Virology **304,** 443–450 (2002)
doi:10.1006/viro.2002.1735

Low-Neurovirulence Theiler's Viruses Use Sialic Acid Moieties on *N*-linked Oligosaccharide Structures for Attachment

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Received April 16, 2002; returned to author for revision May 17, 2002; accepted August 21, 2002

Low-neurovirulence BeAn and DA Theiler's murine encephalomyelitis viruses (TMEV) cause persistent infection in the central nervous system (CNS) of susceptible mouse strains, leading to an inflammatory demyelinating process. A role for a specific virus–cell receptor interaction has been posited to explain why only low- and not high-neurovirulence TMEV cause persistent CNS infections. Low- but not high-neurovirulence TMEV use sialic acid for attachment to mammalian cells, which may contribute to neurovirulence attenuation and viral persistence. Analysis of BeAn virus binding and infection in cells with altered (mutated) cell-surface expression of sialic acid containing glyconjugates indicated that both binding and infection are mediated entirely by N-linked glycoproteins. By contrast, GDVII virus binding and infection appears to be dependent only in part on N-linked glycoproteins and not on O-linked glycoproteins or glycolipids. These results indicate that low-neurovirulence BeAn virus uses a sialic acid moiety expressed on an N-linked carbohydrate of a glycoprotein that serves as the protein entry receptor. © 2002 Elsevier Science (USA)

Key Words: Theiler's murine encephalomyelitis virus; sialic acid; N-linked oligosaccharides; receptor; attachment factor.

INTRODUCTION

Theiler's murine encephalomyelitis virus (TMEV), an enteric pathogen of mice, belongs to the Cardiovirus genus in the *Picornaviridae* family (Pevear *et al.,* 1987). TMEV consists of two groups based on neurovirulence in intracerebrally (ic) inoculated mice; high-neurovirulence strains, such as GDVII, produce acute encephalitis in mice, whereas low-neurovirulence TMEV, such as BeAn and DA, produces persistent central nervous system (CNS) infection of susceptible mouse strains, leading to an inflammatory demyelinating process (Lipton, 1975; Lehrich *et al.,* 1976). Infection of mice with the low-neurovirulence TMEV has been studied extensively because of similarities between the experimental demyelinating disease and multiple sclerosis.

A role for TMEV–cell receptor interaction(s) has been postulated to explain why only the low-neurovirulence TMEV cause persistent infections (Adami *et al.,* 1997; Jarousse *et al.,* 1994). Low-neurovirulence TMEV appear to use sialic acid for attachment (Fotiadis *et al.,* 1991; Zhou *et al.,* 1997, 2000) and a protein entry receptor for internalization into the cell. Virus overlay protein blot assay of ligand binding indicates the use of a 34-kDa protein entry receptor (Kilpatrick and Lipton, 1991). Recently, Hertzler *et al.* (2001) demonstrated that the uridine diphosphate (UDP) galactose transporter found in the *trans*-Golgi apparatus is required for binding and infection of both high- and low-neurovirulence TMEV. Galactose is the penultimate sugar required for addition of terminal sialic acid to glycoproteins and glycolipids (Table 1) and is also a component of the tetrasaccharide linker in proteoglycan synthesis. Thus, loss of galactose could affect virus binding to glycoproteins, glycolipids, and proteoglycans.

To further dissect the role of carbohydrates in TMEV binding and infection, we analyzed BeAn infection of cells with defective surface expression of sialic acid containing glycoconjugates, e.g., N-, O-, and lipid-linked oligosaccharides, and compared the results with those for GDVII virus binding and infection. We find that BeAn virus binding and infection is mediated entirely by N-linked glycoproteins, whereas GDVII virus binding and infection is only partially dependent on N-linked glycoproteins. Indeed, GDVII virus binding and infection was recently shown to depend on heparan sulfate proteoglycans (Reddi and Lipton, 2002).

RESULTS

BeAn virus uses sialic acid moieties for cell attachment

Treatment of BHK-21 cells with *Clostridium perfringens* neuraminidase resulted in greater than 90% reduction of BeAn virus binding as determined by antibody detection of virus binding by flow cytometry and radiolabeled virus

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TABLE 1

Complex Oligosaccharide Structures Dependent on Galactose Linkages

Galactose and sialic acid moiety	Inhibitor	References
N-linked	Tunicamycin	McDowell *et al.,* 1988
	1-Deoxymannojirimycin	Saunier *et al.,* 1982
O-linked	BenzylGalNAc	Huet *et al.,* 1996
Lipid-linked	Fumonisin B1	Wang *et al.,* 1991

binding (Fig. 1), whereas binding of GDVII virus was unaffected (not shown). A typical dose-response to increasing neuraminidase concentrations was observed, with maximal inhibition at 1 mU/ml (not shown). Treatment of BHK-21 cells with *Vibrio cholera* neuraminidase also abrogated BeAn virus binding (not shown). Neuraminidase treatment of BHK-21 cells showed a similar effect on infection (Fig. 2). To determine whether BeAn virus requires cell-surface sialic acid for binding to other mammalian cells, BeAn virus binding to neuraminidase-treated Jurkat cells, a human T lymphocyte line, was examined and reduced by 92% (not shown), and that to Lec-2 and Lec-8 cells, mutant Chinese hamster ovary (CHO) cells lacking the CMP sialic acid and UDP galactose transporters, respectively, was reduced by more than 90% compared to parental CHO cells (Fig. 3). Results of infection of these cells paralleled viral binding (Fig. 2). Thus, consistent with previous results (Fotiadis *et al.,* 1991), cell-surface sialic acid is necessary for attachment and infection of BeAn but not GDVII virus to mammalian cells.

BeAn virus does not use sialic acid on glycolipids or O-linked oligosaccharides for cell attachment

Since loss of the UDP galactose transporter activity in the *trans*-Golgi network eliminates TMEV binding (Hertzler *et al.,* 2001; also see Lec-8 cells in Fig. 3), and the penultimate galactose and terminal sugar sialic acid are found on cell-membrane glycolipids, we examined the role of gangliosides in TMEV attachment. Analysis of BeAn binding in glycolipid-deficient GM95 cells and parental glycolipid-expressing MEB4 cells revealed no significant difference, whereas GDVII virus-binding levels were 2.5-fold higher in the glycolipid-deficient GM95 cells than in parental MEB4 (Fig. 4A). Despite the difference in GDVII virus binding, infection as measured by MTT assay was similar in both cell lines (Fig. 2). In BHK-21 cells treated with the ceramide synthase inhibitor, fumonisin B1, to abrogate ganglioside expression, binding of the lectin Mal I (Wang *et al.,* 1991) was proportionately decreased as expected ($P < 0.01$). Although fumonisin B1 treated BHK-21 cells showed morphological changes (not shown), binding of both TMEV was unaffected (Fig. 4B). Thus, BeAn virus does not attach to sialic acid on glycolipids, while GDVII virus binding is enhanced in the absence of glycolipids. Lack of enhanced GDVII virus binding by fumonisin B1 may be due to less efficient or a difference in glycolipid removal.

The effect of altered cellular O-linked carbohydrate expression on virus attachment and infection was exam-

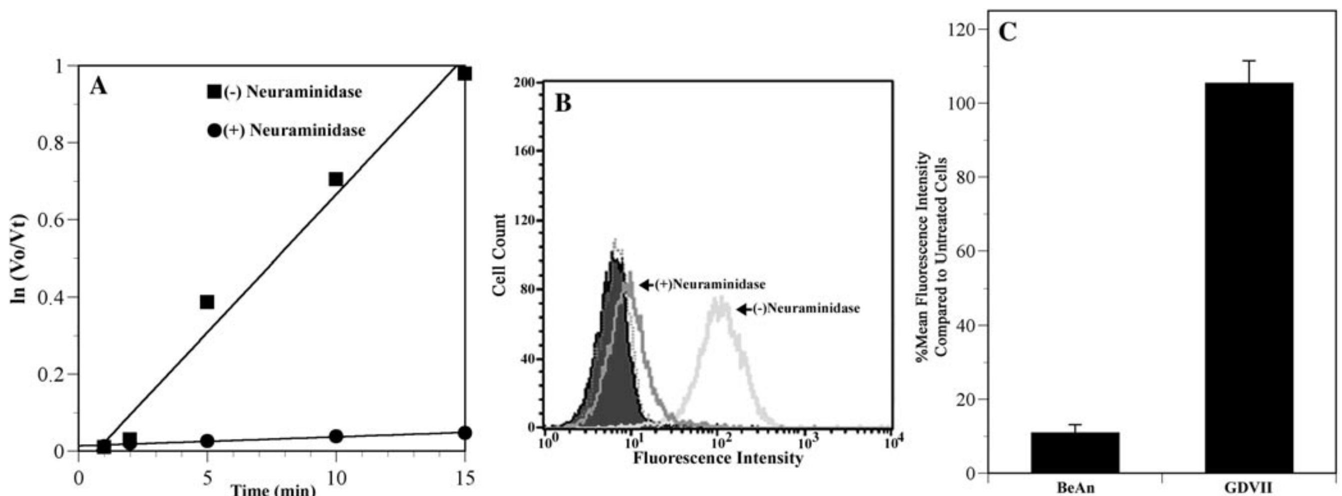

FIG. 1. BeAn virus binding to BHK-21 cells as measured by radiolabeled virus association (A) or flow cytometry (B and C). (A) Purified ^{35}S-labeled BeAn virus (1×10^6 cpm) was incubated with 1×10^6 BHK-21 cells at 4°C for varying times and washed extensively. BHK-21 cells were either treated with 1 mU/ml of *Clostridium perfringens* neuraminidase for 45 min at 37°C (●) or mock treated (■). Cell-associated radioactivity was measured with a scintillation counter and plotted as described (Hertzler *et al.,* 2000); V_0 is the total amount of virus particles added to 1×10^6 cells and V_t is the unattached virus in a period of time. (B) Neuraminidase- or mock-treated BHK-21 cells (1×10^6) were incubated with BeAn virus at an m.o.i. of 10 at 4°C for 45 min, washed, and successively incubated with mAb to TMEV VP1 and goat anti-mouse IgG-FITC. Plot of cell-associated fluorescence measured with a flow cytometer. (C) BeAn and GDVII virus binding to neuraminidase-treated BHK-21 cells shown as mean fluorescence intensity ± SD compared to untreated cells.

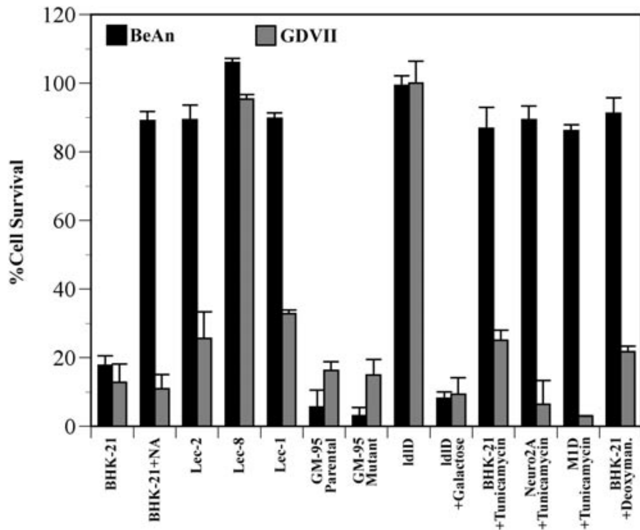

FIG. 2. BeAn and GDVII virus infection was carried out for 20 h in cells treated with listed reagents or in mutant cell lines. Cell viability was measured by MTT assay. All experiments were performed in triplicate and results presented as percentage of cell survival compared to mock-infected cells ± SD. BeAn virus infection was substantially reduced only under conditions in which cell-surface sialic acid or N-linked oligosaccharides were removed. GDVII virus infection was reduced only in conditions in which galactose processing was aberrant and was unaffected by N-linked oligosaccharide processing defects.

ined in ldlD cells, a CHO mutant with defective synthesis of N-linked, O-linked, and lipid-linked glycoconjugates due to loss of UDP-galactose/UDP-galactosamine-4-epimerase activity; this defect is reversible by the addition of galactose and/or *N*-acetylgalactosamine to the culture medium (Kingsley *et al.,* 1986). O-linked glycoproteins require addition of both sugars for proper expression while the other glycoconjugates require only

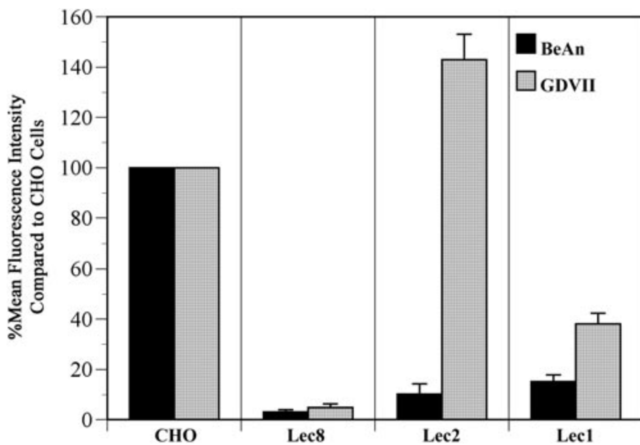

FIG. 3. BeAn and GDVII virus binding to Lec cell mutants. Viral binding was measured by flow cytometry and compared to binding of parental CHO cells. Lec-2 and Lec-8 cells have defects in the CMP sialic acid and UDP galactose transporters, respectively, while Lec-1 cells are defective in N-linked carbohydrate processing. Data are given as % mean fluorescent intensity ± SD.

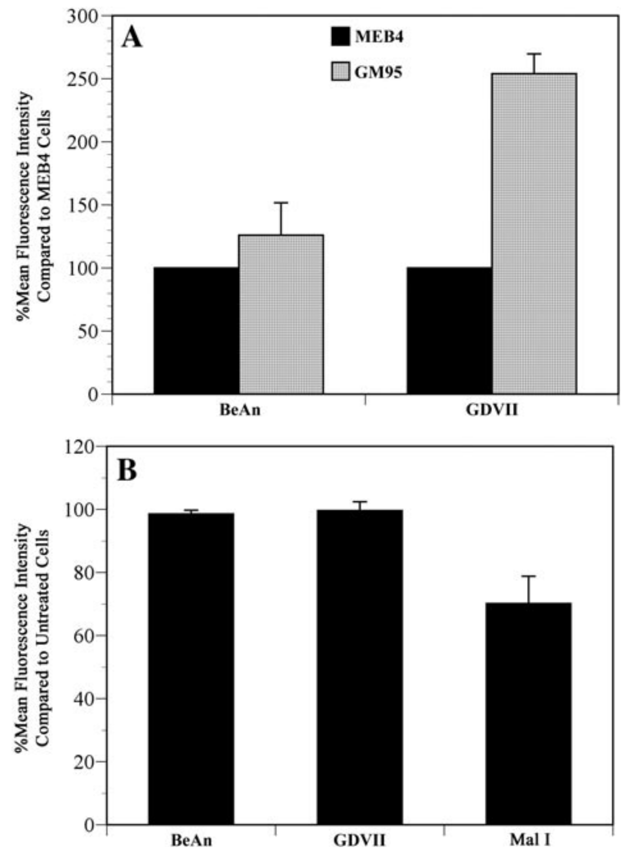

FIG. 4. (A) BeAn and GDVII virus binding to ganglioside-deficient GM-95 cells compared to parental, ganglioside-containing MEB-4 mouse melanoma cells. (B) BeAn virus, GDVII virus, and Mal I binding to fumonisin B1-treated BHK-21 cells. Cells were grown in media containing 10 mM fumonisin B1 for 48 h before viral binding. Viral and lectin binding was measured by flow cytometry, and the mean fluorescent intensity ± SD was determined by comparison with untreated cells.

the addition of galactose. Neither TMEV nor the Mal I lectin bound to ldlD cells grown in the absence of the two sugars, but addition of 20 μM galactose to the culture medium restored binding and infection of both viruses and Mal I (Figs. 2 and 5A). Addition of both 20 μM galactose and 200 μM *N*-acetylgalactosamine to the culture medium did not alter TMEV binding of ldlD cells as compared with addition of galactose alone (not shown). TMEV binding and infection was also examined in HT-29 human colorectal adenocarcinoma cells treated with benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BenzylGalNAc), a metabolic inhibitor that reduces cell-surface sialic acid associated with O-linked carbohydrates (Huet *et al.,* 1996). BenzylGalNAc has been demonstrated to be an effective inhibitor of O-linked glycosylation in HT-29 cells (Huet *et al.,* 1998). After treatment, these cells were morphologically heterogeneous (not shown), and binding of the O-linked carbohydrate-specific lectin jacalin was decreased by ~33% ($P < 0.005$), but binding of both TMEV remained unchanged (Fig. 5B). Similar results were obtained using BenzylGalNAc-

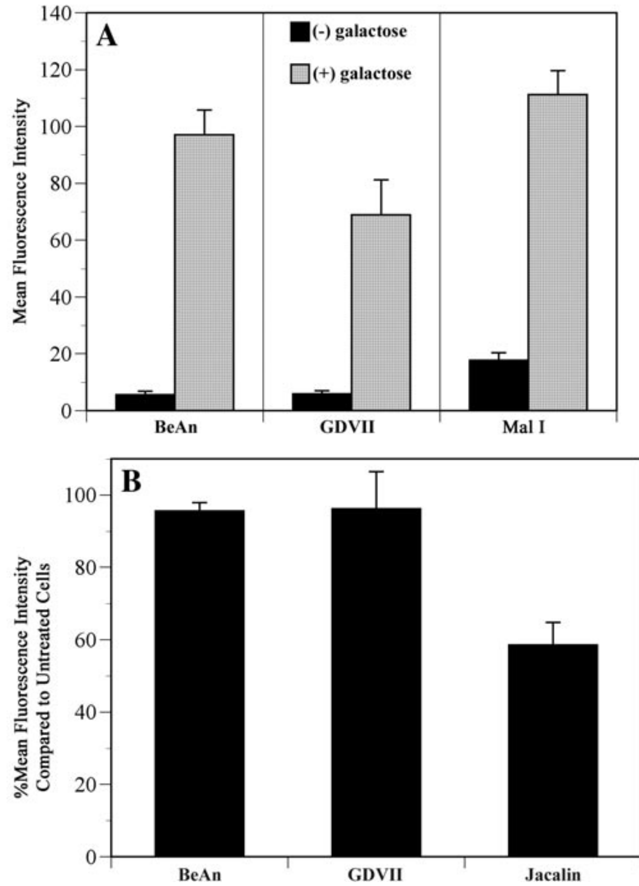

FIG. 5. (A) Virus binding to glycoconjugate-deficient ldlD cells grown in medium in the absence or presence of 20 mM galactose. Without added galactose, ldlD cells do not express N- or O-linked carbohydrates while cells grown in media containing galactose express carbohydrates chains only on N-linked glycoproteins and glycolipids. Restoration of viral binding by addition of galactose only demonstrates that O-linked carbohydrates are not necessary for cell-surface attachment of TMEV. (B) BeAn, GDVII, and jacalin binding to BenzylGalNac-treated HT-29 cells. Cells were grown in medium containing 20 mM BenzylGalNac for 96 h before viral binding. Virus and lectin binding was measured by flow cytometry and compared to untreated cells. Cell-surface O-linked carbohydrates were reduced, as evidenced by reduction in jacalin binding, but BeAn or GDVII virus binding remained unchanged. Viral and lectin binding was measured by flow cytometry and the mean fluorescent intensity ± SD determined by comparison with untreated cells.

treated BHK-21 cells (not shown). Together, these results indicate that neither BeAn nor GDVII virus requires O-linked carbohydrates for cell-surface attachment or for infection.

BeAn virus uses sialic acid on N-linked oligosaccharides for cell attachment

To examine the role of N-linked glycosylation in TMEV binding and infection, BHK-21 cells were treated with tunicamycin, a fungal toxin that blocks assembly of *N*-acetylglucosamine on the lipid carrier dolichol phosphate, or with 1-deoxymannojirimycin, which blocks processing by α-mannosidase I (McDowell and Schwarz, 1988; Saunier *et al.,* 1982); both toxins inhibit early steps in the assembly of N-linked carbohydrates. BeAn virus binding to cells treated with either toxin was decreased by more than 75%, whereas GDVII virus binding was reduced by only 20% (Fig. 6). On the other hand, no inhibition of GDVII virus infection was observed in cells treated with these metabolic inhibitors (Fig. 2). These results indicate that the sialic acid moieties important for BeAn virus attachment and infection are found on N-linked glycoproteins, whereas N-linked glycoproteins probably account for only a minor portion of GDVII virus binding. Further analysis of the role of N-linked oligosaccharides in BeAn virus binding using cell-membrane extracts digested with varying concentrations of PNGase F, an N-linked specific endoglycosidase, or neuraminidase, blotted onto nitrocellulose filters and overlaid with ^{35}S-labeled BeAn virus, revealed abrogation of BeAn vi-

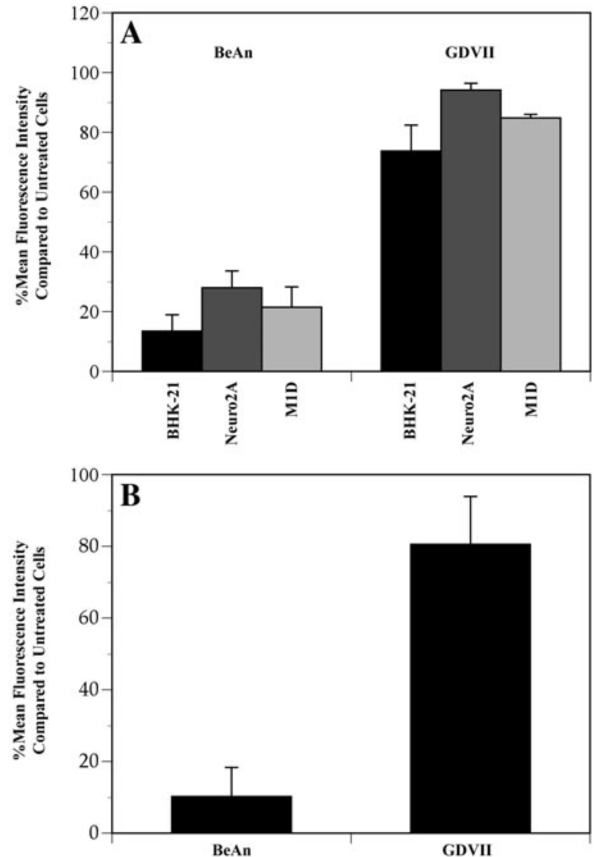

FIG. 6. BeAn and GDVII virus binding to cells treated with tunicamycin or 1-deoxymannojirimycin. (A) Viral binding to BHK-21, Neuro2A, or M1D cells treated with tunicamycin. Cells were grown in medium containing 20 μg/ml tunicamycin for 48 h before viral binding. Virus binding was measured by flow cytometry and the mean fluorescent intensity ± SD determined by comparison with untreated cells. BeAn virus binding was substantially reduced, while that of GDVII was only moderately affected after treatment. (B) Virus binding to BHK-21 cells was carried out as in (A) except that 20 μg/ml of 1-deoxymannojirimycin was used. BeAn but not GDVII virus binding was substantially reduced.

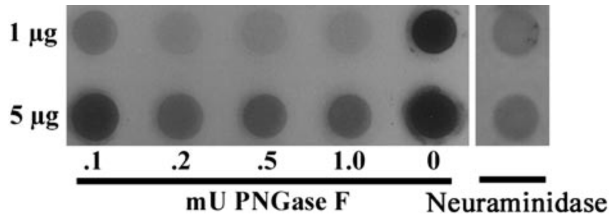

FIG. 7. Binding of ^{35}S-labeled BeAn virus binding to enzyme-treated BHK-21 cell-membrane preparations. BHK-21 membrane proteins were treated with PNGase F or *Clostridium perfringens* neuraminidase and blotted onto nitrocellulose filters and incubated with 1×10^6 cpm of ^{35}S-labeled BeAn virus. Cleavage of N-linked carbohydrates and sialic acid substantially reduced viral binding.

rus binding to membrane extracts digested with either enzyme (Fig. 7). Digestion with *O*-glycanase could not be carried out because prior treatment with neuraminidase is required for enzymatic activity (Umemoto *et al.,* 1977).

BeAn virus uses sialic acid on N-linked oligosaccharides on cell lines representing the principal cellular targets in the mouse CNS

BeAn virus principally replicates in neurons during the acute phase of infection and macrophages/microglia during viral persistence in the mouse CNS (Lipton *et al.,* 1995; Aubert and Brahic, 1995). Therefore, we examined whether BeAn virus utilizes N-linked oligosaccharides for binding and infection of a murine neuronal cell line (Neuro2A neuroblastoma cells) and differentiated murine macrophages (M1D cells). Tunicamycin treatment of both cell lines resulted in a marked decrease in BeAn virus binding and infection; however, there was little effect on GDVII binding and infection (Figs. 2 and 6A). This result suggests that low-neurovirulence TMEV also use sialic acid on N-linked oligosaccharides in infections of the mouse CNS.

DISCUSSION

Members of both TMEV neurovirulence groups require galactose for attachment to cells (Hertzler *et al.,* 2001), but our present results indicate that the galactose residues are present on different molecules. Our study localizes the sialic acid moiety necessary for BeAn virus attachment and infection to a complex type N-linked oligosaccharide structure. The sialic acid residues found on O-linked carbohydrates and glycolipids are not needed for BeAn attachment. Most likely, BeAn virus binds a sialic acid residue with an α2–3 linkage, since (1) sialic acids on N-linked carbohydrates are principally linked to the penultimate galactose residue by this linkage (Dell and Morris, 2001); (2) BeAn binding is blocked by α2–3-linked siallylactose (Zhou *et al.,* 1997); and (3) the lectin *Maackia amurensis* (Mal 1), which recognizes the galacosyl β1,4-*N*-acetylglucosamine linkage alone or with α2,3- but not α2,6-linked sialic acid partially blocks BeAn infection (submitted for publication). In contrast to BeAn virus, GDVII requires galactose residues located in the tetrasaccharide linker of heparan sulfate proteoglycan for attachment (Reddi and Lipton, 2002).

The finding that expression of the UDP galactose transporter (UGT) in a TMEV receptor-negative mutant cell line restored viral binding and infection (Hertzler *et al.,* 2001) pointed to UGT itself as a TMEV receptor protein candidate. Although UGT resides in the *trans*-Golgi network, the transporter may reach the cell surface since other *trans*-Golgi proteins are known to cycle to the plasma membrane in exocytic transport vesicles along with their glycoprotein products and return to the *trans*-Golgi network via the endocytic pathway (Ladinsky and Howell, 1993; Shukla *et al.,* 1999). However, rabbit polyclonal antibodies to human UGT do not block TMEV binding or infection of BHK-21 cells (submitted for publication), suggesting that UGT is not a TMEV receptor protein.

The ability to express receptor proteins in cells has enabled a distinction between attachment and infection, functions that may be mediated by the same or different receptors (Evans and Almond, 1998). We believe that two receptors are necessary for BeAn virus attachment and infection. The involvement of sialic acid residues on a specific subset of cell-surface structures suggests that the sialic acid binding properties of BeAn virus are not promiscuous and couple binding of this carbohydrate to a specific protein receptor. Virus overlay assays have provided evidence for the binding of a protein receptor (Kilpatrick and Lipton, 1991; Libbey *et al.,* 2001). Furthermore, BeAn competitively inhibits the binding of GDVII virus, but GDVII does not competitively inhibit BeAn virus (Fotiadis *et al.,* 1991). The nonreciprocal TMEV blocking results are similar to those reported for reovirus serotype 3 strains, which differ in their capacity to bind sialic acid (Barton *et al.,* 2001). The two TMEV strains may bind different regions of the same protein with BeAn attaching to a more distal site, consistent with the notion of BeAn attachment to both a terminal sialic acid residue and a protein sequence or conformation. Our preliminary observations suggest that BeAn binds to a sialic acid residue linked to a specific protein (model 1); however, the data presented here do not distinguish between this possibility and the virus binding to sialic acid on a glycoprotein and then to another protein entry receptor that is not glycosylated (model 2).

The utilization of carbohydrates for cellular attachment is uncommon among picornaviruses. Virulent or field strains of foot-and-mouth disease virus and echovirus-6 have been shown to rely on heparan sulfate as an attachment factor *in vitro* (Jackson *et al.,* 1996; Goodfellow *et al.,* 2001), and rhinovirus 87 binds sialic acid (Uncapher *et al.,* 1991). These viruses also bind to known protein receptors to gain entry into the cell. However, the simultaneous binding of sialic acid and peptide compo-

nents of the same glycoprotein would be a feature unique to low-neurovirulence TMEV.

Zhou *et al.* (2000) have solved the crystal structure at 3.0 A of the low-neurovirulence DA strain bound to α2,3-siallylactose, revealing the binding site for sialic acid in a positively charged area on VP2 puff B. Four contacts are formed through hydrogen bonds between molecules of *N*-acetyl neuraminic acid and VP2 amino acids 161, 163, and 174, and VP3 residue 232. The three VP2 residues on puff B are located 20–30 A from a depression on the cardiovirion protomer, termed the pit, which is believed to be the docking site for the cellular receptor protein. In fact, mutation of four residues in the BeAn virus pit has indicated that these residues are involved in BeAn virus infection (Hertzler *et al.,* 2000). Clearly, the pit residues are not responsible for attachment to sialic acid, further supporting the use of both sialic acid and a protein for attachment and internalization of low-neurovirulence TMEV. We are currently mutating the three VP2 puff residues that make contact with sialic acid to test for a role in attachment; VP3 residue 232 located in the cleavage dipeptide between VP3 and VP1 cannot be changed.

Our results provide evidence for a differential receptor interaction between high- and low-neurovirulence TMEV. Both viruses may attach to the same protein receptor; however, the disruptions of carbohydrate additions described here only inhibit BeAn attachment. The utilization of different surface structures for cellular attachment may underlie the differences in tissue tropism and neurovirulence for each virus. In this context, it will be interesting to compare posttranslational modifications of neuronal vs glial and macrophage cell membranes, the cells in which low-neurovirulence TMEV persist. Identification of the protein receptor and an understanding of its expression and modification may also help explain the vastly different pathologies caused by the two groups of TMEV.

MATERIALS AND METHODS

Viruses

BeAn and GDVII virus stocks were prepared as clarified lysates in BHK-21 cells, with titers $> 10^8$ PFU/ml, as described (Rozhon *et al.,* 1983). BeAn virus was radiolabeled and purified as described (Hertzler *et al.,* 2000).

Cells

Baby hamster kidney (BHK-21) cells, the Pro-5 variant of CHO cells, CHO glycosylation-deficient mutants Lec-1, Lec-2, and Lec-8 cells, HT-29, human colorectal carcinoma cells, glycoconjugate-deficient ldlD cells, and Neuro2A cells were obtained from American Type Culture Collection. MEB-4 mouse B16 melanoma and GM-95 ganglioside mutant cells (Ichikawa *et al.,* 1994; Saunier *et al.,* 1982) were obtained from the RIKEN Cell Bank (Tokyo, Japan). M1D macrophages were derived from the myeloid precursor M1 cells and maintained as described (Jelachich *et al.,* 1999). BHK-21 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin, 7.5% fetal bovine serum (FBS), and 6.5 mg/ml tryptose phosphate broth (Gibco-BRL) at 37°C in a 5% CO_2 atmosphere. CHO, Lec-1, Lec-2, and Lec-8 cells were grown in minimum essential medium (α-MEM) supplemented with 2 mM L-glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin, and 10% FBS. HT-29 cells were grown in McCoy's 5a medium with 1.5 mM L-glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin, and 10% FBS. ldlD cells were grown in Ham's F12 medium supplemented with 2 mM L-glutamine, 1% ITS+1 (insulin, transferrin, selenium, linoleic acid, and bovine serum albumin (BSA) supplement; Sigma), 100 mg/ml streptomycin, 100 U/ml penicillin, and 5% FBS. MEB-4 and GM-95 cells were grown in DMEM supplemented with 100 mg/ml streptomycin, 100 U/ml penicillin, and 10% FBS. Neuro2A cells were grown in MEM supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10% FBS.

Reagents

V. cholera and *C. perfringens* neuraminidase, tunicamycin, 1-deoxymannojirimycin, BenzylGalNAc, fumonisin B1, and PNGase F were purchased from Sigma. The lectins Mal I and jacalin were purchased from Vector Laboratories. mAb 6F7 to TMEV VP1 was kindly provided by Raymond Roos at the University of Chicago.

Binding assays

Binding of virus to cells in suspension was determined by flow cytometry and with radiolabeled virus as described (Jelachich and Lipton, 2001; Hertzler *et al.,* 2001). Briefly, 10^6 cells were washed with phosphate-buffered saline (PBS), pH 7.2, containing calcium and magnesium, and incubated with virus for 45 min at 4°C. Cells were washed with PBS containing 0.5% FBS (PBS-FACS), blocked with 10 μl of normal goat serum for 15 min at 4°C, and incubated with 1 μg of mAb 6F7 to TMEV VP1 for 30 min at 4°C followed by 1 μg of goat anti-mouse IgG-FITC conjugate for 30 min at 4°C. After three washes with PBS-FACS, cells were fixed with 1% paraformaldehyde and analyzed on a FACSCalibur (Becton-Dickinson, Palo Alto, CA). Lectin binding to cells was determined using 1 μg of biotin-conjugated Mal I or jacalin followed by avidin-FITC. Mean fluorescence intensity (±SD) of triplicate samples was determined and the experiment repeated at least three times. Results from three samples were analyzed using the Student's *t* test.

Binding was also assayed by attachment of ^{35}S-la-

beled BeAn virus to BHK-21 cells. BHK-21 cells were detached from monolayers with PBS without calcium and magnesium, washed, and resuspended to a concentration of 10^6 cells/ml in DMEM containing 20 mM HEPES and 1% BSA and incubated on ice for 1 h before addition of [^{35}S]methionine-labeled virus (20,000 particles/cell). At indicated times, an aliquot of the virus-cell suspension was removed and diluted in DMEM containing 20 mM HEPES before centrifugation at 12,000 *g* for 30 s. Supernatant- and cell-associated radioactivity was determined for triplicate samples in a Beckman LS5000TD scintillation counter and plotted as a percentage of cell-associated counts.

Infectivity assay of cell survival

Cells were grown to 80% confluency in 24-well plates, treated- or mock-treated with reagents as described, followed by infection with BeAn or GDVII virus at an m.o.i. of 10 (absorption for 45 min at 24°C), and incubated in maintenance medium for 20 h. Cell viability was measured by MTT assay as described (Denziot and Lang, 1986).

Extraction of cell membranes

BHK-21 cells (1×10^8) were washed with PBS, resuspended in 10 ml of PBS with the mammalian protease inhibitor cocktail (Sigma), homogenized with 20 strokes of a Dounce homogenizer, and examined for lysis by trypan blue exclusion. The lysate was centrifuged at 2000 *g* for 10 min; the recovered supernatant was centrifuged at 40,000 *g* for 30 min, and the pellet was resuspended in 200 μl of PBS containing 0.1% β-octylglucoside and protease inhibitor cocktail and assayed for protein concentration using the Pierce BCA protocol. Membrane preparations were mock-, neuraminidase-, or PNGaseF-treated at various concentrations.

Dot blots

One or five micrograms of BHK-21 membrane protein preparation was blotted onto nitrocellulose membranes blocked with 5% BSA as described (Kilpatrick and Lipton, 1991). ^{35}S-labeled-BeAn virus (1×10^6 cpm) in a volume sufficient to cover the membrane was added and incubated for 2 h at 24°C and autoradiographed for 12–24 h.

ACKNOWLEDGMENTS

We thank Pat Kallio for technical assistance and Honey V. Reddi for helpful discussions. This work was supported by NIH Grant NS23349.

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