Apoptosis Induction Influences Reovirus Replication and Virulence in Newborn Mice

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Apoptosis is a type of controlled cell death that is essential for development and tissue homeostasis. It also serves as a robust host response against infection by many viruses. The capacity of neurotropic viruses to induce apoptosis strongly correlates with virulence. However, the precise function of apoptosis in viral infection is not well understood. Reovirus is a neurotropic virus that induces apoptosis in a variety of cell types, including central nervous system neurons, leading to fatal encephalitis in newborn mice. To determine the effect of apoptosis on reovirus replication in the host, we generated two otherwise isogenic viruses that differ in a single amino acid in viral capsid protein $\mu 1$ that segregates with apoptotic capacity. Apoptosis-proficient and apoptosis-deficient viruses were compared for replication, dissemination, tropism, and tissue injury in newborn mice and for the capacity to spread to uninfected littermates. Our results indicate that apoptotic capacity enhances reovirus replication in the brain and consequent neurovirulence but reduces transmission efficiency. The replication advantage of the apoptosis-proficient strain is limited to the brain and correlates with enhanced infectivity of neurons. These studies reveal a new cell type-specific determinant of reovirus virulence.

Apoptosis is a form of programmed cell death essential for a variety of physiologic processes, such as embryonic development, metamorphosis, lymphocyte maturation, and the normal turnover of cells (1). Apoptosis can be triggered in response to DNA damage, during infection by a variety of pathogens, or in the absence of growth factors required for cell cycle progression. Interferon-induced suppression of viral replication and subsequent elimination of virus-infected cells by apoptosis constitute an effective mechanism to diminish spread of some viruses (2, 3). However, despite the anti-inflammatory nature of apoptotic cell death, uncontrolled infection with apoptosis-inducing viruses often leads to extensive tissue damage, which has particularly severe consequences in organs with limited regenerative capacity, such as the central nervous system (CNS). Consequently, apoptosis induction influences the virulence of many neurotropic viruses, including herpes simplex virus (4), rabies virus (5), reovirus (6), Sindbis virus (7, 8), and Theiler's murine encephalomyelitis virus (9).

Links between apoptosis induction, viral replication, and dissemination have been reported, but the effect appears to be both virus and cell type specific. For example, Sindbis virus replication in the brain is reduced when apoptosis is blocked by overexpression of Bcl-2 (7). Similarly, reovirus replication is reduced in the brains of mice lacking the apoptosis mediators Bax (10) and Bid (6). In contrast, mutation of baculovirus apoptosis inhibitors leads to reduced replication (11), and inhibition of cell death enhances yields of HIV (12). Overall, it is not well understood whether the net outcome of induction of apoptosis by neurotropic viruses favors the virus or host.

Mammalian orthoreoviruses (reoviruses) are nonenveloped, double-stranded RNA viruses that infect a wide range of hosts in nature but cause disease only in the very young (13). Following peroral inoculation of newborn mice, reovirus replicates in the intestine and disseminates via hematogenous or neural routes to sites of secondary replication, including the central nervous system (CNS) (14). Serotype 3 reovirus strains infect neurons and cause a lethal encephalitis characterized by neuronal apoptosis

and an influx of inflammatory cells (15–18). Other strains of reovirus infect cardiac myocytes and cause myocarditis associated with apoptosis (19, 20). It is not clear whether induction of apoptosis by reovirus enhances overall viral fitness or is an inadvertent effect of the host immune response.

Reovirus replication is initiated by attachment to cell surface glycans (21) and junctional adhesion molecule A (JAM-A), followed by internalization into the endocytic pathway in a mechanism dependent on $\beta 1$ integrin (22–27). In cellular endosomes, reovirus virions undergo stepwise disassembly catalyzed by cathepsin proteases (28, 29). During disassembly, outer-capsid protein $\sigma 3$ is removed and the $\mu 1$ protein is cleaved to form the δ and ϕ fragments, which remain associated with the resultant infectious subviral particles (ISVPs) (30–32). Additional conformational rearrangements of the $\mu 1$ cleavage fragments lead to the formation of ISVP*, which penetrate endosomal membranes and deliver transcriptionally active cores into the cytoplasm (32–34).

The reovirus cell entry events can elicit innate immune responses in some cell types that trigger apoptosis. Reovirus RNA serves as a pathogen-associated molecular pattern (PAMP) that is sensed by the pathogen recognition receptors (PRRs) RIG-I and Mda5 (35). This results in activation of NF- κ B and IRF-3, which leads to production of type I interferon (IFN) or apoptosis, depending on the cell type (36–38). The $\mu 1\phi$ fragment can activate NF- κ B and induce apoptosis when expressed ectopically in cells.

A sequence polymorphism at $\mu 1$ residue 595 in the $\mu 1\phi$ domain of strain type 3 Dearing (T3D) is an important regulator of apoptosis independently of functions in membrane penetration

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(39). Substitution of the isoleucine at residue 595 in $\mu 1\phi$ with a lysine results in diminished apoptosis induction and virulence of T3D following intracranial inoculation of newborn mice (39). It is not known whether variations in apoptosis efficiency associated with polymorphisms in $\mu 1\phi$ affect other aspects of the reovirus-host encounter.

To study the influence of apoptosis induction on reovirus pathogenesis following a natural route of infection, we engineered isogenic viruses that differ only in the $\mu 1\phi$ residue linked to apoptotic capacity and inoculated newborn mice perorally (39). Apoptosis-proficient (AP) and apoptosis-deficient (AD) viruses were compared for virulence, titers at sites of primary and secondary replication, tissue injury in the infected host, and the capacity to spread to uninfected littermates. Our results suggest that apoptotic capacity enhances reovirus replication in the brain but reduces the efficiency with which reovirus is transmitted between hosts. The replication advantage of AP virus is limited to the brain and correlates with enhanced infectivity in cultured neurons. This study reveals that apoptotic capacity is a cell-specific determinant of reovirus virulence.

MATERIALS AND METHODS

Cells and viruses. Murine L929 (L) cells were maintained in Lonza minimal essential medium (SMEM) supplemented to contain 5% fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 25 ng/ml amphotericin B (Sigma). HeLa cells were maintained in Dulbecco's minimal essential medium supplemented to contain 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 25 ng/ml amphotericin B. BHK-T7 cells were grown in Dulbecco's modified Eagle's minimal essential medium (Invitrogen) supplemented to contain 5% FBS, 2 mM L-glutamine, 2% MEM amino acid solution (Invitrogen), and 1 mg/ml Geneticin (Invitrogen).

Primary neuronal cultures were derived from cortices of C57BL/6J day 15 embryos as described previously (14). Neurons were plated at a density of 10^5 cells/well in 24-well plates (Costar) pretreated with 10 mg/ml poly-D-lysine (BD Biosciences) and 1.64 mg/ml laminin (BD Biosciences) solution. Cultures were incubated for 24 h in Neurobasal medium (Gibco) supplemented to contain 10% FBS, 0.6 mM L-glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin. Cultures thereafter were maintained in Neurobasal medium supplemented to contain 10% B27 (Gibco), 50 U/ml penicillin, and 50 μ g/ml streptomycin. One half of the medium volume was replaced with fresh medium every 2 to 3 days. Neurons were cultivated for 5 to 7 days prior to use.

Recombinant reoviruses were generated using plasmid-based reverse genetics (40). BHK-T7 cells were transfected with 10 plasmid constructs encoding the M1 and L2 gene segments from strain type 1 Lang (T1L) and the remaining eight gene segments from strain T3D. An S1 gene plasmid encoding a $\sigma 1$ T249I mutation (40) was used in lieu of the wild-type T3D S1 gene plasmid. A T3D M2 gene plasmid encoding a $\mu 1$ I595K mutation (41) was used to generate a virus with enhanced apoptosis efficiency. After 3 to 5 days of incubation, cells were frozen and thawed three times, and virus was isolated by plaque purification using monolayers of L cells (42). Purified reovirus virions were generated from second- or third-passage L-cell lysate stocks (43). Viral particles were Freon extracted from infected cell lysates, layered onto 1.2- to 1.4-g/cm³ CsCl gradients, and centrifuged at $62,000 \times g$ for 16 h. Bands corresponding to virions (1.36 g/cm³) were collected and dialyzed in virion storage buffer (150 mM NaCl, 15 mM MgCl₂, and 10 mM Tris-HCl [pH 7.4]) (44). The concentration of reovirus virions in purified preparations was determined from the following equivalence: one optical density (OD) unit at 260 nm equals 2.1×10^{12} virions (44). Viral titer was determined by plaque assay using L cells (42). ISVPs were generated by treating 2×10^{11} virion particles with 20 μ g of α -chymotrypsin (Sigma) in a 100- μ l volume of virion storage buffer at

37°C for 60 min (30). Reactions were terminated by the addition of 2 mM phenylmethylsulfonyl fluoride (Sigma).

Sequences of the S1 and M2 gene segments of viruses generated by plasmid-based rescue were determined using RNA purified from virions and the High Pure RNA isolation kit (Roche). Full-length open reading frames were reverse transcribed and amplified using the OneStep reverse transcription-PCR (RT-PCR) kit (Qiagen). Amplification products were analyzed by agarose gel electrophoresis, purified using the QIAquick PCR purification kit (Qiagen), and subjected to sequence analysis.

Assays of reovirus replication and infectivity. Cells were adsorbed with reovirus at a multiplicity of infection (MOI) of 1 (L cells) or 10 (neurons) PFU/cell, washed with phosphate-buffered saline (PBS), and incubated at 37°C for various intervals. Cells were frozen and thawed twice prior to determination of viral titer by plaque assay using L cells (42). Viral yields were calculated using the following formula: $\log_{10}$ yield $tx = \log_{10}(\text{PFU/ml})_{tx} - \log_{10}(\text{PFU/ml})_{t0}$, where tx is the time postinfection. Viral infectivity was determined at 16 or 24 h postadsorption by fluorescence focus assay as described previously (21).

Quantification of apoptosis by AO staining. HeLa cells or L cells (2×10^5) grown in 24-well plates (Costar) were adsorbed with reovirus at room temperature for 1 h and then incubated at 37°C for 24 or 48 h. The percentage of apoptotic cells was determined using acridine orange (AO) staining as described previously (45). Images were collected from >600 cells in two to three fields of view per well by epi-illumination fluorescence microscopy using a fluorescein filter set (Zeiss Photomicroscope III). The percentage of apoptotic cells exhibiting orange nuclei with condensed and fragmented chromatin versus live cells exhibiting structurally normal green nuclei was quantified using an imaging algorithm developed in Borland Delphi 7, an integrated software development environment. Average nucleus size and a fluorescence threshold were defined by the user. Color values from each individual-pixel RGB triplet were obtained and compared to the user-defined threshold. To exclude false-positive signals, an averaged value for surrounding pixels was calculated and compared to the threshold. Following completion of cell enumeration, green nuclei with condensed chromatin (early apoptotic) were manually subtracted from the final count. The process was repeated for orange/red nuclei. Red, structurally normal nuclei also were manually subtracted from the final count.

Detection of caspase-3/7 activity. HeLa cells (10^4) were grown in black, clear-bottom 96-well plates (Costar) and adsorbed with reovirus at room temperature for 1 h. Following incubation of cells at 37°C for 24 h, caspase-3/7 activity was quantified using the Caspase-Glo 3/7 assay (Promega). Staurosporine (10 μ M; Cell Signaling Technologies) was used as a positive control.

Infection of mice. Two- to three-day-old C57BL/6 mice weighing 1.6 to 2.3 g were inoculated perorally (46) or intracranially (47) with purified reovirus diluted in PBS. Titers of virus in the inocula were determined to directly confirm the number of infectious particles in the final dilution. The results of these assays are reported as the inoculating viral dose. For analysis of virulence, mice were monitored for symptoms of disease for 21 days postinoculation and euthanized when moribund. Death was not used as an endpoint in our experiments. For analysis of viral replication, mice were euthanized at various intervals postinoculation, and organs were harvested into 1 ml of PBS and homogenized by freeze-thaw and sonication. Viral titers in organ homogenates were determined by plaque assay using L cells (42). Animal husbandry and experimental procedures were performed in accordance with Public Health Service policy and approved by the Vanderbilt University School of Medicine Institutional Animal Care and Use Committee.

Antibodies and immunofluorescence. Immunoglobulin G (IgG) fractions of rabbit antisera raised against T1L and T3D (48) were purified by protein A Sepharose (21). Mouse anti-neuronal class III β -tubulin (TUJ1) monoclonal antibody was purchased from Covance. Rabbit anti-cleaved caspase-3 monoclonal antibody was purchased from Cell Signaling Technologies. Goat antiactin polyclonal antiserum was purchased

from Santa Cruz Biotechnologies. Alexa fluorophore-conjugated secondary antibodies were obtained from Molecular Probes (Invitrogen), and IRDye-conjugated secondary antibodies were purchased from Li-Cor Biosciences.

Detection of apoptosis in brain lysates. Brains were removed from mice, submerged in 1 ml of PBS, and homogenized by freezing, thawing, and sonication. Brain homogenates were diluted 10-fold in lysis buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris [pH 8], and 1% Nonidet P-40) containing Complete protease inhibitor cocktail [Roche], and 25 μ g of protein extract was resolved by electrophoresis in 10% Tris-glycine gels (Bio-Rad) and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked for at least 1 h in Odyssey blocking buffer (Li-Cor) and incubated with antisera specific for actin and cleaved caspase-3 diluted 1:1,000 in Odyssey blocking buffer at 4°C overnight. Membranes were washed three times with washing buffer (Tris-buffered saline containing 0.01% Tween 20) for 10 min each and incubated with secondary antibodies Alexa Fluor-680-conjugated donkey anti-goat (for actin) and IRDye 800CW-conjugated goat anti-rabbit (for cleaved caspase-3) at 1:2,000 and 1:10,000 dilutions, respectively, in Odyssey blocking buffer at room temperature for 1 h, washed twice with washing buffer and once with PBS for 10 min each, and scanned using an Odyssey infrared imaging system.

Histology. Two- to three-day-old C57/BL6 mice weighing 1.6 to 2.3 g were inoculated intracranially with purified reovirus diluted in PBS. At 10 days postinoculation, mice were euthanized and brains were removed. The right hemispheres were submerged in 10% formalin at room temperature for 24 to 120 h and embedded in paraffin. Consecutive 6- μ m sections were stained with hematoxylin and eosin (H&E) for evaluation of histopathologic changes or processed for immunohistochemical detection of reovirus antigen or the cleaved (active) form of caspase-3. The left hemispheres were processed for plaque assay using L cells (42).

Littermate transmission. Two mice per litter of 6 to 9 2- to 3-day-old C57/BL6 mice were inoculated perorally with 10^3 PFU of purified reovirus and re-placed in original cages with dams and uninoculated littermates. At 10 days postinoculation, both inoculated and uninoculated mice were euthanized, intestines were resected, and viral titers were determined by plaque assay.

Statistical analysis. Experiments were performed in triplicate and repeated at least twice. Representative results of single experiments are shown. Mean values were compared using Student's *t* test, the Mann-Whitney test, Fisher's combined probability test, or the log rank test. Error bars denote the range of data or standard deviation (SD). *P* values of <0.05 were considered to be statistically significant.

RESULTS

Reovirus strains that vary in apoptosis efficiency replicate to comparable titers in cultured cells. Apoptosis-proficient (AP) and apoptosis-deficient (AD) viruses were engineered using reverse genetics (40). The genomes of AP and AD viruses were constructed to contain two gene segments (M1 and L2) from strain T1L and the remaining eight gene segments from strain T3D. The T1L M1 gene is associated with interferon antagonism, and the T1L L2 gene has been linked to reduced interferon sensitivity and enhanced infectivity of reovirus in the mouse intestine (49–51). The S1 genes of both AP and AD viruses were engineered to encode a σ 1 attachment protein with a threonine-to-isoleucine (T249I) substitution, which renders the attachment protein resistant to cleavage by intestinal proteases (52). The T1L M1 and L2 genes and the substitution in σ 1 were incorporated to enhance infectivity in the intestine following peroral inoculation. AP and AD viruses are genetically identical with the exception of an isoleucine or lysine at amino acid position 595 in the μ 1 protein (Fig. 1A). This residue segregates with apoptosis efficiency (41). Sequences of the AP and AD virus S1 and M2 genes were determined

FIG 1 AP and AD viruses replicate to comparable titers in cultured cells. (A) Genomes of apoptosis-proficient (AP) and apoptosis-deficient (AD) viruses are composed of eight segments derived from strain T3D and two segments (M1 and L2) from strain T1L. The S1-encoded attachment protein σ 1 contains a T249I substitution that protects the attachment protein from proteolytic cleavage by intestinal proteases (52). AP and AD viruses are isogenic with the exception of a lysine (AP) or isoleucine (AD) at amino acid 595 in the M2-encoded μ 1 protein. (B and C) HeLa (B) or L (C) cells were adsorbed with an MOI of 1 (HeLa cells) or 0.1 (L cells) PFU/cell of AP or AD virus. At 0, 24, or 48 h postinfection, cells were lysed, and viral titers in lysates were determined by plaque assay. Results are expressed as the mean viral yield for triplicate wells. Error bars indicate SD. Representative results of one of two independent experiments are shown.

using cDNA generated from viral RNA, confirming the σ 1 249 residue and the μ 1 595 residue in each case and the absence of any unintended mutations (data not shown).

To determine the replication efficiency of AP and AD viruses in cultured cells, HeLa (Fig. 1B) and L (Fig. 1C) cells were adsorbed with each virus strain, incubated for 0, 24, or 48 h, and lysed by freeze-thaw. Viral titers in cell lysates were determined by plaque assay. Both viruses achieved comparable yields of ~ 10 -fold at 24 h and ~ 100 -fold at 48 h in HeLa cells and $\sim 2,000$ -fold at 24 h and $\sim 100,000$ -fold at 48 h in L cells, indicating that AP and AD viruses do not differ in the capacity to replicate in these cell types.

AP virus induces enhanced levels of apoptosis compared to those for AD virus in cultured cells. To determine whether AP and AD viruses differ in the capacity to induce apoptosis in cultured cells, HeLa (Fig. 2A) and L (Fig. 2B) cells were adsorbed with each virus at escalating MOIs of AP and AD particles. The particle-to-PFU ratio of the virus preparations used in these experiments was 140 for AP virus and 90 for AD virus, a difference of approximately 1.6-fold. In contrast, the infectivity (fluorescent focus units [FFU]/cell) in L cells is approximately 1.5-fold higher for AP virus than for AD virus (data not shown). Thus, cells were ad-

FIG 2 AP virus induces apoptosis more efficiently than AD virus in cultured cells. (A and B) HeLa (A) or L (B) cells were adsorbed with AP or AD virus at the MOIs shown (in viral particles/cell). At 24 and 48 h postinfection, the percentages of apoptotic nuclei were quantified following AO staining. Staurosporine (ST) (10 μ M) was used as a positive control for panel A. Each result is expressed as the mean percentage of apoptotic cells for triplicate wells. Error bars indicate SD. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.001$ (as determined by Student's *t* test compared with results for AD virus at the same MOI). Representative results from one of two independent experiments are shown. (C) HeLa cells were adsorbed with AP or AD virus at 100,000 particles/cell. At 24 h postinfection, caspase-3/7 activity in cell lysates was quantified. ST (10 μ M) was used as a positive control. Each result is expressed as the mean ratio of caspase-3/7 activity from infected cell lysates compared with that of mock-infected cells for triplicate wells. Error bars indicate SD. ****, $P < 0.001$ (as determined by Student's *t* test). Representative results from one of two independent experiments are shown.

sorbed with comparable numbers of particles as well as infectious units as determined by FFU. At 24 and 48 h postinfection, apoptotic cells were enumerated following AO staining. AP virus induced a significantly higher number of apoptotic cells than AD virus in a dose-dependent manner in both cell types at both time points. To complement the AO staining assay, HeLa cells were adsorbed with each virus strain, and caspase-3/7 activity in cell lysates was quantified at 24 h postinfection using a fluorogenic substrate (Fig. 2C). Levels of caspase-3/7 activity following infection with AP virus were significantly higher than those following infection with AD virus. Thus, AP virus induces apoptosis more efficiently than AD virus in cultured cells.

Apoptotic capacity is required for reovirus neurovirulence and replication in the brain. Apoptotic capacity is associated with the neurovirulence of many neurotropic viruses. To determine whether the differences in apoptosis induced by AP and AD viruses are associated with differences in virulence, newborn mice were inoculated perorally with 10^9 PFU AP or AD virus and monitored for illness for 21 days. Only 70% of the AP virus-infected animals survived, compared with 100% of the AD virus-infected animals (Fig. 3A). These data suggest that apoptotic capacity enhances reovirus virulence.

To determine whether apoptotic capacity influences reovirus replication at the site of primary infection, systemic dissemination, and replication at sites of secondary infection, newborn mice were inoculated perorally with 10^9 PFU AP or AD virus. At 2, 4, 6, 8, and 12 days postinoculation, intestine, spleen, liver, heart, and brain were excised, and viral loads in organ homogenates were determined by plaque assay (Fig. 3B). AP and AD viruses reached comparable titers in the spleen, liver, and heart. AP virus titers in the intestine were significantly higher than those of AD virus at 4 days postinoculation but did not differ at any other time point tested. Most notably, peak titers of AP virus in the brain were approximately 100-fold greater than those of AD virus at 6, 8, and 12 days postinoculation. Protein lysates from three titer-matched

AP or AD virus-infected brains resected at 12 days postinoculation were resolved by SDS-PAGE and immunoblotted using an antibody specific for the cleaved (activated) form of caspase-3 and an antiserum specific for actin as a loading control. Judging from the immunoblot band intensity, brain homogenates from animals infected with AP virus contained considerably greater levels of cleaved caspase-3 than those from animals infected with AD virus, suggesting that AP virus induces apoptosis more efficiently in the brain than AD virus following peroral inoculation (Fig. 3C). Collectively, these data indicate that the efficiency of apoptosis induction is linked to reovirus neurovirulence and replication in the murine brain.

Apoptotic capacity enhances reovirus replication in the CNS. We reasoned that the difference in peak titers of AP and AD viruses in the brain following peroral inoculation might be due to an enhanced capacity of AP virus to either disseminate to the brain or replicate at that site. To distinguish between these possibilities, newborn mice were inoculated intracranially with 10 PFU of AP or AD virus and monitored for illness for 21 days. Remarkably, 90% the AD virus-infected animals survived infection, compared with only 40% of those infected with AP virus (Fig. 4A).

To quantify replication of these virus strains in the CNS, newborn mice were inoculated intracranially with 10 PFU of AP or AD virus. At 4, 8, and 12 days postinoculation, mice were euthanized, brains were resected, and viral titers in brain homogenates were determined by plaque assay. Mean viral titers of AP virus in the brain were significantly higher than those of AD virus (Fig. 4B). Interestingly, viral titers in brain tissue of AD virus-infected mice appeared to display a bimodal distribution. Titers of AD virus in the brains of most infected animals were lower than the limit of detection. However, in a minority of AD virus-infected animals, viral titers in the brain were comparable to those in AP virus-infected animals. Full-length S1 and M2 gene sequences of 10 AD isolates from brain homogenates in which high viral titers were found revealed no nucleotide changes compared with input virus

FIG 3 Apoptotic capacity enhances reovirus neurovirulence and replication in the brain following peroral inoculation. (A) Newborn mice were inoculated perorally with 10^9 PFU AP or AD virus and monitored for illness for 21 days. *, $P < 0.05$, as determined by log rank test. (B) Newborn mice were inoculated perorally with 10^9 PFU AP or AD virus. At the times shown after inoculation, mice were euthanized, organs were excised, and viral titers in organ homogenates were determined by plaque assay. Results are expressed as mean viral titers for 4 to 7 animals for each time point. Error bars indicate SD. *, $P < 0.05$; **, $P < 0.01$ (as determined by Student's t test). (C) Protein lysates from three titer-matched AP virus- or AD virus-infected brains resected 12 days postinoculation were resolved by SDS-PAGE and immunoblotted using antisera against actin and the cleaved (activated) form of caspase-3.

(data not shown), suggesting that the dichotomy in titer is not attributable to reversion of the S1 or M2 mutation.

To investigate whether AP and AD viruses differ in apoptosis efficiency following intracranial inoculation, protein lysates from three titer-matched AP or AD virus-infected brains resected 8 days postinoculation were resolved by SDS-PAGE and immunoblotted using an antibody specific for cleaved caspase-3 and antiserum specific for actin as a loading control. Consistent with our results following peroral inoculation, brain homogenates from AP virus-infected animals contained appreciably greater levels of cleaved caspase-3 than those from AD virus-infected animals, suggesting that AP virus induces apoptosis more efficiently in the brain than AD virus following inoculation by the intracranial route (Fig. 4C). To determine whether induction of apoptosis influences viral protein expression, immunoblots were stripped and reprobed with reovirus polyclonal antiserum. The results suggest that levels of viral proteins were greater in AP virus-infected brains than in AD virus-infected brains (data not shown), consistent with our finding that AP virus replicates more efficiently in the brain than AD virus. Together, these results suggest that apoptotic capacity is not a determinant of reovirus dissemination to the brain but rather influences viral replication at that site.

Tissue injury in the CNS associated with AP and AD viruses.

To assess the influence of apoptosis induction on viral tropism and tissue injury in the nervous system, we inoculated mice with 40 PFU of AP or AD virus and resected brains at 8 days postinfection. The left hemispheres were homogenized for determination of viral titer. The right hemispheres of brains containing comparable loads of AP or AD virus were sectioned, and consecutive sections were stained with H&E, reovirus polyclonal antiserum, or cleaved caspase-3 antibody. Reovirus antigen was detected in the

cerebral cortex, hippocampus, thalamus, and cerebellum of both AP and AD virus-infected brains, consistent with previous reports of neural injury caused by type 3 reovirus (Fig. 5 and data not shown) (6, 14, 39, 53). Areas in the thalamus positive for AP virus antigen displayed extensive tissue injury associated with enhanced cleaved caspase-3 immunoreactivity, whereas areas positive for AD virus antigen displayed little to no tissue damage and little cleaved caspase-3 immunoreactivity (Fig. 5A). Tissue injury also was more extensive in the cerebellum of AP virus-infected animals than that in AD virus-infected animals. In multiple sections from infected animals, brain regions positive for AP virus antigen also stained positive for cleaved caspase-3. In contrast, very little cleaved caspase-3 immunoreactivity was detected in regions that stained positive for AD virus (Fig. 5B). These data are consistent with immunoblotting results showing greater levels of cleaved caspase-3 in whole-brain lysates derived from AP virus-infected mice than in those from mice infected with AD virus (Fig. 4). Examination of an infected region of the cerebellum at higher magnification revealed that Purkinje cells were the predominant cell type infected in the cerebellum of AD virus-infected animals, whereas both Purkinje cells and granule cells contained reovirus antigen in the cerebellum of AP virus-infected animals (Fig. 5C). This observation raises the possibility that apoptosis capacity expands reovirus tropism. Thus, when matched for viral load, AP virus produces more apoptosis and appears to have an expanded distribution in the brain compared with findings for AD virus.

Infectivity and replication of AP and AD viruses in primary cortical neuronal cell cultures. Our data thus far suggest that apoptotic capacity is associated with enhanced reovirus replication in the brain but not in other tissues, suggesting that neurons provide a unique niche for reovirus that requires a proapoptotic

FIG 4 Apoptotic capacity enhances reovirus neurovirulence and replication in the brain following intracranial inoculation. (A) Newborn mice were inoculated intracranially with 10 PFU AP or AD virus and monitored for illness for 21 days. **, $P < 0.01$, as determined by log rank test. (B) Newborn mice were inoculated intracranially with 10 PFU AP or AD virus. At the days postinoculation shown, mice were euthanized, brains were removed, and viral titers in brain homogenates were determined by plaque assay. Each result is expressed as viral titer in the brain of a single infected animal. Horizontal black lines indicate means of log-transformed data. *, $P < 0.05$; ****, $P < 0.001$ (as determined by Mann-Whitney test). (C) Protein lysates from three titer-matched AP virus- or AD virus-infected brains resected 8 days postinoculation were resolved by SDS-PAGE and immunoblotted using antisera against actin and the cleaved (activated) form of caspase-3.

capacity for maximum replication efficiency. It is possible that apoptosis could promote neuronal infection (i.e., viral life cycle events leading to viral protein synthesis), replication (i.e., life cycle events leading to assembly of infectious viral progeny), or some combination of both. To determine whether AP and AD viruses differ in infection of neurons, we cultured primary neurons from the cortices of day 15 embryonic mice, infected those cells with AP or AD virions or ISVPs at an MOI of 1,000 PFU/neuron, and quantified the percentage of infected neurons at 16 and 24 h postinfection by indirect immunofluorescence (Fig. 6A and B). ISVPs were included in these experiments, since these *in vitro* generated disassembly intermediates infect neurons more efficiently (J. L. Konopka-Anstadt, A. J. Pruijssers, and T. S. Dermody, unpublished). A significantly greater percentage of neurons were infected with AP virions and ISVPs than with virions and ISVPs of AD virus, suggesting that AP virus more efficiently

FIG 5 Tissue damage associated with AP and AD virus infection in the murine brain. Newborn mice were inoculated with 40 PFU of AP or AD virus. At 8 days postinoculation, brains were removed, the left hemispheres were homogenized for the determination of viral titer, and the right hemispheres were processed for histology. Consecutive coronal sections of the brain were stained with H&E, polyclonal reovirus antiserum, or an antibody against the cleaved (active) form of caspase-3. Representative sections of brain hemispheres are shown. (A) Low-magnification images of the hippocampal region stained with H&E or reovirus polyclonal serum (scale bars, 2 mm) and higher-magnification image of a boxed region in the thalamus stained for cleaved caspase-3 (scale bars, 250 μm). (B) Higher-magnification image of cerebellum (scale bars, 500 μm). Sections from an AP virus-infected animal are from a brain with a titer of 1.8×10^8 PFU, and sections from an AD virus-infected section are from a brain with a titer of 4.1×10^8 PFU. (C) High-magnification image of cerebellum (scale bars, 250 μm). Arrows indicate Purkinje (P) and granule (g) cells.

FIG 6 Infectivity and replication of AP and AD virus in primary cortical neuronal cell cultures. Primary neuronal cultures were prepared from the cerebral cortices of day 15 embryos and cultured *in vitro* for 5 to 7 days. Cultures were adsorbed with virions or ISVPs of AP or AD virus at an MOI of 1,000 PFU/cell and incubated for 16 or 24 h. Cultures were stained with the neuron-specific marker TUJ1 (red), 4',6-diamidino-2-phenylindole (DAPI) to detect nuclei (blue), and polyclonal reovirus antiserum to detect reovirus antigen (green) and visualized using indirect immunofluorescence microscopy. Neurons infected with AP virions are shown in panel A; neurons infected with AD virions are shown in panel B. The percentages of neurons infected by virions and ISVPs were quantified (C). The results are expressed as the normalized means of data from three independent experiments each performed in triplicate. Error bars indicate SEM. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.001$ (as determined by Fisher's combined probability test). (D) Primary neuronal cultures were adsorbed with virions or ISVPs of AP or AD virus at an MOI of 10 PFU/cell. At 0, 24, or 48 h postinfection, cells were lysed, and viral titers in lysates were determined by plaque assay. Results are expressed as the mean viral yield for triplicate wells. Error bars indicate SD. n.s., not significant. Representative results of one of two independent experiments are shown.

infects primary cortical neurons relative to infection by AD virus (Fig. 6C). To determine whether apoptotic capacity influences reovirus replication in neurons, cultures of primary cortical neurons were infected with AP or AD virions or ISVPs at an MOI of 10 PFU/neuron, and viral titers were determined by plaque assay at 0, 24, and 48 h postinfection (Fig. 6D). Although there was a trend toward higher viral yields after infection with AP virions, no statistically significant differences were observed between yields of AP and AD virions or ISVPs, suggesting that apoptotic capacity is not required for completion of the reovirus replication cycle in primary neuronal cultures. Thus, apoptosis induction appears to be associated with more efficient infection but not an overall replication advantage in neurons.

Apoptotic capacity attenuates transmission between littermates. An important determinant of pathogen fitness is the capacity to spread from host to host. To determine whether apoptotic capacity

influences the efficiency with which reovirus is transmitted between hosts, we inoculated two mice from litters of 6 to 9 mice perorally with 10^3 PFU of either AP or AD virus and placed the animals with their dams and uninfected littermates. Ten days postinoculation, mice were euthanized, intestines were resected, and viral loads were determined by plaque assay (Fig. 7). AD virus replicated to significantly higher titers than AP virus in the intestine of inoculated pups. Moreover, titers of AD virus also were higher than those of AP virus in the intestine of uninoculated littermates. These data suggest that apoptotic capacity attenuates replication in the intestine, which may in turn diminish littermate transmission.

DISCUSSION

In this study, we engineered apoptosis-proficient and apoptosis-deficient reovirus strains to study the function of apoptosis in different stages of reovirus infection in the host following a natural

FIG 7 Apoptotic capacity attenuates viral replication in the intestine and transmission to uninfected littermates. Two newborn mice from litters of 6 to 9 pups were inoculated perorally with 10^3 PFU of AP or AD virus and reunited with dams and uninoculated littermates. At 10 days postinoculation, all mice in the litter were euthanized, intestines were excised, and viral titers in intestinal homogenates were determined by plaque assay. Each data point represents a single animal in three (AP) or four (AD) litters. Horizontal bars indicate the means of log-transformed data. **, $P < 0.01$; ****, $P < 0.001$ (as determined by Student's t test).

route of inoculation. These viruses are genetically identical with the exception of a single residue polymorphism in the $\mu 1$ protein that has been linked to the efficiency of apoptosis induction (39). Our results indicate that the capacity to induce apoptosis contributes to reovirus neurovirulence and leads to higher viral loads in the murine brain. However, enhanced apoptotic capacity is associated with reduced viral loads in the intestine and reduced transmission of reovirus to uninfected littermates. Thus, apoptosis functions in a tissue-specific manner in reovirus pathogenesis.

It is not apparent whether the capacity of reovirus to induce apoptosis enhances viral infectivity and replication in the brain or whether the enhanced infectivity and replication in the brain leads to enhanced apoptosis and neurovirulence. The dichotomy in viral loads produced by AP and AD virus in brains of infected mice suggests that reduced apoptotic capacity dampens productive infection or replication in neurons. Indeed, our studies using cultures of primary cortical neurons suggest that AP virus infects neurons more efficiently than AD virus. In addition, AP virus displayed an expanded distribution in the cerebellum of infected mice compared with that of AD virus. We envision three possible mechanisms to account for the replication differences exhibited by AP and AD viruses. First, the polymorphism in $\mu 1$ may dictate differences in the stability of the virion or the capacity to penetrate endosomal membranes, which in turn might influence the efficiency with which the virus initiates an infectious cycle and evokes proapoptotic signaling. In support of this possibility, yields of AP and AD viruses in primary neuronal cultures are equivalent following infection by ISVPs, which bypass the entry steps following attachment prior to membrane rupture (27). Second, activation of signaling pathways that lead to apoptosis may influence the expression of factors that contribute to viral replication. Such host factors could in turn contribute to cell type-specific susceptibility and resultant expansion of tropism. In this case, since AP virus induces apoptosis more efficiently than AD virus, AP virus may benefit from such proapoptotic signaling networks. Third, apoptotic capacity might be required for virus release from neurons and subsequent infection of neighboring cells. Additional studies are required to discriminate between these possibilities.

Following peroral inoculation, loads of AP and AD viruses

were comparable in the spleen, liver, and heart. However, AP virus reached substantially (~ 100 -fold) higher titers than AD virus in the brain. Viral loads of AP virus in the brain also were higher than those of AD virus following intracranial inoculation. These data are consistent with previous reports that reovirus replication capacity is diminished in brains of mice lacking either of the proapoptotic Bcl-2 family members Bax (10) and Bid (6). Our results do not suggest a role for apoptotic capacity in viral dissemination in the host. Of note, another study from our laboratory indicates that the nonstructural protein $\sigma 1s$ is required for full apoptotic potential in cultured cells and bloodstream dissemination in mice following intramuscular inoculation (54). This finding suggests that different viral determinants are relatively more important for apoptosis induction in different tissues. Therefore, it is possible that $\mu 1$ is essential for apoptosis induction in the CNS, whereas $\sigma 1s$ is essential for apoptosis induction in the muscle to allow bloodstream spread.

The difference in apoptotic capacities displayed by AP and AD viruses is attributable to a single amino acid polymorphism at residue 595 of $\mu 1$. AP virus has a lysine at 595, whereas AD virus has an isoleucine at that position. However, in the context of strain T3D, the isoleucine-to-lysine change at 595 attenuates apoptosis (39). Based on our data and those of the previous study, we think that the I595K polymorphism acts as a toggle for apoptotic capacity depending on the genetic background of the virus. In the case of AP and AD viruses, the M1 and L2 segments are derived from T1L and the S1 gene encodes a threonine-to-isoleucine substitution at residue 249 in $\sigma 1$. It is possible that one or more of these differences account for the change in polarity of I595K with respect to apoptosis efficiency. Identifying the genetic determinants and underlying biochemical mechanisms required for the phenotypic switch is a focus of our current work.

Enhanced apoptotic capacity increases reovirus virulence, infectivity, and replication in the brain, but it reduces replication in the intestine and transmission to uninfected littermates. Thus, enhanced virulence comes at a fitness cost for reovirus. For some viruses, like noroviruses and rotaviruses, virulence, as assessed by production of diarrhea, is linked to transmission. However, for reovirus, it is not apparent how neurovirulence would enhance transmission efficacy. We find it noteworthy that of the seven reovirus M2 gene sequences reported to date all encode an isoleucine at residue 595. Therefore, since lysine 595 has not been found in natural reovirus isolates, it appears that selection pressures modulating reovirus fitness appear to favor transmission efficiency over neurovirulence.

Viruses have evolved a variety of strategies to successfully replicate within a host and transmit to the next host. For some viruses, the induction of apoptosis is beneficial because it aids viral replication in the infected host or transmission to new hosts. For other viruses, apoptosis is detrimental because it results in death of infected cells prior to completion of the viral replication cycle or death of the host before the virus has been transmitted to the next host. For reovirus, enhanced apoptotic capacity may not benefit fitness if that is defined as the efficiency with which the virus replicates in the intestine and spreads to uninfected littermates. These studies suggest that the capacity of a virus to induce apoptosis can have tissue-specific effects on viral replication efficiency that in turn regulate viral virulence and transmission in the environment.

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We declare that there are no conflicts of interest.

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