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Avian Encephalomyelitis Virus Induces Apoptosis Via Major Structural Protein VP3

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Avian encephalomyelitis virus (AEV) strain L_2Z was investigated for its apoptotic activity in specific-pathogen-free chick embryo brain tissue. DNA fragmentation analysis and electron microscopy observation demonstrated that AEV could induce apoptosis in chick embryo brain tissues characterized by chromatin condensation, plasma membrane blebbing, cell shrinkage, and nucleosomal DNA fragmentation after 4 days postinfection. AEV structural protein genes VP1, VP2, and VP3 were transfected into Cos-7 and chick embryo brain (CEB) cells, respectively. The results showed that only VP3 protein was an apoptotic inducer, as demonstrated by DNA fragmentation analysis and TUNEL assay at 24 and 48 h posttransfection. Furthermore, expression of VP3 protein resulted in the activation of caspase-3-like proteases in both cells, which could be inhibited by a caspase-3-like protease-specific inhibitor Ac-DEVD-CHO peptide, suggesting that AEV VP3 protein induces apoptosis through a caspase-3-like protease pathway. In addition, VP3 protein localized to mitochondria in the Cos-7 and CEB cells at 24 h posttransfection observed by confocal microscopy, indicating that mitochondria may play an important role in VP3-induced apoptosis. Taken together, our results show that AEV could induce apoptosis in chick embryo brain tissue, structural protein VP3 could serve as an apoptotic inducer resulting in apoptosis in cell culture through a caspase-3-like protease pathway, which may be related to its localization to mitochondria.

Key Words: avian encephalomyelitis virus (AEV) and VP3 protein; chick embryo brain tissues; apoptosis; DEVDase activity; mitochondria; Cos-7 cells; chick embryo brain (CEB) cells.

INTRODUCTION

Apoptosis, programmed cell death, is a highly conserved, tightly controlled self-destruction process which leads cells to commit suicide in response to a variety of stimuli. It is an active process characterized by particular morphological and biochemical features, including chromatin condensation, plasma membrane blebbing, cell shrinkage, and nucleosomal DNA fragmentation into membrane-bound bodies (Arends and Wellie, 1991). Many viruses induce apoptosis as part of their natural life cycle (O'Brien, 1998; Roulston *et al.,* 1999). Apoptosis may be an important host defense mechanism for eliminating infected cells during viral infection because it induces both the inflammatory response and exposure to the host immune system and degradative enzymes (Roulston *et al.,* 1999). The process of apoptosis is controlled through the expression of a large number of genes, many of which have been identified from a variety of viruses (Roulston *et al.,* 1999; Teodoro and Branton, 1997).

Avian encephalomyelitis virus (AEV), a member of the family Picornaviridae, contains a single-stranded RNA genome of positive polarity and is pathogenic to young chickens, pheasants, quails, and turkeys (Calnek *et al.,* 1997). The virus can result in reduced hatching and infected chicks show characteristic tremors and/or ataxia between 1 and 7 days of age. It has been now shown that the genome with the full length of 7058 bp has a single open reading frame (ORF) encoding a large polyprotein which is proteolytically processed by viral proteases to generate mature proteins (Marvil *et al.,* 1999). Like other picornaviruses, AEV includes also four structural proteins from the P1 region (VP4-VP2-VP3-VP1) and seven nonstructural proteins from the P2 and P3 regions (2A–2C and 3A–3D) of the genome (Marvil *et al.,* 1999). VP4 and VP2 are derived from precursor protein VP0. In poliovirus, the P1 capsid-encoding region has been identified to be related to the attenuation of virulence, virion thermostability, altered host range, *in vitro* cell tropism, persistent infection, and plaque morphology (Muir *et al.,* 1998). Nonstructural proteins in P2 and P3 regions may be associated with polyprotein processing, cleavage and maturing, and viral replication (Muir *et al.,* 1998). However, whether the functions of structural and nonstructural proteins of AEV are similar to that of poliovirus is not clear.

Among the picornaviruses, poliovirus has been shown to induce apoptosis *in vivo* or *in vitro* cell culture (Agol *et al.,* 1998; Ammendolia *et al.,* 1999; Girard *et al.,* 1999; Lopez-Guerrero *et al.,* 2000; Tolskaya *et al.,* 1995). Coxsackievirus B3 causes apoptosis in HeLa cells that is inhibited by zVAD-fmk (a pan-caspase inhibitor) (Carthy

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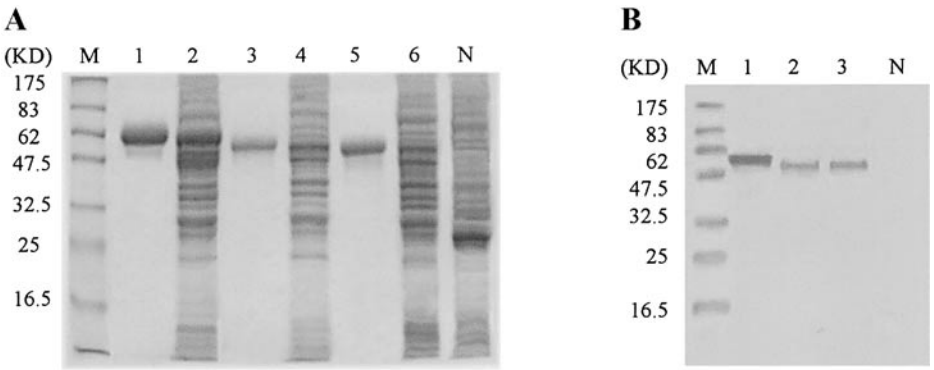


FIG. 1. (A) Expression and purification of AEV VP1, VP0, and VP3 in the prokaryotic expression system. Total cell lysates induced by the corresponding recombinant pGEX4T-3 expressing VP1, VP0, and VP3 on lanes 2, 4, and 6 and purified recombinant proteins VP1, VP0, and VP3 on lanes 1, 3, and 5 were electrophoresed in a 12% SDS–PAGE and stained with Coomassie blue, respectively. (B) Immunoblotting of AEV VP1, VP0, and VP3. The purified recombinant proteins VP1, VP0, and VP3 (lanes 1, 2, and 3) were transferred to nitrocellulose and incubated with chicken serum against AEV. M (protein marker) indicates the molecular mass in kilodaltons. Total cell lysates (N) induced by pGEX4T-3 vector alone was indicated as a negative control.

et al., 1998). Theiler's murine encephalomyelitis virus (TMEV) (Jelachich *et al.,* 1996) and hepatitis A virus (Brack *et al.,* 1996) induce apoptosis that correlates with virulence and therefore may determine whether the infection is persistent or acute. Whether other viruses in this family could cause apoptosis is still unclear. Recently we isolated a field AEV strain from a broiler flock with immune failure in China and documented that it is a virulent strain (Liu *et al.,* 1998). Here, we demonstrated the ability of AEV to induce apoptosis in chick embryo brain tissue after infection, the structural protein VP3 alone can induce apoptosis *in vitro* in Cos-7 and CEB cells. To further confirm the apoptotic pathway induced by VP3 of AEV, the caspase-3-like protease (DEVDase) activity in VP3 transfected Cos-7 and CEB cells was assayed. In addition, study of the subcellular localization of VP3 in the transfected cells was also carried out.

RESULTS

Molecular cloning and sequencing of VP0, VP1, and VP3 of AEV

A field virulent strain of AEV (L_2Z) was isolated during a severe outbreak of AE in northern China (Liu *et al.,* 1998). Several structural proteins of strain L_2Z and their coding sequences, including VP0 (VP4+VP2), VP1, and VP3, were cloned into the *Escherichia coli* vector pGEX4T-3. For each structural gene, four independent clones were completely sequenced. The consensus sequence derived from these four clones was considered the authentic sequence, which have been deposited into the GenBank at accession numbers AF458480 for VP0, AF458481 for VP1, and AF458482 for VP3. No insert or deletion was found in these nucleotide sequences when compared with strain 1143 (Marvil *et al.,* 1999). VP0, VP1, and VP3 of L_2Z shared 95.3, 99.8, and 98.1% nucleotide sequence identity and 97.3, 99.3, and 97.1% amino acid sequence homology, respectively, with the strain 1143 (data not shown).

Expression and characterization of AEV VP0, VP1, and VP3 on *Escherichia coli*

The VP0, VP1, and VP3 genes of the strain L_2Z were expressed in *E. coli* using vector pGEX4T-3 under the control of the *taq* promoter. Each recombinant protein, which fused with a 26-kDa glutathione-*S*-transferase (GST) protein at the N-terminal, was larger than 26 kDa compared to their authentic ones. The recombinant proteins of VP0, VP1, and VP3 were shown to have the respective sizes of 51, 56, and 52 kDa on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 1A). To confirm the viral specificity of the recombinant proteins, Western blotting was performed using the chicken anti-AEV strain L_2Z antiserum (Liu *et al.,* 1998). Consistent with the results from Coomassie blue staining, the same protein bands were detected by the strain L_2Z antiserum on a Western blot (Fig. 1B), indicating their viral specificity. In addition, no signals were detected in the vector control sample when reacted with the antiserum on a Western blot (Fig. 1B).

Induction of apoptosis by AEV

To investigate whether apoptotic cell death was triggered by AEV, the virus-infected chick embryo brain tissues were examined for formation of a DNA ladder. Cellular DNA of L_2Z-infected tissues was extracted at 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 days postinfection (p.i.). The fragmentation of DNA, a characteristic feature of apoptosis, was first observed at 4 days p.i. as a faint, but clearly discernible DNA ladder. As shown in Fig. 2, the intensity of the DNA bands increased considerably thereafter. No DNA fragmentation was detected in mock-infected chick embryo brain tissue. Thus, AEV infection caused the

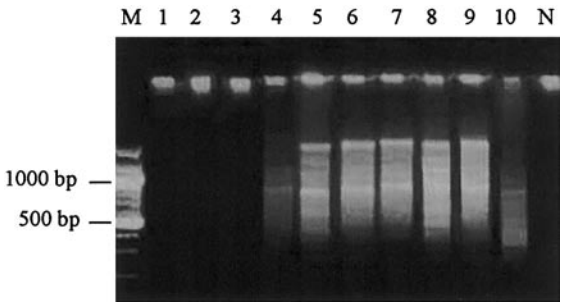


FIG. 2. Time course study of AEV-induced DNA fragmentation in chick embryo brain tissues. At the indicated times after inoculation with AEV strain L_2Z, DNA was isolated from virus- or mock-infected brain tissues and analyzed on 2.0% agarose gel with ethidium bromide. Lane M contains molecular size markers. Lanes 1–10 indicate DNA extracts that were isolated from virus-infected brain tissues at 1–10 days after inoculation, respectively. N indicates DNA that extract was isolated from mock-infected brain tissue at 8 days after inoculation.

cleavage of cellular DNA in the chick embryo brain tissue.

The replication of AEV strain L_2Z *in vivo* resulted in a typical morphological change consistent with apoptosis as illustrated by electron microscopy. Various specific morphological ultrastructure alternations were found in the CNS of infected chick embryos but not in that of mock-infected chick embryos (Fig. 3). The neurons showed shrinkage, membrane blebbing, and nuclear distortion. The chromatin was condensed and collapsed into crescents at the periphery of the nucleus. At later stages of infection, vacuolation and disorganization of the cytoplasm were also observed. The nucleus appeared more indented, and discrete nuclear fragments were sometimes present. Finally, the nucleus became fragmented, although the cell membrane was still present with a marked blebbing.

Identification of VP3 protein as an apoptotic inducer

To discern which protein among the major structural proteins of AEV caused apoptosis, the transient expression of viral structural proteins VP1, VP2, and VP3 in Cos-7 and CEB cells was performed. The recombinant plasmids pcDNA-VP1, pcDNA-VP2, and pcDNA-VP3 were individually transfected and the expression of each protein was demonstrated by fluorescein isothiocyanate (FITC) staining (Figs. 4I and 5) and further confirmed by immunoblotting analysis (Fig. 4II) using guinea pig anti-VP1, VP0, or VP3 polyclonal antibodies, respectively. In pcDNA-LacZ vector-transfected cells, pooled guinea pig anti-VP1, VP2, and VP3 antibodies failed to detect any virus-specific proteins by fluorescrence microscopy (Figs. 4I and 5) or by immunoblotting assay (Fig. 4II). At 24 and 48 h posttransfection, the cellular DNAs were extracted and analyzed by 2.0% agarose gel electrophoresis. As shown in Fig. 6, a distinct laddering effect, indicative of nucleosomal fragmentation, was detected in DNA samples obtained from Cos-7 and CEB cells transfected with pcDNA-VP3. In contrast, no significant DNA degradation occurred in the samples obtained from cells pcDNA-VP1, pcDNA-VP2 (data not shown), or pcDNA-LacZ vector-only-transfected cells (Fig. 6). Apoptosis was induced by transient expression of the VP3 protein as it was further confirmed at the cellular level by TUNEL labeling (Fig. 7). A large number of TUNEL-positive cells (green signal) were detected in pcDNA-VP3-transfected cells at 24 and 48 h posttransfection. However, no TUNEL-positive cells were found in pcDNA-VP1, pcDNA-VP2 (Fig. 7), or pcDNA-LacZ vector-only-transfected cells (Fig. 7).

VP3 protein induces activation of caspase-3-like proteases in Cos-7 and CEB cells

The central effector machinery of apoptosis is composed of cytoplasmic proteases called caspases. To determine whether expression of VP3 protein induced the activation of caspase-3-like proteases, both Cos-7 and CEB cells were transfected with pcDNA-LacZ or pcDNA-VP3 vectors, and lysates of transfected cells were assayed for DEVDase activity. VP3 protein induced an activation of caspase-3-like proteases in both cells. DEVDase activity in lysates from Cos-7 and CEB cells transfected with VP3 was 5.7 and 6.3 nmol/h, respectively (Fig. 8). There was no significant difference in DEVDase activity between Cos-7 and CEB cells expressing VP3 protein. In addition, when VP3-transfected cells were treated with Ac-DEVD-CHO, a peptide inhibitor of DEVDase activity, reduction in VP3 protein-induced apoptosis in both cells was observed.

Localization of VP3 protein to mitochondria of Cos-7 and CEB cells

To examine whether VP3 protein localized to mitochondria, the pcDNA-VP3-transfected Cos-7 and CEB cells were stained with MitoTracker (a mitochondria marker) and an anti-VP3 polyclonal antibodies. Double-label confocal microscopy was performed to explore the subcellular localization of VP3 in more detail. In addition, CEB cells infected with AEV strain L_2Z were also stained for localization of VP3 protein. Cos-7 cells transfected with a VP3 expression vector were stained with an anti-VP3 polyclonal antibody (Fig. 9A) and a MitoTracker dye (Fig. 9B) at 24 h posttransfection, respectively. Overlaying two images indicated that VP3 colocalized with mitochondria (Fig. 9C). Similar results were obtained with transiently transfected CEB cells, as shown in Fig. 9D for VP3 localization and Fig. 9E for mitochondria staining at 24 h posttransfection, respectively. Merged pictures of D and E also showed colocalization of VP3 to mitochondria (Fig. 9F). CEB cells infected with AEV were examined for VP3 localization (Fig. 9G) and mitochondria staining (Fig. 9H) at 24 h postinfection. VP3 protein was also colocal-

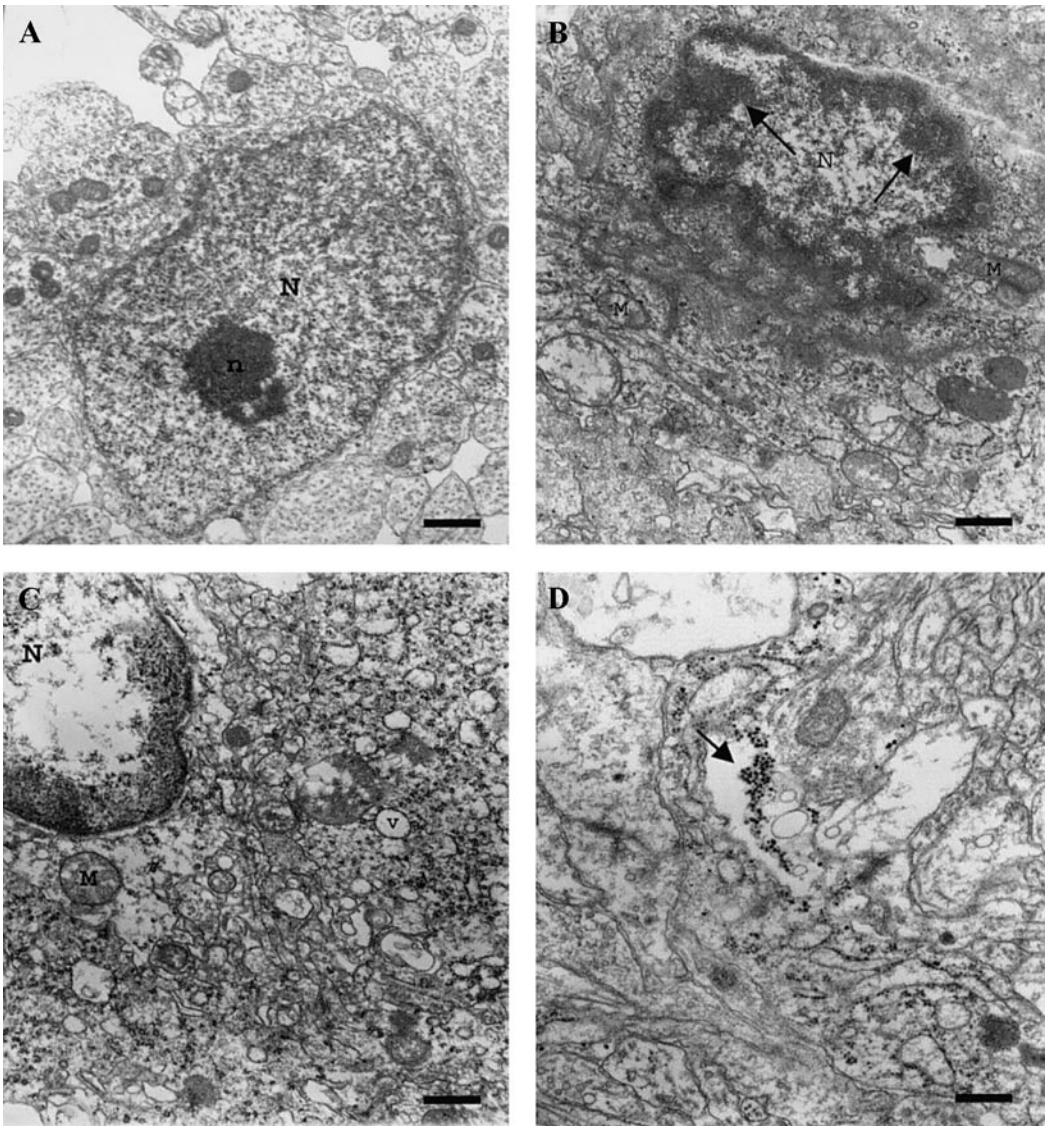


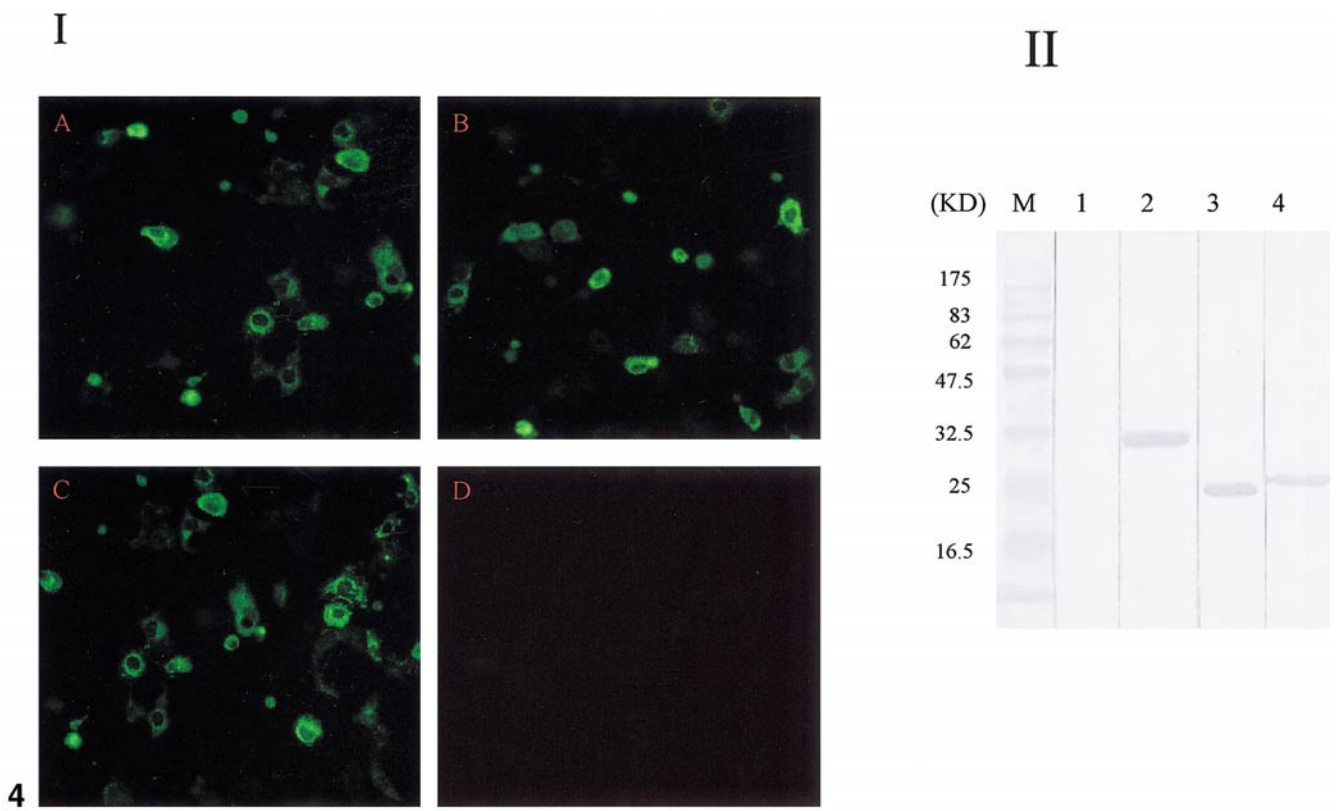
FIG. 3. Electron micrographs of neurons in the CNS of chick embryo brain after infection with AEV strain L_2Z. (A) Chick embryo at 8 days after mock infection; (B–D) chicken embryo at 8 days after infection with AEV. In A, neutron displays a round nucleus (N) with a single electron-dense nucleolus (n). In B, the following specific morphological alterations consistent with apoptosis were observed: aggregates of chromatin (arrows) around the nuclear membrane and cytoplasmic condensation with severely distorted organelles, such as swelling of mitochondria (M). In C, translucent cytoplasmic vacuoles were detected (v). In D, 20–30 nm viral particles (arrow) were scattered or aggregated in the cytoplasm. Bars, 1 μm (A–C) and 500 nm (D).

ized to mitochondria similar to that seen in transfected cells. These results indicated that when VP3 protein was synthesized during both virus infection and transfection, it showed a similar distribution in both Cos-7 and CEB cells.

DISCUSSION

A variety of animal viruses have been shown to induce apoptosis in living organisms and/or cultured cells, which plays an important role in the life cycle of these viruses (Roulston *et al.,* 1999; Shen and Shenk, 1995; Teodoro and Branton, 1997). These viruses have evolved genes that encode proteins that can effectively suppress or delay apoptosis in cells in order to produce sufficient quantities of viral progeny to initiate new rounds of infection (Roulston *et al.,* 1999; Teodoro and Branton, 1997). Apoptosis often occurs at late stages of viral infection, which may represent an important step in the spread of progeny to neighboring cells while evading the host immune system (Teodoro and Branton, 1997). In addition, apoptosis was also considered to be functional by eliminating aberrant cells created by DNA damage or those infected by viral pathogens (Roulston *et al.,* 1999).

In this study, we used a China field isolate of AEV strain L_2Z, which is considered to be a virulent strain (Liu *et al.,* 1998). After inoculation with this strain into 7-day-



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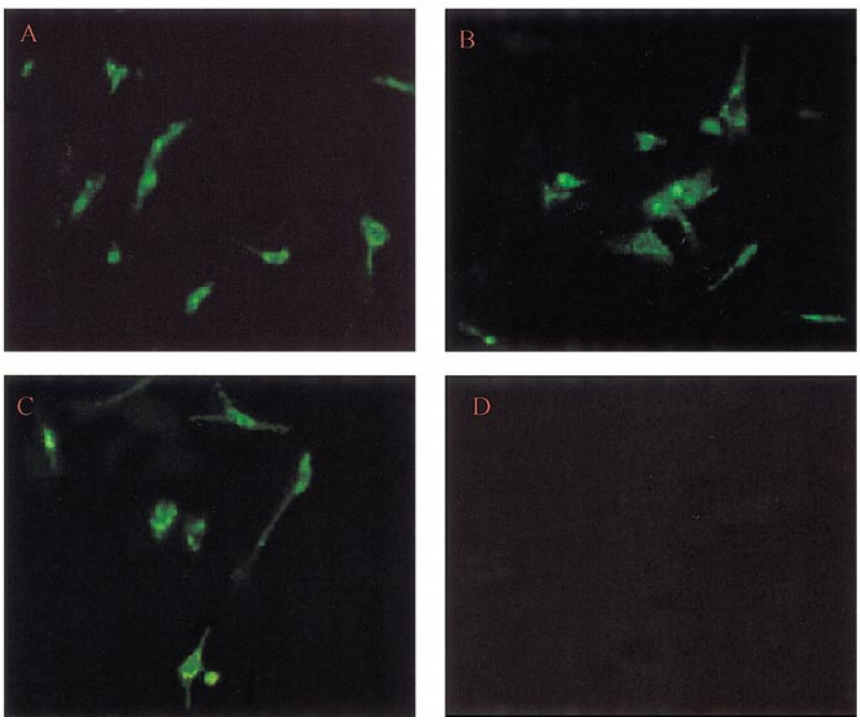

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FIG. 4. Detection of the VP1, VP2, or VP3 expression in transfected Cos-7 cells by IFA staining and immunoblotting at 24 h posttransfection. (I) A, B, C, and D indicate IFA staining of Cos-7 cells after transfection with pcDNA-VP1, pcDNA-VP2, pcDNA-VP3, and pcDNA-LacZ, respectively. The cells were visualized and photographed using fluorescence microscopy. Magnification, ×200. (II) Total cell lysates of transfected Cos-7 cells from above (I) were electrophoresed; transferred to nitrocellulose; and incubated with anti-VP1, -VP0, and -VP3 polyclonal antibodies. Lanes 1, 2, 3, and 4 are Cos-7 cells transfected with pcDNA-LacZ vector, pcDNA-VP1, pcDNA-VP2, and pcDNA-VP3, respectively. M (protein marker) indicates the molecular mass in kilodaltons.

FIG. 5. Detection of the VP1, VP2, or VP3 expression in transfected CEB cells by IFA staining at 24 h posttransfection. A, B, C, and D indicate IFA staining of CEB cells that were transfected with pcDNA-VP1, pcDNA-VP2, and pcDNA-VP3, respectively. The cells were visualized and photographed by a fluorescrence microscopy. Magnification, ×200.

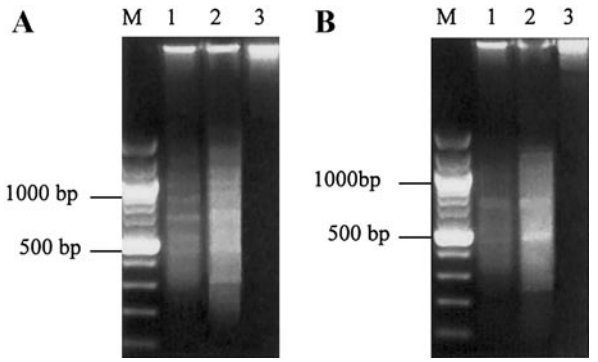

FIG. 6. Detection of cellular DNA fragmentation induced by transient expressing VP3 proteins in Cos-7 (A) and CEB (B) cells. DNA fragmentation was detected using 2.0% agarose gel electrophoresis. DNA samples were extracted from cells transfected with pcDNA-VP3 (lanes 1 and 2) at 24 and 48 h posttransfection and pcDNA-LacZ (lanes 3) at 48 h posttransfection.

old SPF chick embryos, we found that the neurons in the CNS of chick embryo brain showed characteristics of apoptosis from 4 days postinfection as demonstrated by nucleosomal DNA fragmentation analysis and electron microscopy observation (Figs. 2 and 3). The virus could be detected in brains of inoculated embryos at 3–4 days postinfection, and peak titers were found at 6–9 days p.i. (Burke *et al.,* 1965; Calnek and Jehnich, 1959), but whether apoptosis occurring in chick embryo brain is associated with viral replication is still unclear. Additionally, we also found that apoptosis induced by AEV infection occurred in some uninfected bystander cells such as inflammatory cells (data not shown), which are not targeted by AEV (Cheville, 1970; Hishida *et al.,* 1986). Similar observations have been reported in the cases with other viral infections (Girard *et al.,* 1999; Herbein *et al.,* 1998; Oberhaus *et al.,* 1997). Indeed, induction of apoptosis in targeting and nontargeting cells *in vivo* has been reported in viral infections. Reovirus infection of the mouse CNS induces apoptosis in both viral antigen-positive and antigen-negative cells (Oberhaus *et al.,* 1997). Neuron apoptosis induced by Sindbis virus infection is considered to be resulted from aberrant *N*-methyl-D-aspartate (NMDA) receptor stimulation and damage to infected and uninfected neurons (Nargi-Aizenman and Griffin, 2001). From this study, although AEV-induced apoptosis may be associated with the viral pathogenicity, the role of apoptosis in the CNS of chick embryo brain, which is resulted from damage to infected and uninfected cells, is still to be elucidated.

Among the family Picornaviridae, structural VP3 protein has been considered to be related to viral persistent infections, T-cell immunity, and virus–receptor interaction (Luo *et al.,* 1996; Minor, 1992; Yauch *et al.,* 1995). It is unclear if VP3 is related to any other functions. In this study, we have demonstrated that the structural protein VP3 from the AEV strain L_2Z (with a size of 26 kDa) shares 97.1% homology with the VP3 amino acid sequence of the strain 1143 (Marvil *et al.,* 1999) and, by using DNA fragmentation analysis and TUNEL assay, that it can induce apoptosis. VP3 protein is detected in Cos-7 and CEB cells at 24 h posttransfection; TUNEL-positive cells are clearly visible in both Cos-7 and CEB cells. Perhaps VP3 expression induces apoptosis directly by activation of an endogenous cell suicide program, which may serve as a host defense mechanism against viral proliferation. In addition, VP3-induced apoptosis could represent an efficient mechanism to spread AEV into the CNS of infected chick embryos due to AEV-induced apoptosis in chick embryo tissue. However, the protein is to be further identified in virus-infected cells and deletion analysis of its proapoptotic domain has to be carried out.

AEV VP3-induced apoptosis seems to be caspase dependent, based on its activation of caspase-3-like proteases, which could be inhibited by Ac-DEVD-CHO, a peptide inhibitor of DEVDase activity. Caspase-3 cleaves specific substrates at aspartic acid residues to release an endonuclease which migrates to the nucleus, where it can cleave DNA at internucleosomal sites, resulting in DNA fragmentation (Cryns and Yuan, 1998; Earnshaw *et al.,* 1999). There was no significant difference in the activation of caspase-3-like proteases induced by expression of VP3 protein in Cos-7 and CEB cells. Caspases are the central players in apoptosis because they catalyze many steps in the death pathway by cleavage at specific sites containing aspartic acid. Many viruses possess such biochemical mechanisms to evade and/or induce apoptosis in cells which they reside (Roulston *et al.,* 1999). For examples, Langat flavivirus (LGTV) or its envelope protein alone is able to induce apoptosis in mouse neuroblastoma (Neuro-2a) through a caspase-3-like protease pathway (Prikhod'ko *et al.,* 2001). Coxsackievirus B3, an enterovirus in the family Picornaviridae, also induces apoptosis in HeLa cells after infection and is dependent on caspase-3 protease activity (Carthy *et al.,* 1998).

To demonstrate a link of apoptosis program, we decided to explore colocalization of VP3 and mitochondria using MitoTracker and anti-VP3 antibody. Unlike conventional fluorescent stains for mitochondria which are readily sequestered by actively respiring organelles, the MitoTracker contains a thiol-reactive chloromethyl moitey and can react with accessible thiol groups on peptides and proteins to form an aldehyde-fixable conjugate. Therefore, cells stained with the red MitoTracker dye can be fixed with paraformaldehyde and then labeled with a primary guinea pig antibody against VP3 and goat antiguinea pig FITC-conjugate secondary antibody. Using double staining and confocal microscopic analysis, we demonstrated that the VP3 protein of AEV localizes mainly to mitochondria in transfected Cos-7 and CEB cells. Furthermore, VP3 protein was also localized to mitochondria of CEB cells after infection of AEV. The association of VP3 with mitochondria

seems to be an intrinsic property of the viral protein rather than an artifact of overexpression. The mitochondrion is a key organelle which generates cellular energy and controls apoptosis by releasing death-promoting proteins into the cytoplasm (Everett *et al.,* 2001). Recent data suggest that viral infections or other stimuli result in a precipitous collapse of electrochemical potential ($\Delta\Psi$m) and loss of selective ion permeability in the mitochondrion, leading to the formation of mitochondria permeability transition pores and the release of apoptosis-initiating factors, triggering the latent activity of caspases and produce apoptosis (Golstein, 1997; Wyllie, 1997). It has been reported that hepatitis B virus X protein localizing to mitochondrion of cultured human hepatoma cells led to alteration of mitochondrial transmembrane potential and indirectly induce apoptosis (Rahmani *et al.,* 2000). Therefore, the association of VP3 with mitochondria suggests that VP3 is capable of influencing mitochondrial functions. However, whether apoptosis induced by VP3 protein of AEV is directly associated with the localization of VP3 protein to mitochondria is to be further identified.

In conclusion, this study demonstrates that avian encephalomyelitis virus could result in apoptosis in chick embryo brain tissues by using DNA fragmentation analysis and electron microscopy. The structural protein VP3 is identified to be an apoptotic inducer when expressed in Cos-7 and CEB cells and activated through the caspase-3-like protease apoptotic pathway, which may be related to the localization of VP3 protein to mitochondria.

MATERIALS AND METHODS

Cells and virus

Primary chick embryo brain (CEB) cells were prepared from 12-day-old specific-pathogen-free (SPF) embryonated eggs as described previously (Nicholas *et al.,* 1987; Sato *et al.,* 1971). The primary CEB cells were maintained in growth medium consisting of MEM supplemented with 20% fetal bovine serum (FBS), 5% L-glutamine, 100 U/ml of penicillin G, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. The monolayer CEB cells after 7–8 days postseeding were used for subculture. Secondary CEB cells from subculture were used for transient expression of recombinant plasmids. Mammalian continuous cell Cos-7, used for transient expression assay, was maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO), supplemented with 10% fetal bovine serum (FBS) and 100 U/ml of penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. AEV virulent strain L_2Z was isolated from a broiler flock with immune failure and titrated in SPF chick embryos as described (Liu *et al.,* 1998).

Preparation of monospecific antibodies against VP0, VP1, and VP3 of AEV

Viral RNA from chicken embryo brain tissue infected with AEV strain L_2Z was extracted for reverse transcription–polymerase chain reaction (RT–PCR) of VP0, VP1, and VP3 genes. The primers for the amplifications of VP0, VP1, and VP3 genes were derived from the genome of the AEV vaccine strain 1143 (Marvil *et al.,* 1999). These primers are VP0(3): 5′-CCCTCGAGCTGTGCGACCAAAGGGCTAAG-3′; VP0(5): 5′-CCCTCGAGCTGTGCGACCAAAGGGCTAAG-3′; VP1(5): 5′-CCCCCGGGTTGGGAAAGAGGATGAAGGAGGA-3′; VP1(3): 5′-CCCTCGAGCTCTTCTACCAACTCGTCATC-3′; VP3(5): 5′-CGGAATTCCATGATGCGCAACGAATTTCGAC-3′; and VP3(3): 5′-CCCTCGAGTTGAGCAAAAACTGAGGTGT-TGG-3′, respectively. To facilitate cloning of the PCR fragments, *Sma*I/*Xho*I and *Eco*RI/*Xho*I restriction enzyme sites (underlined) were incorporated into the forward and reverse primer sequences, respectively. The digested DNA fragments of VP0, VP1, and VP3 genes were cloned into expression vector pGEX4T-3 (AMRAD), and the GST fusion proteins were induced and purified by standard techniques as recommended by the manufacturer.

To produce specific antibodies against the VP0, VP1, and VP3 proteins, two female guinea pigs were administered 200–250 μg intramuscularly of the purified proteins emulsified with adjuvant MONTANIDE ISA 70 (Seppic, Paris, France). Guinea pigs were boosted twice with the same quantities of antigen emulsion at 2-week intervals. Ten days after the final booster injection, the animals were bled and the antibody titers and specificities were determined by Western blotting with *E. coli*-expressed AEV antigens.

Transient expression of VP1, VP2, or VP3 proteins of AEV *in vitro*

To prepare recombinant eukaryotic expression plasmids, the coding sequence of the VP1, VP2, and VP3 genes was amplified by PCR reaction using three pairs of oligonucleotide primers from plasmids pGEX4T-3-VP1, -VP0, or -VP3. The sequences of the primers used for these three gene amplifications were as follows: VP1(5): 5′-GGGGTACC-TATGTTGGGAAAGAGGATGAAGGAGGA-3′; VP1(3): 5′-CC-CTCGAGTCACTCTTCTACCAACTCGTCATC-3′; VP2(5): 5′-GGGGTACCTATGACTGAATCTTATGCTGGCCCTG-3′; VP2(3): 5′-CCCTCGAGTCACTGTGCGACCAAAGGGCTAAG-3′; VP3(5): 5′-GGGGTACCTATGATGCGCAACGAATTTC-GAC-3′; and VP3(3): 5′-CCCTCGAGTCATTGAGCAAAAACT-GAGGTGTTGG-3′. The *Kpn*I/*Xho*I fragments VP1, VP2, and VP3 were directionally cloned between *Kpn*I and *Xho*I sites of eukaryotic expression vector pcDNA4 (Invitrogen), downstream of the human cytomegalovirus (HCMV) promoter, to obtain pcDNA-VP1, -VP2, and -VP3. They were analyzed by sequencing to confirm that no errors were introduced as a result of PCR amplification.

In vitro expression of the pcDNA-VP1, pcDNA-VP2, and

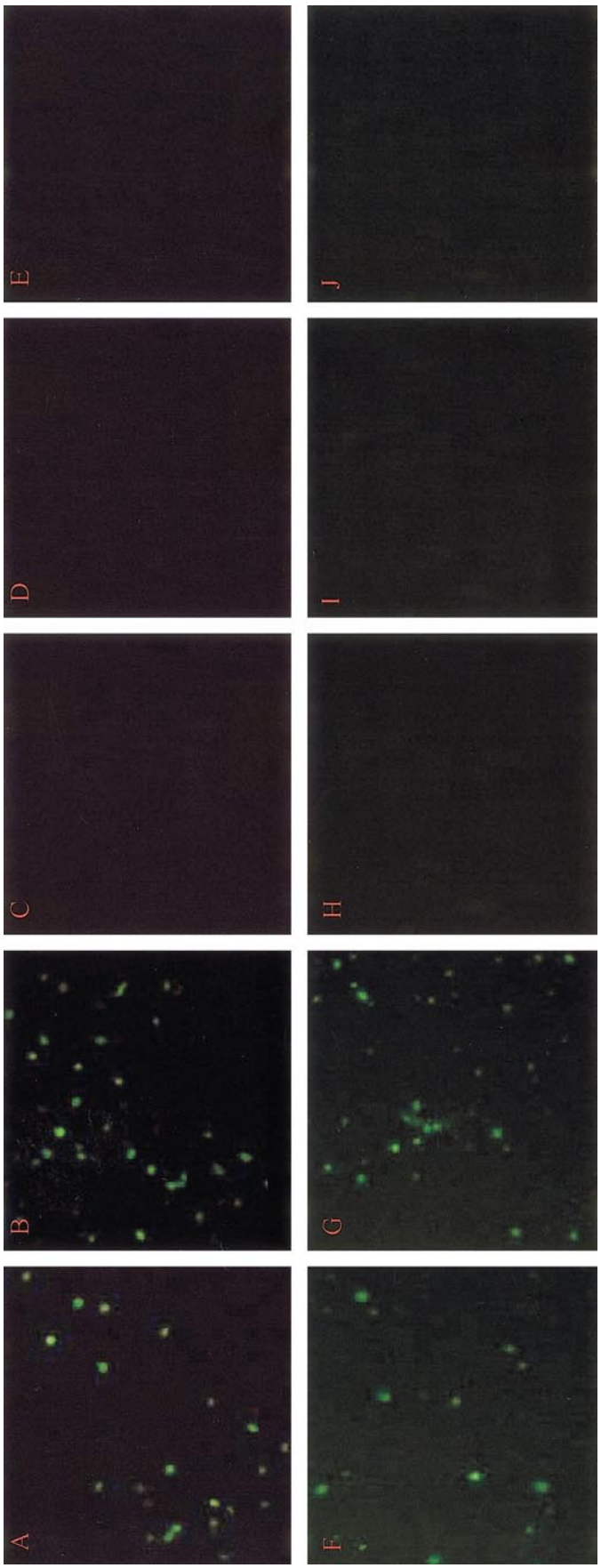

FIG. 7. Detection of apoptosis in Cos-7 and CEB cells by TUNEL labeling. Cos-7 or CEB cells were transfected with the expression vector pcDNA-VP3. At 24 and 48 h posttransfection, the cells were fixed and analyzed for detection of apoptosis by TUNEL staining, which detects the DNA breakage (green signal). A and B and then F and G indicate TUNEL labeling of Cos-7 and CEB cells that were transfected with pcDNA-VP3 at 24 and 48 h, respectively. C, D, and E and H, I, and J indicate Cos-7 and CEB cells that were transfected at 48 h with pcDNA-VP1, pcDNA-VP2, and pcDNA-LacZ, respectively. The cells in A through J were visualized and photographed using fluorescrence microscopy. Magnification, ×200.

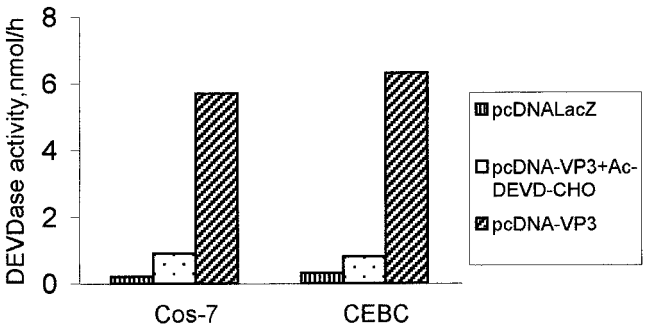

FIG. 8. AEV VP3 protein induces activation of caspase-3-like proteases in both Cos-7 and CEB cells. Cells were transfected with plasmids pcDNA-VP3 and pcDNA-LacZ. Cell lysates were harvested 24 h after transfection and assayed for DEVDase activity using the colorimetric substrate Ac-DEVD-pNA. In addition, cells transfected with pcDNA-VP3 were treated with Ac-DEVD-CHO. The pcDNA-LacZ was used as a negative control and to maintain equal plasmid DNA concentrations for each of the transfections. Results are means ± SD from duplicated experiments.

pcDNA-VP3 constructs were tested in transient expression experiments using Cos-7 and CEB cells. Cos-7 or CEB cells, grown in chamber slides (IWAKI), were transfected with pcDNA-LacZ vector only, pcDNA-VP1, pcDNA-VP2, or pcDNA-VP3 (0.6 μg of plasmid per well) using LipofectiminePlus (GIBCO/BRL) as described in the manufacturer's protocol. Indirect immunofluorescence assay was carried out to determine the expression of VP1, VP2, or VP3. Briefly, at 24 and 48 h posttransfection, the cells were fixed with precooled absolute ethanol for 30 min at room temperature (RT). The fixed cells were incubated with guinea pig anti-VP1, anti-VP2, or anti-VP3 antibodies diluted in phosphate-buffered saline (PBS)-T buffer with 1% bovine serum albumin (BSA) for 1 h at RT and then followed by incubation with FITC-conjugated antiguinea pig IgG secondary antibody (DAKO) diluted in PBS-T buffer with 1% BSA for another hour at RT. The plates were washed three times and observed under an immunofluoscence microscopy. The expressed proteins VP1, VP2, and VP3 were further confirmed using Western blotting according to standard technique.

TUNEL assay

Apoptosis was detected using the Fluorescein Apoptosis Detection System (TUNEL) Kit (Promega) following the instructions of the manufacturer. Briefly, Cos-7 or CEB cells grown on chamber slides (IWAKI) were transfected as described above. At 24 and 48 h posttransfection, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 at RT. After equilibration for 10 min at RT, the equilibrated areas were overlaid with 50 μl of terminal deoxynucleotidytransferase (TdT) incubation buffer and incubated at 37°C for 60 min. Reactions were stopped by immersing in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min at RT. After being washed three times with PBS, the slides were examined under a fluorescence microscope.

DNA fragmentation analysis

Low-molecular-weight DNA was isolated from tissue or cells using an Apoptotic DNA Ladder Kit (Roche) following the manufacturer's instructions. The tissues or cells were harvested and resuspended in binding/lysis buffer [6 M guanidine–HCl, 10 mM urea, 10 mM Tris–HCl, and 20% Triton X-100 (pH 4.4)] and incubated for 10 min at RT. After incubation, isopropanol was added and the mixture was vortexed. The mixture was then placed in a filter tube and centrifuged for 1 min at 8,000 rpm at RT. The upper reservoir was washed with washing buffer and centrifuged twice. After centrifugation for 1 min at 13,000 rpm, the nucleic acid was dissolved in prewarmed (70°C) elution buffer, centrifuged, and collected. Equal amounts of DNA were resolved on a 2% agarose gel containing ethidium bromide.

Caspase-3-like protease (DEVDase) activity assay

Colorimetric assay of caspase-3-like proteolytic activity was performed using an ApoAlert Caspase-3 Colori-

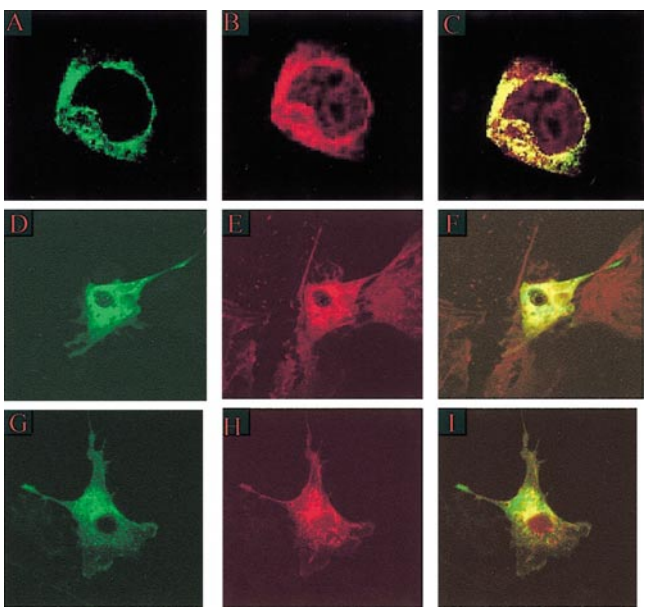

FIG. 9. Subcellular localization of AEV VP3 protein with MitoTracker, a mitochondria marker. Cos-7 (A, B, and C) or CEB (D, E, and F) cells were transfected with pcDNA-VP3 plasmid, while CEB (G, H, and I) cells were infected with AEV strain L_2Z. They were then stained with MitoTracker and labeled with an anti-VP3 polyclonal antibody. Cos-7 cell expressing VP3 protein (image A, green) and localization at mitochondria (image B, red) were merged and showed orange or yellow (image C) at 24 h posttransfection. CEB cell expressing VP3 protein (images D and G, green) and localization at mitochondria (images E and H, red) were merged and showed orange or yellow (images F and I) at 24 h posttransfection and infection, respectively. Cells were amplified 63 × 15 times and viewed in a confocal microscope.

metric Assay Kit (Clontech Laboratories). Cos-7 or CEB cells (2–3 × 10^6) were seeded in 25 × 25 cm flasks. Twenty-four hours later, cells were transfected with 2 μg of pcDNA-VP3 or pcDNA-LacZ vector. Twenty-four hours after transfection, cells were harvested at 2000 *g* for 5 min. Cells (2–3 × 10^6) were lysed in 50 μl of lysis buffer on ice for 10 min and centrifuged at 12,000 *g* for 3 min and the supernatant was collected. Supernatant (50 μl) was added to an equal volume of 2× reaction/dithiothreitol buffer supplemented with Ac-DEVD-pNA (50 μM) and incubated at 37°C for 1 h and the optical density at 405 nm was determined. Values for nanomoles of pNA released expressed per hour were calculated from those observed in A_{405} values using a standard curve.

Localization of VP3 protein to mitochondria

Cos-7 or CEB cells grown on the chamber slides (IWAKI) were transfected with pcDNA-VP3 plasmid as described above. For infection, CEB cells were incubated with AEV strain L_2Z for 45 min at 37°C at 1000 EID_{50} (50% embryo infection dose) and added to MEM for incubation. Following the incubation at 37°C, cells at 24 h posttransfection or infection were washed with PBS and stained with MitoTracker (Molecular Probes) at a concentration of 100 to 375 nM for 30 min at 37°C and fixed for 30 min at RT with 4% paraformaldehyde in PBS. After fixation, the cells were blocked by PBS-T with 1% BSA at RT for 1 h. Primary antibody, anti-VP3 antibody, was diluted in PBS-T and incubated with the cells for 1 hr at RT. After washing with PBS, the cells were incubated with goat antiguinea pig FITC-conjugate (DAKO, Demark) diluted in PBS-T for 1 h at RT. The cells were washed three times with PBS, rinsed in dH_2O, dried and mounted with fluorescence mounting media, and examined using an LSM 510 confocal laser scanning microscope (Zeiss, Germany) with a Plan-Novofluar 63×/1.2 water objective.

Electron microscope

Seven-day-old SPF chick embryos were inoculated with 1000 EID_{50} of AEV strain L_2Z in 0.2 ml of 0.1 M PBS (pH 7.4) by the yolk sac route. At 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 days postinoculation, brains were collected for DNA fragmentation analysis and electron microscopy observation. For EM samples, brain tissues were fixed with 2.5% glutaraldehyde and subsequently with 1% OsO_4 and embedded into EPON-812. Ultrathin sections were examined with a Hitachi H-700 electron microscope.

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REFERENCES

Agol, V. I., Belov, G. A., Bienz, K., Egger, D., Kolesnikova, M. S., Raikhlin, N. T., Romanova, L. I., Smirnova, E. A., and Tolskaya, E. A. (1998). Two types of death of poliovirus-infected cells: Caspase involvement in the apoptosis but not cytopathic effect. *Virology* **252,** 343–353.

Ammendolia, M. G., Tinari, A., Calcabrini, A., and Superti, F. (1999). Poliovirus infection induces apoptosis in CaCo-2 cells. *J. Med. Virol.* **59,** 122–129.

Arends, M., and Wellie, A. (1991). Apoptosis: Mechanism and roles in pathology. *Int. Rev. Exp. Pathol.* **32,** 223–254.

Barco, A., Feduchi, E., and Carrasco, L. (2000). Poliovirus protease $3C^{pro}$ kills cells by apoptosis. *Virology* **266,** 352–360.

Burke, C. N., Krauss, H., and Luginbuhl, R. E. (1965). The multiplication of avian encephalomyelitis virus in chicken embryo tissues. *Avian Dis.* **9,** 104–108.

Calnek, B. W., and Jehnich, H. (1959). Studies on avian encephalomyelitis. I. The use of a serum-neutralization test in the detection of immunity levels. *Avian Dis.* **3,** 95–104.

Calnek, B. W., Luginbuhl, R. E., and Helmboldt, C. F. (1997). Avian encephalomyelitis. *In* "Diseases of Poultry," pp. 571–581. Iowa State University Press, Ames, IA.

Carthy, C. M., Granville, D. J., Watson, K. A., Anderson, D. R., Wilson, J. E., Yang, D., Hunt, D. W. C., and McManus, B. M. (1998). Caspase activation and specific cleavage of substrates after coxsackievirus B3-induced cytopathic effect in HeLa cells. *J. Virol.* **72,** 7669–7675.

Cheville, N. F. (1970). The influence of thymic and bursal lymphoid systems in the pathogenesis of avian encephalomyelitis. *Am. J. Pathol.* **58,** 105–125.

Cryns, V., and Yuan, J. (1998). Proteases to die for. *Genes Dev.* **12,** 1551–1570.

Despres, P., Frenkiel, M.-P., Ceccaldi, P.-E., Duarte Dos Santos, C., and Deubel, V. (1998). Apoptosis in the mouse central nervous system in response to infection with mouse-neurovirulent dengue viruses. *J. Virol.* **72,** 823–829.

Earnshaw, W. C., Martins, L. M., and Kaufmann. (1999). Mammalian caspases: Structure, activations, and functions during apoptosis. *Annu. Rev. Biochem.* **68,** 383–424.

Everett, H., and McFadden, G. (2001). Viral proteins and the mitochondrial apoptotic checkpoint. *Cyt. Growth Fact. Rev.* **201,** 181–188.

Girard, S., Couderc, T., Destombes, J., Thiesson, D., Delpeyroux, F., and Blondel, B. (1999). Poliovirus induces apoptosis in the mouse central nervous system. *J. Virol.* **73,** 6066–6072.

Golstein, P. (1997). Controlling cell death. *Science* **275,** 1081–1082.

Hishida, N. Y., Odagiri, Y., Kotani, T., and Horiuchi, T. (1986). Morphological changes of neurons in experimental avian encephalomyelitis. *Jap. J. Vet. Sci.* **48,** 169–172.

Liu, J., Liu, Y. C., Yao, W. G., Zhang, F. L., Zhou, J., and Liu, S. G. (1998). Isolation and identification of avian infectious encephalomyelitis virus. *Beijing Agr. Sci.* **1,** 32–35.

Luo, M., Toth, K. S., Zhou, L., Pritchard, A., and Lipton, H. L. (1996). The structure of a highly virulent Theiler's murine encephalomyelitis virus (GDVII) and implications for determinations of viral persistence. *Virology* **220,** 246–250.

Marvil, P., Knowles, N. J., Mockett, A. P. A., Britton, P., Brown, T. D. K., and Cavanagh, D. (1999). Avian encephalomyelitis virus is a picornavirus and is most closely related to hepatitis A virus. *J. Gen. Virol.* **80,** 653–662.

Minor, P. D. (1992). The molecular biology of poliovaccines. *J. Gen. Virol.* **73,** 3065–3077.

Muir, P., Kämmerer, U., Korn, K., Mulders, M. N., Pöyry, T., Weissbrich, B., Kandolf, R., Cleator, G. M., and van Loon, A. M. (1998). Molecular typing of Enteroviruses: Current status and future requirements. *Clin. Microbiol. Rev.* **11,** 202–227.

Nargi-Aizenman, J. L., and Griffin, D. E. (2001). Sindbis virus-induced neuronal death is both necrotic and apoptotic and is ameliorated by *N*-methyl-D-asparate receptor antagonists. *J. Virol.* **75,** 7114–7121.

Nicholas, R. A. J., Ream, A. J., and Thornton, D. H. (1987). Replication of avian encephalomyelitis virus in chick embryo neuroglial cell cultures. *Arch. Virol.* **96,** 283–287.

Oberhaus, S. M., Smith, R. L., Clayton, G. H., Dermody, T. S., and Tyler, K. L. (1997). Reovirus infection and tissue injury in the mouse central

nervous system are associated with apoptosis. *J. Virol.* **71,** 2100–2106.

O'Brien, V. (1998). Viruses and apoptosis. *J. Gen. Virol.* **79,** 1833–1845.

Prikhod'ko, G. G., Prikhod'ko, E. A., Cohen, J. I., and Pletnev, A. G. (2001). Infection with Langat Flavivirus or expression of the envelope protein induces apoptotic cell death. *Virology* **286,** 328–335.

Rahmani, Z., Huh, K.-W., Lasher, R., and Siddiqui, A. (2000). Hepatitis B virus X protein colocalizatizes to mitochondria with a human voltage-dependent anion channel, HVDAC3, and alters its transmembrane potential. *J. Virol.* **74,** 2840–2848.

Sato, G., Kamada, M., Miyamae, T., and Miura, S. (1971). Propagation of non-egg-adapted avian encephalomyelitis virus in chick embryo brain cell culture. *Avian Dis.* **15,** 326–333.

Shen, Y., and Shenk, T. E. (1995). Viruses and apoptosis. *Curr. Opin. Genet. Dev.* **5,** 105–111.

Teodoro, J. G., and Branton, P. E. (1997). Regulation of apoptosis by viral gene products. *J. Virol.* **71,** 1739–1746.

Tolskaya, E. A., Romanova, L. I., Kolesnikova, M. S., Ivannikova, T. A., Smirnova, E. A., Raikhlin, N. T., and Agol, V. I. (1995). Apoptosis-inducing and apoptosis-preventing functions of poliovirus. *J. Virol.* **69,** 1181–1189.

Tsunoda, I., Kurtz, C. I. B., and Fujinami, R. S. (1997). Apoptosis in acute and chronic central nervous system disease induced by Theiler's murine encephalomyelitis virus. *Virology* **228,** 388–393.

Wyllie, A. (1997). Clues in the p53 murder mystery. *Nature* **389,** 237–238.

Yauch, R., Kerekes, K., Saujani, K., and Kim, B. S. (1995). Identification of a major T-cell epitope within VP3 amino acid residues 24 to 37 of Theiler's virus in demyelination-susceptible SJL/J mice. *J. Virol.* **69,** 7315–7318.