NOTES

Apoptosis Plays an Important Role in Experimental Rabies Virus Infection

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Cultured rat prostatic adenocarcinoma (AT3) cells infected with the challenge virus standard (CVS) strain of fixed rabies virus showed characteristic morphologic features of apoptosis, evidence of oligonucleosomal DNA fragmentation, and expression of the Bax protein. CVS-infected *Bcl-2*-transfected AT3 cells did not demonstrate these features. Adult ICR mice inoculated intracerebrally with CVS showed morphologic changes of apoptosis, DNA fragmentation, and increased Bax expression in neurons, with changes most marked in the hippocampus and cerebral cortex. Ultrastructurally, some neurons demonstrated morphologic features more typical of necrosis. These studies provide evidence that apoptosis plays an important role in the pathogenesis of rabies virus infection.

Neurotropic viruses spread to the central nervous system, infect neural cells, and produce clinical illness by causing dysfunction or death of a population of these cells (7). There is recent evidence that viruses may cause death of cells by apoptosis (2, 6, 20, 22), which is a tightly regulated energy-requiring process that is orchestrated by a genetic program (3). We have found evidence that apoptosis plays an important role in producing cell death in rabies virus infection of cultured cells and in the brains of experimentally infected mice.

Rat prostatic adenocarcinoma cells transfected with pZipNeo (AT3) and with pZip-*bcl-2*/neo containing the human *bcl-2* open reading frame (AT3-Bcl-2), which were obtained from Diane Griffin (Johns Hopkins University, Baltimore, Md.) (12), were infected with the challenge virus standard (CVS) strain of rabies virus at a multiplicity of 10 PFU per cell. At serial time points, cellular pellets were prepared and either fixed in 4% paraformaldehyde and embedded in paraffin or fixed in 0.5% glutaraldehyde and 2.0% paraformaldehyde and infiltrated with Jembed resin. Immunoperoxidase staining for rabies virus antigen (10) was demonstrated in 19.3% of the cells at 24 h after viral adsorption and in a gradually diminishing proportion thereafter (2.6% at 5 days) (Fig. 1A). Viral adsorption was also performed over a wide range of multiplicities of infection, and these studies indicated that CVS infection of AT3 cells was nonpermissive (data not shown). Infected AT3 cells developed discrete condensations of nuclear chromatin that were often localized adjacent to the nuclear envelope (marginalization) and cytoplasmic shrinkage (Fig. 2A). These morphologic features of apoptosis developed between 3 to 5 days after infection. Ultrastructurally, cells typically showed intact cytoplasmic membranes and organelles and some cells demonstrated cytoplasmic blebbing (Fig. 2B). Swelling of organelles was observed in a minority of cells with typical nuclear features of apoptosis. In addition, some cells exhibited a less discrete pattern of chromatin condensation. Oligonucleosomal DNA fragmentation was assessed in situ in sections by using the terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) method (9). TUNEL staining was demonstrated in CVS-infected AT3 cells (Fig. 1B), but CVS inactivated with β-propiolactone prior to adsorption of the virus on the cells did not result in increased TUNEL staining over the low levels in mock-infected AT3 cells. TUNEL staining gradually increased in infected AT3 cells and became maximal 3 days after infection, with 7.9% of the cells being TUNEL positive (Fig. 3).

AT3-Bcl-2 cells infected with CVS under identical conditions demonstrated immunostaining for rabies virus antigen in about 40% fewer cells than in infected AT3 cells. The morphologic features of apoptosis were minimal in AT3-Bcl-2 cells and much less TUNEL staining was observed in these cells (Fig. 3). Immunostaining for the Bax protein (polyclonal anti-Bax protein from Santa Cruz Biotechnology, Santa Cruz, Calif.) was demonstrated in many CVS-infected AT3 cells (Fig. 1C) 3 to 5 days after viral adsorption but not in mock-infected AT3, AT3-Bcl-2 cells, or CVS-infected AT3-Bcl-2 cells.

The biological importance of apoptotic cell death was also examined in vivo in 6-week-old ICR mice inoculated intracerebrally with 9.3×10^5 PFU of CVS. The mice developed neurologic signs of rabies, including paralysis, and death occurred 7 to 9 days after inoculation. Mouse brains fixed in 4% paraformaldehyde and embedded in paraffin were examined at daily intervals after inoculation. Light microscopy showed typical apoptotic morphology beginning 4 days after inoculation, and this became more prominent as the disease advanced. Typical changes in neurons included multiple discrete clumps of nuclear chromatin (Fig. 2C) and cytoplasmic shrinkage. Apoptosis was first observed and most marked in pyramidal neurons of the hippocampus and in neurons scattered in the neocortex (Fig. 2C). Subsequently, less severe changes were observed in multiple other areas, including the basal ganglia, thalamus, and brain stem. Morphologic changes were much less conspicuous in the cerebellum, despite prominent infec-

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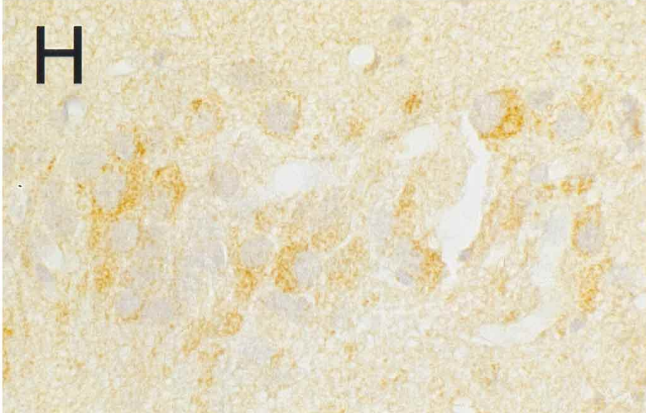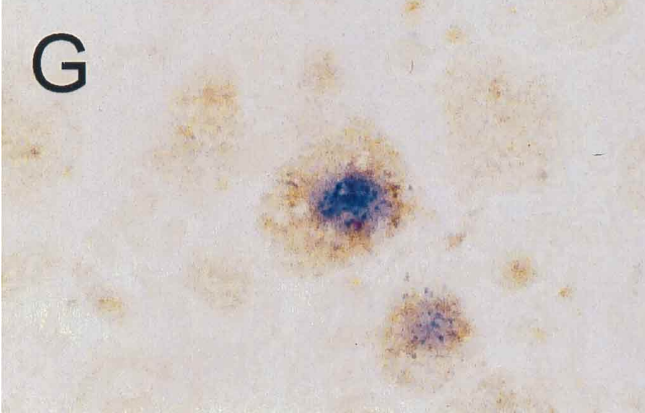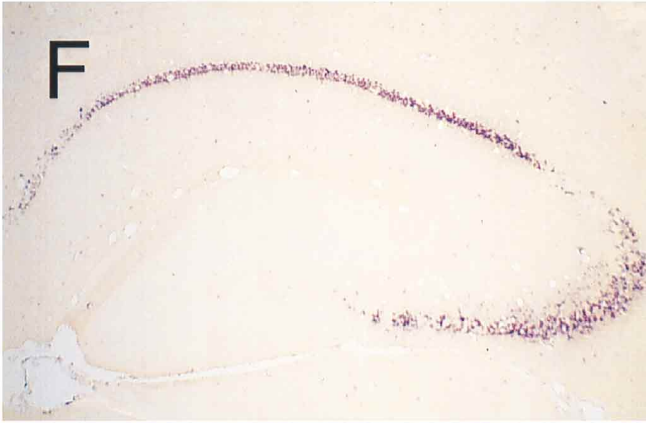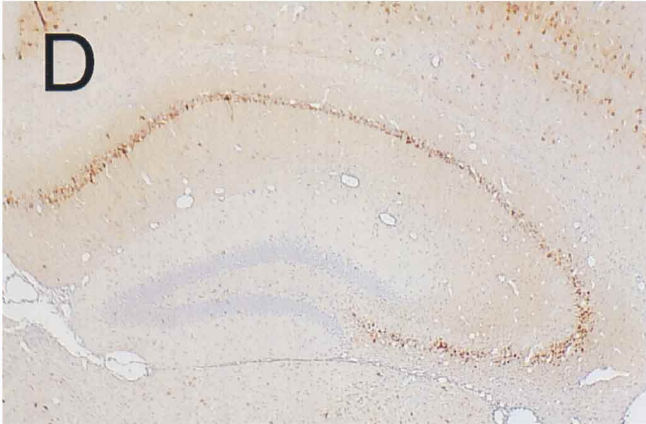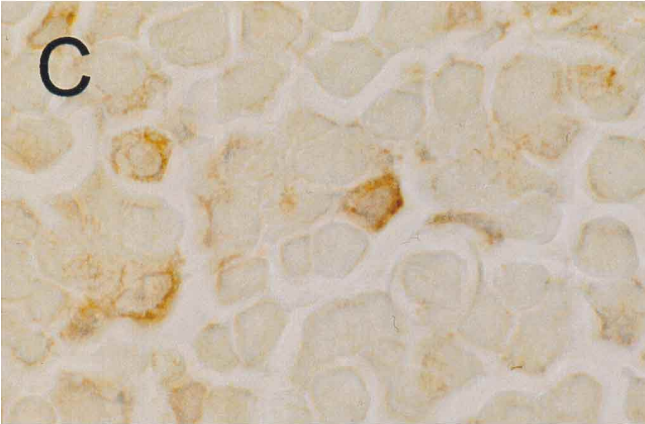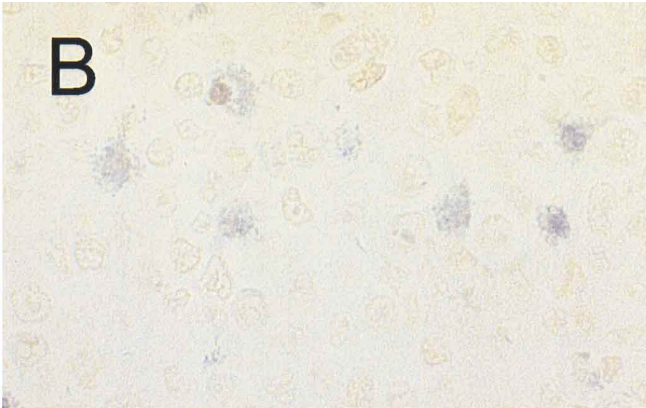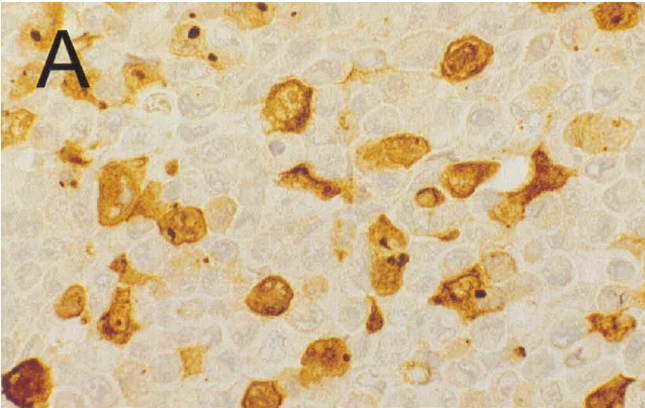
FIG. 1. (A) Immunostaining for rabies virus antigen in AT3 cells 2 days after adsorption of CVS, showing antigen in the cytoplasm of many cells. (B) TUNEL staining of CVS-infected AT3 cells 3 days after adsorption of CVS, showing evidence of oligonucleosomal DNA fragmentation in the cells. (C) Immunostaining for the Bax protein in CVS-infected AT3 cells, showing antigen in the cytoplasm. (D) Immunostaining for rabies virus antigen in the hippocampus of a mouse 7 days after intracerebral inoculation with CVS, showing antigen in pyramidal neurons and in cortical neurons. Neurons in the dentate gyrus do not demonstrate staining. (E) TUNEL staining of a tissue section pretreated with DNase I (positive control), showing prominent staining of nuclei of the hippocampus, including cells in the dentate gyrus. (F) TUNEL staining in the hippocampus of a mouse 7 days after intracerebral inoculation with CVS. Marked staining is present in pyramidal neurons, but not in neurons of the dentate gyrus. Note the similarity in the distribution of TUNEL staining (F) and rabies virus antigen (D). (G) Double staining of a pyramidal neuron in the CA3 region of the hippocampus of a mouse 9 days after intracerebral inoculation with CVS, which shows TUNEL staining in the nucleus and immunostaining for rabies virus antigen in the cytoplasm. (H) Immunostaining for Bax protein in pyramidal neurons (CA3 region) 5 days after intracerebral inoculation with CVS. Magnifications: A, ×370; B, ×470; C, ×840; D, E, and F, ×40; G, ×2,600; H, ×520.
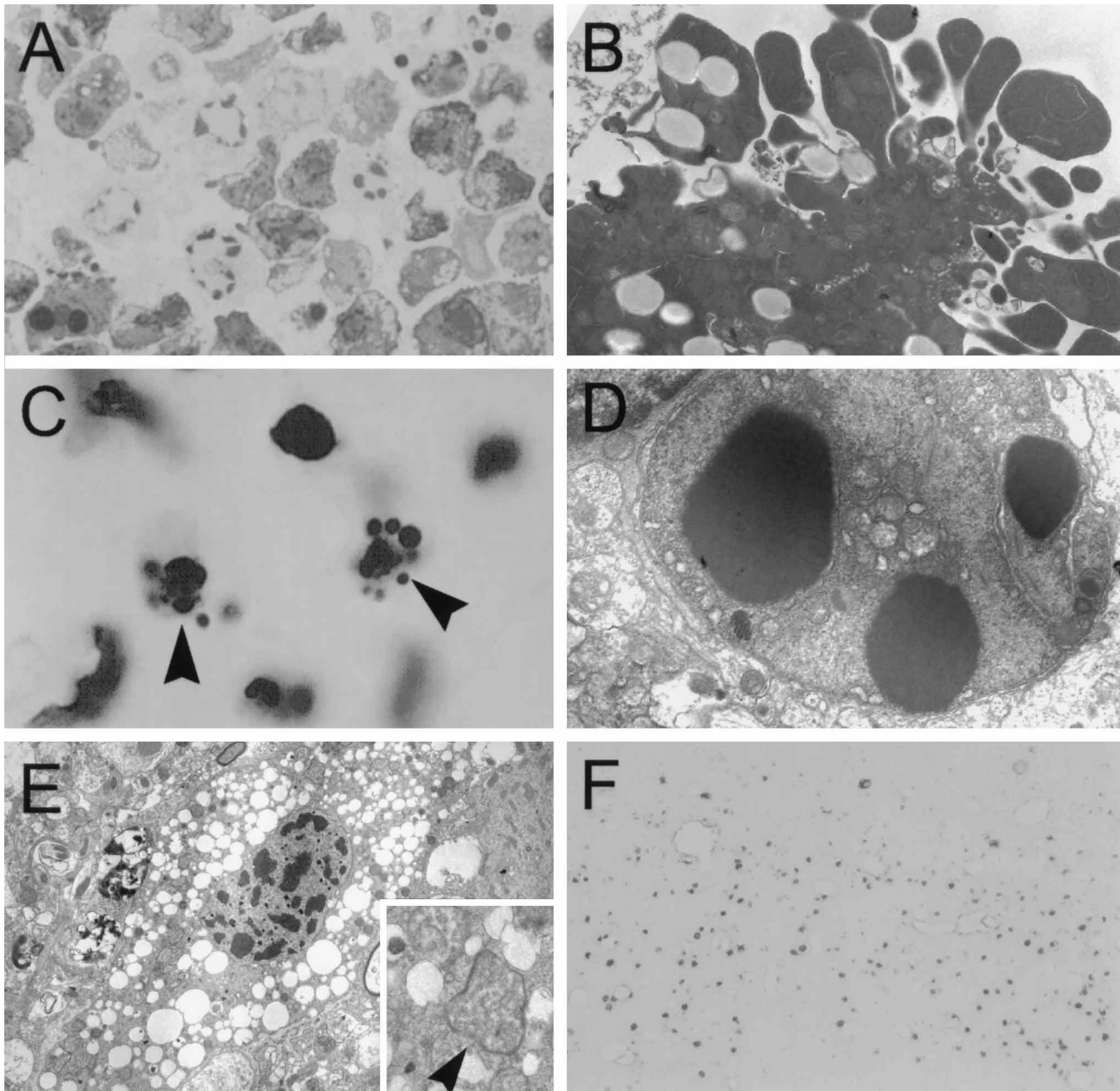

FIG. 2. (A) CVS-infected AT3 cells 5 days after adsorption of virus. Typical morphologic changes of apoptosis are demonstrated in many cells, with condensation of nuclear chromatin, which is frequently distributed in multiple discrete masses at the nuclear margin. Cytoplasmic shrinkage is also seen. Toluidine blue staining. (B) CVS-infected AT3 cell with multiple cytoplasmic blebs that contain electron-dense cytoplasm and intact organelles. (C) Neurons in the cerebral cortex 8 days after inoculation with CVS. There are multiple condensations of nuclear chromatin in two cells (arrowheads). Cresyl violet staining. (D) Hippocampal pyramidal neuron 5 days after intracerebral inoculation with CVS, with three discrete masses of condensed chromatin and cytoplasmic shrinkage with mildly enlarged mitochondria and endoplasmic reticulum and an intact plasma membrane. (E) Hippocampal pyramidal neuron showing a pattern of irregular chromatin condensation, marked cytoplasmic vacuolation, and prominent enlargement and focal loss of membrane integrity (arrowhead) in mitochondria (inset). (F) TUNEL staining in many cortical neurons 9 days after intracerebral inoculation with CVS. Magnifications: A, ×1,100; B, ×8,600; C, ×2,100; D, ×10,800; E, ×2,900 and ×18,400 (inset); F, ×120.
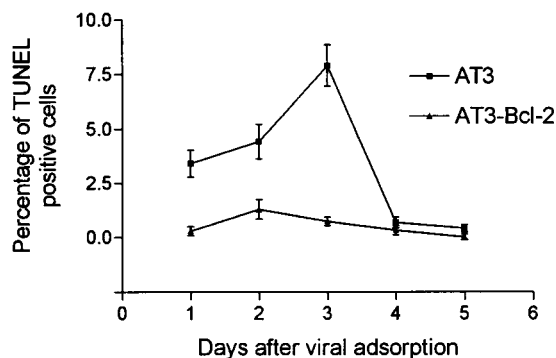
FIG. 3. CVS-induced oligonucleosomal DNA fragmentation. AT3 cells and AT3-Bcl-2 cells were infected with CVS or mock infected. Results are shown as the percentage of virus-infected cells demonstrating TUNEL staining minus the percentage of mock-infected cells demonstrating staining. Error bars indicate standard errors of the mean.

tion of Purkinje cells, which was demonstrated with immunostaining for rabies virus antigen.

Ultrastructurally, there was a range in the morphologic alterations of dying cells. Many neurons had discrete dense nuclear chromatin accumulations and condensed cytoplasm (Fig. 2D), which is in keeping with apoptotic cell death. In these cells changes in mitochondria and endoplasmic reticulum ranged from minimal to moderate enlargement. However, other scattered cells exhibited a much more irregular pattern of chromatin condensations, frequently in association with cytoplasmic vacuolation, organelle swelling, and focal loss of both organelle and plasma membrane integrity (Fig. 2E). These features are not typical of apoptosis and are more characteristic of necrotic cell death (11).

TUNEL staining of brain sections showed prominent staining of neurons in topographic regions with apoptotic morphologic changes and immunostaining for rabies virus antigen (Fig. 1D and F). In contrast, TUNEL staining of tissue sections pretreated with DNase I (positive control) demonstrated strong staining of nuclei in all regions of the brain (Fig. 1E). TUNEL staining of neurons developed 5 days after inoculation and gradually increased as the disease progressed. Staining was particularly prominent in pyramidal neurons of the hippocampus and in cortical neurons (Fig. 1F and 2F). Smaller numbers of neurons demonstrated TUNEL staining in other areas, including the basal ganglia, diencephalon, and brain stem. Neurons were double labeled by the TUNEL method in combination with immunostaining for rabies virus antigen (Fig. 1G), directly establishing that rabies virus-infected neurons underwent apoptotic cell death. In general, there was a good correlation with the distribution of TUNEL staining and rabies virus antigen. However, despite strong immunostaining for rabies virus antigen in cerebellar Purkinje cells, TUNEL staining was markedly less in the cerebellum, with staining in only a small number of scattered cells, at least some of which were likely inflammatory cells.

Increased immunoreactivity for the Bax protein over the normal quantities found in uninfected control animals was demonstrated in topographic areas with prominent apoptotic morphologic changes and strongly positive TUNEL staining, including pyramidal neurons of the hippocampus (Fig. 1H) and cortical neurons. Expression was maximal between days 4 and 6 postinoculation, which was prior to the development of maximal morphologic changes of apoptosis and TUNEL staining,

indicating that the Bax protein may be important in modulating the apoptotic process in this model.

These in vitro and in vivo studies demonstrate that rabies virus infection is associated with both morphologic and biochemical evidence of apoptosis. Although apoptosis is now a widely recognized mechanism by which both RNA and DNA viruses may kill cells in cell culture (20), the pathogenetic significance of apoptosis in encephalitis is only well established in a small number of viral infections (9, 13, 18).

CVS infection of cultured AT3 cells resulted in prominent morphologic features of apoptosis. Since morphologic changes are necessary to establish the presence of an apoptotic process, CVS infection of AT3 cells was a good in vitro model to study rabies virus-induced apoptosis. Because CVS inactivated with β-propiolactone prior to viral adsorption did not result in apoptosis, binding of rabies virus to its unknown cellular receptor is not sufficient to initiate the apoptotic process and a cytoplasmic event is likely required. However, we cannot exclude the possibility that β-propiolactone significantly reduces the binding of CVS to AT3 cells.

A number of genes in the *bcl-2* family are known to exert a modulating effect on apoptosis (2, 5, 21). AT3-Bcl-2 cells were found to be resistant to CVS-induced apoptosis. Although there was a mild reduction in their susceptibility to infection, it is unknown whether viral replication or viral protein synthesis was also inhibited. The marked protective effect of *bcl-2* expression on CVS-induced cell death of the AT3 cells supports the importance of an apoptotic mechanism for cellular injury in this in vitro model. The Bcl-2 protein can heterodimerize with the Bax protein, and in the rheostat model it is the relative ratios of Bcl-2 and Bax heterodimers to homodimers that determine susceptibility to apoptotic cell death (17, 23). Expression of the Bax protein was detected in CVS-infected AT3 cells at a time when there were morphologic features of apoptosis and maximal TUNEL staining. Induction of Bax and other proteins likely plays an important role in producing apoptosis in CVS-infected AT3 cells.

In CVS-infected mice, there were morphologic changes of apoptosis in multiple regions of the brain. Although the changes had typical morphologic features of apoptosis by light microscopy, at the level of electron microscopy there was a spectrum of changes from apoptosis to necrosis. However, the lack of uniform classical ultrastructural features of apoptosis is in concert with the findings in other neurologic diseases, including cerebral ischemia and excitotoxic animal models (quinolinic acid-injected rat striatum), in which there is evidence that apoptotic cell death plays an important pathogenetic role (2, 4, 14–16, 19).

The morphologic features of apoptosis and TUNEL staining were fairly widespread, but were most prominent in pyramidal neurons of the hippocampus and in the cerebral cortex. Despite prominent expression of rabies virus antigen in cerebellar Purkinje cells (8), morphologic changes and TUNEL staining were much less in this location. We have made similar observations in experimental infection of mice with Venezuelan equine encephalitis virus (9). We speculate that expression of a modulating protein, possibly in the Bcl-2 family, may protect these cells.

Adle-Biassette et al. (1) recently described apoptotic neurons on the basis of positive TUNEL staining in the hippocampus and brain stem of a human patient with rabies and AIDS. The in vitro and in vivo evidence presented in these models in combination with the recent observations by Adle-Biassette et al. (1) strongly support an important pathogenetic role of apoptosis in producing the neurologic disease in rabies. Future therapeutic efforts should be directed toward inhibiting the

apoptotic process, and such an approach may prove useful in the treatment of rabies and other infectious diseases.

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