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Herpes simplex virus type 1–induced encephalitis has an apoptotic component associated with activation of c-Jun N-terminal kinase

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Herpes simplex virus type 1 (HSV-1) triggered apoptosis in hippocampal cultures, as determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and immunohistochemistry with antibody specific for the large fragment of activated caspase 3. The levels of phosphorylated (activated) c-Jun N-terminal kinase (JNK) were also increased in HSV-1–infected hippocampal cultures as were the levels of activated c-Jun, its target. JNK activation was involved in HSV-1–induced apoptosis as evidenced by apoptosis inhibition with the JNK inhibitor SP600125. HSV-2 activated the mitogen-activated protein kinase/extracellular regulated protein kinase (MEK/ERK) survival pathway and did not trigger apoptosis in hippocampal cultures. The MEK specific inhibitor U0126 inhibited ERK activation and caused a significant increase in the percent TUNEL$^+$ cells in HSV-2–infected cultures, indicating that the failure of HSV-2 to trigger apoptosis is due to its ability to activate the MEK/ERK survival pathway. JNK was also activated in brain tissues from patients with HSV-associated acute focal encephalitis (HSE) that were positive for HSV-1 antigen. JNK activation correlated with apoptosis, as determined by immunohistochemistry with antibody to activated caspase 3 or cleaved poly (ADP-ribose) polymerase (PARP). The data suggest that HSE has an apoptotic component that may contribute to disease pathogenesis. *Journal of NeuroVirology* (2003) **9,** 101–111.

Keywords: apoptosis; encephalitis; ERK; HSV-1; JNK

Introduction

Herpes simplex virus (HSV)-induced encephalitis (HSE) is generally caused by HSV type 1 (HSV-1) in adults and older children (Whitley, 1997; Dennett *et al*, 1997; Kleinschmidt-Demasters and Gilden, 2001), where it accounts for 10% to 20% of all cases of viral encephalitis. It is characterized by severe destruction of temporal and frontal lobe structures, including limbic mesocortices, amygdala, and hippocampus, and is associated with high mortality (60% to 70%) and morbidity in survivors (Beers *et al*, 1993; Koskiniemi *et al*, 1996; Whitley, 1997). Antiviral therapy reduces the mortality rate to approximately 30%, but survivors may still have severe neurological impairment (Skoldenberg, 1996; Whitley, 1997). Traditionally, HSE has been associated with necrotic cell death resulting from virus replication and inflammatory changes/cerebral edema secondary to virus-induced immune response (Sobel *et al*, 1986; Thompson *et al*, 2000). However, there is a relatively poor correlation between virus burden in the brain and the severity of histological changes and neurological symptoms (Ando *et al*, 1993). Moreover, a small, but notable, number of HSE patients with central nervous system (CNS) symptoms are negative for HSV-1 DNA early in the course of infection (Studahl *et al*, 1998; Weil *et al*, 2002), suggesting that

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factors other than virus replication are involved in cell death/disease pathogenesis.

Necrosis is an unregulated cell death process that occurs upon exposure to extremely unphysiological stimuli; it is often accompanied by extensive tissue damage and an intense inflammatory response. By contrast, apoptosis is a tightly regulated cell death process that is primarily mediated by cysteine proteases (known as caspases), which are activated by the cleavage of inactive zymogens (procaspases). Caspase 3 is a key executioner of apoptosis. It is responsible for the proteolytic cleavage of key proteins, such as the poly (ADP-ribose) polymerase (PARP) that is involved in DNA repair. PARP cleavage into an inactive 85-kDa fragment (p85PARP) is a crucial event in the cell commitment to undergo apoptosis (Johnson-Webb *et al*, 1997). Signal transduction pathways are linked to the apoptotic machinery. Activation of the c-Jun N-terminal kinase (JNK) is associated with increased expression of proapoptotic proteins and is a crucial event in neuronal cell apoptosis (Bossy-Wetzel *et al*, 1997; Honig and Rosenberg, 2000; Kaplan and Miller, 2000; Morishima *et al*, 2001). However, apoptotic signals are overridden by activation of the mitogen-activated protein kinase/extracelluar regulated protein kinase (MEK/ERK) or phosphatidylinosite 3′ kinase/protein kinase B (PI3-K/Akt) survival pathways (Leu *et al*, 2000; Levresse *et al*, 2000; Eilers *et al*, 2001).

Apoptosis and necrosis often coexist in CNS diseases (Ankarcrona *et al*, 1995). Alternatively, they occur in a temporal sequence or follow a certain spatial distribution (Ankarcrona *et al*, 1995; Charriaut-Marlangue *et al*, 1996; Leist and Nicotera, 1998). It has been suggested that apoptosis and necrosis are the extremes of a continuum of possible types of cell death, such that increasing the intensity of the insult (exposure time or concentration) can alter cell death from apoptotic to necrotic (Leist and Nicotera, 1998). Because HSV-1 infection induces apoptosis in non-neuronal cells (Wilson *et al*, 1997; Tropea *et al*, 1998; Aubert *et al*, 1999; Raftery *et al*, 1999), our studies asked whether (i) HSV-1 can also trigger apoptosis in hippocampal cultures, and (ii) HSE has an apoptotic component.

Results

HSV-1, but not HSV-2, induces apoptosis in hippocampal cultures

Two series of experiments were done in order to examine whether HSV-1 and/or HSV-2 induces apoptosis in hippocampal cultures. First, primary hippocampal cultures were mock infected or infected with 10 plaque-forming units (pfu) per cell of HSV-1 or HSV-2 for 0.5, 4, 8, 16, or 24 h and assayed by TdT-mediated dUTP nick-end labeling (TUNEL), an assay that is widely accepted as indicative of apoptosis (Gavrieli *et al*, 1992; Gold *et al*, 1994). In

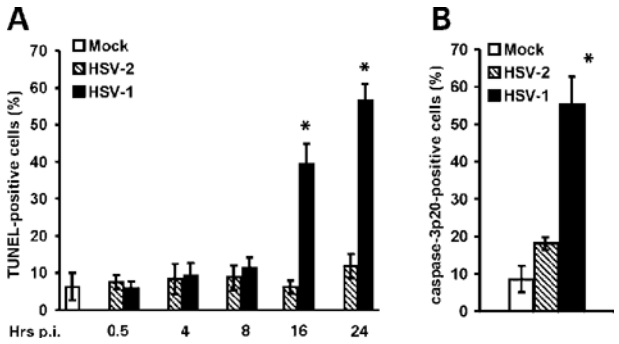

Figure 1 HSV-1, but not HSV-2, triggers apoptosis in hippocampal cultures. (**A**) Hippocampal cultures were mock-infected or infected with HSV-1 or HSV-2 (moi = 10) and analyzed by TUNEL at 0.5, 4, 8, 16, and 24 h p.i. Results represent the average of three independent experiments and are expressed as mean percent TUNEL$^+$ cells ≈ *SEM* ($^*P < .001$ by ANOVA). (**B**) Duplicates of mock-infected and 24-h infected hippocampal cultures in (**A**) were stained in immunohistochemistry with caspase3p20 antibody. Results represent the average of three independent experiments and are expressed as mean percent caspase3p20$^+$ cells ≈ *SEM* ($^*P < .01$ by ANOVA).

HSV-1–infected cultures, the percent of TUNEL$^+$ cells, estimated as described in the Materials and Methods section, increased as a function of time post infection (p.i.). It was similar to that in mock infected cultures (6.3% ≈ 3.8%) at 0.5, 4, and 8 h p.i. (5.9% ≈ 1.8%, 9.2% ≈ 3.5%, and 11.3% ≈ 2.9%, respectively), but much higher at 16 h p.i. (39.4% ≈ 5.5%) and reached maximal levels (56.4% ≈ 4.5%) at 24 h p.i. ($P < .001$ by analysis of variance [ANOVA] versus mock- and HSV-2–infected cultures [6.3% ≈ 1.7% and 11.8% ≈ 3.2% at 16 and 24 h p.i., respectively]) (Figure 1**A**). In the second series of experiments, duplicates of the mock or 24-h virus-infected cultures were assayed for caspase 3 activation by immunohistochemistry with antibody (D175) specific for the large cleavage fragment of caspase 3 (caspase 3p20) (Ouyang *et al*, 1999; Perkins *et al*, 2002a). The percent caspase3p20$^+$ cells, estimated as described in Materials and methods, was significantly higher for HSV-1 (55.4% ≈ 7.4%) than HSV-2 (18.1% ≈ 1.7%) or mock (8.6% ≈ 3.5%) infected cultures ($P < .01$ versus HSV-2– and mock-infected cultures by ANOVA) (Figure 1**B**), and normal rabbit serum was negative (data not shown). Collectively, the data indicate that HSV-1, but not HSV-2, triggers apoptosis in hippocampal cultures. Apoptosis was unrelated to virus replication, because HSV-1 and HSV-2 replicate equally well in these cells, with growth initiating at 3 h p.i. (0 h is at the end of adsorption) and maximal titers seen at 15 to 24 h p.i. (Figure 2).

TUNEL-positive HSV-1–infected hippocampal cells are neurons

To estimate the proportion of neurons in hippocampal cultures, duplicate cultures (uninfected or infected with HSV-1 or HSV-2 for 24 h) were assayed by TUNEL using immunohistochemistry with alkaline

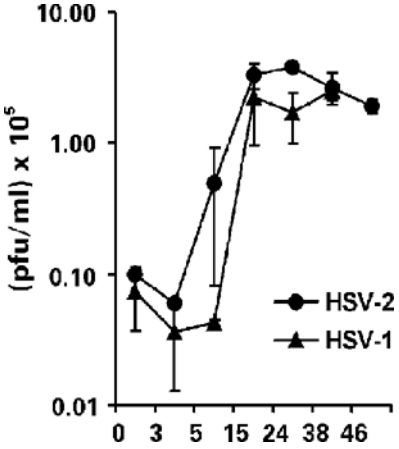

Figure 2 HSV-1 and HSV-2 replicate equally well in hippocampal cultures. Single-step growth assays were done in hippocampal cultures infected with HSV-1 or HSV-2 (10 pfu/cell). Results are expressed as mean pfu/ml ≈ *SEM*.

phosphatase (AP) and the percent positive cells estimated as described in Materials and methods or examined by double immunofluorescence with fluorescein isothiocyanate (FITC)-labeled dUTP (TUNEL) and phycoerythrin (PE)-labeled TuJ1 (specific for postmitotic neurons [Fereira and Caceres, 1992]). Antibodies to glial fibrillary acidic protein (GFAP) and galactosyl ceramide (GalC), which are respectively specific for astrocytes and oligodendrocytes, served as controls. Approximately 60% to 65% of the cells in the HSV-1–infected cultures were TUNEL$^+$ cells (Figure 3**C**) as compared to 2% to 6% of the cells in HSV-2 (Figure 3**B**) or uninfected (Figure 3**A**) cultures. Consistent with previous reports that the majority of cells in hippocampal cultures are neurons (Banker and Cowan, 1977; Perkins *et al*, 2002a), approximately 87% to 93% of the cells stained with TuJ1 antibody. GFAP staining was seen in 6% to 9% of the cells and GalC antibody was negative, as previously described for such cultures (Bertolinni *et al*, 1997). As shown in Figures 3**D–F**, for a microscopic field that contains a high proportion of TUNEL$^+$ cells (Figure 3**E**), these were TuJ1 positive (Figure 3**F**). Consistent with previous reports (Brazelton *et al*, 2000), TuJ1 staining (PE) localized in the cell bodies and projections (Figure 3**D**), whereas FITC staining (TUNEL) was primarily nuclear (Figure 3**E**, **F**). GFAP staining cells were TUNEL negative (data not shown). These findings indicate that TUNEL$^+$ cells in HSV-1–infected hippocampal cultures are neurons, and are consistent with previous reports for the apoptotic HSV-2 mutant ICP10ΔPK (Perkins *et al*, 2002a).

HSV-1 activates the JNK/c-Jun pathway in hippocampal cultures

Previous studies have shown that HSV-1 activates (phosphorylates) JNK in non-neuronal cells (McLean and Bachenheimer, 1999; Zachos *et al*, 1999). To

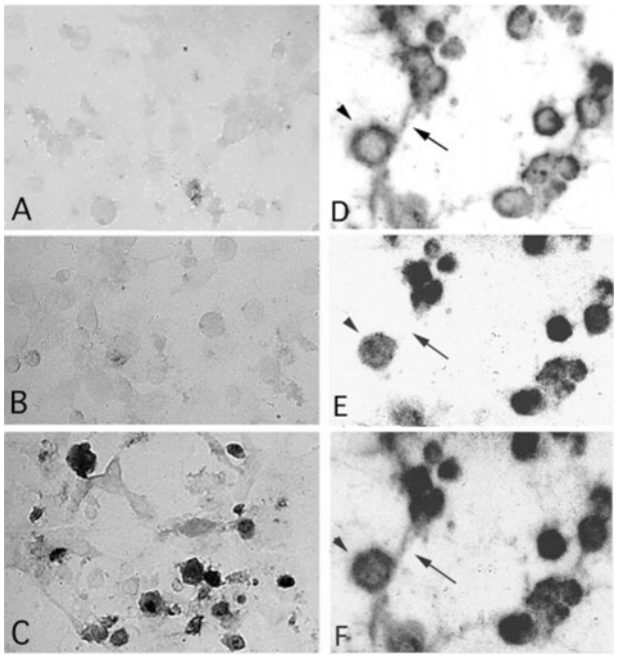

Figure 3 TUNEL$^+$ cells in HSV-1–infected hippocampal cultures are neurons. (**A–C**) Primary hippocampal cultures, uninfected (**A**) or infected with 10 pfu/cell of HSV-2 (**B**) or HSV-1 (**C**) were assayed for TUNEL$^+$ cells at 24 h p.i. using immunohistochemistry with alkaline phosphatase. (**D**) Microscopic field from slide of HSV-1–infected culture containing TUNEL$^+$ cells stained with PE-labeled TuJ1 antibody. Arrow indicates staining dendrites. Arrowhead indicates cytoplasmic TuJ1 localization. (**E**) FITC-dUTP staining of microscopic field shown in (**D**) indicates that TUNEL is intranuclear. Arrow and arrowhead indicate location of TuJ1 staining in panel (**E**). (**F**) TuJ1 and TUNEL colocalization.

examine whether JNK is also activated in virus-infected hippocampal cultures, extracts of cultures mock-infected or infected with 10 pfu/cell of HSV-1 or HSV-2 (used as control) were immunoblotted with antibody specific for activated (phosphorylated) JNK1/2/3 (P-JNK1/2/3) at 0.5 and 24 h p.i. Antibody to the nonphosphorylated JNK1/2/3 served as control. JNK1 was the only isotype expressed in mock-infected cultures, and it was not phosphorylated (Figure 4**A**, lane 1). As shown in Figure 4**A**, for 24-h infected cultures, HSV-2 induced low levels of JNK2/3 expression and JNK1/2 activation (Figure 4**A**, lane 2). By contrast, HSV-1 induced expression of the three JNK isotypes and caused intense activation (phosphorylation) of JNK1/2 (Figure 4**A**, lane 3). Similar results were obtained at 0.5 h p.i. (data not shown), indicating that JNK activation precedes apoptosis. The results are not an artefact due to improper gel loading or other technical difficulties, because actin levels were virtually identical in all cell extracts (Figure 4**A**).

c-Jun, a P-JNK target (Kaplan and Miller, 2000; Eilers *et al*, 2001), was also activated in HSV-1–infected cultures. Duplicates of the mock- or virus-infected cultures were immunoblotted with antibody to c-Jun phosphorylated on Ser^{63} [P-Jun(Ser63)] or

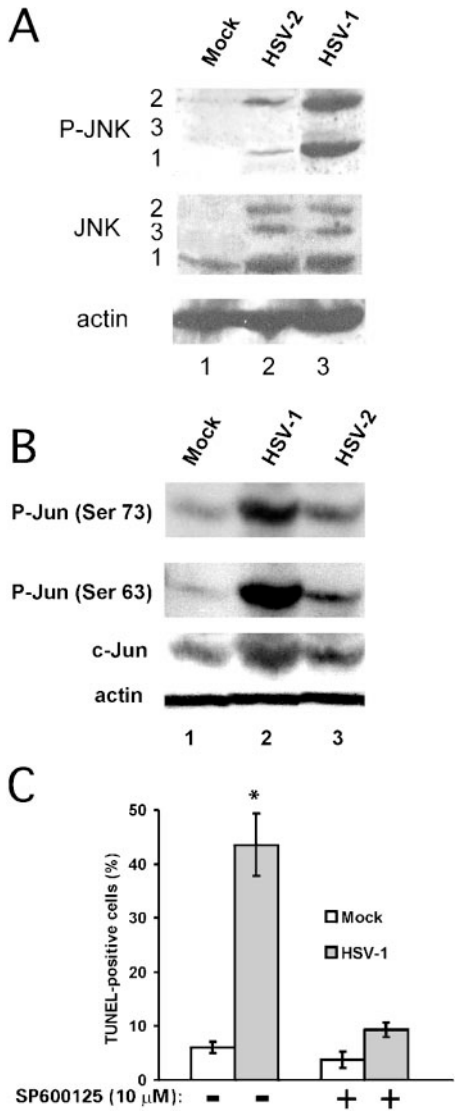

Figure 4 HSV-1–mediated apoptosis depends on JNK/c-Jun activation. (**A**) Hippocampal cultures were mock infected (lane 1) or infected with 10 pfu/cell of HSV-2 (lane 2) or HSV-1 (lane 3). Proteins in cell extracts obtained at 24 h p.i. were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with P-JNK1/2/3 antibody. The blot was stripped, reprobed with antibody specific for JNK1/2/3, restripped, and reprobed with actin antibody. Similar results were obtained for cell extracts obtained at 0.5 h p.i. (**B**) Duplicates of cultures mock infected (lane 1) or infected with HSV-1 (lane 2) or HSV-2 (lane 3) were immunoblotted with antibodies to P-Jun (Ser63), P-Jun (Ser73), and nonphosphorylated c-Jun or actin, as in (**A**). (**C**) Hippocampal cultures were mock-infected or infected with 10 pfu/cell of HSV-1 in the absence (≈) or presence (+) of 10 μM of the JNK inhibitor SP600125 and assayed by TUNEL at 24 h p.i. The results of three independent experiments are expressed as mean TUNEL$^+$ cells ≈ *SEM* ($^{\approx}P < .001$ by ANOVA).

Ser^{73} [P-Jun(Ser73)], both of which are phosphorylated by P-JNK (Minden and Karin, 1997). Antibody to nonphosphorylated c-Jun was studied in parallel and served as control. The levels of P-Jun(Ser63) and P-Jun(Ser73) were increased dramatically in HSV-1–(Figure 4**B**, lane 2) relative to mock- (Figure 4**B**, lane 1) infected cultures. Their increase in HSV-2–infected cultures (Figure 4**B**, lane 3) was significantly less robust. Consistent with previous findings that c-Jun is subject to positive autoregulation (Minden and Karin, 1997; Eilers *et al*, 2001), the levels of nonphosphorylated c-Jun were also increased in HSV-1–infected cultures (Figure 4**B**). The data indicate that HSV-1, but not HSV-2, induces robust activation of the JNK/c-Jun pathway in cultured hippocampal neurons.

The JNK inhibitor SP60025 blocks HSV-1–induced apoptosis in hippocampal cultures

To examine the relationship between HSV-1–induced JNK activation and apoptosis, we took advantage of previous finding that JNK is inhibited by the anthrapyrazolone SP600125 (Bennett *et al*, 2001; Han *et al*, 2001), a finding confirmed in our laboratory by the observation that SP600125 inhibits c-Jun activation in HSV-1–infected hippocampal cultures (Perkins *et al*, 2003). Specifically, hippocampal cultures were mock-infected or infected with 10 pfu/cell of HSV-1 in the absence or presence of 10 μM of SP600125 (Calbiochem, La Jolla, CA), and assayed by TUNEL at 24 h p.i. The percentage of TUNEL$^+$ cells was similar in SP600125 treated or untreated mock-infected cultures (3.8% ≈ 1.5% and 6% ≈ 1%, respectively), but it was significantly ($P <$ .001, by ANOVA) decreased in HSV-1–infected cultures treated with SP600125 (9.3% ≈ 1.4%) than in untreated HSV-1–infected cultures (43.6% ≈ 5.8%) (Figure 4**C**). We interpret the data to indicate that JNK activation is involved in HSV-1–induced apoptosis.

The failure of HSV-2 to trigger apoptosis is due to MEK/ERK activation

Having shown that HSV-2 does not induce apoptosis in hippocampal cultures, we wanted to know whether this is related to its ability to activate MEK/ERK (Smith *et al*, 2000). Hippocampal cultures were mock infected or infected with 10 pfu/cell of HSV-2 or HSV-1 (used as control) and immunoblotted with antibody to the phosphorylated (activated) ERK1/2 (P-ERK1/2). Relative to mock-infected cultures (Figure 5**A**, lane 1), HSV-2 infection caused a significant increase in the levels of P-ERK1/2 (Figure 5**A**, lane 3). We conclude that ERK activation was MEK dependent, because the levels of P-ERK1/2 were not increased in cultures infected with HSV-2 in the presence of 20 μM of the MEK-specific inhibitor U0126 (Favata *et al*, 1998) (Figure 5**A**, lane 2). P-ERK1/2 levels were not increased in cultures infected with HSV-1 in the absence (Figure 5**A**, lane 4) or presence (Figure 5**A**, lane) of U0126. The levels of actin were similar in all cultures (Figure 5**A**), indicating that the altered P-ERK1/2 levels are not due improper gel loading or technical artefacts.

To determine whether MEK/ERK activation is required for the antiapoptotic activity of HSV-2, hippocampal cultures were mock-infected or infected

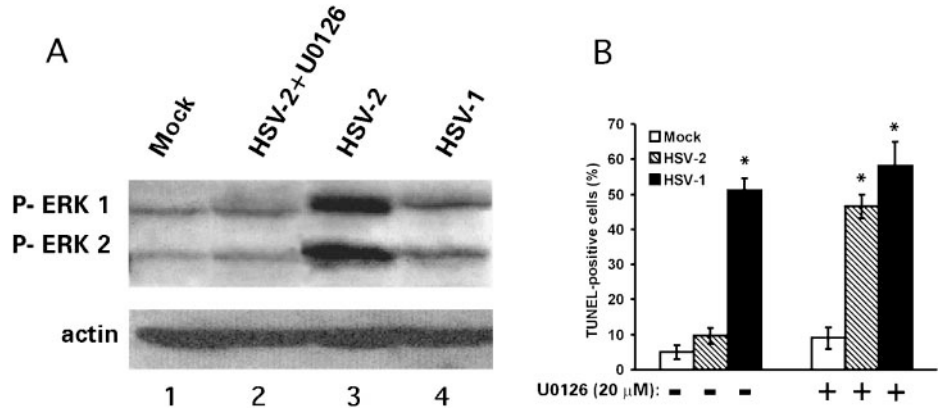

Figure 5 MEK/ERK activation is required for HSV-2 antiapoptotic activity. (**A**) Hippocampal cultures were mock-infected (lane 1) or infected with 10 pfu/cell of HSV-2 in the presence (lane 2) or absence (lane 3) of 20 μM of U0126, or with 10 pfu/cell of HSV-1 (lane 4) and immunoblotted with antibody to P-ERK1/2. They were striped and reprobed with actin antibody. (**B**) Hippocampal cultures were mock-infected or infected with HSV-2 or HSV-1 (moi = 10) in the absence (≈) or presence (+) of 20 μM of U0126 and assayed by TUNEL at 24 h p.i. The results of three independent experiments are expressed as mean TUNEL$^+$ cells ≈ *SEM* ($^{\approx}P < .001$ by ANOVA).

with HSV-2 (10 pfu/cell) in the absence or presence of 20 μM of U0126 and examined by TUNEL at 24 h p.i. The percent TUNEL$^+$ cells in HSV-2–infected cultures treated with U0126 (46.5% ≈ 3.4%) was significantly ($P < .001$ by ANOVA) higher than that in similarly infected, but untreated cultures (9.7% ≈ 2.2%). However, U0126 had no effect on TUNEL development in mock-infected cultures (5% ≈ 2% and 9% ≈ 3.1% TUNEL$^+$ cells in untreated and U0126 treated cultures, respectively) nor in cultures infected with HSV-1 (51.2% ≈ 3.3% and 58.1% ≈ 6.8% for untreated and U0126 treated, respectively) (Figure 5**B**). The data indicate that MEK/ERK activation is required for the antiapoptotic activity of HSV-2, but not for the survival of mock-infected hippocampal neurons, at least under these experimental conditions. This interpretation is consistent with previous reports that the basal maintenance of hippocampal neurons depends on the PI3-K/Akt pathway (Crowder and Freeman, 1998; Perkins *et al*, 2002a).

JNK and caspase 3 are activated and PARP is cleaved in HSE brains

Having shown that HSV-1 activates JNK and triggers apoptosis in cultured hippocampal neurons, we wanted to know whether these findings extend to HSE. Serial sections of HSE brains were obtained from eight adults diagnosed with HSE at the Armed Forces Institute of Pathology, Washington DC, USA, during 1975 to 1985. Diagnosis was based on clinical criteria (fever, lethargy, disorientation, and seizures) and temporal lobe involvement in the presence of a HSV-1 diagnosis confirmed by virus isolation, antigen detection, and/or immune electron microscopy. Brain tissues from two adult patients without neurological or psychiatric histories (normal brains) were also obtained as controls. Hematoxylin-eosin (H&E) staining in our department revealed varying degrees of viral encephalitis in the HSE (but not normal) brains that was characterized by perivascular lymphoid infiltrates, microglial nodules, and neuronophagia. Most HSE cases also demonstrated foci of hemorrhage/subacute hemorrhagic necrosis with a macrophage-rich inflammatory infiltrate and 5/8 cases had HSV-characteristic Cowdry A intranuclear inclusions in the setting of encephalitis. HSV infection was confirmed in our laboratory, with all the HSE brain tissues staining with a monoclonal antibody (MAb) specific for HSV-1. This is shown for one of these (patient 1828476) in Figure 6**A**. The tissues did not stain with a MAb specific for HSV-2 (as shown in Figure 6**B** for patient 1828476), and normal brain tissues were negative (data not shown).

Serial sections of the HSE brain tissues also stained with antibodies to P-JNK, activated caspase 3 (caspase3p20), and the 85-kDa cleaved fragment of PARP (p85PARP). Positive signals were seen for all the antibodies in the same 6/8 (75%) tissues. Staining with P-JNK1/2 (Figure 7**A**), caspase3p20 (Figure 8**A**, **B**), and p85PARP (Figure 9**A**) antibodies was localized in neurons, and in some tissues, also in glial cells and scattered macrophages. Staining was not seen in normal brain tissues (Figures 7**B**, 8**C**, 9**B**) and normal rabbit serum was negative (data not shown). Upon counterstaining with hematoxylin, neurons staining with caspase3p20 antibody were counted and results expressed as mean percent caspase-3p20$^+$ cells ≈ *SEM*. The percent caspase-3p20$^+$ cells in the six staining

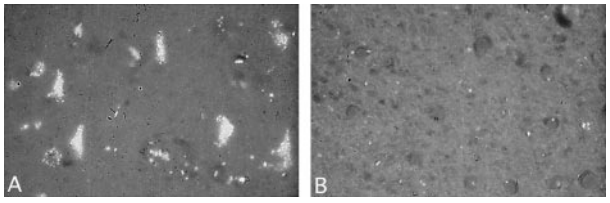

Figure 6 HSV-1, but not HSV-2, is associated with HSE. Immunofluorescent staining of HSE brain tissue (patient 1828476) with MAb specific HSV-1 (**A**) or HSV-2 (**B**). Similar results were obtained for all HSE brain tissues.

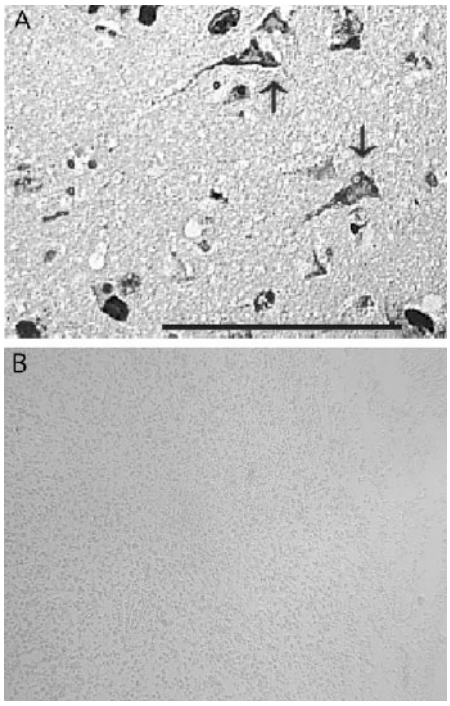

Figure 7 JNK is activated in HSE brain tissues. (**A**) Immunohistochemistry of brain tissue from HSE patient 1537066 with P-JNK antibody. Positive neurons are indicated by *arrows*. Similar results were obtained for 6/8 HSE brain tissues. (**B**) Immunohistochemistry of normal brain tissue with P-JNK antibody. Bar = 100 μm.

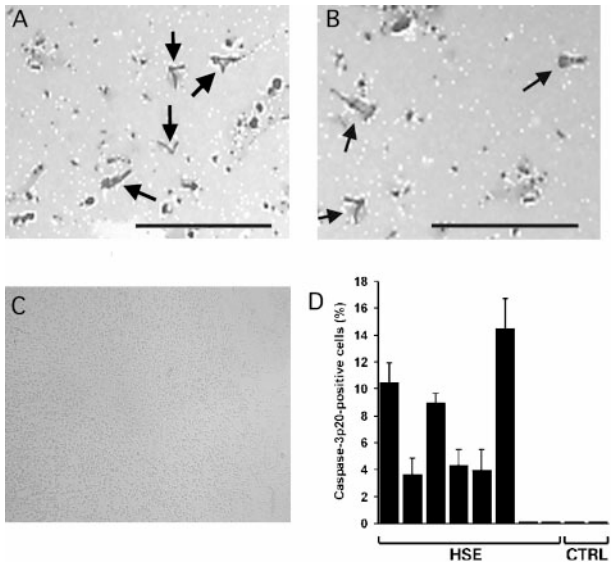

Figure 8 Caspase 3 activation in HSE brain tissues. Immunohistochemistry of brain tissues from HSE patients 1580213 (**A**) and 1671191 (**B**) stained with antibody specific for cleaved (active) caspase3 (caspase3p20). Similar results were obtained for the other four HSE brain tissues positive for P-JNK. Positive signal was detected in neurons (*arrows*) glial cells, and occasionally in cells of blood origin (macrophages). (**C**) Section of normal brain tissue stained with caspase3p20 antibody is negative. (**D**) HSE brain samples processed as in (**A**) were counterstained with hematoxylin. Cells were counted and the results expressed as mean percent caspase3p20$^+$ cells $\approx$ *SEM*. HSE tissue from patient 1537066 has the highest percentage of positive cells. Bar = 100 μm.

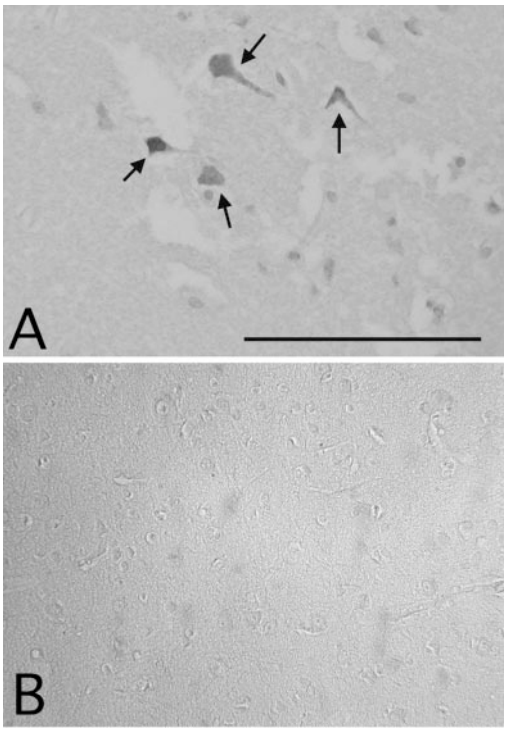

Figure 9 HSE is associated with PARP cleavage. Sections of brain tissue from HSE patient 1524648 (**A**) or normal brain (**B**) were stained with antibody specific for the cleaved PARP (p85PARP). Similar results were obtained for all six P-JNK–positive HSE brain tissues. Neurons are indicated by *arrows*. Bar = 100 μm.

HSE brains ranged between 4% (patient 1474368) and 14.5% (patient 1537066) (Figure 8**D**). Because serial sections were stained with the antibodies to HSV-1, P-JNK, caspase3p20, and p85PARP, it is possible to conclude that (i) apoptosis was localized in the area of HSV infection, and (ii) there was a good correlation between apoptosis and JNK activation. Cellular colocalization studies are ongoing.

Discussion

HSE is a severe focal disease that accounts for 10% to 20% of viral encephalitis cases among adults and older children in the United States. Most HSE cases are caused by HSV-1 (Skoldenberg, 1996; Whitley, 1997; Dennett *et al*, 1997; Kleinschmidt-Demasters and Gilden, 2001), with HSV-2 infection of the CNS commonly restricted to a self-limiting, nonfatal meningitis (Bergstrom *et al*, 1990, 1991; Sauerbrei *et al*, 2000). The mechanism responsible for the different outcome of CNS infection with the two HSV serotypes is still unknown. Cytopathological manifestations of HSE include lytic effects resulting from virus replication in neurons and glial cells (Graham and Lantos, 1997), and those caused by inflammatory cells. However, the severity of histopathological changes and neurological symptoms does not correlate well with the viral burden in the brain (Ando

et al, 1993; Studahl *et al*, 1998; Weil *et al*, 2002), suggesting that mechanisms other than virus-induced necrotic cell death are also involved in disease pathogenesis.

Apoptosis is a tightly regulated process that is necessary for the proper development of the nervous system. When inappropriate, either in timing or extent, apoptosis can trigger CNS disease or account for progression of neurodegeneration in various diseases, including stroke, trauma (Honig and Rosenberg, 2000), and virus infection (Hardwick, 1997). Apoptosis involves nuclear and cytoplasmic condensation, intranucleosomal DNA cleavage, and blebbing of the cell into membrane-bound apoptotic bodies. It is mediated by proteases (caspases) that are activated by the cleavage of zymogens (procaspases). Caspase 3 is a key executioner of apoptosis. It is activated by cleavage of the procaspase into a 17- to 20-kDa fragment that, in turn, cleaves proteins that are involved in DNA repair (*viz*. PARP). PARP cleavage into an inactive 85-kDa fragment (p85PARP) is a crucial event in the cell commitment to undergo apoptosis (Johnson-Webb *et al*, 1997). JNK, and its target c-Jun, play an important role in triggering neuronal apoptosis (Morishima *et al*, 2001; Xu *et al*, 2001; Shoji *et al*, 2001). JNK/c-Jun targets that could account for increased apoptosis are the Fas ligand (Morishima *et al*, 2001) and the death cytokine tumor necrosis factor (TNF)-μ (Ishizuka *et al*, 1997; Hoffmeyer *et al*, 1999), as well as transcription factors, such as p53 and c-Myc, that acquire proapoptotic functions when they are phosphorylated by JNK (Milne *et al*, 1995; Fuchs *et al*, 1998; Noguchi *et al*, 1999). Expression of proapoptotic members of the Bcl-2 protein family, such as Bax, is also increased by a JNK/p53-dependent mechanism (Kaplan and Miller, 2000). Accumulating evidence indicates that the MEK/ERK and PI3-K/Akt survival pathways can override apoptotic signals, including those generated by activated JNK/c-Jun (Leu *et al*, 2000; Levresse *et al*, 2000).

HSV-1 and HSV-2 have an overall DNA homology of 50%, with genome regions of very high (80% to 90%) homology and others with significantly lower ($\approx$40%) homology (Aurelian, 2000). One of the regions of limited homology is the first one third of the gene for the large subunit of ribonucleotide reductase (R1), respectively known as ICP10 and ICP6 for HSV-2 and HSV-1. At least in HSV-2 this minigene codes for a serotype-specific serine-threonine protein kinase (PK) (Chung *et al*, 1989; Cooper *et al*, 1995). The HSV-2 PK (ICP10 PK) activates the MEK/ERK survival pathway in non-neuronal and neuronal cells (Smith *et al*, 2000; Perkins *et al*, 2002a), and it inhibits caspase 3–mediated apoptosis in cultured CNS neurons (Perkins *et al*, 2002a, 2002b). By contrast, HSV-1 activates JNK (McLean and Bachenheimer, 1999; Zachos *et al*, 1999) and triggers apoptosis (Tropea *et al*, 1995; Wilson *et al*, 1997; Aubert *et al*, 1999; Raftery *et al*, 1999) in non-neuronal cells in culture, but the apoptotic viral gene is still unknown.

Our data indicate that HSV-1 triggers apoptosis also in hippocampal cultures, as determined by both TUNEL and caspase 3 activation. Apoptosis was not seen before 8 h p.i. and it reached maximal levels at 24 h p.i. Apoptotic cells were neurons, as evidenced by double immunofluorescent staining using FITC-labeled dUTP (TUNEL) and PE-labeled TUJ1 antibody, which is specific for a neuronal protein (Fereira and Caceres, 1992). Although 6% to 9% of the cells in these cultures stained with GFAP antibody, they were TUNEL negative, suggesting that astrocytes do not become apoptotic under the experimental conditions used in these studies. The JNK/c-Jun pathway was also activated in HSV-1–infected hippocampal cultures, as evidenced by a significant increase in the levels of P-JNK and P-Jun(Ser^{63})/P-Jun(Ser^{73}). P-JNK levels were increased as early as 0.5 h p.i. and remained equally elevated at 24 h p.i., indicating that the pathway is activated before apoptosis and remains activated throughout. We conclude that JNK activation is required for HSV-1–triggered apoptosis, because the percentage of $TUNEL^{+}$ cells in HSV-1, but not mock-infected, cultures was significantly decreased by treatment with SP600125, an inhibitor of JNK activation (Bennett *et al*, 2001; Han *et al*, 2001). However, the genes and programs of gene expression downstream of JNK and c-Jun that control apoptosis in HSV-1–infected hippocampal neurons, and the function of the neuron-specific JNK3 isotype (Minden and Karin, 1997), the expression of which is increased by both HSV-1 and HSV-2, are still unknown.

By contrast to HSV-1, HSV-2 does not trigger apoptosis in hippocampal neurons, although it induces low levels of JNK/c-Jun activation. We conclude that the ability of HSV-2 to block apoptosis is due to the activation of the MEK/ERK survival pathway, because it is known to counteract JNK activation (Leu *et al*, 2000; Levresse *et al*, 2000) and we have previously shown that the pathway is activated by the HSV-2 specific protein ICP10 PK (Smith *et al*, 2000; Perkins *et al*, 2002a, 2002b). Indeed, the levels of activated (phosphorylated) ERK1/2 (P-ERK1/2) were significantly higher in hippocampal cultures infected with HSV-2 than in mock-infected cultures, an increase that was not seen in cells infected with HSV-1. ERK activation was inhibited by U0126, which is a MEK-specific inhibitor (Favata *et al*, 1998), and U0126 treatment of HSV-2–infected cultures caused a significant increase in the proportion of apoptotic cells, suggesting that MEK/ERK activation is involved in the antiapoptotic activity of HSV-2. We believe that activation occurs upstream of caspase 3 and it involves the activation of Ras, because (i) Ras/MEK/ERK are activated in HSV-2–infected non-neuronal cells (Smith *et al*, 2000), and (ii) activation of Ras effector pathways protects neurons from apoptosis induced by various stimuli (Lin *et al*, 1995; Erhardt *et al*, 1999). The proportion of apoptotic cells in mock-infected cultures was not increased by U0126 treatment, consistent with our previous report

that the MEK/ERK pathway is not involved in the survival of uninfected neurons, the basal maintenance of which depends on the PI3-K/Akt pathway (Perkins *et al*, 2002a). Presumably, HSV-2 uncouples cell survival from PI3-K activity by activating the MEK/ERK survival pathway.

The correlation between HSV-1 infection, activation of JNK/c-Jun, and apoptosis extends to HSE brains. Staining with antibodies to P-JNK1/2/3, caspase3p20, or p85PARP was seen in most of the studied HSE brains (6/8 [75%]), and there was a strong correlation between the results obtained with the three antibodies and the presence of HSV infection at the site of apoptosis. Because apoptotic cells are rapidly cleared out *in vivo* (Johnson-Webb *et al*, 1997), the percentage of staining neurons (estimated for caspase3p20 antibody) is likely to represent a 'snapshot' in time rather than being indicative of the real extent of apoptosis in HSE brains. Ongoing immunofluorescence studies are designed to confirm that JNK activation and apoptosis (caspase3p20/p85PARP staining) colocalize with HSV-1 antigen.

Collectively, our data indicate that HSV-1 triggers apoptosis involving activation of the JNK/c-Jun pathway in hippocampal cultures and demonstrate the presence of apoptotic components in HSE brains. Although we cannot exclude the possibility that apoptosis is a secondary effect due to ischemia/hypoxia/increased intracranial pressure, it was seen at the sites of HSV infection, and the data are consistent with the contribution of both necrotic and apoptotic neuronal cell death to the pathogenesis of encephalitis caused by other viruses (Nargi-Aizenman and Griffin, 2001). The mechanism connecting JNK activation to apoptosis is still unclear and may involve death (e.g., TNF-μ) receptors and/or aberrant stimulation of glutamate receptors. Notwithstanding, the data suggest that HSE patients with severe neurological impairment in the presence of adequate antiviral therapy (Skoldenberg, 1996; Whitley, 1997) might benefit from combined antiviral and antiapoptotic therapies.

Materials and methods

Antibodies

The following antibodies were purchased and used according to the manufacturer's instructions: polyclonal antibodies to cleaved caspase-3 (D175, recognizes the activated [caspase3p20] species) (Cell Signaling Technology, Beverly, MA, USA); PARP p85 fragment (recognizes the 85-kDa caspase-cleaved fragment of PARP) (Promega, Madison, WI, USA); P-JNK1/2/3 (recognizes the dually phosphorylated forms of JNK1/2/3 and JNK1/2/3 [recognizes the nonphosphorylated JNK1/2/3]) (Promega); ERK (recognizes ERK1/2) (Oncogene, Cambridge, MA, USA); the dually phosphorylated (active) forms of ERK1/2 (P-ERK1/2) (Promega); and FITC-conjugated MAbs specific for HSV-1 or HSV-2 (HSV Typing Kit, Baxter Diagnostics, Isaquah, WA, USA). Antibodies to c-Jun phosphorylated on Ser^{63} [P-Jun(Ser63)] or Ser^{73} [P-Jun(Ser73) and nonphosphorylated c-Jun (part of the PhosphoPlus c-Jun [Ser63] II c-Jun [Ser73] Antibody Kit) were purchased from Cell Signaling Technology (Beverly, MA).

Cells and viruses

Primary cultures of cells dissociated from the hippocampi of 16- to 19-day-old rat fetuses (Sprague-Dawley) were prepared as described (Perkins *et al*, 2002a, 2002b). Cells were plated at a density of approximately 750,000/2 ml on collagen coated 35-mm dishes (Nunc, Rochester, NY) or glass coverslips precoated with poly-L-lysine (Sigma, St. Louis, MO, USA). Cultures were used when 6 days old. They consisted of 83% to 90% nondividing cells, determined with the 5-bromo-2′-deoxyuridine labeling and detection kit (Roche Molecular Biochemicals, Indianapolis, IN, USA), that were identified as neurons by immunohistochemistry with neuron-specific class III μ-tubulin (TUJ1) antibody. HSV-2 (G) and HSV-1 (F) were used as previously described (Smith *et al*, 2000). Cultures were infected with HSV-1 (strain F) or HSV-2 (strain G).

Single-step virus growth assays

Primary hippocampal cultures were infected with HSV-1 or HSV-2 at (moi) = 10 pfu/cell. Adsorption was for 1 h at 36.5℃. After adsorption, the virus inoculum was removed and the cultures were overlaid with growth medium and reincubated at 36.5℃ (0 h in growth curve). Cells and supernatants were harvested at 2 to 48 h after adsorption, frozen, and thawed and assayed for virus titers by plaque assay (Aurelian, 2000). Results are expressed as mean pfu/ml $\approx$ *SEM*.

Immunoblotting assay

Immunoblotting was done as previously described (Perkins *et al*, 2002a). Briefly, cells were lysed with RIPA buffer (20 mM Tris-HCl, pH 7.4, 0.15 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with phosphatase and protease inhibitors cocktails (Sigma) and sonicated for 30 s at 25% output power using a Sonicator/Ultrasonic Processor (Misonix, Farmingdale, NY, USA). Total protein was determined by the bicinchoninic assay (Pierce, Rockford, IL, USA) and proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The blots were incubated (1 h, 37℃) in TN-T buffer (0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween 20) containing 1% bovine serum albumin (BSA) to block nonspecific binding and exposed (overnight; 4℃) to the appropriate antibodies (diluted in TN-T buffer with 0.1% BSA). After three washes with TN-T

buffer, the blots were incubated with Protein A–Peroxidase for 1 h at room temperature. Detection was with ECL reagents (Amersham Life Science, Arlington Heights, IL, USA) and exposure to high performance chemiluminescence film (Hyperfilm ECL, Amersham). Quantitation was by densitometric scanning using the Bio-Rad GS-700 Imaging Densitometer.

Immunofluorescent and immunoperoxidase staining
Sections of paraffin-embedded brain tissues were deparafinized through a series of xylene-ethanol washes. For immunofluorescence, they were incubated (1 h; 37≈C) with FITC-conjugated HSV-1– or HSV-2–specific MAbs and counterstained with Evans blue according to the manufacturer's instructions. Stained cells were visualized with an epifluorescent confocal microscope fitted with an argon ion laser (Zeiss LSM 410) (Aurelian, 2000; Perkins *et al*, 2002a). The DAKO LSAB 2 Kit, HRP (DAKO) was used for immunoperoxidase staining. Cells and tissue sections were exposed overnight (4≈C) to primary antibodies and immunolabeled cells were detected using streptavidin-biotin method according to the manufacturer's instructions. Counterstaining was with Mayer's hematoxylin (Sigma) (Perkins *et al*, 2002a). At least 300 cells were counted and results are expressed as percent positive cells ≈ *SEM*.

TUNEL
The In Situ Cell Death Detection Kit-AP (Roche Molecular Biochemicals) was used according to the manufacturer's instructions. Briefly, cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (pH 7.4) for 1 h at room temperature, followed by permeabilization in 0.1% Triton X-100 (in 0.1% sodium citrate) for 2 min on ice. DNA breaks were labeled by addition of terminal deoxynucleotidyl transferase (TdT) and nucleotide mixture (containing FITC-conjugated dUTP) and incubation for 60 min at 37≈C. Coverslips were mounted in PBS/glycerol and cells were analyzed by fluorescence microscopy. After extensive washes in PBS, cells were incubated for 30 min at 37≈C with an anti-FITC antibody conjugated with AP. Chromogenic reaction was carried out by adding AP substrate solution (0.4 mg/ml nitroblue tetrazolium chloride [NBT] and 0.2 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt [BCIP] [Roche Molecular Biochemicals] in 0.1 M Tris-HCl, pH 9.5, 0.05 M $MgCl_2$, 0.1 M NaCl, and 1 mM levamisole) for 10 min at room temperature. Coverslips were mounted in PBS/glycerol and analyzed by light microscopy. Apoptotic cells (characterized by a dark nuclear precipitate) and nonapoptotic cells (unstained or displaying a diffuse, light, and uneven cytoplasmic staining) were counted in five randomly chosen microscopic fields (containing at least 250 cells). The percentage of apoptotic mock-infected cells was subtracted from each average. Results are expressed as percent apoptotic cells ≈ *SEM*.

Brain samples
Postmortem samples of brain tissues (temporal lobe and hippocampus) were obtained from eight adults diagnosed with HSE and two adult patients without neurological or psychiatric histories (normal brain) seen at the Armed Forces Institute of Pathology, Washington DC, USA, during 1975 to 1985. Tissues were fixed in 10% formalin (pH 7.6), embedded in paraffin, sectioned at 6 μm and mounted on glass slides. For routine histopathological examination, sections were stained with H&E.

Statistical analyses
One-way ANOVA with Tukey-Kramer post-test was done using GraphPad InStat version 3.01 for Windows 95/NT, GraphPad Software, San Diego, CA, USA.

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