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Short communication

Theiler's murine encephalomyelitis virus attachment to the gastrointestinal tract is associated with sialic acid binding

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DA and GDVII are strains of Theiler's murine encephalomyelitis virus (TMEV). DA virus mutant DApB encodes VP2 puff B of GDVII, whereas DApBL2M contains VP1 loop II of GDVII with a point mutation in VP2 puff B. Neuraminidase treatment of cells inhibited infection by DA and DApB, but not GDVII or DApBL2M viruses; sialic acid (SA) binding correlated with virus persistence. In virus binding assays to intestine sections, all four TMEVs bound goblet cells and the mucus of the epithelium that was SA dependent. Therefore, differences in SA composition on different cell types can affect tropism and infection. *Journal of NeuroVirology* (2009) **15,** 81–89.

Keywords: CNS autoimmune demyelinating diseases; multiple sclerosis; intestinal mucosa; mucins; *N*-acetylneuraminic acid; virus receptors

Several viruses, including paramyxoviruses and coronaviruses, have the ability to bind sialic acid (SA) *in vitro* (Compans and Herrler, 2005). Although its role in pathogenesis *in vivo* remains largely unknown, SA-binding has been associated with the ability to cause disease (Helander *et al*, 2003; Barton *et al*, 2003; Forrest and Dermody, 2003; Komagome *et al*, 2002; Liu *et al*, 1998). For example, influenza viruses attach to cells by the viral hemagglutinin binding to sialosaccharides (van Riel *et al*, 2006). The porcine coronavirus, transmissible gastroenteritis virus (TGEV), which is a cause of a fatal diarrhea in newborn piglets, agglutinates erythrocytes *in vitro* due to SA-binding activity (Krempl *et al*, 1997). TGEV containing mutations within the S glycoprotein loses hemagglutinating activity and has reduced enteropathogenicity. Wild-type TGEV binds more efficiently to cells than viral mutants lacking SA-binding activity (Schwegmann-Weβels *et al*, 2002). Similarly, reovirus type 1 Lang (T1L) binds selectively to M cells with glycoconjugates containing SA in the intestinal epithelium (Helander *et al*, 2003; Barton *et al*, 2003; Forrest and Dermody, 2003; Komagome *et al*, 2002; Liu *et al*, 1998).

Picornaviruses are positive-strand RNA viruses, and cause infection of the enteric and respiratory systems. In natural picornavirus infection in humans, viruses are able to spread from the gut to the central nervous system (CNS), causing meningitis and encephalitis (Melnick, 1996). However, how these viruses disseminate is not well studied. Theiler's murine encephalomyelitis virus (TMEV) belongs to the family *Picornaviridae* (Roos, 2002; Tsunoda and Fujinami, 1996, 1999; Tsunoda and Fujinami). TMEV is divided into two subgroups: GDVII and TO, based upon neurovirulence. In nature, TMEV transmission occurs by the fecal-oral route, resulting in an unapparent infection of the gut (Lipton *et al*, 2006); however, on occasion, TMEV is able to infect the CNS (Theiler, 1934). We do not know how TMEV infects in the gut and spread into the CNS, although enteric TMEV infection can be a good model system to study how enteric virus disseminates into the CNS in humans.

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Experimentally, intracerebral (i.c.) GDVII virus infection of mice causes an acute fatal encephalitis, whereas i.c. infection with the TO subgroup, including Daniels (DA) and BeAn viruses, results in a chronic inflammatory demyelinating disease in the CNS (Villarreal *et al*, 2006). TMEV encodes four capsid proteins: VP1 to VP4, and has unique loop structures that are made up of connections of the β strand. There are four large loops between the CD strands of VP1 (loops I and II) and the EF strands of VP2 (puffs A and B). Exposed amino acids on all four loops have been shown to be important disease determinants (McCright *et al*, 1999, 2002; McCright *et al*.; Tsunoda *et al*, 2001; Wada *et al*, 1998). Several differences between the TO and GDVII subgroups are located in VP1 loop II and VP2 puff B. These two surface features are located spatially near each other, close to the proposed receptor binding site, ''pit,'' or depression surrounding the fivefold axis of TMEV (Grant *et al*, 1992; Luo *et al*, 1996; McCright *et al*, 1999; Tsunoda *et al*, 2001).

Although the two subgroups of TMEV may share a common receptor (Fotiadis *et al*, 1991), the TO subgroup binds α2,3 SA moieties on *N*-linked oligosaccharides at the surface of susceptible cells (Fotiadis *et al*, 1991; Shah and Lipton, 2002; Zhou *et al*, 1997, 2000; Zhou *et al*.). However, the attachment to *N*-linked oligosaccharides accounts for only a portion of GDVII virus binding (Shah and Lipton, 2002; Lipton *et al*, 2006). Atomic modeling of DA virus complexed with sialyllactose was consistent with SA-binding to a positively charged pocket, termed ''gap,'' on the capsid surface (Zhou *et al*, 2000). The gap is formed by the interaction between VP1 loop II and VP2 puff B and is shaped differently between GDVII and TO subgroups (Zhou *et al*, 1997). SA has been suggested to interact directly with VP2 puff B: VP2-161, -163, and -174 of DA virus (Zhou *et al*, 2000) and VP2-161 and -174 of BeAn virus (Kumar *et al*, 2003). Amino acids comprising VP1 loop II likely influence SA- binding, although they have no direct interactions with SA residues. GDVII virus has been shown to use the proteoglycan heparin sulfate, but not SA, as a coreceptor (Shah and Lipton, 2002; Lipton *et al*, 2006). Although it is not clear how differences in SA-binding between GDVII versus TO subgroups contribute to differing biological activities *in vivo*, the requirement of virus binding to SA on cells could contribute to the different pattern of disease observed between TO versus GDVII virus infection.

Previously, we reported neuropathology caused by two DA mutant viruses: DApB and DApBL2M (Tsunoda *et al*, 2001, 2007; Tsunoda *et al*.). DApB virus encodes the VP2 puff B of GDVII virus with one conservative change from GDVII virus (A173V). DApBL2M virus contains the VP1 loop II of GDVII virus and has a point mutation (S171R) in VP2 puff B. Both mutant viruses are on the background of DA virus. Although all viruses replicated to similar titers *in vitro*, i.c. infection of mice with GDVII, DA, DApB, and DApBL2M viruses resulted in distinct CNS pathology. During the acute stage, mice infected with GDVII virus had large numbers of apoptotic neurons with a relative lack of T-cell infiltration in the brain and all infected mice died (Tsunoda *et al*, 2007). Similarly, the two DA virus mutants induced higher levels of apoptosis in neurons than wild-type DA virus, although levels of inflammation were similar between DA and DA virus mutants (Tsunoda *et al*, 2007). During the chronic stage, DA and DApB induced demyelinating disease with virus persistence in the white matter of the spinal cord, whereas DApBL2M caused gray matter lesions in the brain without demyelination (Tsunoda *et al*, 2001). These data suggest that conformational differences via interaction of VP1 loop II and VP2 puff B between GDVII and DA viruses play an important role in TMEV disease.

We first tested the contribution of VP1 loop II and VP2 puff B in SA-binding *in vitro* and correlated this with virus replication. TMEV has been shown to cause cytopathic effects (CPEs), but not apoptosis, in permissive baby hamster kidney (BHK)-21 cells, whereas TMEV causes apoptosis with no or limited CPEs in restrictive cells (Jelachich and Lipton, 1996). BHK-21 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Binding of TMEV to SA was tested using treatment with neuraminidase (sialidase) to remove cell surface SA prior to virus adsorption/infection (Fotiadis *et al*, 1991; Jnaoui *et al*, 2002). BHK-21 cell monolayers grown in 24-well plates were washed with serum-free DMEM at pH 5.5 and then incubated for 1 h at 37°C, pH 5.5 with α2-3,6,8-neuraminidase (*Vibrio cholerae* sialidase, Calbiochem) at 20 and 200 mU/ml. Cells were washed and infected with TMEV at a multiplicity of infection (MOI) of 3. After a 1-h virus adsorption, cells were washed and cultured in DMEM containing 2% FBS. CPEs were monitored for 24 h.

Neuraminidase treatment inhibited CPEs of BHK-21 cells induced by DA virus, whereas treatment had no effect on GDVII virus infection (Figure 1). Interestingly, reduced CPEs by neuraminidase treatment was also seen in DApB virus infection, but not in DApBL2M virus infection. Virus titers were also compared in TMEV-infected cell cultures, with or without SA digestion by neuraminidase, after one replication cycle. At 8 and 24 h post infection (p.i.), infected cells and supernatant fluids were collected, and viral titers were determined by plaque assay (McCright *et al*, 1999). Neuraminidase treatment prior to DA and DApB virus infection reduced virus titers by more than 90%, versus mock treated control cultures at 8 h p.i. (Figure 2a). A dose-dependent reduction of virus titers by neuraminidase treatment was also seen in DA and DApB virus infection at 24 h p.i. (Figure 2b). In contrast, in GDVII or DApBL2M virus infections, no significant reductions

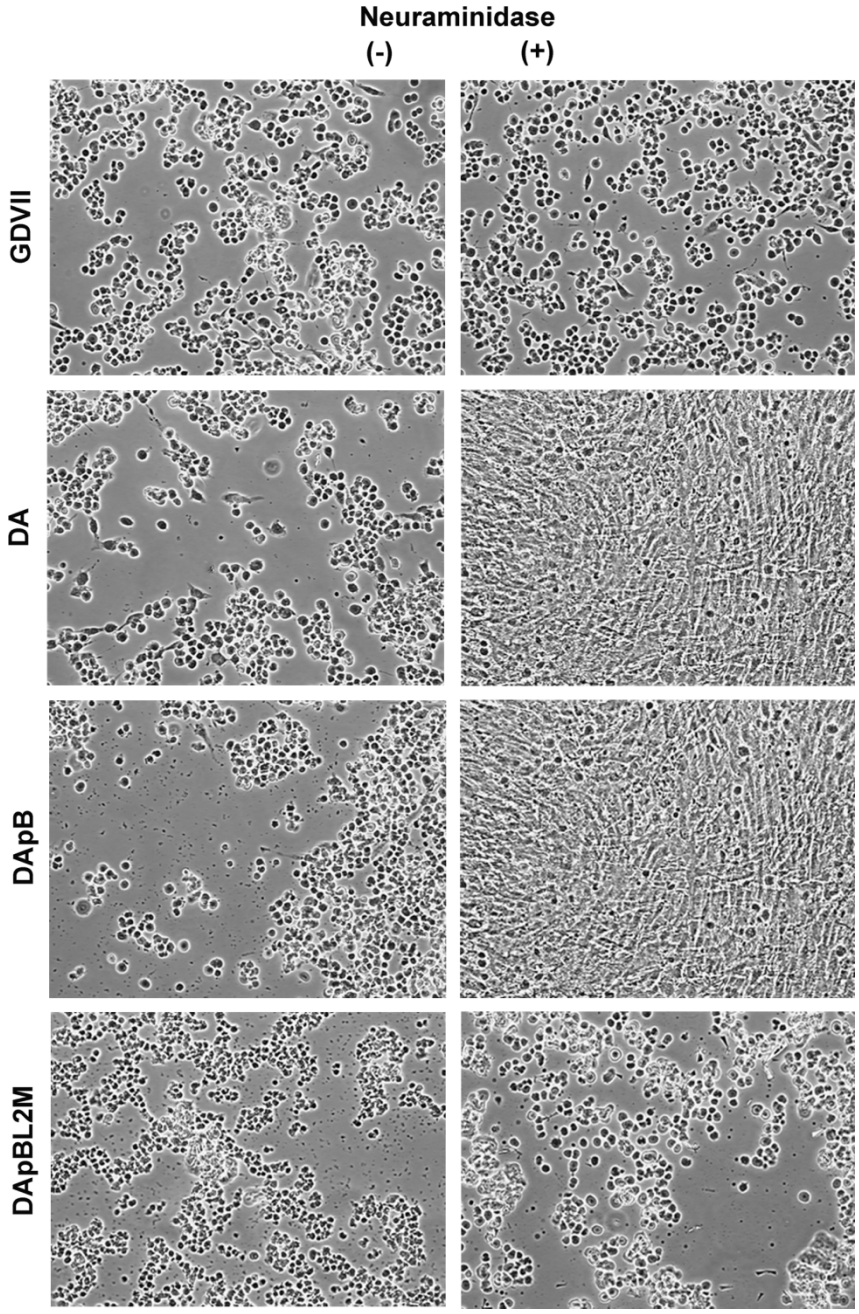

Figure 1 Influence of neuraminidase treatment on cytopathic effects (CPEs) by TMEV infection. At 24 h p.i., neuraminidase treatment (*right panels*) did not inhibit CPEs in cultures infected with GDVII virus, but did inhibit CPEs in cultures infected with DA virus, compared with untreated cultures (*left panels*). Reduced CPEs by neuraminidase treatment was also seen in DApB virus infection. The inhibitory effect of neuraminidase was almost abolished for DApBL2M virus. Magnification $\times 43$.

in virus titer by neuraminidase treatment were observed at 8 or 24 h p.i. These results are compatible with previous findings where CPE of BHK-21 cells was reduced in BeAn and DA, but not in GDVII, virus infections by addition of sialyllactose, the first three sugar molecules of common oligosaccharides on the surface of mammalian cells (Fotiadis *et al*, 1991; Shah and Lipton, 2002; Zhou *et al*, 1997, 2000), In those experiments, the reduction in the ability of TMEV to infect cells was associated with SA-dependent virus attachment to BHK-21 cells. Therefore, our results predict that VP1 loop II and VP2 puff B influence the requirement for viral attachment to SA on cells. However, it should be noted that, even after high doses of neuraminidase treatment, a substantial amount of virus replication was detected in all TMEV infections; blocking of SA binding did not completely abrogate virus infection/attachment.

Although TMEV has been isolated from the gastrointestinal tract, the cell types in the intestine that the

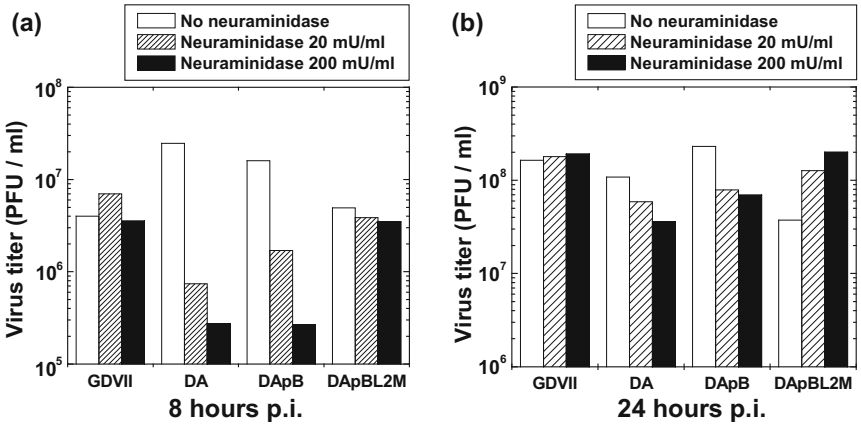

Figure 2 Effect of neuraminidase treatment on virus replication. Virus titers were determined in TMEV-infected cultures with or without 20 or 200 mU/ml of neuraminidase at 8 (**a**) or 24 (**b**) h p.i. Neuraminidase treatment reduced the virus titers in DA and DApB virus infections. No significant reduction of the virus titers was seen in GDVII or DApBL2M virus infections. The histogram shows one representative experiment of four independent experiments.

virus binds are unknown (Theiler, 1941). Therefore, we analyzed the binding of TMEV to murine intestinal epithelium, the natural target for this virus, using virus overlay assays, which have been widely used to test tissue attachment of microbes, such as *Helicobacter pylori* and influenza virus H5N1 (Falk *et al*, 1993; van Riel *et al*, 2006; Helander *et al*, 2003) (Figure 3). We harvested the intestine from naïve mice without washing the intestine. We fixed the intestine in 4% paraformaldehyde, embedded in paraffin, and made 4-μm paraffin sections. Intestine sections were deparaffinized, washed three times with phosphate-buffered saline (PBS), blocked with PBS containing 3% normal donkey serum for 30 min, and incubated with DMEM containing DA, GDVII, DapB, or DApBL2M virus, at a final concentration of 1×10^9 plaque-forming units (PFU)/ml for 1 h. Sections were incubated with antibody against TMEV (Tsunoda *et al*, 2001), followed by secondary tetramethyl rhodamine isothiocyanate

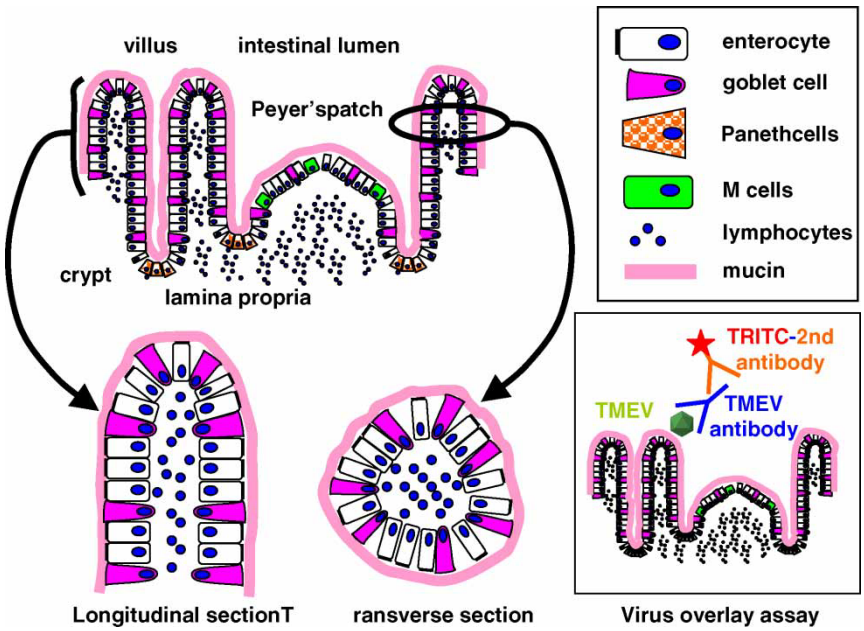

Figure 3 Intestine section preparation and a virus overlay assay. Intestinal epithelium is composed of enterocytes, goblet cells, Paneth cells, and M cells. The intestinal lymphocytes disseminate beneath the epithelium and also between epithelial cells. Lymphocytes are also found within more organized structures, including the Peyer's patch. The luminal surface of the intestine is covered with mucin produced by goblet cells, and the mucin can be stained purple-magenta by PAS. Intestine was harvested from uninfected mice, and was fixed with 4% paraformaldehyde and embedded longitudinally or transversely in paraffin. Deparaffinized sections were incubated with TMEV for 1 h, followed by rabbit anti-TMEV antibody and TRITC-conjugated secondary antibody against rabbit IgG. Consecutive sections from one naïve mouse were used for one set of experiments.

(TRITC) conjugated anti-rabbit immunoglobulin G (IgG).

All four TMEVs showed a similar pattern of attachment to the intestine (Figure 4a to d). Virus was associated with the mucus at the surface of the intestine and bound to goblet cells. Goblet cells are specialized epithelial cells that synthesize and secrete mucins. This suggests that virus attached to the mucus produced by goblet cells. Virus binding to Peyer's patches was similar; virus was found at the

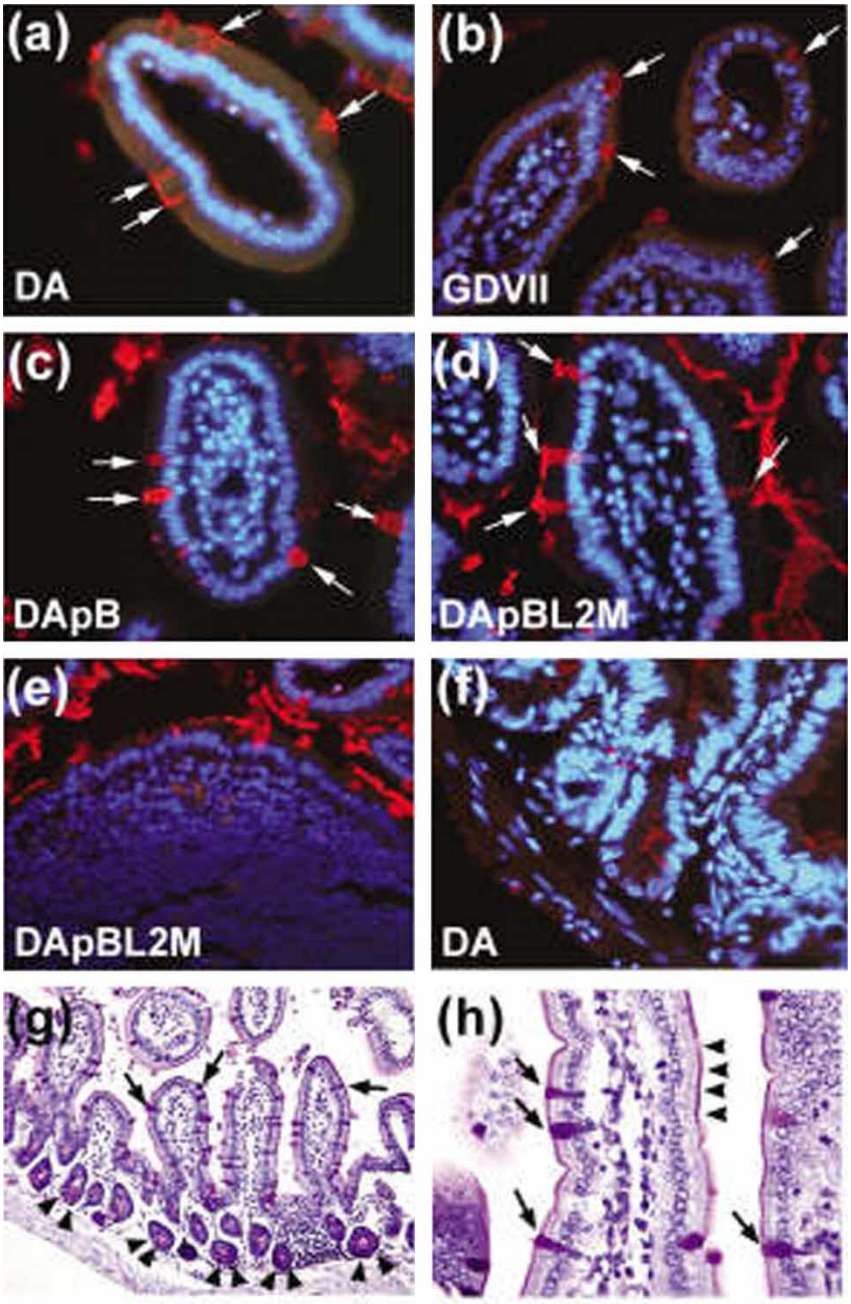

Figure 4 (**a**–**f**) Virus overlay assays. Intestine sections were incubated with DA (**a, f**), GDVII (**b**), DApB (**c**), or DApBL2M (**d, e**) virus, followed by rabbit antibody against TMEV and TRITC-conjugated anti-rabbit IgG. (**a**–**d**) Transverse sections showed that all four TMEVs (*red*) bound cytoplasm of goblet cells (*arrow*). (**e, f**) In longitudinal sections, virus binding was also seen at the surface of the Peyer's patch (**e**) and the crypt (**f**) of the intestine. For nuclear staining, DAPI (*blue*) was used. (**g, h**) PAS stained mucins purple-magenta in longitudinal sections. Sections were counter stained with hematoxylin (*blue*). (**g**) Goblet cells in the intestinal villi (*arrow*) and cells at the base of the crypts (*arrowhead*) were PAS positive. (**h**) The luminal surface (arrowhead) of the enterocytes was PAS positive due to the presence of a glycocalyx and a surface layer of goblet cell (*arrow*)–derived mucus. Note the entire cytoplasm of goblet cells were filled with PAS-positive mucin. The difference in the number of lymphocytes in each villus is normal anatomical variance (for example, in **g**, most villi contained many lymphocytes, whereas two villi at the right side contained few lymphocytes). Magnification (a–d, f, h) $\times 210$, (e) 140, (g) $\times 70$.

surface of epithelia and in the cell bodies of goblet cells (Figure 4e). Virus was also seen bound to cells in the crypt of the intestine (Figure 4f). We did not see autofluorescence in the intestine sections (Figure 5b) nor positive staining in negative controls, such as incubation with TMEV antibody and secondary antibody without virus overlay (Figure 5d), incubation with secondary antibody alone, and incubation with normal rabbit serum instead of TMEV antibody. The identification of goblet cells was confirmed by periodic acid–Schiff (PAS) staining where mucins stain purple-magenta (Figure 4g, h). The presence of mucins on the intestine sections were confirmed on the luminal surface of the

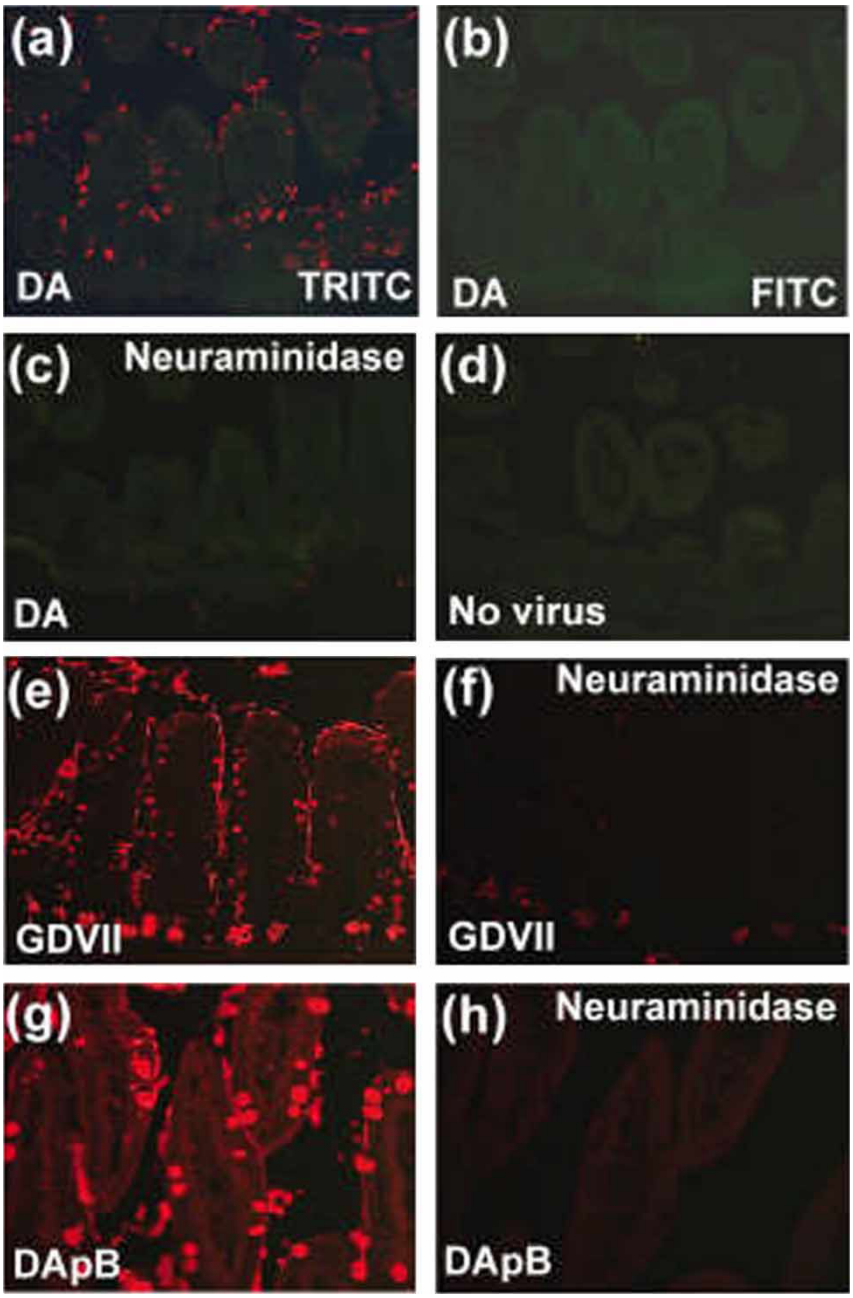

Figure 5 Effect of neuraminidase treatment on virus binding. (**a**–**c**) Intestine sections were incubated with DA virus. Virus binding (*red*) was visualized with rabbit antibody against TMEV, followed by incubation with TRITC-conjugated anti-rabbit IgG. The fluorescence was not due to autofluorescence of sections, because it was detectable by the filter for TRITC (**a**) but not by the filter for FITC (**b**). (**c**) Treatment with neuraminidase before DA virus overlay abrogated virus binding. (**d**) Negative control. Sections were incubated without virus, followed with antibody against TMEV and TRITC-conjugated anti-rabbit IgG. No staining was seen. GDVII (**e, f**) or DApB (**g, h**) virus was incubated with intestine sections without (**e, g**) or with (**f, h**) neuraminidase pretreatment. Neuraminidase abrogated GDVII and DApB virus binding. DApBL2M virus overlay assays resulted in similar binding to those in GDVII and DApB viruses (data not shown). Magnification (a–f) $\times 100$, (g, h) $\times 200$ to (a–f) $\times 70$, (g, h) $\times 140$.

intestinal villi as well as in the cytoplasm of goblet cells (Figure 4h).

Because all four TMEVs showed similar binding to the intestinal tissue, this suggested that all four TMEVs use a similar receptor/co-receptor. To test whether virus attachment depended on SA-binding, prior to incubation with TMEV, tissue sections were incubated in serum-free DMEM containing neuraminidase at 20 mU/ml at pH 5.5 for 1 h at 37°C. In contrast to virus attachment on BHK-21 cells, the binding of all four TMEVs was significantly diminished (Figure 5). These results indicate that all four TMEVs attach to mucin-producing goblet cells in a SA-dependent fashion. In neuraminidase-treated sections, we sometimes detected faint residual virus binding to cells, presumably Paneth cells, near the crypt in all four TMEVs for unknown reasons (Figure 5c, f). However, because the area is not on the surface of the intestine, it is unlikely that TMEV uses these cells for entry during enteric infection.

We do not know the physiologic role of SA-binding by TMEV in the intestine. Goblet cell binding by virus via SA was shown in TGEV infection (Schwegmann-Weβels *et al*, 2003). As was suggested in TGEV infection (Schultze *et al*, 1996), binding of sialylated macromolecules may increase the stability of virions and thus facilitate survival in the gastrointestinal tract, protecting the virus from inactivation due to low pH and proteases (Krempl *et al*, 2000; Lipton *et al*, 2005). Virus binding to mucin via SA may also allow the virus to remain longer in the intestine. This would make it easier to interact with the cellular receptor initiating intestinal infection, thus preventing the loss of virions due to intestinal peristalsis (Schwegmann-Weβels *et al*, 2003). In addition, because VP1 loop II and VP2 puff B of TMEV contain antibody binding sites (Tsunoda and Fujinami, 2007), SA-binding of virus may also prevent TMEV from neutralization by antibody against TMEV in the gastrointestinal tract.

In contrast, mucus is known to act as a barrier in the mucosal immune system. It regulates mucosal microbiota by secreted antibodies and mucus (Cone, 2005). To infect the intestinal epithelium, virions must pass through the mucus blanket and glycocalyx on the gastrointestinal epithelium to reach cellular receptors on the apical surface of the epithelial cells (Schwegmann-Weβels *et al*, 2003). Microorganisms, including viruses, are known to be trapped by mucus. In the mucus, aggulutination and neutralization by antibodies, especially polyvalent IgA and IgM, can occur. Mucin, the primary component of the mucus layer, can also inhibit SA-dependent virus-cell attachment, resulting in suppression of virus infection (Chen *et al*, 1993).

Currently, we cannot explain why GDVII and DApBL2M viruses have differences in SA dependency for attachment to BHK-21 cells versus intestine tissues. Interestingly, wheat germ aggulutinin (WGA), which specifically binds to SA residues, reduces the BHK-21 cell binding of both BeAn and GDVII viruses *in vitro*, although neuraminidase treatment has no effect on GDVII virus binding to BHK-21 cells (Fotiadis *et al*, 1991; Kilpatrick and Lipton, 1991). However, our studies using intestinal tissue as substrate in binding assays should be more relevant to what is occurring *in vivo*.

The requirement for SA in viral attachment could also be different between the gastrointestinal tract versus the CNS. Jnaoui *et al* (2002) hypothesized that VP1 loop II and VP2 puff B of TMEV contact the carbohydrate moiety of the receptor, thus altering TMEV tropism and pathogenicity. They demonstrated decreased ability by two DA virus mutants, KJ6 virus with mutations in VP1 loop II and VP2 puff B and OT11 virus with mutations in VP1 loop II, to persist in the CNS. Sialyllactose inhibition studies found decreased SA usage for entry by KJ6 virus. In addition, mice infected for 6 days with GDVII virus mutant KJ38, which has three mutations in VP2 puff B and two in VP1 (VP1-51 and -195), had a dramatic decrease in the number of infected cells in the CNS compared to mice infected with wild-type GDVII virus. Sialidase treatment of cells *in vitro* reduced infection by KJ38 virus, indicating binding of KJ38 virus to SA.

In summary, we demonstrated that DApBL2M virus, which has mutations in both VP1 loop II and VP2 puff B, showed decreased SA-binding similar to that of GDVII virus. Our results support the hypothesis that VP1 loop II and VP2 puff B form the SA-binding site, termed ''gap.'' Virus overlay assays indicated that not only DA and DApB but also GDVII and DApBL2M viruses bound to mucus, which is produced in the cell bodies of goblet cells and spreads onto the surface of epithelium, in the intestine. This attachment was dependent upon the presence of SA. Our report is the first to show that the requirement for SA in viral attachment is different among host cell types and this could lead to differences in cell tropism and disease.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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