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## Apoptotic cell death is an important cause of neuronal injury in experimental Venezuelan equine encephalitis virus infection of mice

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**Abstract** Mice develop a fatal encephalomyelitis after infection with the Trinidad donkey strain of Venezuelan equine encephalitis (VEE) virus. Adult mice were inoculated intraperitoneally with VEE virus and the brains were examined at different time points. Morphological changes were assessed by histological staining. VEE virus antigen was detected with immunoperoxidase staining, and DNA fragmentation was evaluated in situ using the terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) method. VEE antigen was found in many areas of the brain and it was prominent in neurons. There were mild associated inflammatory changes. DNA fragmentation was demonstrated in many of these areas using TUNEL. In areas with TUNEL staining, morphological neuronal changes ranged from nuclear chromatin condensations to nuclear and cellular fragmentation, which are characteristic of apoptosis. There is strong morphological and biochemical evidence of apoptotic cell death in this experimental model of VEE virus infection.

**Key words** Apoptosis · Arbovirus · Encephalitis · Pathogenesis

### Introduction

Venezuelan equine encephalitis (VEE) virus is an arthropod-borne virus of the *Togaviridae* family. VEE virus infection causes an acute systemic illness and encephalo-

myelitis in humans and horses in the Americas, and it represents an important public health problem [16, 17]. Experimental VEE virus infection of mice is an excellent model for the central nervous system infection that occurs in humans, and studies on this model may give important insights into the pathogenesis of viral encephalitides caused by arboviruses and other viruses.

Apoptosis is a process by which cells undergo physiological cell death in response to diverse stimuli. It is a normal process in embryonic development, maturation of the immune system, and in normal tissue turnover [2, 12, 14]. Recently, a large quantity of evidence has accumulated showing that apoptosis plays an important role in the pathogenesis of diverse diseases, including cancer, autoimmune diseases, neurodegenerative disorders, and viral infections [15]. Razvi and Welsh [13] recently reviewed the importance of apoptosis in a variety of viral infections, including those caused by adenoviruses, Epstein-Barr virus, human immunodeficiency virus, influenza virus, and unconventional viruses. In addition, Griffin and co-workers [6, 8] have demonstrated an important role of apoptotic cell death in the age-dependent mortality and neurovirulence of Sindbis virus, which is also a member of the *Togaviridae* family.

To determine the role of apoptotic cell death in experimental VEE virus infection in mice, histopathological changes and the distribution of VEE virus antigen were assessed in the brain at different time points. In addition, evidence of oligonucleosomal DNA fragmentation, which is a biochemical marker of apoptosis [1, 11], was sought in neural cells of the brain.

### Materials and methods

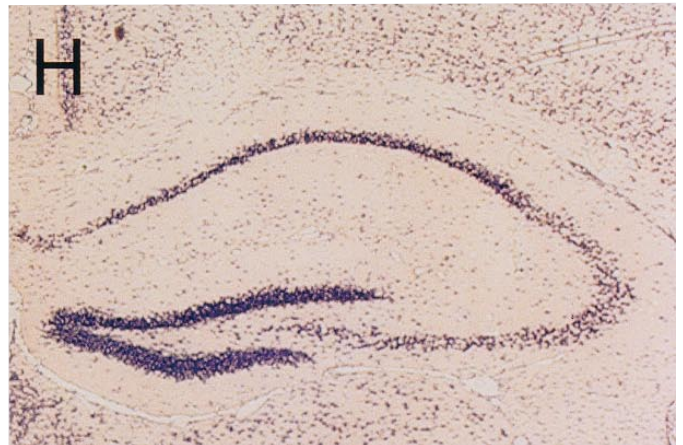
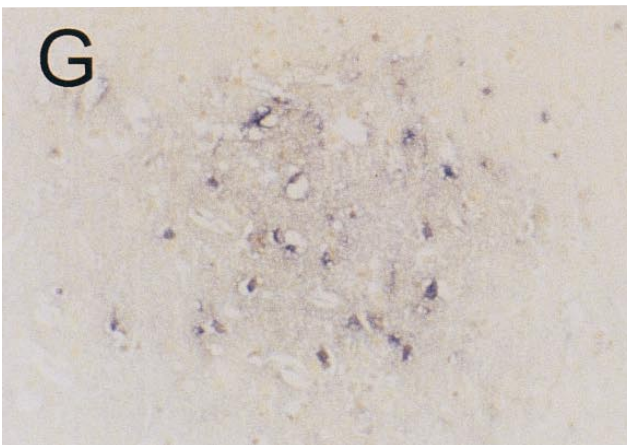
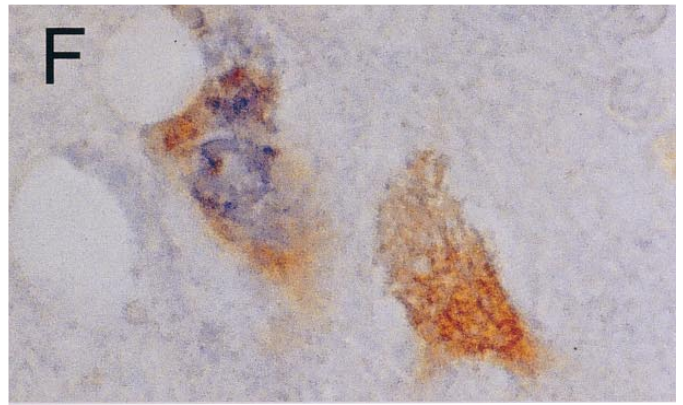
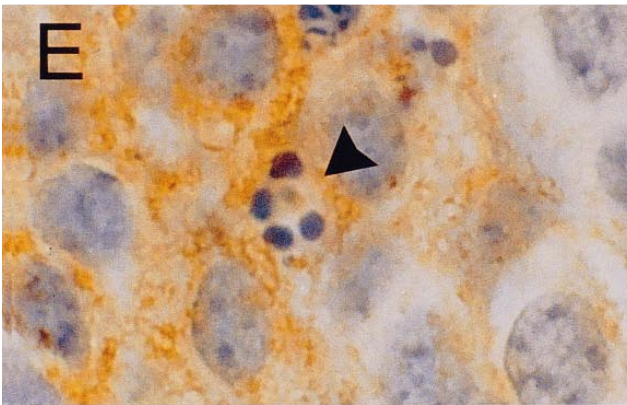
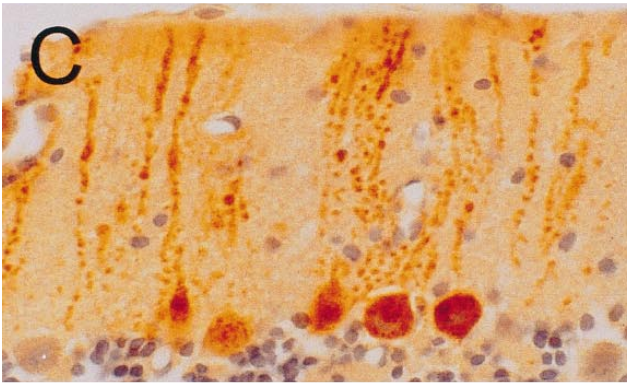
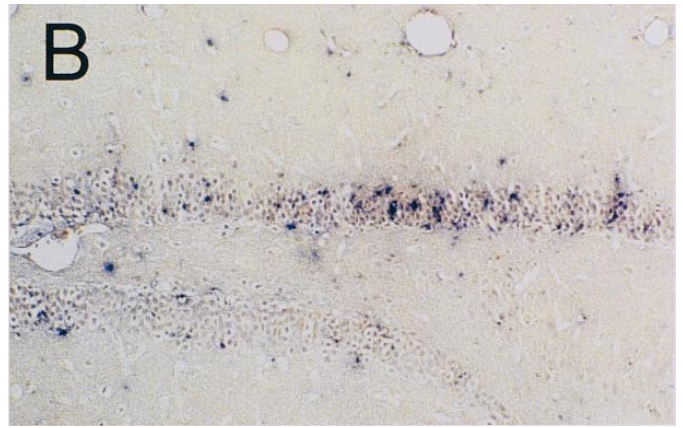
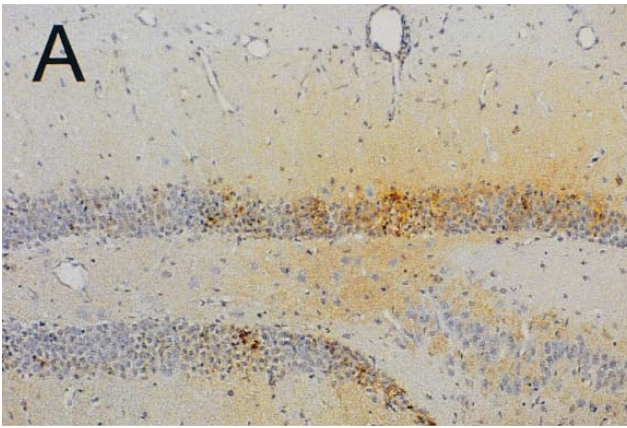
#### Virus, animals, and inoculations

The Trinidad donkey strain of VEE virus was used to inoculate 6- to 8-week-old male C57BL/6 mice as previously described [7]. A viral dose of  $1.0 \times 10^4$  plaque-forming units (pfu) of plaque-purified virus, grown in BHK cells, was administered intraperitoneally. Uninfected control mice were inoculated with phosphate-buffered saline (PBS).

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### Preparation of tissue sections

Mice were anesthetized with methoxyflurane and perfused with buffered 4% paraformaldehyde. The brains of three infected mice were removed at daily intervals and immersion-fixed in the same fixative for 18 h at 4°C. Tissue sections (6 µm) were prepared in the coronal plane after dehydration and embedding in paraffin. Tissues for histological examination were stained with cresyl violet.

### Immunoperoxidase staining

Sections were stained for VEE virus antigen by the avidin-biotin-peroxidase complex method, as previously described, using polyclonal rabbit anti-VEE virus serum (diluted 1:200) as primary antibody [7]. Tissues from uninfected mice were used as controls.

### DNA nick end labeling of tissue sections

DNA fragmentation was assessed *in situ* in tissue sections using the terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) method of MacManus et al. [9] with modifications. Proteolytic digestion was performed in 0.016% pepsin (Boehringer Mannheim, Mannheim, Germany) in 0.01 N HCl at 37°C for 15 min. Following three washes in PBS, sections were labeled using terminal transferase for 1 h at 37°C in a buffered solution containing 0.1 M potassium cacodylate (pH 7.2), 2 mM cobalt chloride, 0.2 mM dithiothreitol, 1 µM digoxigenin-11-dUTP (Boehringer Mannheim), 9 µM dATP, and 0.1 U/µl terminal transferase (Gibco BRL, Burlington, Ontario).

Slides were blocked with blocking reagent (Boehringer Mannheim) for 30 min at room temperature and then incubated for 1 h with a 1:5000 dilution of polyclonal sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim) in 3% milk powder, 0.5% Tween 20, 50 mM TRIS-HCl, 200 mM NaCl (pH 7.4). Incubation with a substrate solution containing nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt (X-phosphate) (Boehringer Mannheim) was performed for 4 h, and the reaction was stopped with 10 mM TRIS-HCl (pH 8.0) and 1 mM EDTA. Positive control sections were prepared with enzymatic DNA fragmentation with 1 µg/ml DNase I (Pharmacia Biotech, Baie d'Urfe, Quebec) for 10 min at 37°C. Terminal transferase was omitted during end labeling for negative controls. Selected slides were double-labeled with the above TUNEL method followed by immunostaining for VEE virus antigen without counterstaining.

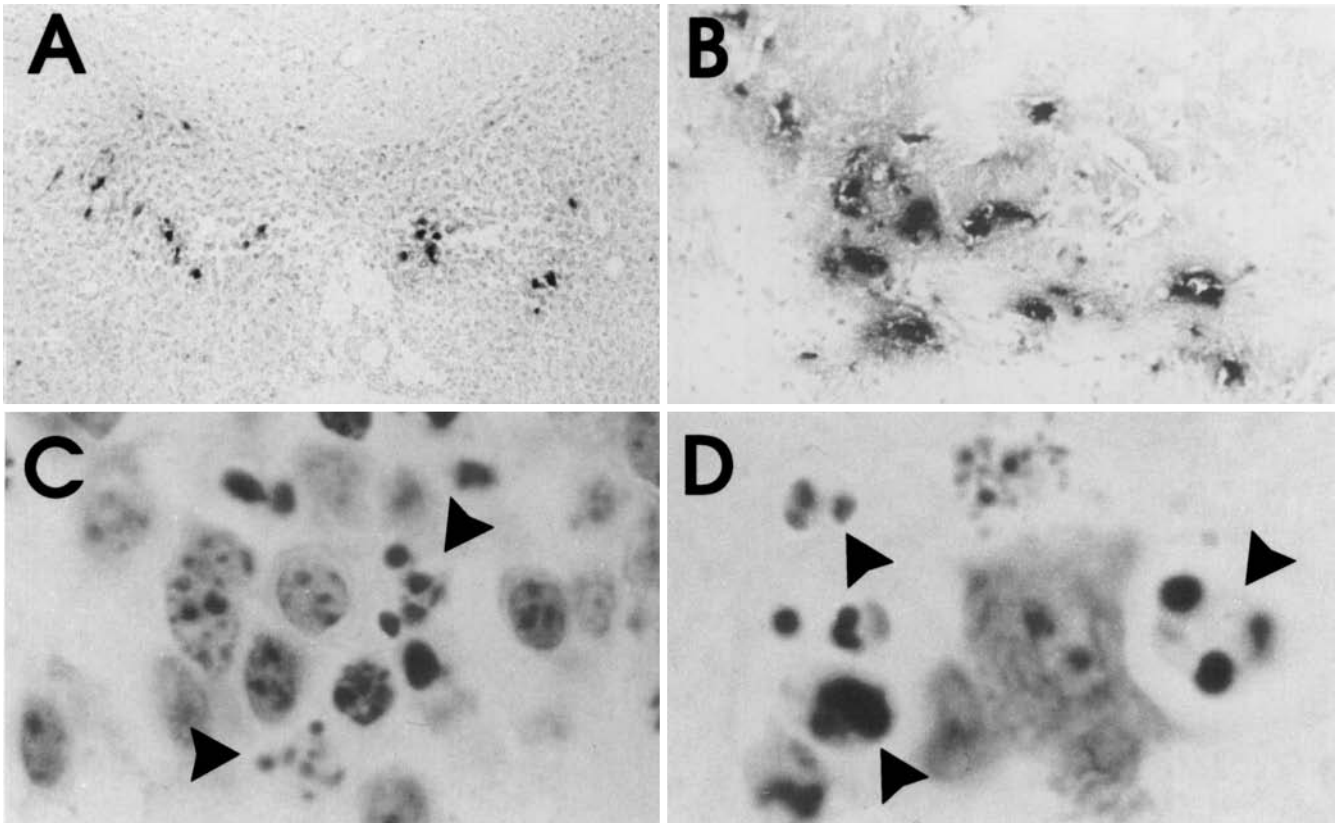
◀ **Fig. 1A, B** Venezuelan equine encephalitis (VEE) virus antigen (A) and terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) staining (B) in the dentate gyrus of the hippocampus of a mouse 5 days after intraperitoneal inoculation of VEE virus. The distribution of stained cells using these methods is very similar. **C, D** In contrast, in the cerebellum viral antigen is present in Purkinje cells and their dendritic processes in the molecular layer and in Bergmann astrocytes (C) and TUNEL staining is absent (D) at 5 days post inoculation (p.i.). **E** Viral antigen is present in the cytoplasm of neurons in the dentate gyrus of the hippocampus and a neuron demonstrating multiple nuclear chromatin condensations and cytoplasmic shrinkage (arrowhead). **F** A thalamic neuron (left) demonstrates both TUNEL staining and VEE virus antigen 7 days p.i. **G** Prominent TUNEL staining is seen in the cerebral cortex of a VEE-infected mouse 7 days p.i. **H** TUNEL-stained section of uninfected hippocampus that was pretreated with DNase I (positive control), demonstrating strong nuclear staining. **A, C, E** Immunoperoxidase-hematoxylin; **D** Nomarski optics, **F** Immunoperoxidase and TUNEL staining, **G, H** TUNEL staining. **A, B** × 120; **C, D** × 510; **E** × 1900; **F** × 2200; **G** × 240; **H** × 40

## Results

The distribution of VEE virus antigen, TUNEL staining, and morphological changes in VEE virus-infected mice are illustrated in Figs. 1 and 2. VEE virus antigen was first detected in a perinuclear distribution in neurons in the cerebral isocortex and pyriform cortex 3 days after inoculation. By the next day antigen was observed in the hippocampus (greater involvement of the dentate gyrus than pyramidal neurons), thalamus, basal ganglia, and Purkinje cells of the cerebellum. Neurons were more prominently infected than glial cells, and at this time antigen was observed in perikarya and in processes. By day 5 post inoculation (p.i.) the distribution of antigen was widespread and extensive (Fig. 1A, C, E). There was involvement of the brain stem and cerebellum, which included Purkinje cells, Bergmann astrocytes, and their processes in the molecular layer (Fig. 1C) and multiple foci in the granular cell layer. At later time points (days 6–8) antigen was observed in a larger number of cells and it was more apparent in glial and inflammatory cells.

Strong TUNEL staining was observed on tissue sections pretreated with DNase I (Fig. 1H) and uninfected tissues did not demonstrate staining. TUNEL staining was first detected in the brain at 5 days p.i. Staining was present in large neural cells with morphological features of neurons in the cerebral cortex, hippocampus (especially the dentate gyrus, Fig. 1B), thalamus, basal ganglia (Fig. 2B), and brain stem. Staining was observed in the nucleus and also in the cytoplasm of cells, possibly because leakage of fragmented DNA occurs across the nuclear membrane [3]. There was also mild diffuse staining in the neuropil around stained cells (Figs. 1G, 2B), which may be explained by diffusion of fragmented DNA into neural processes. Staining was prominent in neural cells by day 8 p.i. There was very good correlation between the brain regions that demonstrated viral antigen and TUNEL staining (Fig. 1A, B). Neurons were observed that demonstrated both TUNEL staining and viral antigen (Fig. 1F). These findings suggest that VEE virus infection induces apoptotic cell death in the brain and that a subpopulation of infected neural cells undergo apoptosis. However, TUNEL staining was not observed in the cerebellum (Fig. 1D) despite the presence of viral antigen in multiple cell types (Fig. 2C, D).

Morphological features of apoptotic cell death were observed in many regions of the brain in which TUNEL staining was demonstrated, including the cerebral cortex, hippocampus, basal ganglia, and diencephalon. However, these changes were less marked in the cerebellum, which demonstrated negative TUNEL staining, and changes were not observed in Purkinje cells. Typically, many cells exhibited multiple nuclear chromatin condensations and cytoplasmic shrinkage in affected areas (Figs. 1E; 2C, D). At later time points, mild multifocal neuronal cell loss was observed in areas with TUNEL staining. No Purkinje cell loss was observed. There were mild mononuclear inflammatory infiltrates in the parenchyma and leptomeninges.



**Fig. 2** **A, B** TUNEL staining in the subiculum (**A**), which is involved bilaterally, and globus pallidus (**B**) of VEE-infected mice (7 and 5 days p.i., respectively). **C, D** Morphological features of apoptotic cell death, including nuclear chromatin condensations and cytoplasmic shrinkage (*arrowheads*), can be seen in the dentate gyrus of the hippocampus (**C**) and globus pallidus (**D**) 5 days p.i. **A, B** TUNEL staining; **C, D** cresyl violet. **A**  $\times 160$ , **B**  $\times 220$ , and **C**  $\times 2200$ , **D**  $\times 2800$

Areas with apoptotic changes were observed both with and without inflammatory infiltrates.

## Discussion

These studies provide strong evidence that there is extensive apoptotic cell death in the brain in experimental VEE virus infection in mice. Gold et al. [4, 5] have reported that the TUNEL method is superior to the in situ end labeling method using DNA polymerase I for the detection of DNA fragmentation in apoptosis. TUNEL staining may occur in very late stages of necrosis, although labeling is probably less marked in this setting [4, 10]. In the present study, TUNEL staining was demonstrated in neural cells in topographic regions that showed prominent infection on the basis of viral antigen distribution. In addition, there were typical morphological features of apoptotic cell death in neural cells in these regions. The combination of both typical morphological and biochemical features of apoptosis is important in establishing a pathogenetic role for apoptotic cell death in this model. The presence of TUNEL

staining and viral antigen in the same neurons indicates that at least a subset of the infected cells undergo apoptotic cell death. However, apoptosis in this model may be mediated, in part, by indirect mechanisms that do not require infection of the cells undergoing apoptotic cell death. It is possible that Purkinje cells, which did not demonstrate morphological features or biochemical markers of apoptotic cell death, synthesize one or more inhibitors of apoptosis. It is uncertain whether apoptosis also occurs in glial cells or uninfected neurons in this model.

A large amount of evidence is accumulating that apoptosis is a common mechanism by which viruses cause death of host cells, although studies on in vivo models of central nervous system infections are very limited [6, 8, 13]. The widespread occurrence of apoptotic cell death in the brain may explain the neurological disease of VEE virus infection and its fatal outcome. A better understanding of the cellular mechanisms of apoptosis in viral infections may lead to effective new therapies for these infections.

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