection and the inhibition of rotavirus binding mediated by this protein were sensitive to deglycosylation. Other milk components that have been found to inhibit rotavirus infection are collectins [69], lactoferrin [70], and lactadherin [71]. Recently, it was shown that the bovine macromolecular whey protein fraction contains an efficient and versatile activity against rotavirus infection [71].

Furthermore, in recent years several synthetic sialyl-containing compounds have been tested for their capacity to inhibit rotavirus infection. The first attempt involved the synthesis and evaluation of various *S*-sialosylglycosides [72]. The inhibition activity of these compounds was strain dependent, blocking the infectivity of two NA-sensitive strains (NCDV and SA11), but not that of the NA-resistant human strain Wa. In subsequent studies various groups have synthesized different Sia-containing compounds with blocking activity against both, NA-sensitive and—resistant rotavirus isolates. Among these, lactose-based sialylmimetics showed a low, but significative inhibitory activity against both NCDV and Wa strains, which represent NA-sensitive and NA-resistant strains, respectively [74]. Sialylphospholipid, synthesized from *N*-acetylneuraminic acid and phosphatidylcholine, was capable of inhibiting the infectivity of rotavirus strains SA11 and MO [73]. Another chemically synthesized compound tested for its protective effect against rotavirus infection was sulfated sialyllipid [70]. Its addition during the virus adsorbtion period resulted in the inhibition of infection of various human rotavirus strains (Wa, DS-1, S-2, MO and Hosokawa). The prophylactic oral administration to mice of 10 μg of sialyllipid three times a day, prevented the development of diarrhea after experimental infection. These results suggest that the design and synthesis of inhibitors which mimic the cellular Sia-containing recognition sites is a viable approach to obtain effective anti-rotavirus treatments.

Conclusions

The perception of the relevance of Sia for rotavirus cell infection has evolved over time. It has shifted from the original idea that it was likely that all rotavirus isolates of human and animal origin bound to Sia, to the other end that proposed that only some rotaviruses of animal origin need Sia to infect the cells [27]. The determination of the crystal structure of the protease-resistant rotavirus VP8 core complexed to Sia, and the detailed analysis of the binding characteristics of different rotavirus isolates to Sia-containing compounds, have changed this idea again. Currently, it seems that many, if not most rotavirus strains might indeed bind Sia, since although most rotavirus strains are NA-resistant (at least to the NA commonly tested), this does not mean that these strains do not use Sia for cell attachment, since Sia moieties that are internal in oligosaccharide structures are less or not sensitive to NA [37]. Furthermore, the fact that many rotavirus strains apparently do not need to bind to Sia to infect cells in static culture conditions, does not imply that binding to these acid sugars is not important for enterocytes infection in the gut, in a more dynamic environment. Under these conditions, a rapid (probably non-specific) binding to gut-abundant Sia might stop the travel of the virus in the intestine, to then search locally for more specific receptors. The low affinity and high valency of the rotavirus–Sia interaction makes Sia a good candidate for such a non-specific, but potentially important, primary interaction. The Sia requirement of NA-resistant rotavirus strains for cell infection needs to be further explored. Regardless of the biological relevance of the Sia-virus interaction, the use of glycoconjugates of natural or synthetic origin seems to offer the possibility to develop alternative therapeutic approaches for the control of the diarrhea caused by rotavirus. Such treatments will be particularly important for immunocompromised patients, in which the use of live attenuated rotavirus vaccine is not recommended.

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