Isolation and Molecular Characterization of a Novel Type 3 Reovirus from a Child with Meningitis

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Mammalian reoviruses are nonenveloped viruses that contain a segmented, double-stranded RNA genome. Reoviruses infect most mammalian species, although infection with these viruses in humans is usually asymptomatic. We report the isolation of a novel reovirus strain from a 6.5-week-old child with meningitis. Hemagglutination and neutralization assays indicated that the isolate is a serotype 3 strain, leading to the designation T3/Human/Colorado/1996 (T3C/96). Sequence analysis of the T3C/96 S1 gene segment, which encodes the viral attachment protein, α1, confirmed the serotype assignment for this strain and indicated that T3C/96 is a novel reovirus isolate. T3C/96 is capable of systemic spread in newborn mice after peroral inoculation and produces lethal encephalitis. These results suggest that serotype 3 reoviruses can cause meningitis in humans.
Table 1. Reovirus strains used for T3C/96 S1 gene sequence analysis.

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* Strains are named according to the following scheme: serotype/species of origin/place of origin/strain designation/year of isolation [70].

of neurotropism [14, 18] segregate with the viral S1 gene, which encodes the viral attachment protein [19, 20]. The σ1 protein which determines the CNS cell types that serve as targets for reovirus infection, presumably by its capacity to bind receptors expressed on specific CNS cells.

The σ1 protein is a fibrous trimer with an elongated tail domain that projects away from the virion surface [21–23]. T1 and T3 σ1 contain receptor-binding domains in both the tail and head regions. A domain in the T3 σ1 tail binds α-linked sialic acid [24, 25], and another domain in the head of both T1 and T3 σ1 binds functional adhesion molecule 1 (JAM1) [26]. The T1 σ1 tail also binds cell-surface carbohydrate [25]. As a consequence of a sequence polymorphism in the tail of T3D/55 σ1, the sialic acid– and JAM1-binding domains are dissociable by treatment of virions with intestinal proteases, such as trypsin or chymotrypsin [27, 28], which may determine the attenuated virulence of T3D/55 after oral inoculation [29].

Engagement of reovirus receptors also induces postbinding signaling events that influence disease pathogenesis. Reovirus induces apoptosis in cultured cells [30–33], including neurons [34], and in vivo [35, 36]. Neurovirulent strains induces apoptosis to a greater extent than nonneurovirusin strains [30, 31, 34, 37]. Analysis of T1L53 X T3D/55 reassortant viruses indicates that differences in apoptosis efficiency are determined primarily by the σ-encoding S1 gene [30, 31, 37], which suggests that receptor engagement influences the magnitude of the apoptotic response. In concordance with this idea, the most apoptogenic reovirus strains bind to both sialic acid [37] and JAM1 [26].

In the present study, we report the isolation of a T3 reovirus designated T3/Human/Colorado/1996 (T3C/96) from the CSF of a 6.5-week-old child with meningitis. The T3C/96 S1 gene sequence was determined and compared to all previously reported T3 reovirus S1 gene sequences. The capacity of T3C/96 to bind sialic acid and JAM1 and to cause encephalitis in mice was assessed. The results indicate that T3C/96 is a novel T3 reovirus capable of systemic spread to the CNS after peroral inoculation of newborn mice and provide direct evidence that T3 reovirus can be neurovirulent in humans.

MATERIALS AND METHODS

Cells, viruses, and antibodies. Spinner-adapted murine L929 (L) cells grown in suspension or on monolayer cultures and HeLa cell monolayers were maintained as described elsewhere [30, 32]. Prototype reovirus strains T1L/53 and T3D/55 and reovirus field isolate strains (table 1) are laboratory stocks. Virus titers were determined by use of plaque assay [38], and purified virus and particle concentrations were determined, as described elsewhere [21, 39]. Antibodies used included murine σ1-specific monoclonal antibody (MAb) 5C6 (T1 σ1) [40], MAb 9B5G (T3 σ1) [41], and JAM1-specific MAb 10.4 [42].

Electron microscopy. Supernatant of infected rhesus monkey kidney (RMK) cells was clarified by centrifugation at 1000 g for 10 min at 22°C. Clarified supernatant was placed in an Airfuge EM-90 rotor (Beckman Coulter) containing a Formvar-coated grid (Electron Microscopy Sciences) and was centrifuged at 100,000 g for 30 min at 22°C. The grid was stained with 2% phosphotungstic acid (1 min) and was examined by use of a Zeiss EM10 electron microscope.

Hemagglutination (HA) assays. Puriﬁed reovirus virions (10¹¹ particles) were serially diluted in 50 mL of PBS in 96-well round-bottom microtitre plates (Corning-Costar). Calf eryth-
rocytes (Colorado Serum) were washed twice with PBS and were resuspended at a concentration of 1% (vol/vol) in PBS. Erythrocytes (50 μL) were added to wells containing virus and incubated for 2 h at 4°C.

Neutralization assays. T1 and T3 σ1-specific MABs were serially diluted 2-fold in gelatin saline and were incubated with 10^7 pfu/mL of T3C/96 virions for 1 h at 37°C. Samples were titrated in duplicate on L-cell monolayers by use of plaque assay [38]. Data are presented as the percentage of control plaque-forming units (virions untreated by antibody).

Sequence analysis of the S1 gene. The S1 gene segment of T3C/96 was amplified by use of reverse-transcriptase polymerase chain reaction (RT-PCR), using primers complementary to the 5′ and 3′ nontranslated regions (NTRs) [43], and then was cloned into the pCR 2.1 vector (Invitrogen) and sequenced by use of T4 DNA polymerase (Sequenase 2.0; United States Biochemical). Sequences of the NTRs were determined by use of direct dsRNA sequencing, using purified viral genomic RNA [44]. The S1 gene nucleotide sequences of independent isolates of T3C/96 were determined in independent laboratories and were found to be identical.

Phylogenetic analysis of S1 gene nucleotide sequences. Phylogenetic trees were constructed from variation in the σ1-encoding S1 gene nucleotide sequences by use of the neighbor-joining algorithm (phylogenetic analysis program; MacVector 2001, version 7.1.1; Accelrys). Branching orders of the phylogenograms were verified statistically by resampling the data 1000 times in a bootstrap analysis, using the branch and bound algorithm (MacVector).

Fluorescent-focus assays of viral infectivity. HeLa cell monolayers (2 × 10^3 cells/well) were pretreated with PBS, coxsackie and adenovirus receptor (CAR)–specific MAB Rm6B (20 μg/mL) [45, 46], Arthrobacter ureafaciens neuraminidase (40 mU/mL; ICN Biomedicals), or JAM1-specific MAB J10.4 (20 μg/mL) [47], to permit completion of a single round of viral replication. Cells were fixed with 1 mL of methanol for 30 min at −20°C. Fixed monolayers were incubated with 1:200 dilution of goat anti-rabbit immunoglobulin conjugated with Alexa Fluor 546 fluorophore (Molecular Probes). Monolayers were washed twice with PBS/0.5% Triton X-100, and infected cells were visualized by indirect immunofluorescence by use of a Zeiss Photomicroscope III microscope modified for fluorescence microscopy. Infected cells were identified by the presence of intense cytoplasmic fluorescence that was excluded from the nucleus. No background staining of uninfected control monolayers was detected. Reovirus antigen-positive cells were quantitated by counting fluorescent cells in 3 random fields of view/well in triplicate at a 20× magnification.

Mice and inoculations. ND4 Swiss Webster mice aged 2–3 days with an average weight of 2 g (Harlan) were inoculated either intracranially or perorally with purified virus. Before inoculation, all mice from simultaneously delivered litters were pooled and randomly subdivided into litters of 8–11 mice/dam. For intracranial inoculations, 5 μL of purified virus diluted in gelatin saline was delivered into the right cerebral hemisphere by use of a Hamilton syringe and a 30-gauge needle [48]. For peroral inoculations, 50 μL of purified virus diluted in gelatin saline was delivered into the stomach by passage of a polyethylene catheter (0.61 mm in diameter (BD Biosciences) through the esophagus [49]. At various times after inoculation, mice were killed, and brains were harvested into 2 mL of gelatin saline. Brains were homogenized by freezing (−70°C) and thawing (37°C), which was then followed by sonication. Virus titer in brain homogenates was determined by plaque assay [38].

The LD₅₀ of reovirus was determined by use of ND4 Swiss Webster mice aged 2–3 days. Litters of mice were inoculated either intracranially or perorally with a single dose of reovirus and were checked daily for survival. Moribund mice were killed. The LD₅₀ of reovirus was calculated by use of the method of Reed and Muench [50]. All animal experiments were performed under institutionally approved protocols in Association for Assessment and Accreditation of Laboratory Animal Care–approved facilities.

Histological and immunohistochemical staining for reovirus antigen. At various times after intracranial inoculation of neonatal mice, mice were killed. Brains were fixed in 10% buffered formaldehyde for 24–48 h at 4°C, paraffin embedded, and thin sectioned. Deparaffinized tissue samples were stained with hematoxylin-eosin.

For immunohistochemical staining, deparaffinized tissue samples were rehydrated by incubation in PBS for 20 min at room temperature. Endogenous peroxidase was quenched by incubation in 0.3% hydrogen peroxide in methanol for 30 min. Tissue samples were blocked by incubation in PBS containing 1.5% normal goat serum for 20 min. After the blocking solution was removed, tissue samples were incubated for 30 min in rabbit antireovirus serum (1:800 dilution) which was then followed by sonication. Virus titer in brain homogenates was determined by plaque assay [38].
for 45 s with hematoxylin, dehydrated in 95% ethanol, air dried, and mounted under coverslips by use of Accu-Mount 60 reagent (Baxter Healthcare).

RESULTS

Case report. A 6.5-week-old female infant who was delivered vaginally at 35 weeks gestation had a 3-day history of irritability, decreased appetite, vomiting, and high-pitched cry. After vital signs were notable for temperature of 38.8°C (pr), heart rate of 166 beats/min, respiratory rate of 36 breaths/min, and blood pressure of 72/30 mmHg. The child was in mild respiratory distress, with grunting while breathing room air. Her neck was supple, chest and cardiac examinations were normal, and her abdomen was soft and nontender. Neurological examination was remarkable for lethargy, normal cranial nerves, muscle tone, and reflexes. An episode consistent with a generalized seizure was noted in the emergency department.

Her hematocrit was 27.7%, white blood cell (WBC) count was 9500 cells/mm³, and platelet count was 412,000 platelets/mm³. CSF contained a WBC count of 22 cells/mm³ (77% neutrophils, 9% bands, and 14% lymphocytes), a protein level of 67 mg/dL, and a glucose level of 44 mg/dL. The serum glutamic oxaloacetic transaminase level was 64 IU/L (normal, <60 IU/L), and the conjugated bilirubin level was 0.5 mg/dL (normal, <0.3 mg/dL). Alkaline phosphatase, serum glutamate pyruvate transaminase, and gamma glutamyltransferase levels were normal. Bacterial cultures of blood and CSF were sterile. CSF PCR assay was negative for enteroviruses and herpes simplex virus. During her 5 days of hospitalization, the patient developed transient abdominal distension and watery diarrhea. No erythrocytes or leukocytes were observed after microscopy of stool samples, and stool cultures were negative for enteric pathogens, as were tests for rotavirus antigen and electron microscopy for rotavirus particles. She gradually recovered and was discharged from the hospital without obvious neurological sequelae.

Isolation and virological characterization of a novel T3 reovirus strain. CSF was inoculated onto primary human diploid fibroblast (MRC-5), human epithelial carcinoma (Hep-2), and primary RMK cell monolayers. The RMK cell cultures developed a granular, nonsloughing pattern of cytopathic effect (CPE). Supernatant of these cells was used to infect MRC-5 cells, which developed similar CPE. Electron microscopy of infected cells demonstrated icosahedral nonenveloped viral particles characteristic of mammalian reovirus (figure 1A).

The original RMK cell lysate was used to inoculate flasks of murine L cells, which developed a characteristic reovirus CPE. Plaque assay of lysates demonstrated round 1–2 mm plaques. Electrophoretic analysis of infected L-cell lysates demonstrated the presence of 10 viral gene segments (figure 1B). The largest

Figure 1. Strain characterization of T3C/96. A, Electron micrograph of T3C/96 after growth in primary rhesus monkey kidney cells. B, Electrophoretic analysis of reovirus strains T1L/53, T3C/96, and T3D/55. Approximately equal numbers of reovirus particles were resolved by acrylamide gel electrophoresis. Viral double-strand RNA (dsRNA) gene segments were visualized after staining of the gel with ethidium bromide. Position of the serotype-determining S1 gene segment is indicated for each strain. C, Neutralization of T3C/96 with α1-specific monoclonal antibodies (MAbs). T3C/96 virions were incubated for 1 h at 37°C with either the T1α1-specific MAb 5C6 or the T3 α1-specific MAb 9BG5 at the concentrations shown. Viral titer was determined by plaque assay, using L cells. Data are the mean of 2 experiments.
of the S-class gene segments (S1) migrated slowly in the acrylamide gel, a characteristic of T3 reovirus strains (figure 1B). Virus produced after infection of L cells agglutinated bovine erythrocytes (HA observed at virus concentrations ≥6.25 × 10⁴ pfu/well), which is characteristic of HA produced by T3 reovirus (data not shown) [51, 52]. The CSF isolate was efficiently neutralized by T3 σ1-specific MAb 9BG5 (>50% plaque reduction at 5 ng/mL), but not by T1 σ1-specific MAb 5C6 (no plaque reduction at ≤500 μg/mL) (figure 1C). The new reovirus isolate was designated T3C/96.

**Sequence analysis of the T3C/96 S1 gene.** The reovirus S1 gene, encoding viral attachment protein σ1 [19, 20], is the major genetic determinant of neurovirulence in infected mice [14, 15]. Therefore, we determined the sequence of the T3C/96 S1 gene (GenBank accession no. AY302467). The 5′ and 3′ NTRs of all T3 reovirus S1 genes sequenced to date are highly conserved [44]. RT-PCR primers complementary to the NTRs were designed to permit amplification of the entire T3C/96 S1 gene from infected L-cell lysates. A PCR product of the expected size (≈1.4 kb) was obtained. The S1 gene cDNA was cloned and sequenced. NTRs of the T3C/96 S1 gene were directly sequenced by use of dsRNA as template. Nucleotide sequences of the prototype T3D/55 and the novel T3C/96 S1 genes shared 69% positional identity, which provided sequence confirmation of the assignment of this new isolate as a T3 strain.

To define the evolutionary relationship of the T3C/96 S1 gene with the S1 genes of other reovirus strains sequenced to date, we constructed phylogenetic trees by use of variation in the S1 gene nucleotide sequences and the neighbor-joining algorithm (figure 2). The most noteworthy feature of the S1 phylogenetic tree is that the S1 gene sequence of T3C/96 is substantially divergent from all other T3 strains analyzed. However, the T3C/96 S1 gene is more closely related to the S1 genes of the other T3 strains than to those of either T1 or T2 strains. A phylogenetic tree generated by use of the maximum likelihood method (Phylogeny Inference Package) [53] had a topology identical to the tree generated by using the neighbor-joining algorithm (data not shown). Therefore, T3C/96 is the first member of a highly divergent clade of T3 reovirus.

Comparison of the deduced amino acid sequences of the T3D/55 and T3C/96 σ1 proteins confirms their evolutionary relationship, with the sequences sharing 74.3% positional identity (figure 3A). Secondary structure predictions for the 2 proteins are strikingly similar (data not shown). Notably, both T3D/55 and T3C/96 σ1 possess amino-terminal regions with high α-helical predictions and contain regions of sequence between amino-terminal residues 200–300 with high β-sheet predictions.

The deduced aa sequence of T3C/96 σ1 protein was compared with that of T3D/55 across known functional domains (figure 3B). The region between aa residues 198 and 204 has been linked genetically [24, 52, 54] and biochemically [25] to the capacity of T3D/55 to bind sialic acid. The sequence of T3C/96 σ1 was identical to that of T3D/55 across this region, which is consistent with the capacity of T3C/96 to produce HA and suggests that T3C/96 uses α-linked sialic acid as a coreceptor. Sequence polymorphism at aa 249 influenced the susceptibility of T3 σ1 protein to cleavage by intestinal proteases [28]. T3C/96 encoded a hydrophobic isoleucine at aa 249, which is a characteristic of all T3 strains that possess protease-resistant...
Figure 3. Sequence analysis of the T3C/96 σ1 protein. A, Alignment of deduced amino acid sequences of the σ1 proteins of T3D/55 and T3C/96. T3D/55 σ1 protein aa residues 1–455 are shown by use of the single-letter aa code. aa Residues in T3C/96 σ1 that are identical to the T3D/55 sequence are indicated by dashes. aa Positions are numbered above the sequences. aa Residues in the predicted sialic acid–binding domain [24, 25, 52, 54] are underlined. A sequence that confers sensitivity to cleavage by intestinal proteases [28] is circled. aa Residues identified to be important for neuronal tropism [55, 56] are boxed. B, Functional domains of σ1 protein. JAM1, junctional adhesion molecule 1.

σ1 proteins [28]. Two aa residues in the σ1 head domain (aa 340 and 419) have been genetically implicated in reovirus neurotropism [55, 56]. These aa residues are identical in the T3D/55 and T3C/96 σ1 proteins, and aa residues in the immediate vicinity of these 2 sites are highly conserved. Thus, the divergent strain T3C/96 conserves several domains of σ1 known to be important for reovirus neurovirulence.

**T3C/96 receptor utilization.** To determine whether T3C/96 is capable of using sialic acid and JAM1 as functional receptors, HeLa cells were treated with neuraminidase, JAM1-specific MAb J10.4, or both neuraminidase and MAb J10.4 before adsorption with T3C/96. Infected cells were quantitated by use of indirect immunofluorescence, using an antireovirus serum (figure 4). Compared to either untreated cells or cells treated with CAR-specific MAb RmC8 as a control, treatment of cells with neuraminidase to remove cell-surface sialic acid resulted in a 61% reduction in the number of infected cells. Treatment of cells with JAM1-specific MAb J10.4 resulted in a 47% reduction in the number of infected cells. However, when cells were treated with both neuraminidase and MAb J10.4, infection by T3C/96 was virtually abolished. Therefore, T3C/96 is capable of using both sialic acid and JAM1 as receptors, which confirms predictions made by analysis of its σ1 protein sequence.

**Pathogenesis of T3C/96 in mice.** To test the capacity of T3C/96 to infect CNS tissue, newborn mice were inoculated intracranially with either T3C/96 or T3D/55 and were killed at various times after inoculation. Infectious virus present in homogenized brain tissue samples was quantitated by use of plaque assay (figure 5). Similar to T3D/55, T3C/96 replicated efficiently in CNS tissues, producing yields 10,000-fold greater than input 4 days after inoculation and sustaining high titers in the brain up to 12 days after infection. These data indicate that T3C/96 can infect and grow to high titer in the murine CNS.

To assess the neurovirulence of T3C/96, litters of newborn mice were inoculated intracranially with increasing doses (10^1–10^6 pfu/mouse) of either T3D/55 or T3C/96, and mortality was monitored daily for 21 days after inoculation (figure 6A). T3D/55 is neurovirulent after intracranial infection, displaying an LD₅₀ of ∼100 pfu, which is consistent with previously studies, as described elsewhere [38, 57, 58]. Infection with T3C/96 also resulted in lethality, although with somewhat reduced virulence, compared with T3D/55 (LD₅₀, ∼3000 pfu). These results indicate that T3C/96 is virulent after direct inoculation of the virus into the brain.

To determine whether T3C/96 is virulent after a natural route of infection, newborn mice were inoculated perorally with 10⁵, 10⁶, or 10⁷ pfu/mouse of either T3D/55 or T3C/96 and were monitored daily for mortality (figure 6B). In contrast to results obtained after intracranial infection, we found that only T3C/96 was virulent after peroral inoculation. At the highest dose of virus used, no mice survived infection with T3C/96, whereas 90% of T3D-infected mice survived. Thus, T3C/96 can disseminate from the murine intestine to the CNS and produce a lethal infection.

To assess pathologic changes associated with T3C/96 infection in the CNS, brain section samples derived from mice killed 2, 4, and 6 days after intracranial inoculation with either 10⁴
Figure 4. Effect of neuraminidase and junctional adhesion molecule 1 (JAM1)–specific monoclonal antibody (MAb) on growth of T3C/96. HeLa cells (2 × 10^6 cells) were pretreated with PBS, coxsackie and adenovirus receptor (CAR)–specific MAb RmcB (20 μg/mL) [45, 46], neuraminidase (40 mU/mL), JAM1–specific MAb J10.4 (20 μg/mL) [42], or neuraminidase and MAB J10.4 before adsorption with T3C/96 at an MOI of 1 fluorescent focus unit (ffu)/cell. After incubation for 20 h, cells were fixed and permeabilized with methanol. Newly synthesized viral proteins were detected by incubating cells with polyclonal rabbit antireovirus serum, followed by incubation with anti–rabbit immunoglobulin Alexa-546 serum for visualization of infected cells by indirect immunofluorescence. A, Representative fields of view are shown. B, Reovirus antigen–positive cells were quantitated by enumerating fluorescent cells in 3 random fields of view/well in triplicate. Data are mean fluorescent focus units for 3 wells. Error bars indicate SDs. ffu, Fluorescent-forming units.
Figure 5. Growth of T3C/96 in mice after intracranial inoculation. ND4 Swiss Webster mice (aged 2–3 days) were inoculated intracranially with 100 plaque-forming units (pfu) of either T3C/96 or T3D/55. At the indicated days after inoculation, mice were killed, and brains were collected. Brain tissue samples were homogenized by sonication, and titers of virus present in homogenates were determined by use of plaque assay. Each data point represents the average virus titer of 2–4 brains. Error bars indicate SD.

pfu of T3C/96 or gelatin saline were examined after staining with hematoxylin-eosin (figure 7A, 7C, 7E, and 7G; data not shown). Brain section samples derived from mice infected with T3C/96 demonstrated evidence of meningoencephalitis (figure 7A and 7E; data not shown). Inflammatory infiltrates were detected primarily in the cerebral cortex, hippocampus, diencephalon, and brain stem. Morphologically, inflammatory cells were mostly lymphocytes and macrophages/microglia, with some plasma cells and neutrophils. The extent of inflammation increased with time after virus inoculation, with the most extensive inflammation observed 6 days after inoculation (data not shown).

To define the extent and location of reovirus antigen in the CNS of T3C/96-infected mice, section samples derived from mice killed 2, 4, and 6 days after inoculation with either 10⁴ pfu of T3C/96 or gelatin saline were examined after staining with a reovirus-specific antiserum (figure 7B, 7D, 7F, and 7H; data not shown). Immunohistochemical staining for reovirus protein demonstrated immunoreactive neurons in brain section samples derived from T3C/96-infected, but not mock-infected, mice. Antigen-positive neurons were detected primarily in the cerebral cortex, hippocampus, diencephalon, and brain stem. Similar to the observed inflammatory changes, the number of antigen-positive cells increased with time after virus inoculation (data not shown). Most cells demonstrating immunohistochemical evidence of reovirus protein were detected in inflamed foci. These observations indicate that intracranial inoculation of T3C/96 leads to encephalitis and reovirus protein expression in newborn mice. Therefore, T3C/96 is capable of neurovirulent infection.

DISCUSSION

The present study is the first molecular characterization of a reovirus strain isolated directly from the CSF of an infant with symptoms and signs consistent with meningitis. The strain isolated, T3C/96, is a novel T3 reovirus, as determined by its HA capacity, neutralization profile, and S1 gene sequence. Molecular analysis of the T3C/96 S1 gene indicates that this strain is the most divergent T3 reovirus isolated to date. Despite this divergence, key functional domains of σ1 are conserved in this strain. T3C/96 encodes an isoleucine at position 249 in σ1, a polymorphism that has been linked to the capacity of T3 reovirus strains to infect the murine intestine and to spread from the intestine to the CNS [28]. In addition, key aa residues in the σ1 head domain implicated in T3 neural tropism and neu-
Figure 7. Inflammation and reovirus protein expression in the brain of newborn mice infected with T3C/96. ND4 Swiss Webster mice (2–3 days) were inoculated intracranially with either $10^4$ pfu of T3C/96 (A, B, E, and F) or gelatin saline (C, D, G, and H). At 6 days after inoculation, brain tissue samples were harvested, paraffin embedded, sectioned, and stained with hematoxylin-eosin (A, C, E, and G) or stained for reovirus antigen by use of a polyclonal antireovirus serum (B, D, F, and H). Sections are from the upper brain stem (A–D) and the cortex (E–H). Brown staining indicates reovirus antigen.
rovirolence [55, 56] are conserved. The conservation of aa residues involved in sialic acid binding [24, 25, 52, 54] in T3C/96 S1 suggests that binding to this carbohydrate may be important in neurovirulence, a hypothesis supported by the finding that the capacity to bind sialic acid enhances reovirus spread from the murine intestine to the CNS [59].

Experimental infections of mice indicate that T3C/96 is neurotropic and neurovirulent. T3C/96 productively infects the murine CNS, producing lethal infection after direct intracranial inoculation. However, in striking contrast to T3D/55, T3C/96 is also virulent after peroral inoculation, which may be related to a sequence polymorphism in S1 at aa 249. Why do reovirus infections of humans rarely produce disease? Analysis of the T3C/96 S1 sequence suggests that the low incidence of serious illness associated with reovirus infection may be caused, in part, by a requirement for a discrete set of viral biochemical characteristics that permit neural spread of enteric reovirus infections. Specifically, a protease-resistant S1 molecule may be required for both efficient growth in the intestine and spread to secondary sites of replication, including the CNS. In addition, the capacity to bind sialic acid also may function to enhance spread to the CNS in infected humans [59]. Finally, specific sequences may be required in receptor-binding domains of the S1 head to permit efficient infection of CNS neurons. It is conceivable that viral strains lacking any of these characteristics would be nonpathogenic in humans.

Host factors also play an important role in determining the outcome of reovirus infection. Studies using mice indicate that reovirus virulence strongly correlates with host age. Newborn mice are exquisitely susceptible to reovirus CNS infection, whereas adult mice support limited viral growth and show no histopathological evidence of CNS injury even after intracranial inoculation of large doses of virus [17]. The capacity of reovirus to invade the CNS from a peripheral site of inoculation also declines rapidly with age [60]. In addition to host age, host immune responses can influence susceptibility to reovirus infection. Administration of reovirus-specific antibodies by either transplacental transfer [61] or intraperitoneal inoculation [38, 57, 58] protects newborn mice against fatal reovirus infection of the CNS. The rarity of human neurological infection with reoviruses could reflect the fact that exposure to nonneurovirulent reovirus generates protective immune responses, which, in turn, prevent neurological disease after subsequent exposure to "neurovirulent" strains. Neurological disease would occur only when a nonimmune susceptible individual was exposed to a virus containing the appropriate set of neurovirulence determinants.

In addition to reovirus, other members of the Reoviridae family have been associated with CNS disease in humans. Rotavirus, an important cause of gastroenteritis in children (reviewed in [62]) has been implicated in a few cases of encephalitis [63, 64], as has Colorado tick fever virus, a member of the Coltivirus genus of the Reoviridae [65]. The patient reported in the present study provides evidence that reovirus also can cause human CNS disease.

Acknowledgments

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Reference

17. Tardieu M, Powers ML, Weiner HL. Age-dependent susceptibility to
34. Richardson-Burns SM, Kominsky DJ, Tyler KL. Reovirus-induced neuronal apoptosis is mediated by caspase 3 and is associated with the activation of death receptors. J Neurovirol 2002; 8:365–80.