Journal of NeuroVirology (1998) 4, 407–414
© 1998 Journal of NeuroVirology, Inc.
http://www.jneurovirol.com

Apoptosis induction in brain during the fixed strain of rabies virus infection correlates with onset and severity of illness

Sompone Theerasurakarn and Sukathida Ubol

Department of Microbiology, Faculty of Science, Mahidol University, RAMA VI Road, Bangkok 10400, Thailand

Viruses such as HIV, influenza, picornavirus and others are known stimulators of apoptosis. This individual cellular elimination is a preferential host defense in regenerative tissues. In contrast, if this death occurred in nonregenerating cells, such as neurons of the central nervous system, may result in disease. The target cell for rabies virus is the neuron. Here we studied the outcome of the interaction between rabies virus (CVS-11) and mouse brain cells. Replication of rabies virus in suckling mouse brain cells resulted in brain cell apoptosis, detected by DNA fragmentation and *in situ* apoptosis within 25 h after infection and before evidence of intracerebral immune activation. Cell death occurred simultaneously with rabies virus replication. There were clinical signs of illness in infected newborn mice within 24 h after the appearance of DNA fragmentation and before infiltration by lymphocytes. This suggested that onset of illness started independently of the immune function. This conclusion was supported by the occurrence of massive apoptosis followed by paralysis in rabies virus-infected immunosuppressed mice. Direct, viral-induced, neuronal apoptosis was the earliest death mechanism detected in these mice. We propose that pathogenesis of this fixed strain of rabies virus in mice begins with the induction of apoptosis by rabies virus replication. Cerebral damage may then be amplified by immunological mechanisms plus an additional unidentified factor. This is followed by increased permeability of the blood brain barrier.

Keywords: rabies virus; hydrophobia; rabies pathogenesis; apoptosis

Introduction

There are two major types of cell death: apoptosis and necrosis (Sanders and Wride, 1995). Apoptosis is an active process which occurs through an evolutionarily conserved program. Cells undergoing apoptosis are characterized by membrane blebbing, cellular shrinkage, chromatin condensation, and chromosomal DNA fragmentation into oligonucleosomes (Schwartzman and Cidlowski 1993; Jones *et al*, 1989). In the final stage, an apoptotic cell breaks down into apoptotic bodies (Wyllie 1981). This type of death involves activation of certain enzymes and the upregulation of specific genes. Apoptisis is considered an individual cell death since it does not induce damage to neighboring cells. In contrast to apoptosis, necrosis depends mainly on autolytic activities of intrinsic cellular enzymes. Death by necrosis induces damage to nearby cells.

Viruses have recently been shown to trigger apoptosis. Human immunodeficiency virus (HIV) was postulated to cause immune suppression by inducing programmed cell death of CD4+ lymphocytes (Laurent-Crawford *et al*, 1991). Influenza virus can trigger apoptosis in alveolar cells and bronchial/bronchiolar epithelial cells (Mori *et al*, 1995). Sindbis virus (SV) can induce neuronal cell death by apoptosis (Levine *et al*, 1993; Ubol *et al*, 1994). Apoptosis is considered a natural host defense mechanism, since it may play a role in decreasing the rate of viral spread from infected cells (Clem and Miller, 1993). However, after a long adaptation to host cells, viruses seem to be able to overcome this type of host defense. For example, a virus with a large genome, such as baculovirus, takes a certain amount of time to generate progeny. Therefore, baculovirus has acquired the p53 gene to block apoptosis and ensure complete viral progeny

Correspondence: S Ubol
Received 24 April 1997; revised 14 January 1998; accepted 9 March 1998

production (Clem and Miller, 1993, 1994). Viruses with a small genome will complete replication and release viral progeny before the death of host cells by apoptosis (Levine *et al*, 1993). Inefficient viral blockage and apoptotic induction of nonregenerating cells could be responsible for disease progression. Lewis *et al* (1996) have shown that the ability of SV to induce apoptosis in neurons correlates with mortality in mice.

The pathogenesis of rabies includes encephalitis and paralysis. This may be the consequence of the direct effect of the virus on infected neurons combined with activation of immune mediators. The direct role of rabies virus in host cell damage is unclear. There is no evidence of necrosis or neurophagia in the infected brains of immunosuppressed mice (Smith *et al*, 1982). However, direct damage by rabies virus on neural cells has been shown *in vitro*. Replication of rabies virus strain 1820B in embryonic chick myotubes induces morphological changes consistent with apoptosis, including myotube shrinkage, small surface blebs, and irregularities under phase microscopy (Baer and Lentz, 1991). Using advanced flow cytometry, Marcovistz *et al* (1994) were able to detect apoptosis outside the nervous system of rabies virus infected mice. This raises the possibility that a part of the pathogenesis of rabies infection may be due to induction of apoptosis in infected neurons. In this study a fixed strain of rabies virus, CVS-11, was selected since it is derived from street rabies virus and is believed to induce pathophysiologic changes similar to infection by street rabies virus. We studied changes in brain cells of suckling mice, immunocompetent and immunosuppressed adult mice during infection by CVS-11 in order to answer whether rabies virus infection can lead to apoptosis of brain cells and whether apoptotic induction in rabies virus infected brain cells correlates with the severity of infection.

Results

Replication of rabies virus in mouse neuroblastoma cells, an undifferentiated neuronal cell line, caused apoptosis within 48 h after infection as shown by DNA fragmentation in Figure 1A. This provided evidence that rabies virus could induce apoptosis in target cells. A similar experiment was performed in suckling mice to answer whether this phenomenon occurred in the central nervous system *in vivo*. As shown in Figure 1B, DNA laddering was first detected from infected brains at 25 h post infection, while sham inoculated mice did not show any signs of DNA fragmentation. This experiment was repeated three times with three sets of mice and the same results were obtained that apoptotis of infected brains could be detected as early as 25 h after infection. Apoptosis in rabies virus infected brain was confirmed by the *in situ* TUNEL assay. As shown in Figure 2B and C, infected brains at 24 h and 72 h post infection exhibited abundant signals of nuclear TUNEL staining while TUNEL assays on brains of control mice were uniformly negative (Figure 2A). These results showed that neuronal apoptosis could be triggered during rabies virus infection.

Apoptosis occurred *in vitro* as a direct effect of rabies virus on target cells. However, in infected suckling mice, this phenomenon might be due to many factors such as immune mediators. CD_8^+-lymphocytes and certain cytokines like TNf have been shown to induce apoptosis in target cells (Kagi *et al*, 1994; Lowin *et al*, 1994; Walsh *et al*, 1994; Ware *et al*, 1996). Upregulation of inflammatory cytokine genes in infected brain was studied, since these soluble molecules may be responsible for progression to programmed cell death. By using semiquantitative RT–PCR, upregulation of mRNA, for IL-1β and TNFα was detected at 48 h after infection while the expression of IFNγ mRNA was increased by 36 h (Figure 3A and B). This, intracerebral upregulation of apoptotic related cytokine genes occurred after DNA laddering.

To determine any effect of the cellular immune response on apoptotic induction in rabies virus infected mice, the correlation between lymphocyte infiltration and appearance of DNA laddering was monitored. As showed in Table 1, infected brain cells underwent apoptosis within 25 h after infec-

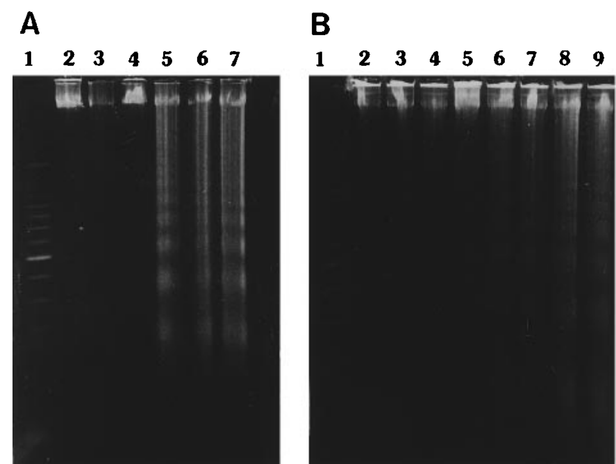

Figure 1 (**A**) Detection of DNA laddering of rabies virus-infected mouse neuroblastoma cells (harvested at 24, 48, 60 and 72 h after infection; lanes 4, 5, 6 and 7 respectively). The neuroblastoma cells were infected with rabies virus at an MOI of 1. Lanes 2 and 3 represented an uninfected culture harvested at 48 and 72 h after culture initiation. Lane 1 is a molecular weight marker. (**B**) DNA fragmentation analysis of rabies virus-infected suckling mouse brains at 12, 18, 25, 48, 60 and 72 h after infection (lanes 4, 5, 6, 7, 8 and 9) and sham inoculated mouse brain at 12 and 72 h (lanes 2 and 3). Lane 1 is a molecular weight marker.

tion. The histopathological examination of an extravascular lymphocyte infiltration of rabies virus-infected brains at 18, 25 and 48 h after infection were not observed. This data revealed that apoptotic of brain cells was not driven by infiltrated lymphocytes. Moreover mice clearly showed signs of illness within 24 h after brain cells entered programmed cell death and before the appearance of lymphocyte infiltration. These results suggested that neuronal damages due to apoptotic death of brain cells initiated disease development which was independent to the activities of immunological mediators.

Information obtained from study in rabies virus-infected suckling mice suggested that apoptosis is an earliest detected death of brain cells and is independent of immune mediators. To disprove the role of the immune responses as a apoptotic inducer and as a primary cause of rabies, development of rabies was studied in rabies virus-infected, immunosuppressed, adult mice in comparison to immunocompetent mice. Infected adult mice had paralysis by day 7. Immunosuppressed mice developed sign of illness in the presence of an undetectable level of an antibody and cellular responses to rabies which were detected by RIFFIT and lymphocyte stimulation respectively (data not shown). By contrast, massive apoptosis was found in their brains (Figure 4A and C). The intensity of the apoptotic signal was not different between infected immunosuppressed and immunocompetent mice as shown in Figure 4A–D.

The ability of rabies virus to activate apoptosis in nerve cells directly was studied by determining the correlation between amount of DNA fragmentation and growth of rabies virus in infected brains. The kinetics of rabies virus replication in infected brains was studied by Fluorescent Foci staining. The

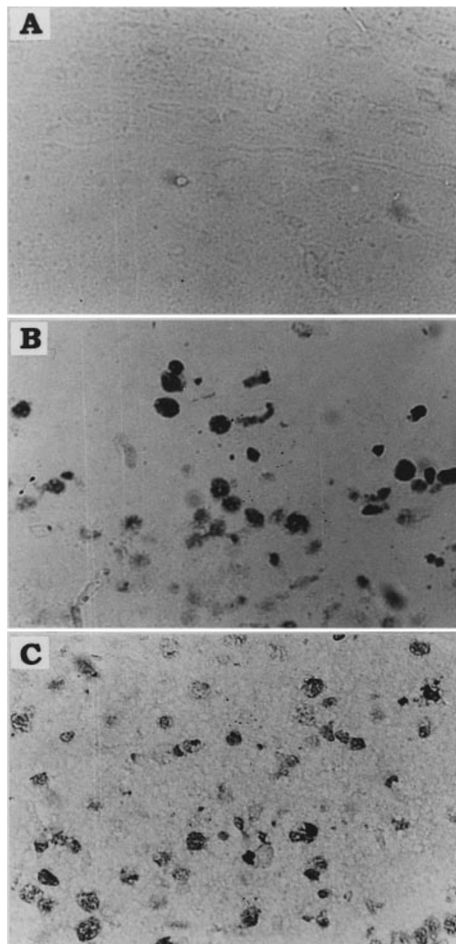

Figure 2 *In situ* detection (TUNEL assay) of apoptotic cells in suckling mouse brains infected with CVS-11 at 24 and 72 h post infection (**B** and **C**). The TUNEL assay on brain tissues from sham inoculated mice is negative control (**A**). Original manification ×200.

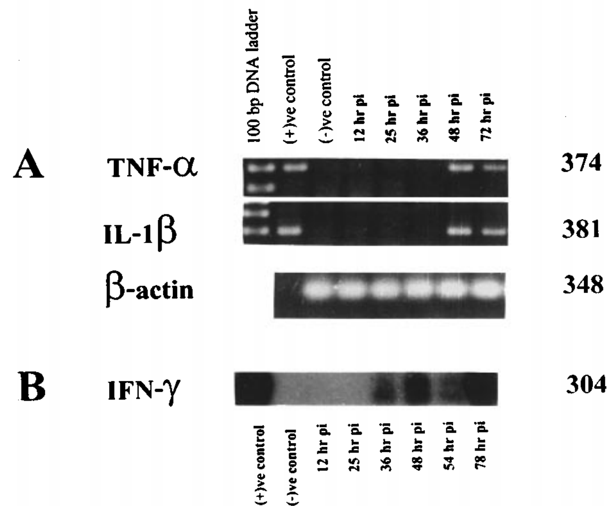

Figure 3 Detection of mRNA coding for inflammatory cytokines by semiquantitative RT–PCR. The mRNA for IL-1β and TNFα (A) and IFNγ (B) were reverse transcribed, amplified, and analysed by electrophoresis. For IFNγ, the amplified products were hybridized with ^{32}P-IFNγ probe. The amount of cDNA used at each time point was standardized to the level of the β-actin gene.

Table 1 Correlation between apoptosis induction, lymphocyte infiltration, and severity of infection in CVS-11-infected suckling mice

Hours post infection	*DNA fragmentation*	*Lymphocyte infiltration*[a]	*Severity of infection*[b]
18	No	No	–
25	Yes	No	–
48	Yes	No	+/++
60	Yes	ND	+++
72	Yes	ND	++++

[a]Lymphocyte infiltration was determined by the presence or absence of perivascular cuffing. [b]Severity of infection was graded as follows: –=absence of clinical signs; +/++=severe illness; ++++=moribund. ND=not done

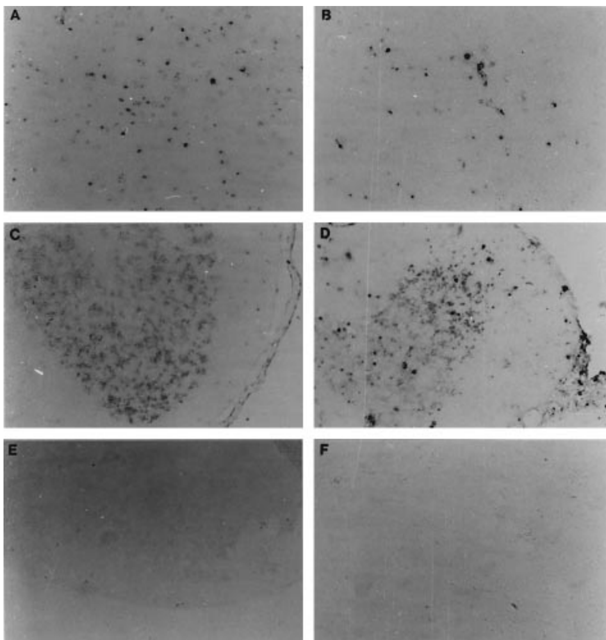

Figure 4 TUNEL assay in immunosuppressed and immunocompetent mouse brains infected with CVS-11 at day 7 after infection. Apoptotic cells are abundant in cerebellum and cerebrum of immunosuppressed (**A** and **C**) and immunocompetent mice (**B** and **D**). **E** and **F** are negative controls of cerebellum and cerebrum respectively. Original magnification ×100.

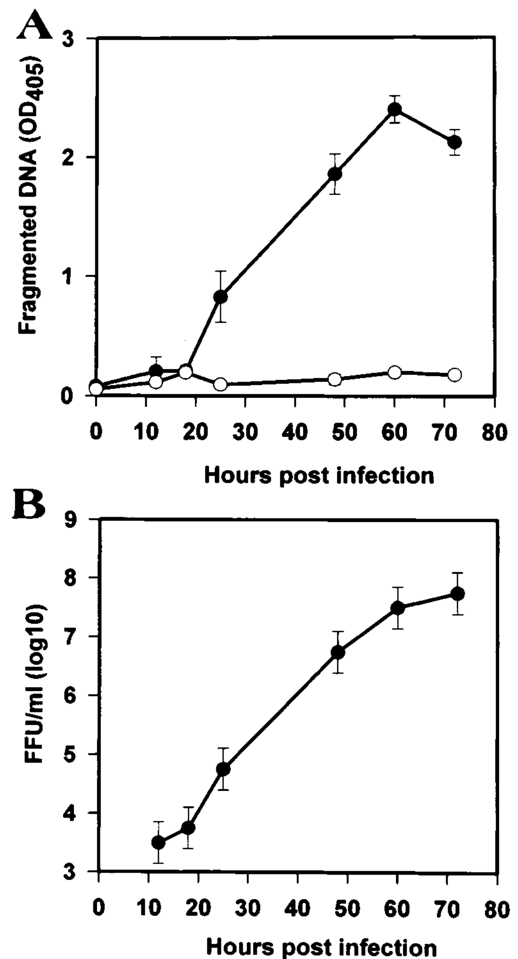

Figure 5 (**A**) Quantitation of fragmented DNA of rabies virus–infected suckling mouse brains at 12, 18, 25, 48, 60 and 72 h after infection by photometric-enzyme immunoassay (●-●). DNA from sham inoculated mouse brains were used as a negative control (○-○). The amount of fragmented DNA was expressed as $(OD_{405})_{brain\ cells} - (OD_{405})_{blank}$. (**B**) Kinetics of rabies virus replication in suckling mouse brain. Rabies virus infected brains were harvested and the amount of virus was detected by fluorescent staining of BHK-21 cells.

amount of fragmented DNA was quantitated by ELISA assay. As shown in Figure 5A and B, amount of DNA fragmentation was increased in parallel to production of viral progenies in infected brain. DNA from sham inoculated mice showed no fragmentation. Suggesting that replication of rabies viruses is required for an activation of DNA degradation process.

Discussion

This study demonstrated for the first time that a fixed strain of rabies virus triggered apoptosis in newborn mice and in immunosuppressed and immunocompetent adult mice brain cells. This was detected by DNA laddering and by *in situ* apoptotic staining. In newborn mice, induction of apoptosis occurred before lymphocyte infiltration and activation of cytokine production in infected brains. This finding indicated that immune components were not necessary for the progression of neural cells to programmed cell death after rabies virus infection. Indeed, the appearance of apoptosis and the increasing amount of fragmented DNA correlated with viral replication in the brain (Figures 1 and 5). Suggesting that rabies virus repliction is important in causing apoptotic death of infected brain cells. Our data was supported by information reported by Jackson and Rossiter (1997) in which rabies virus-infected neurons were cells that underwent apoptosis. Moreover, their report also demonstrated a good correlation between the distributions of apoptotic nuclei and rabies virus antigens. A similar phenomenon was found in Sindbis virus-induced apoptosis in neuroblastoma cells in which flow cytometry indicated no evidence of apoptosis until viral replication had reached the stage of new virion formation (Ubol *et al*, 1996).

This study also demonstrated that signs of illness in infected suckling mice developed after brain cells entered programmed cell death and before activation of immune responses. This data suggested that the onset of acute encephalitis in

newborn mice during rabies virus infection may be dependent on the ability of the virus to directly induce brain cell apoptosis. Moreover, incresing of severity of illness correlated to the increasing amount of fragmented DNA (Figure 5 and Table 1). Our findings are similar to reports for Sindbis virus (SV) infection. SV, an alphavirus, causes age-dependent encephalitis. Young mice are more susceptible to SV infection than older mice (Johnson *et al*, 1972). It has been demonstrated that the age affect on susceptiblity is not due to an inadequate or immature immune response in the newborn mice but rather due to the increased susceptibility of immature neurons to apoptosis induction (Levine *et al*, 1993; Wesselingh *et al*, 1994; Griffin, 1995). Experimentally induced over expression of antiapoptotic gene, bcl-2, inhibits apoptosis and protects newborn mice from fatal infection by SV (Levine *et al*, 1996). This supports the hypothesis that acute encephalitis in SV-infected newborn mice starts with apoptotic death of infected neurons which is triggered during SV replication. In rabies, age-dependent illness is not significant, both newborn and adult mice die of rabies. However, newborn mice die faster. Our data showed that apoptosis is not only a major mechanism of brain death in newborn mice, but that it can also be triggered in immunosuppressed and immunocompetent adult mice.

Rabies in adult mice and humans manifests as two clinical forms: paralysis and encephalitis. Immunosuppressed mice and athymic mice develop encephalitic illness. This encephalitis occurs concomitantly with increased viral replication and destruction of neurons in the absence of detectable immune mediators (Iwasaki *et al*, 1977; Smith *et al*, 1982). Therefore, encephalitic rabies in adult mice seems to be a direct effect of the rabies virus. Paralytic rabies is found in immunocompetent mice only. Passive transfer of immune sera and activated lymphocytes to immunosuppressed mice results in paralysis and early death (Smith *et al*, 1982; Sugamata *et al*, 1992).

Information from the mouse model contradicts what has been reported in human rabies. Hemachudha *et al* (1988, 1989) have found a defect in activation of the immune process in human paralytic rabies patients, while encephalitic patients have an active immune response to rabies. This raises questions whether the immune responses is significant in rabies pathogenesis and whether the pathogenesis processes differ in mice and man. A study in immunosuppressed adult mice showed that illness can be developed due to apoptosis of brain cells progressing to paralysis in the absence of a T and B cell response. This suggested that factors such as the strain of rabies virus and individual imbalances of neuronal function and other unidentified factor may determine clinical manifestation.

Our study led to the hypothesis that pathogenesis in mice infected with this fixed strain of rabies virus may be divided into two phases. The first phase is the result of interaction between rabies virus and host cells. Rabies virus disseminates from the primary infection site, enters and replicates in the CNS leading to apoptosis of brain cells. Pathogenesis begins as a consequence of viral induced neuronal death. Infiltration of immunological components through the blood–brain barrier and activation of intrinsic local immune responses may play a dual function. Immune responses may limit viral spread. However, these immunological factors may also amplify the severity of brain death. Such an immunological attack on brain cells, including damage from other unidentified mechanisms may represent a second phase of illness. Since this study was performed by using a laboratory adapted strain of rabies virus whether it reflects situation in natural rabies virus infection is unknown.

Materials and methods

Cell cultures

Mouse neuroblastoma cells (N18) were obtained from The Johns Hopkins University. Both N18 cells and BHK-21 cells were cultured in DMEM supplemented with 10% FBS, pennicillin, and streptomycin.

Virus

A fixed strain of rabies virus (CVS-11) from The Queen Soavabha Memorial Institute was used in this study. Rabies viruses in brain suspension were propagated in N18 cells. Supernatant fluid of infected cultures was clarified by centrifugation at 2000 r.p.m., at 4°C for 5 min and stored in aliquots at −70°C. Stock viruses were assayed by Fluorescent Foci staining on BHK-21 cells and had titers of approximately 10^7 p.f.u./ml.

Animal infection

Litters of 1-day-old Swiss albino mice were inoculated intracerebrally with diluted rabies virus (0.02 ml of 10^5 p.f.u./ml). Mice received diluent only were used as a negative control. The infected mice were chloroform euthanized and brains were harvested at various times after infection. These harvested brains were used as samples for apoptotic detection, upregulation of proinflammatory cytokine genes, and lymphocyte infiltration studies. Because of a small size of 1-day-old mouse brain, each of the above experiments was performed on different mice within the same litter.

Detection of apoptosis in rabies virus infected brains and infected mouse neuroblastoma cells

Infected brains, one mouse brain at each time point, were homogenized in lysis buffer containing 100 mM Tris-HCl pH 8.0, 0.5% SDS and 25 mM

EDTA. Homogenates were then treated with proteinase K (0.1 mg/ml) overnight at 56°C. Total DNA was extracted with phenol-chloroform and precipitated with absolute ethanol. RNA was eliminated from DNA by RNase digestion (0.1 mg/ml) for 1 h at 37°C. Extracted DNA was analysed by 1.8% agarose gel electrophoresis. DNA from cells undergoing apoptosis give a characteristic DNA laddering of 180–200 bp fragments.

Detection of chromosomal cleavage in rabies virus infected mouse neuroblastoma cells was performed by a similar procedure. The infected cells were harvested at various times as indicated and were lysed in lysis buffer. The lysates were treated and processed as described.

Detection of apoptosis in immunosuppressed mice
Four-week-old male and female adult mice were immunosuppressed by intraperitoneal injection with three doses of Cytoxan (3 mg/mouse, Mead Johnson), on day 1, 4 and 7 after virus inoculation. Brains were harvested at various times and fixed with 4% paraformaldehyde for 48 h and embedded in paraffin and sectioned. Sections were used for apoptosis detection by the TUNEL assay.

Lymphocyte infiltration study
Rabies-virus-infected suckling mice were chloroform euthanized without perfusion. Brains were removed, paraformaldehyde fixed for 48 h, embedded in paraffin and sectioned. Sequential sections were stained by hematoxylin and eosin. Extravascular infiltration of lymphocytes was evaluated by the presence of perivascular cuffing observed using light microscopy. Five constitutive sections from fore brain, mid brain, and hind brain were examined. Twenty fields were examined for each section.

Detection of upregulation of proinflammatory cytokine mRNA by RT–PCR
Infected suckling mouse brains were homogenized in RNase-free PBS. Total RNA was isolated from homogenized brains using TRIZOL (GIBCO) as recommended by the manufacturer. The portion of extracted RNA was subjected to first strand cDNA synthesis as described by Wesselingh *et al* (1994). In brief, 20 μg of extracted RNA was used in a 20 μl reaction mixture for cDNA synthesis containing 2.5 units of AMV reverse transcriptase, reverse transcriptase specific buffer as supplied by the manufacturer, 1 mM of each dNTP, 20 μg/ml oligo dT and 1 unit of RNase inhibitor. The first strand cDNA was synthesized at 42°C for 1 h. After completion of synthesis, the reaction was diluted to 100 μl with distilled water and was used for each PCR reaction.

The PCR reaction (50 μl) contained 200 μM of each dNTP, 2.5 units Taq polymerase (Perkin-Elmer), buffer as supplied by the manufacturer, 1 μM of each specific primer (Wesselingh *et al*, 1994). A Gene Amp PCR system 2400 thermal cycle (Perkin-Elmer, Cetus Corp) was used. The PCR conditions were optimized for each set of primers and the products were analysed by gel electrophoresis. The purified cDNA from Sindbis virus infected mouse brains (kindly provided by Diane Griffin, Johns Hopkins University) and the cDNA from PHA-stimulated mouse spleen cells were used as positive controls. The β-actin gene was used as an internal control.

For detection of IFNγ mRNA, the amplified products were hybridized with the ^{32}P-IFNγ probe as described by Wesselingh *et al* (1994). Specifically, 20 μl of PCR product was electrophoresed through 1.8% agarose gel and transferred onto a membrane (Nytran-plus, S&S). Filters were prehybridized in hybridizing buffer containing 5 × SSC, 0.1% blocking reagent (Boehringer Mannheim), 0.1% N-laurylsarkosine and 0.02% SDS at 50°C for 2 h. Hybridization was performed in the same buffer at 60°C overnight. An oligonucleotide probe specific to IFNγ (Wesselingh *et al*, 1994) was radiolabelled with ^{32}P-ATP using T4 kinase. After hybridization, filters were washed three times in 2 × SSC and 0.1% SDS at 60°C and finally in 0.1 × SSC and 0.1% SDS at the same temperature before autoradiography.

Virus titration
Viruses from infected brains were determined by fluorescent staining of foci of infected BHK-21 cells. Briefly, infected brains were harvested, homogenized and centrifuged at 2000 r.p.m. for 5 min at 4°C. Then 50 μl of tenfold dilutions of the supernatant were inoculated onto BHK-21 cells in a 96 well plate. After overnight incubation, infected cultures were washed, fixed with cold 70% acetone, and stained with fluorescein-conjugated anti-rabies antibody (Becton Dickinson Company, USA). The number of fluorescent positive cells in each well at each dilution were counted. The viral titer was expressed as number of fluorescent foci per milliliter.

TUNEL assay
The TUNEL (termianl deoxynucleotidyltransferase-mediated dUTP nick end labeling) staining was used to detect apoptotic nuclei by the addition of fluorescein-labeled dUTP to the endonucleolytically cleaved DNA ends by terminal transferase. It was performed as recommended by the manufacturer (Boehringer Mannheim). Briefly, sections of infected-paraffinized brains were deparaffinized, rehydrated, and 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 50 μl of a TUNEL reaction mixture containing, deoxynucleotidyl transferase, nucleotide mixture (including fluorescein-labeled dUTP), and reaction buffer for 60 min

at 37°C. The reaction was terminated by washing samples twice in PBS. Then, 50 μl of anti-fluorescein antibody conjugated with alkaline phosphatase was added to the treated tissue section and incubated for 60 min at 37°C. The sections were then washed twice with PBS and color was developed by addition of 4-nitroblue tetrazolium chloride and X-phosphate as substrates.

Quantitation of fragmented DNA in rabies virus infected mouse brain by ELISA assay
The amount of fragmented DNA was quantitated by photometric enzyme-immunoassay (Cell Death Detection ELISA plus, Boehringer Mannheim) as recommended by the manufacturer. Briefly, 250 mg of fresh frozen infected brains were homogenized in lysis buffer supplied by the manufacturer. Cytosolic fractions were prepared from homogenates by centrifugation at 200 *g* for 10 min. Supernatant was used as an antigen in ELISA detection. 20 μl of supernatant was added into streptavidin coated microtitier plate. 80 μl of the immunogent (anti-histone biotin and anti-DNA-POD) was added to each well and incubated for 2 h at room temperature on shaker. The unbound immunoagents were removed by washing. The amount of fixed fragmented DNA was qualified by the POD retained in the fixed immunocomplex. POD was determined photometrically with ABTS (2,2′-Azino-di[3-ethylbenzhiazolin-sulfonate]) as substrate. The color was measured at 405 nm against substrate solution as a blank.

Acknowledgements

We thank Dr Henry Wilde (The Queen Soavabha Memorial Institute) for helpful discussions and critical reading of this manuscript. We also thank Dr Witaya Thamavit for microscopic observations of brain sections and Dr TW Flegel for editing the manuscript. This work was supported by The Thailand Research Fund.

References

Baer GM, Lentz TL (1991). Rabies pathogenesis to the central nervous system. In *The Natural History of Rabies.* Baer GM (ed), 2nd edition. CRC Press: Boston, pp 105–118.

Clem RJ, Miller LK (1994). Control of programmed cell death by the baculovirus gene. *Mol Cell Biol* **14:** 5212–5222.

Clem RJ, Miller LK (1993). Apoptosis reduces both the *in vivo* infection of a baculovirus. *J Virol* **67:** 3730–3738.

Griffin DE (1995). The Gordon Wilson Lecture: Unique interactions between viruses, neurons and the immune system. *Trans Amer Clin & Climato Assoc.* **107:** 89–98.

Hemachudha T (1989). Rabies. In *Handbook of Clinical Neurology.* Vinken PJ, Bruyn GW, Klawans HL (eds) Elsevier Science: Amsterdam, pp. 383–404.

Hemachudha T, Phanuphak P, Sriwanthana B (1988). Immunologic study of human encephalitic and paralytic rabies. A preliminary of 16 patients. *Am J Med* **84:** 673–677.

Iwasaki Y, Gerland W, Clark HF (1997). Role of host immune response in the development of either encephalitic or paralytic disease after experimental rabies infection in mice. *Infect Immun* **18:** 220–225.

Jackson AC, Rossiter JP (1997). Apoptosis plays an important role in experimental rabies virus infection. *J Virol* **71:** 5603–5607.

Johnson RT, McFarland HF, Levy SE (1972). Age-dependent resistance to viral encephalitis: Studies of infection due to resistance to Sindbis virus in mice. *J Infect Dis* **125:** 257–262.

Jones DP, McConkey DJ, Micotera P, Orrenius S (1989). Calcium-activated DNA fragmentation in rat liver nuclei. *J Biol Chem* **264:** 6398–6403.

Kagi D, Vignaux F, Ledermann B, Burki K, Depraetere V, Nagata S, Hengartuer H, Golstein P (1994). Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* **265:** 528–530.

Laurent-Crawford AG, Krust B, Muller S, Riviere Y, Rey-Cuille MA, Bechet JM, Montagnier L, Hovanessian AG (1991). The cytopathic effect of HIV is associated with apoptosis. *Virology* **185:** 829–839.

Levine B, Goldman JE, Jiang HH, Griffin DE, Hardwick JM (1996). Bcl-2 protects mice against fatal alphavirus encephalitis. *Proc Natl Acad Sci USA* **93:** 4810–4815.

Levine B, Huang Q, Isaacs JT, Reed JC, Griffin DE, Hardwick JM (1993). Conversion of lytic to persistent alphavirus infection by the bcl-2 cellular oncogene. *Nature* **361:** 739–742.

Levine B, Huang Q, Isaacs JT, Reed JC, Griffin DE, Hardwick JM (1993) Conversion of lytic to persistent alphavirus infection by the bcl-2 cellular oncogene. *Nature* **361:** 739–742.

Lewis J, Wesselingh SL, Griffin DE, Hardwick JM (1996). Alphavirus-induced apoptosis in mouse brains correlates with neurovirulence. *J Virol* **70:** 1828–1835.

Lowin B, Hahne M, Mattmann C, Tschopp J (1994). Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature* **370:** 650–652.

Marcovistz R, Bertho AL, Matos DCS (1994). Relationship between apoptosis and thymocyte depletion in rabies-infected mice. *Brazilian J Med Biol Res* **27:** 1599–1603.

Mori I, Komatsu T, Takeuchi K, Kazuya N, Sudo M, Kimura Y (1995). *In vivo* induction of apoptosis by influenza virus. *J Gen Virol* **76:** 2869–2873.

Sanders EJ, Wride MA (1995). Programmed cell death in development. *Inter Rev Cytol* **163:** 105–153.

Schwartzman RA, Cidlowski JA (1993). Apoptosis: The biochemistry and molecular biology of programmed cell death. *Endocrine Reviews* **14:** 133–151.

Smith JS, McClelland CL, Ried FL, Bear GM (1982). Dual role of the immune response in street rabies virus infection of mice. *Infect Immun* **35:** 213–221.

Sugamata M, Miyazawa M, Mori S, Spangrude GJ, Ewalt LC, Lodmell DL (1992). Paralysis of street rabies virus-infected mice is dependent on T lymphocytes. *J Virol* **66:** 1252–1260.

Ubol S, Park S, Budihardjo I, Desnoyers S, Montrose MH, Poirier GG, Kaufmann SH, Griffin DE (1996). Temporal changes in chromatin, intracellular calcium and poly (ADP-ribose) polymerase during Sindbis virus-induced apoptosis of neuroblastoma cells. *J Virol* **70:** 2215–2220.

Ubol S, Tucker PC, Griffin DE, Hardwick JM (1994). Neurovirulent strains of alphavirus induced apoptosis in bcl-2-expressing cells; role of a single amino acid change in the E2 glycoprotein. *Proc Natl Acad Sci USA* **91:** 5202–5206.

Walsh CM, Matloubian M, Lin C-C, Ueda R, Kurahara CG, Christensen JL, Huang MTF, Young JDE, Ahmed R, Clark WR (1994). Immune function in mice lacking the perforin gene. *Proc Natl Acad Sci USA* **91:** 10854–10858.

Ware CF, VanArsdale S, VanArsdale TL (1996). Apoptosis mediated by the TNF related cytokine and receptor families. *J Cell Biochem* **60:** 47–55.

Wesselingh SL, Levine B, Fox RJ, Choi S, Griffin DE (1994). Intracerebral cytokine mRNA expression during fatal and nonfatal Alphavirus encephalitis suggests a predominant type 2 T cell response. *J Immunol* **152:** 1289–1297.

Wyllie AH (1981). Cell death: a new classification separating apoptosis from necrosis. In: *Cell death in biology and pathology,* Bowen ID, Lockshin RA (eds). Chapman & Hall: London, pp 9–34.