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Alphavirus-Induced Apoptosis in Mouse Brains Correlates with Neurovirulence

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Sindbis virus induces apoptotic cell death in cultured cell lines, raising the possibility that apoptosis of infected neurons and other target cells in vivo may contribute to the resulting disease and mortality. To investigate the role of apoptosis in Sindbis virus pathogenesis, infected mouse brains were assayed by the in situ terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling technique and for DNA ladder formation. Infection with recombinant Sindbis virus strain 633 resulted in widespread apoptosis in newborn mouse brains and spinal cords, but few apoptotic cells were observed following infection of 2-week-old animals. This finding correlates with the age-dependent mortality observed in mice. The more neurovirulent virus TE, which differs from 633 by a single amino acid in the E2 glycoprotein, induced significant apoptosis in brains and spinal cords of 2-week-old animals, consistent with its ability to cause fatal disease in older animals. Double-labeling experiments demonstrated that the apoptotic cells were also infected with Sindbis virus. Thus, Sindbis virus-induced apoptosis appears to be a direct result of virus infection and is likely to reflect pathogenic mechanisms for other viruses.

Infection of cultured cells with a wide variety of viruses, including herpesviruses (22, 25, 26, 28), parvoviruses (38), retroviruses (9, 30, 36, 39, 41), paramyxoviruses (8), myxoviruses (12, 20, 42), alphaviruses (33, 49), and picornaviruses (44), results in activation of a programmed cell death pathway. This built-in cell suicide program is required for normal embryonic development and tissue homeostasis and may also be an important host defense mechanism to eliminate virus-infected cells (27). In contrast, virus-induced activation of programmed cell death in a nonreplenishing cell population such as neurons may result in disease. To understand viral disease mechanisms, it will be important to determine whether viruses activate the cell death pathway (apoptosis) or kill cells by lethal parasitism (necrosis).

Sindbis virus infection of mice causes age-dependent illness. Newborn mice are readily killed, but 2-week-old and older animals usually recover from encephalomyelitis following infection with a standard laboratory strain, AR339 (43). This phenomenon is not dependent on the host T- and B-cell responses, since Sindbis virus-infected SCID (severe combined immunodeficient) mice also exhibit age-dependent susceptibility to Sindbis virus (45a). These observations raise the possibility that a cellular factor protects mature neurons from virus-induced apoptosis in vivo (18). A single amino acid change at position 55 in the Sindbis virus E2 glycoprotein can convert a virus that fails to kill 2-week-old mice into a more neurovirulent strain that is lethal for 2-week-old mice (47). E2 is a transmembrane glycoprotein that heterodimerizes with a second transmembrane glycoprotein, E1, to form the virion spikes. The recombinant virus, TE, contains a histidine at E2 position 55 and has a 97% mortality rate in 2-week-old mice. In contrast, the recombinant virus 633, which has a glutamine at position 55, fails to kill 2-week-old animals (47, 49). Thus, the ability of neurovirulent Sindbis virus strains to kill mice may be a direct result of their ability to overcome a protective host factor and induce apoptosis. Here we demonstrate that the ability of Sindbis virus to induce apoptosis in neurons in vivo is directly related to mortality in mice. Thus, Sindbis virus induces apoptotic cell death in an age-dependent manner that is virus strain specific.

MATERIALS AND METHODS

Animals and viruses. Recombinant Sindbis viruses TE and 633 were constructed as previously described (49). Litters of 1- and 14-day-old CD-1 mice (virus antibody free; Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were inoculated intracerebrally in the right hemisphere with 1,000 PFU of Sindbis virus in 30 μl of Hanks balanced salt solution (HBSS). At the indicated days postinoculation, brains from 2-week-old mice were collected and divided along the mid-sagittal plane. The right sides were used for determining virus titers, and the left sides were fixed in 4% paraformaldehyde for 48 h, embedded in paraffin, and sectioned. Because of the small size of 1-day-old mouse brains, virus titers and tissue sectioning were performed on different mice within the same litter. In situ hybridization assays were performed on two mouse brains for each virus and one control mouse brain at each time point postinfection (24, 48, and 72 h for 1-day-old mice; 48, 72, and 96 h for 2-week-old mice). For virus titrations, brains were frozen, suspended as 10% homogenates in HBSS, and stored at −80°C. Virus titers were determined in a standard plaque assay on BHK-21 (baby hamster kidney) or N18 (mouse neuroblastoma) cells.

In situ hybridization. Precautions were taken throughout to prevent contamination with RNase. Five-micrometer tissue sections on Superfrost Plus slides (Fisher) were deparaffinized, rehydrated, and permeabilized with 0.1% Triton X-100 (Sigma) in phosphate-buffered saline (PBS) for 15 min. Sections were treated with proteinase K (10 μg/ml; Boehringer Mannheim) in 100 mM Tris-HCl–50 mM EDTA (pH 8.0) at 37°C for 10 min, washed in PBS for 5 min, and then postfixed in fresh 4% paraformaldehyde for 3 min. To reduce the nonspecific binding of the probe to other reactive groups, tissues were acetylated by incubation in 0.1 M triethanolamine (pH 8.0) (Sigma), while acetic anhydride

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(Sigma) was added dropwise to a final concentration of 0.25% with constant stirring.

Under RNase-free conditions, tissue sections were incubated in 2× SSC (300 mM NaCl, 30 mM sodium citrate) for 10 min at room temperature and prehybridized for 30 min at 37°C in hybridization solution (6× SSC, 1× Denhardt's solution, 0.1 mM ATP, salmon sperm DNA [400 mg/ml], 2 mM sodium pyrophosphate, 0.2% sodium dodecyl sulfate [SDS]) plus 35% formamide without probe. Two 19-mer antisense oligonucleotide probes to the Sindbis virus E2 glycoprotein (nucleotides 8852 to 8833 and 8912 to 8893) were labeled according to the Boehringer Mannheim protocol for 3′ tailing of oligonucleotides with digoxigenin-11-dUTP-dATP. These probes do not span the single-nucleotide difference between TE and 633 viruses. One hundred microliters of probe cocktail (10 μl of each probe reaction mix in 3 ml of hybridization solution plus 35% formamide) was added to each slide and incubated in a humid chamber at 45°C overnight. Following hybridization, tissue sections were washed once in 6× SSC for 10 min at room temperature, twice in 2× SSC–0.1% SDS at 55°C for 5 min, and twice in 0.5× SSC–0.1% SDS at 55°C for 5 min, with a final rinse in 2× SSC for 5 min at room temperature.

The probe was detected by using alkaline phosphatase-conjugated antidigoxigenin Fab fragments with the Genius 3 nonradioactive nucleic acid detection kit from Boehringer Mannheim. Blocking buffer contained 2% normal lamb serum (NLS; Gibco) and 0.3% Triton X-100. One hundred microliters of a 1:500 dilution of antibody in buffer 1 (Boehringer Mannheim) plus 1% NLS and 0.3% Triton X-100 was added to each slide and incubated in a humid chamber for 2 to 3 h at room temperature. The color reaction was developed with 4-nitroblue tetrazolium chloride, 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate), and levamisole for 4 to 18 h in the dark. Slides were dehydrated, rinsed in Histo-Clear (National Diagnostics, Atlanta, Ga.), and mounted in Permount (Fisher).

TUNEL assay. The TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling) technique detects endonucleolytically cleaved DNA by the addition of labeled dUTP to DNA ends by terminal transferase. Tissue sections were deparaffinized, rehydrated, permeabilized by incubation in 10 μg of proteinase K per ml for 15 min at room temperature, and washed twice for 5 min in PBS. Tissues were covered with 40 μl of labeling mix (25 mM Tris-HCl [pH7.2]), 0.2 M potassium cacodylate, 5 mM cobalt chloride, 30 U of terminal deoxynucleotidyltransferase, 0.6 nmol of digoxigenin-11-dUTP, 0.15 mM dATP) and incubated in a humid chamber at 37°C for 60 min. The reaction was terminated by incubation in 2× SSC for 15 min at room temperature. Digoxigenin-labeled sections were treated as described above except that the blocking buffer contained 0.1% Triton X-100, antibody diluent contained 2% NLS and 0.1% Triton X-100, and incubation with antidigoxigenin antibody was for 30 min. The Boehringer Mannheim Genius nucleic acid detection system for blue staining of alkaline phosphatase activity with 4-nitroblue tetrazolium chloride and X-phosphate was incubated for 10 to 30 min according to the vendor's protocol. The slides were washed, dehydrated, and mounted as described above. TUNEL assay controls included omission of terminal transferase or labeled nucleotide.

Double labeling. The in situ hybridization protocol was completed as far as the wash steps following the overnight hybridization with digoxigenin-labeled probe. Then the TUNEL reaction was performed with 0.6 nmol of biotin-16-dUTP (Boehringer Mannheim) as far as the wash steps after the labeling reaction. Next, the antibody treatment and color reaction for detection of the digoxigenin-labeled probe with 4-nitroblue tetrazolium chloride and X-phosphate was incubated for a few hours instead of the usual overnight incubation to prevent background blue staining. Subsequently, sections were blocked in PBS plus 2% NLS and 0.2% Triton X-100 for 30 min at room temperature, and the Vectastain Elite-ABC kit (Vector Laboratories) was used for detection of biotin incorporation onto the ends of fragmented DNA. Peroxidase-conjugated avidin-biotin complexes were visualized with diaminobenzidine as the substrate according to the vendor's protocol.

DNA ladders. Brains were collected 1, 2, and 3 days after inoculation of 1-day-old CD-1 mice with 633 virus. Using a protocol kindly provided by Carlos Portera-Cailliau (40), DNA was extracted and end labeled with terminal transferase and digoxigenin-11-ddUTP (Boehringer Mannheim). Five-microgram samples were electrophoresed on a 1.8% agarose gel, transferred to a Hybond-N nylon membrane (Amersham Life Science), and visualized by using the Boehringer Mannheim Genius system with alkaline phosphatase-conjugated antibody and Lumi-phos Plus as the chemiluminescent alkaline phosphatase substrate.

RESULTS

Virus growth in mouse brains. We compared the brains of 2-week-old CD-1 mice injected intracranially with the recombinant Sindbis virus TE or 633. These viruses were derived from DNA clones that differ only at codon 55 of the E2 glycoprotein (47). Although these viruses have similar growth kinetics in cultured BHK cells used for propagating virus (47), the avirulent 633 virus replicated less efficiently than the neurovirulent TE virus in 2-week-old mouse brains (Fig. 1). The

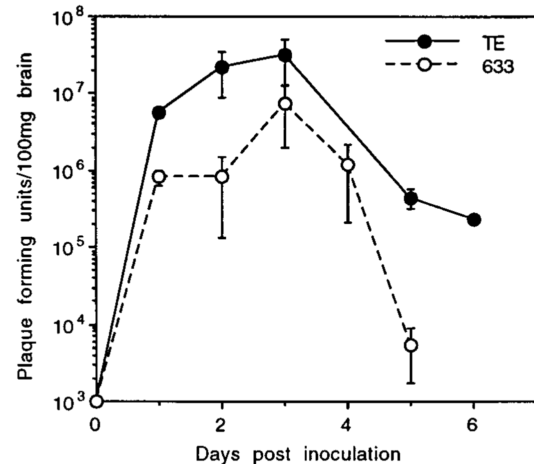

FIG. 1. Sindbis virus production in mouse brains. Two-week-old mice were inoculated intracerebrally with 1,000 PFU of recombinant virus TE or 633. Virus titers obtained from two mice for each virus in each of two independent experiments are indicated as geometric means + standard errors.

peak virus titers for both viruses occurred on day 3 after infection, when the titer for TE exceeded that of 633 by approximately fivefold. Thus, the level of viral replication in mouse brains correlated with mortality.

Induction of apoptosis in mouse brains by virulent virus. To determine if TE and 633 could induce apoptotic cell death in mouse brains, we used the in situ TUNEL technique (14). In two independent experiments, brains from 2-week-old mice infected with the virulent TE virus exhibited nuclear TUNEL staining (Fig. 2B and D). In situ hybridization was performed on adjacent tissue sections with a digoxigenin-labeled oligonucleotide probe specific for viral RNA (Fig. 2A and C). The abundance of signal in both the TUNEL and the in situ hybridization assays was greatest on day 3 after infection, correlating with the virus titers shown in Fig. 1. In addition, only those areas of the brain that stained by in situ hybridization for viral RNA were positive in the TUNEL assay, suggesting that the cells undergoing apoptosis were either the same cells infected with Sindbis virus or their near neighbors. There were 1,072 in situ hybridization-positive cells counted in 12 fields from two experiments, compared with 913 TUNEL-positive cells in the identical areas of adjacent sections. Occasional cells were of sufficient size to span adjacent sections (arrows in Fig. 2C and D). The in situ hybridization signal was cytoplasmic, consistent with the replication cycle of Sindbis virus. The size and morphology of the majority of TUNEL- and hybridization-positive cells were consistent with other studies showing that Sindbis virus primarily infects neurons (23, 31). In situ hybridization and TUNEL assays on brains of mice inoculated with HBSS alone were uniformly negative (Fig. 2E and F, respectively).

To determine if the cells dying by apoptosis were also infected with Sindbis virus, tissue sections from TE-infected brains were double labeled to detect both TUNEL (brown stain) and in situ hybridization (blue stain) signals (Fig. 3). Cell counts indicated that 78 to 92% of the viral RNA-positive cells in a field were also TUNEL positive, indicating that the Sindbis virus-infected cells were those dying by apoptosis. Not all cells positive for viral RNA were stained by the TUNEL assay, consistent with the frequency of in situ and TUNEL-positive cells shown in Fig. 2. Perhaps infected neurons in vivo are detectable by in situ hybridization before they appear positive

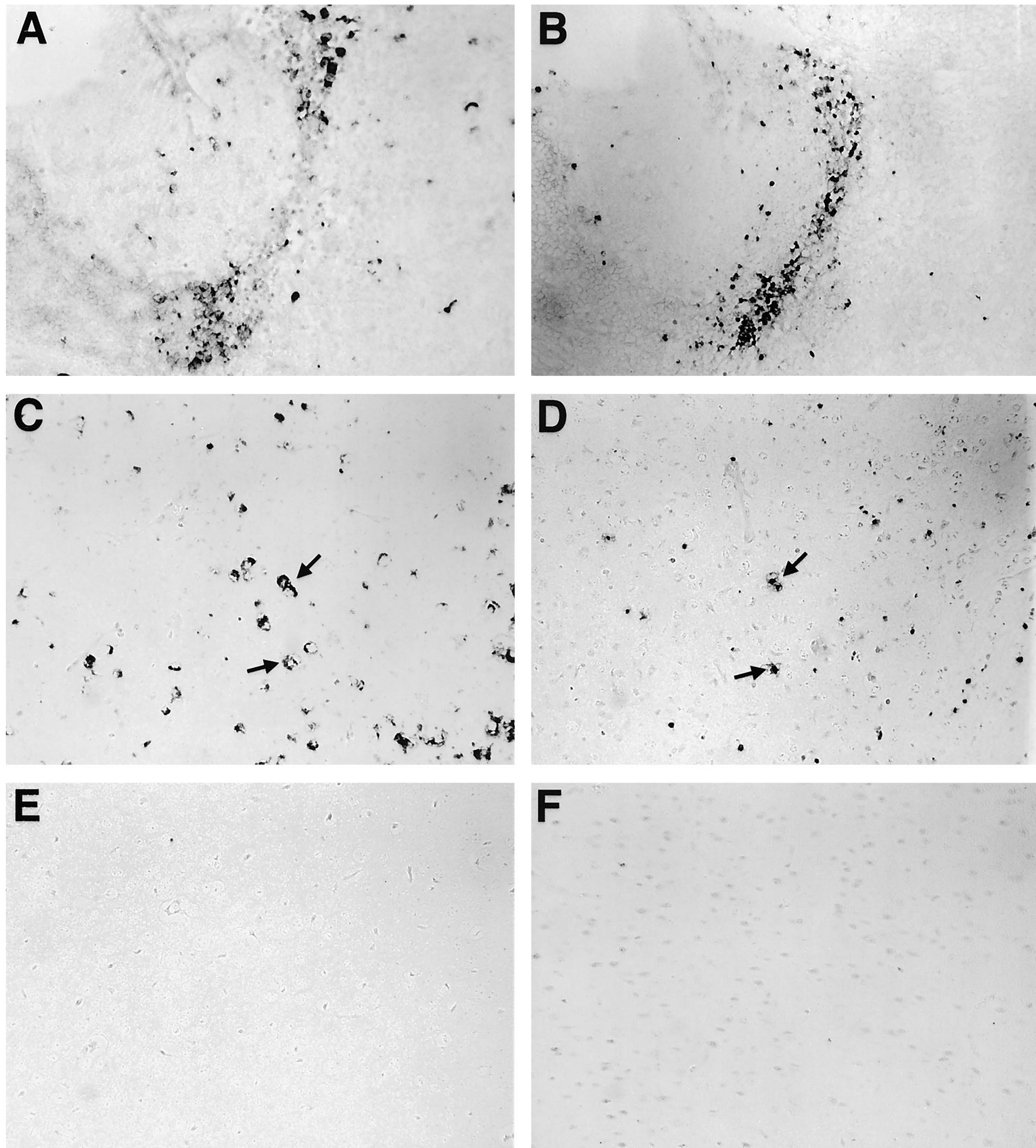

FIG. 2. Correlation between viral RNA production and induction of apoptosis in brains of 2-week-old mice infected with TE virus. (A and C) Viral RNA was detected by in situ hybridization in brains harvested 3 days postinoculation in two independent experiments. (B and D) Apoptosis was detected by TUNEL assay in tissue sections adjacent to those shown in panels A and C, respectively. (E) In situ hybridization for viral RNA and (F) TUNEL assay on brains from control mice inoculated with HBSS. Original magnification, ×40.

in a TUNEL assay. Analysis of cultured cells indicates that progeny virus production precedes the appearance of apoptotic morphology by several hours (33, 45). However, we cannot eliminate the possibility that some virus-infected cells of the brain recover from infection without undergoing apoptosis.

Although the TUNEL assay can also detect nonspecific degradation of DNA during necrotic cell death (16), the TUNEL-positive nuclei in these sections exhibited morphology unique to apoptosis. Characteristic of late-stage apoptosis, many nuclei were fragmented into apoptotic bodies. The arrow in Fig. 3A marks a cell that contains a TUNEL-positive, fragmented nucleus. Apoptotic cells ultimately bleb apart into small bodies of nuclear and cytoplasmic material (27). This disintegration of the cell results in TUNEL-positive apoptotic bodies that are not associated with an intact cell (Fig. 3B). The morphology observed in these sections is consistent with that observed by

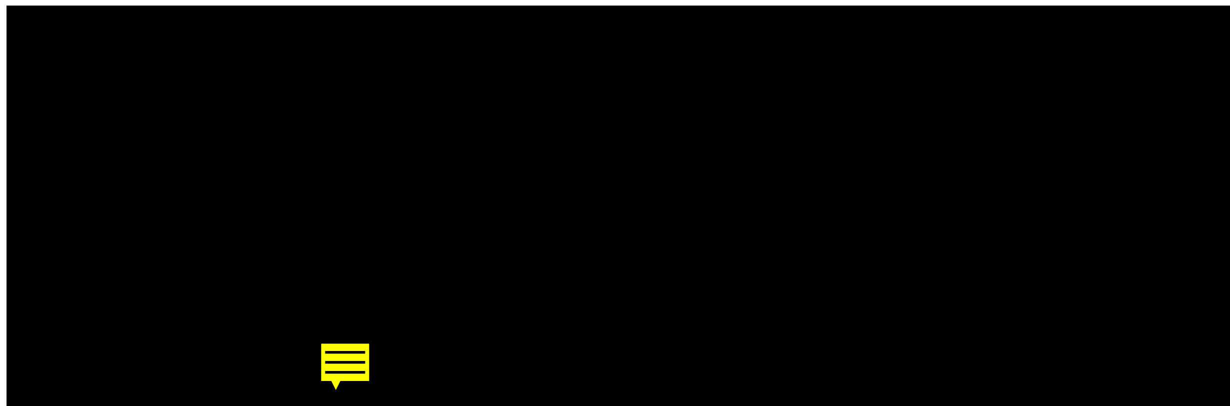

FIG. 3. Colocalization of viral RNA and apoptosis in the brains of TE-infected mice. Tissue sections were double labeled by in situ hybridization for viral RNA (blue stain) and by the TUNEL reaction for DNA fragmentation (brown stain). The arrow in panel A marks a single infected cell containing a fragmented nucleus. Panel B is a photographic enlargement of an adjacent area of the same section showing a fragmented, apoptotic nucleus that is being expelled from the infected cell. ab indicates a stray apoptotic body. Original magnification, ×63.

electron microscopy of Sindbis virus-infected cell cultures (see Fig. 1 of reference 33). Unlike cultured cells, apoptotic bodies present in tissues may be engulfed by adjacent cells. Engulfment of apoptotic bodies by neighboring, normally nonphagocytic cells is typical of the noninflammatory apoptotic process in tissues (27). Several genes responsible for engulfment of apoptotic cells have been identified by genetic studies in the nematode *Caenorhabditis elegans*, in which cells undergo apoptosis normally but are not engulfed by their neighbors (7). However, the intense focal apoptosis in Sindbis virus-infected brains observed in these sections presumably results in apoptotic cell debris that persists because of the lack of a sufficient number of healthy adjacent cells to dispose of the debris, eventually leading to the inflammatory response characteristic of Sindbis virus-induced encephalitis (37).

Age-dependent apoptosis. In contrast to the results obtained with TE virus, TUNEL-positive cells were rarely observed in animals infected with the less virulent 633 virus. Even areas of the brain that were positive for viral RNA by in situ hybridization (Fig. 4A) were often devoid of TUNEL-positive cells (Fig. 4B). In two whole-brain sections from two experiments, there were 35 in situ-positive cells, compared with 2 TUNEL-positive cells in adjacent sections. In situ hybridization results were consistent with virus titers in that fewer viral RNA-positive cells were observed in 633-infected brains than in TE-infected brains. No positive cells were detected in 633-infected animals on days 2 and 5 postinfection, again correlating with low virus titers by plaque assay. One of eight 633-infected mice had easily detectable TUNEL staining and correspondingly more virus by in situ hybridization, indicating that 633 virus was capable of inducing apoptosis when it replicated to sufficient levels (Fig. 4C and D). In a whole-brain section from this mouse, there were 115 in situ-positive cells, compared with 113 TUNEL-positive cells in the adjacent section. The brain from this mouse also contained the highest titer of virus by plaque assay compared with other 633-infected mice. Although not expected from the short duration between inoculation and harvest times, there was a possibility that the glutamine codon at position 55 of E2 had mutated to the histidine codon found in the virulent TE virus, thereby explaining the TUNEL staining in this one particular mouse. After all, the histidine mutation was originally identified because it occurred spontaneously with passage of virus through mouse brain (17, 32). RNA was prepared from the brain homogenate of the mouse in question, and the sequence analysis of cDNA generated by reverse transcription-PCR confirmed that this virus had maintained the glutamine at position 55. However, we cannot eliminate the possibility that another mutation allowed this virus to replicate to higher titers. In situ hybridization and TUNEL assays on brains inoculated with HBSS alone were negative (Fig. 4E and F). Results of analyses of spinal cords from TE- and 633-infected animals by in situ hybridization and TUNEL were similar to the results obtained with brain tissue (data not shown). Taken together, these data indicate that the less virulent 633 virus has a reduced ability to induce apoptosis in mouse brains because of its reduced ability to replicate.

Although virus 633 has a 0% mortality rate in 2-week-old mice, it kills 100% of newborn mice (47). Therefore, we predicted that 633 virus would induce apoptosis in newborn mouse brains. TUNEL-positive cells were readily detected at 2 days after inoculation of 1-day-old mice with 633 virus (Fig. 5B). TUNEL staining consistently colocalized with areas of the brain that were positive for viral RNA by in situ hybridization on adjacent sections (Fig. 5A). The abundance of 633 virus RNA was consistent with the 10-fold-higher titers of 633 virus in newborns (4.4×10^8 PFU/100 mg of brain) compared with peak titers observed with either virus in 2-week-old animals (Fig. 1). As expected, no viral RNA was detected in brains of HBSS-inoculated mice by in situ hybridization (Fig. 5C). Rare TUNEL-positive cells were observed in HBSS-inoculated brains, perhaps reflecting developmental apoptosis (6) (Fig. 5D).

Activation of an endogenous endonuclease that cleaves chromatin between nucleosomes during apoptotic cell death produces characteristic 180- to 200-bp DNA ladders on agarose gels (27). To confirm that the rapid mortality observed in newborns resulted from apoptosis rather than necrosis, we used a solution-phase end-labeling assay to detect DNA ladders (40). Ladders were readily observed 2 and 3 days after inoculation of 1-day-old mice with 633 virus, confirming that Sindbis virus-induced cell death in vivo was apoptotic (Fig. 6). A longer exposure (two rightmost lanes) also detected ladders at 1 day postinoculation. The weak background observed in HBSS-inoculated animals was not detected in an uninoculated mouse, indicating that the trauma of intracerebral inoculation may induce a low level of apoptosis.

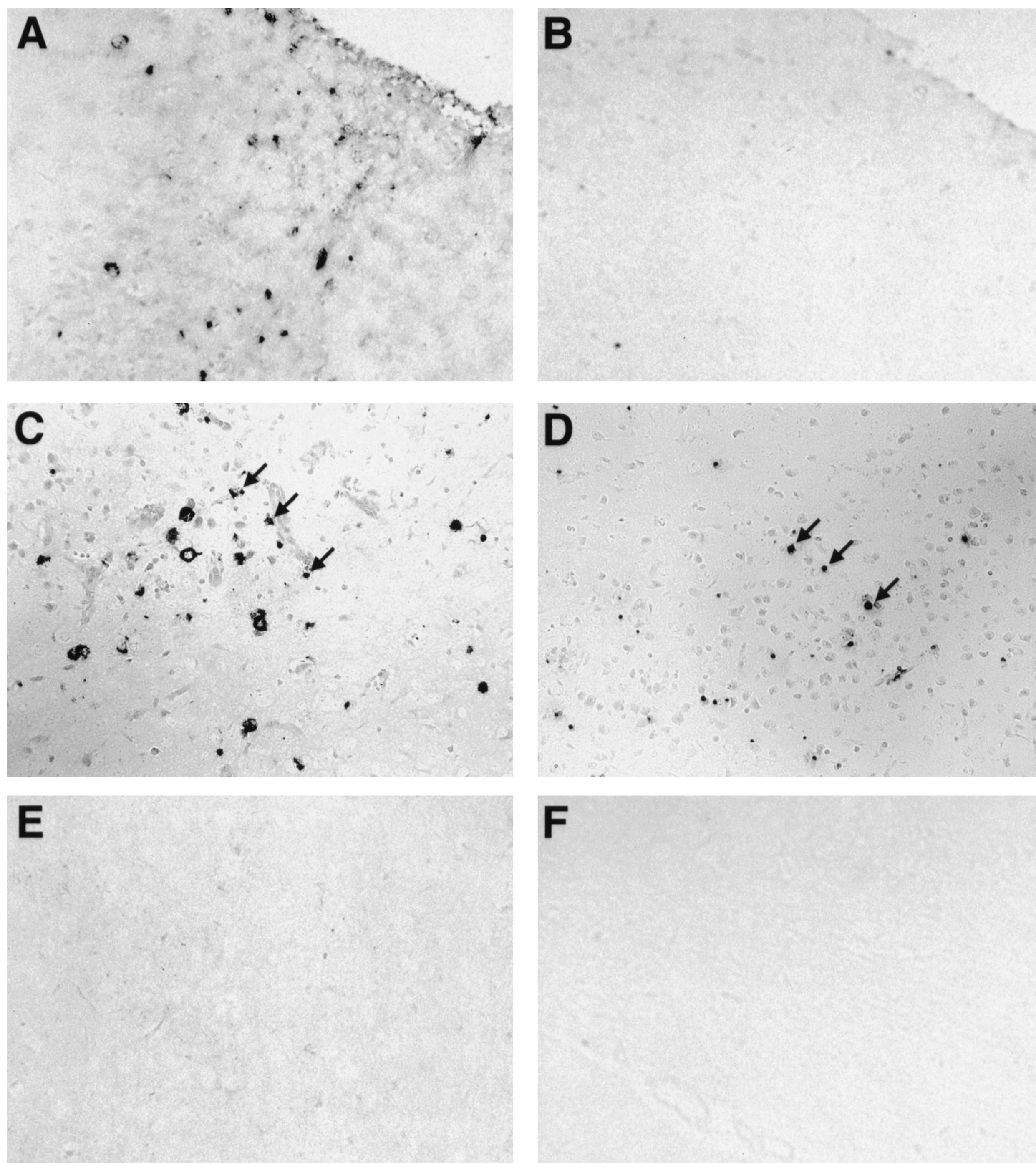

FIG. 4. Detection of viral RNA and apoptotic cells in brains of 633-infected mice. Viral RNA was detected by in situ hybridization (A and C) and apoptotic cells were detected in a TUNEL assay (B and D) on tissues collected 3 (A and B) or 4 (C and D) days postinoculation in two separate experiments. (E) In situ hybridization for viral RNA and (F) TUNEL assay on brain tissue from control mice inoculated with virus diluent alone. Original magnification, ×40.

DISCUSSION

Although Sindbis virus replication and progeny virus production per se may be sufficient to kill cells by lethal parasitism if given time, an alternate mechanism of cell death appears to predominate. Upon infection of a variety of cell lines, Sindbis virus triggers a cellular response that activates a programmed cell death pathway resulting in apoptosis (33, 49). It has been hypothesized that apoptotic cell death may be an important host defense mechanism for eliminating infected cells, thereby slowing the spread of virus. In support of this hypothesis is the observation that some viruses with large genomes have acquired genes that block apoptosis. The p35 and *iap* genes of baculovirus and the E1b 19k gene of adenovirus inhibit apoptosis, and mutations in these genes significantly reduce the production of progeny virus (4, 5a, 19). In contrast, Sindbis virus, which encodes only two polyproteins, appears to thrive in

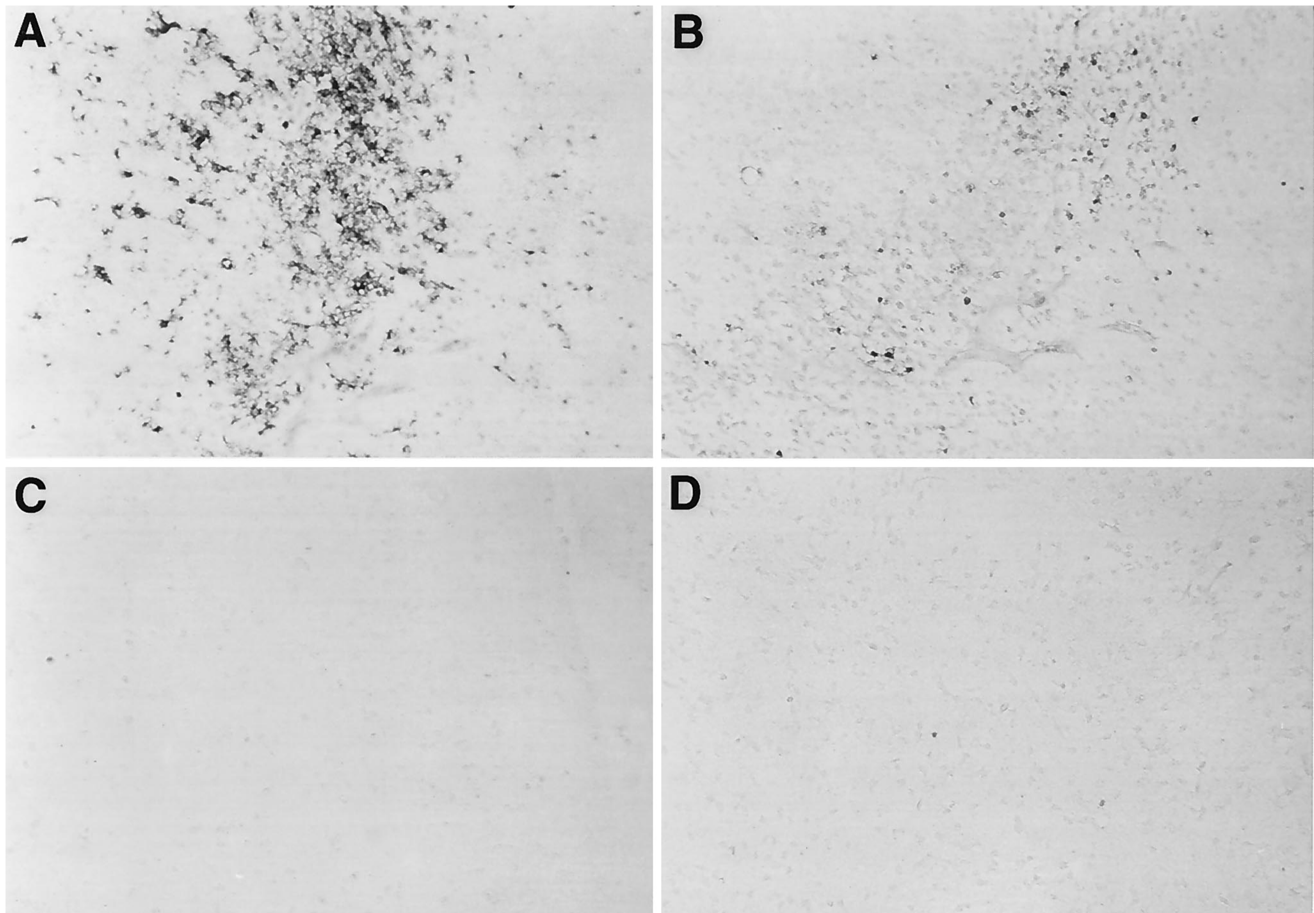

FIG. 5. Recombinant virus 633 induces widespread apoptosis in newborn mice. In situ hybridization (A and C) and TUNEL (B and D) assays were performed on adjacent brain sections prepared 2 days after inoculation of 1-day-old mice with 633 virus (A and B) or virus diluent alone (C and D). Original magnification, ×40.

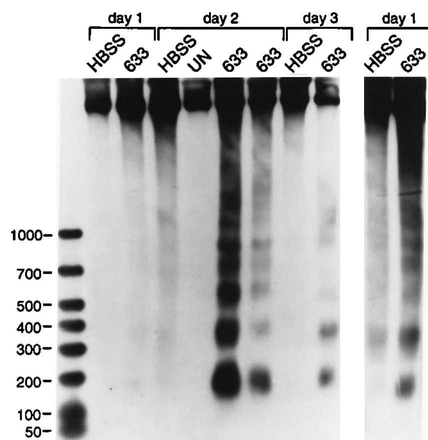

FIG. 6. Detection of DNA ladders in Sindbis virus-infected mouse brains. Brains were harvested 1, 2, and 3 days following inoculation of newborn mice with buffer alone (HBSS) or with recombinant virus 633 or from an uninoculated mouse (UN). Duplicate 633-inoculated mouse brains were harvested on day 2. DNA was end labeled by addition of digoxigenin-ddUTP with terminal transferase and analyzed on an agarose gel. DNA size markers are indicated in nucleotides.

apoptotic cells perhaps in part because its replication cycle is completed prior to cell death. Thus, Sindbis virus-induced apoptosis in vivo could potentially be responsible for the observed disease and mortality. Compounding the issue, Sindbis virus primarily targets neurons, thus eliminating a postmitotic cell population that cannot be regenerated. We found that Sindbis virus-induced apoptosis in mouse brains and spinal cords correlated with mortality. The neurovirulent TE virus, which causes hindlimb paralysis and fatal encephalomyelitis in 2-week-old animals, induced apoptosis that was readily detected by the in situ TUNEL technique. However, with the less virulent 633 virus, TUNEL-positive cells were rarely observed in infected brains. In contrast, 633 produced abundant apoptosis in the tissues of newborn animals, corresponding with a 100% mortality rate for 633 in newborns. Thus, the ability to induce apoptosis correlated with neurovirulence, suggesting that apoptosis may be an important factor in the pathogenesis of Sindbis virus infection.

The molecular mechanisms of age-dependent neurovirulence are not known and may be due to a combination of viral and cellular factors. Although neurovirulent strains of Sindbis virus infect a larger number of cells in mouse brains, virulent and avirulent viruses exhibit the same cell and tissue distribution (13, 23). Therefore, cell tropism is probably not a factor for viruses differing at E2 position 55. Developmentally regulated expression of a viral receptor in mouse brains could potentially explain age-dependent susceptibility. This possibil-

ity is supported by the observation that a putative Sindbis virus receptor is more abundant at 16 h than at 96 h after birth (48). It is conceivable that an amino acid difference at E2 position 55 in TE virus could increase the efficiency of binding to a receptor or other required membrane component that is down regulated with age. A recent study indicates that E2 position 55 influences binding to BHK and N18 cells but probably has a greater influence on virus uncoating (46). These differences, however, are not sufficient to alter the outcome of in vitro infection of the two cell lines tested, as both are readily killed by Sindbis virus infection.

We reported previously that AT-3 cells expressing human *bcl-2* are not killed by infection with 633 virus even though they are efficiently killed by TE virus, thereby mimicking the outcome of infection of 2-week-old mice (49). Although the effect of *bcl-2* on cellular components that modulate viral binding, entry, and uncoating are not known, other intracellular factors affecting viral replication may also be important. The possibility that *bcl-2* functions at a stage following viral entry and uncoating is supported by the observation that *bcl-2* can inhibit apoptosis in cultured cells infected with recombinant Sindbis viruses carrying a copy of the *bcl-2* gene (3). Furthermore, *bcl-2* expressed from a recombinant Sindbis virus also protects mice from fatal encephalitis (31). A stop codon inserted into the *bcl-2* gene within the viral genome abolishes its protective effect in vitro and in vivo. In these experiments, *bcl-2* is likely to be working at an intracellular step because *bcl-2* is not expressed until the viral replication cycle is already under way. Although *bcl-2* is expressed in neurons (11, 21, 29), there is no evidence to date that endogenous *bcl-2* protects 2-week-old mice from Sindbis virus.

The reduced ability of 633 virus to induce apoptosis correlated with reduced 633 virus production in mouse brains (Fig. 1) and in AT3Bcl2 cells (49). Although cell surface interactions could potentially explain these results by reducing the number of cells that become infected, an equally plausible hypothesis is that antiapoptotic cell factors alter the intracellular environment to make it less supportive of viral replication. The latter possibility is supported by the finding that the recombinant Sindbis virus expressing *bcl-2* grows to lower titers than the *bcl-2stop* virus in mouse brains (31). Perhaps the single amino acid change in TE virus has a stabilizing effect on E2 or improves the function of E2 through some other mechanism in an antiapoptotic intracellular environment. In either case, it needs to be determined if the reduction in viral replication efficiency is an inconsequential side effect of the antiapoptotic state or whether protection from virus-induced apoptosis is a direct consequence of the reduced levels of virus production. Either mechanism could explain the observed correlation between the number of apoptotic cells and the amount of virus present in mouse brains.

Sindbis virus RNA and apoptotic morphology colocalized in double-labeling experiments, indicating that the virus directly triggered the apoptotic pathway. The molecular mechanism by which virus infection activates the death pathway is unknown. Cessation of cellular RNA synthesis has been suggested to be the stimulus for baculovirus-induced cell death (5). It has been postulated that nonpermissive poliovirus infection activates the death pathway by shutting off host protein synthesis (44). These hypotheses are supported by the finding that the uninfected host cells for these viruses are induced to undergo apoptosis by treatment with metabolic inhibitors. Thus, these cells apparently require ongoing synthesis of a protective protein to avoid activating the death pathway. Although Sindbis virus also has the capacity to inhibit host protein synthesis, cultured neurons are protected from apoptosis by inhibitors of RNA and protein synthesis, suggesting that they require the expression of new genes to facilitate the death pathway (35). Genes that are activated during neuronal cell death are beginning to be identified (10), and it will be interesting to determine if Sindbis virus also activates these genes. The molecular mechanisms by which Sindbis virus activates the death pathway may vary in different cell types. Sindbis virus activates the transcription factor NF-κB shortly after infection (34). Treatment of cells with NF-κB-binding oligonucleotide decoys that prevent activation of downstream genes by NF-κB can also block Sindbis virus-induced cell death in AT-3 cells but not N18 cells (34). Thus, NF-κB appears to play a role in Sindbis virus-induced activation of the death pathway in some cells. The requirement for new gene expression to activate the death pathway seems contrary to the observation that Sindbis virus inhibits host protein synthesis, as measured by [^{35}S]methionine incorporation. Yet evidence that NF-κB plays a role in virus-induced cell death indicates that synthesis of at least some cell factors escapes the inhibitory effects of the virus.

Virus-induced apoptosis has been implicated in other disease states. Chicken anemia virus, a small single-stranded DNA virus, causes complete depletion of the thymic cortex in hatchlings (24). While epithelial cells in the thymus are spared, loss of cortical thymocytes through apoptosis is probably due to direct infection by this lymphotropic virus since virus particles were observed within engulfed apoptotic bodies (24). Measles virus also induces apoptosis in thymocytes following infection of human thymus tissue implanted into SCID mice (2). In contrast to chicken anemia virus, measles virus is found primarily in the thymic epithelial stroma, with no evidence of measles antigens in thymocytes; thus, apoptosis appears to result from an indirect mechanism. Although several retroviruses are apparently capable of directly inducing apoptotic cell death in cultured cells (30, 39, 41), apoptosis of $CD4^+$ T cells and neurons in AIDS pathogenesis appears to be indirect, as these cells are largely uninfected (1, 10, 15).

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