Replication-incompetent virions of Japanese encephalitis virus trigger neuronal cell death by oxidative stress in a culture system

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It has been shown that replication of the Japanese encephalitis virus (JEV) can trigger infected cells to undergo apoptosis. In the present study, it is further demonstrated that replication-incompetent virions of JEV, obtained by short-wavelength ultraviolet (UV) irradiation, could also induce host-cell death. It was found that UV-inactivated JEV (UV-JEV) caused cell death in neuronal cells such as mouse neuroblastoma N18 and human neuronal NT-2 cells, but not in non-neuronal baby hamster kidney BHK-21 fibroblast or human cervical HeLa cells. Only actively growing, but not growth-arrested, cells were susceptible to the cytotoxic effects of UV-JEV. Killing of UV-JEV-infected N18 cells could be antagonized by co-infection with live, infectious JEV, suggesting that virions of UV-JEV might engage an as-yet-unidentified receptor-mediated death-signalling pathway. Characteristically, mitochondrial alterations were evident in UV-JEV-infected N18 cells, as revealed by electron microscopy and a loss of membrane potential. N18 cells infected by UV-JEV induced generation of reactive oxygen species (ROS) as well as the activation of nuclear factor kappa B (NF-κB), and the addition of anti-oxidants or specific NF-κB inhibitors to the media greatly reduced the cytotoxicity of UV-JEV. Together, the results presented here suggest that replication-incompetent UV-JEV damages actively growing neuronal cells through a ROS-mediated pathway.

INTRODUCTION

Japanese encephalitis virus (JEV), a member of the family Flaviviridae, is a mosquito-borne RNA virus that causes acute encephalitis in humans. Epidemiological studies have shown that JEV infection leads to a high mortality rate, especially in south-east Asia (Burke & Monath, 2001). JEV is an enveloped virus that contains a single-stranded, positive-sense RNA genome of approximately 11 kb. Receptor-mediated endocytosis, partly mediated by the viral envelope glycoprotein binding to highly sulfated heparin sulfate on the cell surface, is used by JEV to initiate infection (Su, C. M. et al., 2001). After entry, the viral genome encodes a poly-protein that is subsequently cleaved into a number of structural and non-structural proteins by host and viral proteases (Lindenbach & Rice, 2001).

Immediately after virus infection, robust host responses are initiated to limit virus replication, including specific immune reactions and intracellular defensive mechanisms. To cope with such powerful antiviral effects, many viruses have evolved different strategies to avoid host destruction until sufficient progeny have been produced (Roulston et al., 1999). Since apoptosis may serve as an intracellular defence mechanism to restrict virus production, especially at the early stage of infection, several viruses have evolved genes able to counteract the apoptotic cascade during infection. On the other hand, some viruses encode proteins that are able to induce apoptotic processes at the late stages of the virus life cycle, from which the viruses may conceivably benefit due to promotion of their spreading without causing noticeable immune responses. We have demonstrated that JEV infection induces severe cytopathic effects (CPEs) in different types of cultured cells and the replication of JEV appears to be essential for induction of apoptosis in those infected cells, albeit at a late time of infection (Liao et al., 1997). The cross-talk between virus and host cell at an earlier stage of JEV infection remains largely elusive.

Oxidative stress has been implicated in the pathophysiology of many neurological diseases (Gilgun-Sherki et al., 2001; Maher & Schubert, 2000). Excessive production of reactive oxygen species (ROS), free radical derivatives of molecular oxygen and hydrogen peroxide, may damage various intracellular macromolecules, which leads to oxidative stress often accompanied by loss of cell function, and apoptosis and/or necrosis (Nordberg & Arner, 2001). In addition, ROS
may trigger a variety of signalling pathways that involve transcriptional activation and protein phosphorylation (Kamata & Hirata, 1999; Suzuki et al., 1997). ROS are primarily generated by mitochondria, which possess an antioxidant system capable of neutralizing the damaging effects of ROS under normal conditions (Yu, 1994). When excessive amounts of ROS result, nucleic acids, proteins and lipids are extensively modified by oxidation, thereby giving rise to mitochondrial dysfunction (Richter et al., 1995). Of these, an increase in mitochondrial membrane permeabilization (MMP) may constitute a common event in cell death by both apoptosis and necrosis (Green & Reed, 1998; Kroemer et al., 1998; Kroemer & Reed, 2000). Several studies have shown that viral infections can generate ROS and induce oxidative stress, such as in the case of human immunodeficiency virus (HIV) (Baruchel & Wainberg, 1992; Israel & Gougerot-Pocidalo, 1997), Sendai virus (Peterhans, 1979), cytomegalovirus (CMV) (Speir et al., 1996), influenza virus (Akaike et al., 1990), hepatitis B virus (HBV) (Hagen et al., 1994), dengue virus serotype 2 (Jan et al., 2000), JEV (Raung et al., 2001) and tobacco mosaic virus (Allan et al., 2001). It has been shown that generation of ROS in the target cells in response to infection plays a role in virus replication and pathogenesis (Everett & McFadden, 2001a, b; Schwarz, 1996).

Nuclear factor kappa B (NF-κB) was one of the first transcription factors shown to be regulated by ROS and is often considered to be a primary sensor of oxidative stress in cells (Kamata & Hirata, 1999; Sen & Packer, 1996; Suzuki et al., 1997). The activation of NF-κB occurs via the phosphorylation and degradation of an inhibitory protein, IkB-α, thereby releasing NF-κB from the cytoplasm and allowing its translocation into the nucleus. NF-κB binds to nuclear κB elements of target genes and activates transcription of genes mediating cell growth, differentiation, inflammation, oncogenesis, pro- and anti-apoptotic reactions, etc. (Baeuerle & Baltimore, 1996; Barkett & Gilmore, 1999; Hatada et al., 2000; Karin & Lin, 2002; Mogensen & Paludan, 2001). In addition, NF-κB activation is also a hallmark of most infections (Barkett & Gilmore, 1999; Mogensen & Paludan, 2001). At the early stages of viral infections, such as for herpes simplex virus (HSV), CMV, Epstein–Barr virus (EBV), HIV (Mogensen & Paludan, 2001) and reovirus (Barton et al., 2001), NF-κB can be activated by the interaction between viral envelope glycoproteins and cellular receptors. This NF-κB activation can either increase virus replication because the viruses have NF-κB-binding sites in their promoter, to enhance viral pathogenicity, or to block or promote apoptosis. Some viruses may block apoptosis by NF-κB activation in infected cells (Barkett & Gilmore, 1999); in contrast, certain viruses may induce apoptosis through NF-κB activation, such as in infection by sindbis virus (Lin, K. I. et al., 1995), dengue virus (Marianneau et al., 1997) and reovirus (Barton et al., 2001).

To study the effect of JEV replication on host cells, we used ultraviolet-inactivated JEV (UV-JEV) as the control; unexpectedly, we found that the virions of replication-defective JEV could cause a unique cell death of the target cells. This killing event occurred only in actively growing cells and appeared to be cell-type-dependent, i.e. only the vigorously growing neuronal cells were vulnerable to assault by UV-JEV. Both ROS generation and NF-κB activation were observed in UV-JEV-treated mouse neuroblastoma N18 cells. Blocking either ROS generation or NF-κB activation could readily suppress the cell death induced by UV-JEV. The possible mechanisms and the significance of such JEV-replication-independent cytotoxicity are discussed in the present study.

METHODS

Virus, cells and chemicals. A plaque-purified Taiwanese JEV strain, RP-9, was employed throughout this study (Chen et al., 1996b). Virus propagation was carried out in C6/36 cells grown in RPMI 1640 medium containing 5% fetal bovine serum (FBS; GIBCO, BRL). N18, a mouse neuroblastoma cell line (Amano et al., 1972) (a kind gift from Dr D. E. Griffin, John Hopkins University, Baltimore, MD, USA), was grown in RPMI 1640 medium containing 5% FBS. NT-2, a human neuronal precursor cell line, was cultured in Opti-MEM (GIBCO) supplemented with 10% FBS. The HeLa cell line was cultured in RPMI 1640 medium containing 10% FBS. Lactacystin and a specific NF-κB peptide inhibitor, SN50, were both from BioMol. MnTBAP [Mn(III) tetrakis (4-benzoic acid) porphyrin chloride], a cell-permeable superoxide dismutase (SOD) mimetic, Cu/Zn SOD and N-acetyl-l-cysteine (NAC) were obtained from Calbiochem. Glutathione monooethyl ester was from OXIS.

Purification and UV inactivation of JEV. For virus purification, JEV supernatant was clarified at 8000 r.p.m. for 30 min, then centrifuged through a 20% sucrose cushion at 27 000 r.p.m. for 3.5 h at 4°C. The pellet was resuspended in RPMI 1640 medium and the viral titre [plaque-forming units per millilitre (p.f.u. ml⁻¹)] was determined by a plaque assay on BHK-21 cells as described previously (Chen et al., 1996b). JEV inactivation was carried out with a Stratalinker 2400 (Stratagene) using short-wavelength UV radiation (UVC, 254 nm) at a distance of 5 cm for 30 min on ice. Virus inactivation was verified by plaque assay, indicating its infectious titre had been reduced by more than 10⁻⁵-fold, and immunofluorescence assay using anti-JEV antibodies (Chen et al., 1996a), showing no positive staining (data not shown).

Lactate dehydrogenase (LDH) assay. Cell viability was assessed by the release of the cytoplasmic enzyme LDH using a commercial kit (Cytotoxicity Detection Kit; Boehringer Mannheim) according to the manufacturer’s instructions. Percentage of LDH release was calculated as [(experimental LDH—medium control)/(total cellular LDH—medium control)] x 100. Percentage of cytotoxicity was determined by using the formula [(experimental LDH—medium control)/(UV-JEV LDH—medium control)] x 100.

Measurement of NF-κB activity by luciferase reporter assay. N18 cells were stably transfected with the reporter plasmid pNF-κB-Luc (Stratagene), which carried the luciferase gene downstream of NF-κB-binding sites. At various experimental time points, cells were harvested, lysed and the luciferase activity (counts per second (c.p.s.)) was determined using a Luciferase Assay System kit purchased from Promega.

Measurement of intracellular ROS by 2',7'-dichlorofluorescin (DCF) fluorescence. This was done using a fluorescent probe, 2',7'-dichlorofluorescin diacetate (DCFH-DA), purchased from Molecular Probes. Briefly, DCFH-DA diffuses through the cell membrane and is
enzymically hydrolysed by intracellular esterases to the highly fluorescent compound DCF. N18 cells were loaded with 2 μM DCFH-DA for 30 min, and washed with PBS twice before the treatment. The fluorescence intensity was monitored on a spectrophotometer (Fluoroskan Ascent; Labsystems) using 485 nm excitation and 538 nm emission.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay.** Apoptosis-induced DNA strand breaks were end-labelled with dUTP by terminal deoxynucleotidyl transferase (TdT) using the In situ Cell Death Detection kit (Boehringer Mannheim) according to the manufacturer’s instructions. The labelled cells were observed under a Leica fluorescence microscope.

**Hoechst 33258 staining of cells.** Cell monolayers were fixed with 70 % ethanol at 4 °C for 1 h, then washed three times with PBS before treatment with Hoechst 33258 (Molecular Probes) for 15 min at room temperature. Cells were then washed three times with PBS and observed under a Leica fluorescence microscope.

**Detection of the mitochondrial membrane potential (ΔΨm).** The ΔΨm was determined by using a DePsipher kit (Trevigen); this kit uses a unique cationic dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) to indicate the loss of the mitochondrial potential. Healthy cells will appear red following aggregation of the DePsipher dye within the mitochondria; in cells with disrupted mitochondrial potential, the dye remains in the cytoplasm as a green fluorescent monomeric form.

**Electron microscopy.** Cells were collected as pelleted, washed with RPMI 1640 medium and fixed with freshly prepared 2.5 % glutaraldehyde in RPMI 1640 medium for 90 min at room temperature. After fixation, the pellets were washed three times in RPMI 1640 medium, post-fixed with 1 % osmium in distilled water for 90 min and then dehydrated in a graded series of ethanol. The dehydrated pellets were embedded in LR white acrylic resin. Sections were double-stained by floating on a fresh 50 % aqueous saturated solution of uranyl acetate, rinsed twice with distilled water and stained immediately with lead citrate. Thin-sections were examined under a JEM 1200 EX electron microscope.

**Immunofluorescence and confocal laser scanning microscopy.** Cells were transfected with plasmid pEYFP-Mito (Clontech), which encodes a fusion protein of enhanced yellow fluorescent protein (EYFP) with the targeting sequence from subunit VIII of cytochrome c oxidase. Transfected cells were untreated (control) or treated with UV-JEV (m.o.i. 30) for 5 h. The cells were rinsed twice with PBS and fixed with 2 % formaldehyde for 30 min followed by permeabilization with 0.5 % Triton X-100 for 10 min. Subsequently, staining for cytochrome c was performed with a mAb against cytochrome c (Pharmingen clone 6H2.B4) and probed with a Cy3-conjugated secondary antibody (Jackson ImmunoResearch). Images were collected using a ZEISS LSM5 Pascal confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) equipped with an argon laser attached to a Zeiss Axiosvert-100 M microscope with a LD-Althroplan 40 x oil-immersion objective lens. EYFP was measured using 488 nm excitation and 505 nm emission, and Cy3 was measured using 543 nm excitation and 590 nm emission.

**Western immunoblot analysis.** Cells were lysed in SDS sample buffer (62.5 mM Tris/HCl, pH 6-8, 2 % SDS, 10 % (v/v) glycerol, 50 mM DTT, 0.1 % bromophenol blue) containing a cocktail of protease inhibitors. Proteins were separated by SDS-PAGE and then transferred to a nitrocellulose membrane (Hybond-C Super; Amersham). Non-specific-antibody-binding sites were blocked with 5 % skim milk in TBS-T (25 mM Tris, 0-8 % NaCl, 2-68 mM KCl, pH 7-4, with 0-1 % Tween 20) and membranes were reacted with the primary antibody. Blots were then treated with a horseradish-peroxidase-conjugated secondary antibody (Amersham) and developed using the ECL system (Amersham).

**RESULTS**

**A unique cell death induced by UV-JEV in neuronal cells**

Previously, we have reported that replication capability is essential for JEV to trigger infected cells to undergo apoptosis (Liao et al., 1997). To further characterize the molecular events initiated by JEV infection, we used UV-JEV as the control. Unexpectedly, we observed a unique type of cell death, which was quite distinct from the death triggered by replication-competent JEV (Liao et al., 1997) in murine neuroblastoma N18 cells. As shown in Fig. 1(A), at 7 h post-infection (p.i.), N18 cells were abnormally rounded-up by UV-JEV, in a dose-dependent manner, but not by UV-irradiated culture medium derived from mock-infected C6/36 cells (Fig. 1B); this is in great contrast to the infectious JEV-infected N18 cells (Fig. 1B), which only showed noticeable CPEs after overnight infection (Liao et al., 1997). The induction of cell death by UV-JEV was further confirmed by the trypan-blue exclusion method (Fig. 1C), which indicated that the viability of UV-JEV-treated N18 cells was greatly impaired when compared to the mock-infected cells. As measured by release of the cytoplasmic enzyme LDH into the culture medium, an increase of cytotoxicity of UV-JEV-infected N18 cells as the incubation proceeded was also noted (Fig. 1D).

Furthermore, this type of cell death appeared to only occur within the population of actively growing cells, since only the cells in low density (Fig. 2A) and under no serum starvation (Fig. 2B) were shown to be readily killed by UV-JEV. We next examined whether the cytotoxicity induced by UV-JEV was cell-type-dependent. The susceptibility to UV-JEV killing was compared in four different cell lines: mouse neuroblastoma N18, human neuronal NT-2, baby hamster kidney fibroblast BHK-21 and human cervical HeLa cells. As shown in Fig. 2(C), UV-JEV could cause cell death in neuronal N18 and NT-2 cells, but not in non-neuronal BHK-21 and HeLa cells. Thus, these results indicate that infection by a high dose of UV-JEV specifically damages actively growing neuronal cells.

**JEV infection suppressed cytotoxicity induced by UV-JEV in N18 cells**

Since there was no viral protein synthesized in the UV-JEV-infected N18 cells, any early steps of the virus life cycle before translation, such as attachment, entry and/or uncoupling, could result in UV-JEV-induced cell death. To determine whether binding of the putative JEV receptor could transmit the death signal from UV-JEV, we performed a competition experiment through co-infection of the target cells with JEV and UV-JEV. As shown in Fig. 3, in the presence of live JEV, the extent of cell death triggered by UV-JEV (m.o.i. 30) was found to be reduced, as the number of viable cells gradually increased as JEV titres rose, indicating that co-infection of target cells with replication-competent JEV could suppress the cell death induced by UV-JEV. Nevertheless, this
experiment does not define if the molecular basis for the above phenomenon is due to a pure competition for the putative virus receptor(s) or to stimulation of certain anti-apoptotic processes by JEV replication, or to both.

**N18 cells treated with UV-JEV underwent caspase-independent cell death**

To identify the mechanism underlying the UV-JEV-induced cell death, N18 cells were infected with UV-JEV (m.o.i. 40) for 2, 4 or 6 h, and the resulting cells were analysed by TUNEL assay for apoptosis-induced DNA strand breakage. As compared to the mock-infected cells, positive TUNEL staining gradually became slightly evident 6 h after UV-JEV infection (Fig. 4A). Using Hoechst 33258 staining, some cells with apoptosis-induced chromatin condensation could be also detected (Fig. 4B). A cell-permeable pan-caspase inhibitor, z-VAD-fmk, was used to further examine whether caspases were involved in this cell death. We found that treatment with z-VAD-fmk (50 μM) had no protective effect on the cell death induced by UV-JEV (data not shown), indicating that the majority of UV-JEV-treated cells died from a caspase-independent pathway. Since release of mitochondrial cytochrome c could result in the activation of caspase-9, which in turn activates other caspases (Green & Reed, 1998; Kroemer & Reed, 2000), we then analysed whether cytochrome c was released in UV-JEV-infected cells. As revealed by confocal microscopy (Fig. 4C), the immunostaining signal of cytochrome c co-localized with the mitochondrion-targeted EYFP (pEYFP-Mito; Clontech), both in the mock-infected (Fig. 4C, top panel) and the UV-JEV-infected (m.o.i. 30 for 5 h) N18 cells (Fig. 4C, middle and bottom panels), which was in great contrast to a positive control for cytochrome c release, cells treated with TNF-α plus cycloheximide (Fig. 4D). These data suggest that cytochrome c release was not a precursory event in UV-JEV-induced cell death and that UV-JEV may primarily trigger the target cells to undergo necrosis in a cytochrome-c- and caspase-independent manner.

**Oxidative stress induced by UV-JEV in N18 cells**

Oxidative stress has long been thought to be an important mediator causing neurotoxicity (Maher & Schubert, 2000).
To investigate whether the cytotoxicity of UV-JEV on N18 cells was mediated by oxidative stress, we tested whether NAC, a potent antioxidant, could block the cell death triggered by UV-JEV. As shown in Fig. 5(A), NAC substantially increased the numbers of viable UV-JEV-treated N18 cells in a dose-dependent manner. Rapid intracellular ROS generation, which could be suppressed by addition of NAC, was confirmed by DCF fluorescence detection in the UV-JEV-infected but not in the mock- or JEV-infected N18 cells (Fig. 5B). In addition, other antioxidants (Fig. 5C, D) such as glutathione, a major reductant found in all eukaryotic cells, MnTBAP, a mimetic form of mitochondrial Mn SOD (Faulkner et al., 1994), and catalase decreased the cytotoxicity induced by UV-JEV. Cytosolic Cu/Zn SOD (Nordberg & Arner, 2001) (50 and 200 U ml⁻¹; Fig. 5C) also suppressed UV-JEV-induced cytotoxicity, despite the fact that it was much less effective than mitochondrial Mn SOD. These results strongly suggest that UV-JEV could trigger intracellular ROS generation in N18 cells, most probably through a mitochondrial pathway.

Fig. 2. Cytotoxicity induced by UV-JEV was dependent on the cell density, cell type and cell growth conditions. (A) N18 cells, seeded at the cell densities shown on the top of each panel, were infected with UV-JEV (m.o.i. 30) for 7 h before the cell morphology was observed under phase-contrast microscopy. (B) Subconfluent N18 cells cultured in media with or without 2% FBS were infected with UV-JEV (m.o.i. 40). At 24 h p.i., the percentage of cytotoxicity was determined by LDH assay, as described in Methods. Values are shown as the mean ± SD for two independent wells. (C) Subconfluent N18 (●), NT-2 (■), BHK-21 (×) and HeLa (△) cells were infected with UV-JEV (m.o.i. 40) and LDH release was determined at the indicated time points p.i. LDH release was determined as described in Methods. Values are shown as the mean ± SD for two independent experiments.
UV-JEV induced loss of Δψm in N18 cells

Disruption of Δψm has been demonstrated to be a causative factor for the occurrence of necrosis and apoptosis, and is often associated with oxidative stress (Kowaltowski et al., 2001). As determined by DePsipher staining, which can serve as an indicator for mitochondrial activity, we found an apparent Δψm reduction following infection with UV-JEV (m.o.i. 40 for 5 h) (Fig. 6A). Furthermore, the phenomena of mitochondrial swelling and distortion could be clearly visualized by electron microscopy of the cells infected with UV-JEV (m.o.i. 40 for 5 h) (Fig. 6B). Taken together, these results illustrate that UV-JEV could cause mitochondrial damage in N18 cells, most probably through a loss of Δψm.

UV-JEV-induced NF-κB activation in N18 cells could be suppressed by anti-oxidant NAC

NF-κB is considered to be a primary sensor for oxidative stress in cells (Kamata & Hirata, 1999; Suzuki et al., 1997) and NF-κB activation has been reported in JEV-infected BHK-21 cells (Liao et al., 2001). Here, we further examined whether UV-JEV could also trigger NF-κB activation in N18 cells. Using an NF-κB-dependent luciferase reporter plasmid that was stably expressed in N18 cells, time- and dose-dependent NF-κB activation was observed in UV-JEV-infected N18 cells to levels slightly lower than those of JEV-infected cells (Fig. 7A). As revealed by Western blotting (Fig. 7B), degradation of the NF-κB inhibitor, IκB-α, became evident starting from 30 to 120 min p.i. (lanes 5, 8, 11 and 14), and such degradation of IκB-α was greatly inhibited by NAC treatment (lanes 6, 9, 12 and 15).

Moreover, NAC treatment could also block NF-κB activation in the UV-JEV-treated N18 cells as measured by luciferase activity (Fig. 7C). These results strongly suggest that UV-JEV can activate NF-κB signalling pathways through a ROS-dependent mechanism in N18 cells. To further study the role of NF-κB in UV-JEV-induced cell death, two additional NF-κB inhibitors were used, lactacystin, a specific proteasome inhibitor blocking degradation of IκB-α, and SN50, an NF-κB-specific cell-permeable peptide inhibitor, which inhibits translocation of the NF-κB active complex into the nucleus (Lin, Y. Z. et al., 1995). As shown in Fig. 8, treatment of N18 cells with either lactacystin (Fig. 8A) or SN50 (Fig. 8B) considerably reduced UV-JEV cytotoxicity. These findings imply that UV-JEV-induced NF-κB activation may play a detrimental role in neuronal N18 cells.

DISCUSSION

In viral infection, apoptotic signals can be initiated extracellularly by adsorption of virus particles to receptors on the plasma membrane. This receptor-mediated death signalling has been documented for the avian leucosis-sarcoma virus, which binds to a TNF-receptor-related protein (Brojatsch et al., 1996), and reovirus, which binds to the moiety of junction adhesion molecule and sialic acid (Barton et al., 2001; Connolly et al., 2001). Attachment of non-infectious viruses, such as reovirus (Tyler et al., 1995), vaccinia virus (Ramsey-Ewing & Moss, 1998), herpesvirus (Hanon et al., 1998) and HIV (Esser et al., 2001), to their receptors on cell surfaces may also be proapoptotic. In the present study, we have demonstrated that virions of UV-inactivated, replication-incompetent JEV were capable of inducing cell death in actively growing neuronal cells (Figs 1 and 2), most probably through a ROS-dependent (Fig. 5), NF-κB-mediated (Figs 7 and 8) pathway. Since the virions of heat-denatured JEV failed to cause neuronal cell death, and by SDS-PAGE analysis the E proteins of UV-JEV could still be detected by an anti-E mAb (data not shown), the binding between conformationally correct JEV virions and the putative cellular receptors might be important in triggering the death signalling. Alternatively, the cell killing induced by UV-JEV may be merely due to such virions possessing a large amount of free radicals generated by UV irradiation, which can directly injure the cellular proteins on the surface and/or the lipid bilayers of target cells. However, UV-JEV failed to kill the target cells cultured under growth-arrest conditions (Fig. 2B), which seems to argue against the direct-damage theory. Free radicals can be generated either by the direct cleavage of chemical bonds or by electron transfer reactions. In most biological systems the latter process seems to predominate, although the former can be significant when the protein is directly exposed to the energetic radiation of γ rays or UV (Hawkins & Davies, 2001). Since the JEV particles used in this study have been partially purified from other cellular proteins before UV inactivation and the UV-treated medium control did not exhibit any cytotoxicity to the neuronal cells, we believe this
A type of killing effect is a JEV-specific phenomenon in our experimental system. Owing to the observation that co-infection with infectious JEV could diminish UV-JEV cytotoxicity to neuronal cells (Fig. 3), we favour the supposition that specific receptor binding is required for UV-JEV to transmit a death signal to kill the target cells. Therefore, in JEV-infected cells, multiple intracellular organelles might be involved in the virus-induced pathogenesis, i.e. not only endoplasmic reticulum stress (Su, H. L. et al., 2002) but also a receptor-mediated mitochondrial death signal could be triggered.

Binding of UV-inactivated viruses to target cells has been shown to activate various cellular genes. For example, UV-inactivated virions of Human cytomegalovirus (HCMV) on human lung fibroblasts (Carlquist et al., 1999) and respiratory syncytial virus on mouse alveolar macrophages (Stadnyk et al., 1997) could stimulate the expression of interleukin-6 as efficiently as their infectious counterparts. In addition, UV-inactivated, non-replicating HSV-1 appears to trigger early and transient synthesis of alpha/beta interferon in mouse regional lymph nodes when delivered in the dermis of the ear (Riffault et al., 2000). Also, the binding of either purified live or UV-inactivated HCMV to the cell surface can quickly upregulate the expression of the cellular transcription factors Sp1 and NF-kB (Yurochko et al., 1997). In this study, we also found that the virions of UV-inactivated, non-replicating JEV were able to activate the

Fig. 4. Characteristics of UV-JEV-infected N18 cells. N18 cells infected with UV-JEV (m.o.i. 40), mock-infected and the DNase I-treated control were analysed by TUNEL assay (A) or stained with Hoechst 33258 (B) 7 h p.i. The arrowheads indicate the cells with condensed chromatin. (C) Localization of cytochrome c to mitochondria in UV-JEV-infected N18 cells. N18 cells transfected with pEYFP-Mito (Clontech) were mock-infected (top panel) or infected with UV-JEV (middle and bottom panels) (m.o.i. 30 for 5 h), stained with anti-cytochrome c antibody (red) and analysed using a confocal microscope. (D) N18 cells transfected with pEYFP-Mito (Clontech) were mock-treated (left panel) or treated with rhTNF-α (50 ng ml⁻¹) (R&D Systems) plus cycloheximide (1 μg ml⁻¹) (Sigma) (right panel) for 5 h. Images of cells under phase-contrast (a), EYFP-Mito (b) and cytochrome c (c), and merged images of (b+c) and (a+b+c) are shown.
NF-κB pathway in a ROS-dependent manner (Fig. 7). Whether UV-JEV is also capable of triggering certain cellular events such as those triggered by the viruses described above remains to be determined. Our preliminary results indicated that UV-JEV was not able to induce beta interferon production in a cultured cell system, probably due to a failure of
UV-JEV to activate interferon regulatory factor-3 phosphorylation (data not shown). The potential activation of other cytokines by UV-JEV remains elusive. Several flaviviruses have been shown to activate the NF-κB signalling cascade (Jan et al., 2000; Kesson & King, 2001; Liao et al., 2001; Marianneau et al., 1997), which probably plays a crucial role in the life cycle of flaviviruses. Both infectious JEV and UV-JEV activated NF-κB (Fig. 7A); however, infectious JEV did not induce early cell death whereas UV-JEV did, suggesting that distinct downstream NF-κB-dependent genes might be activated by JEV and UV-JEV. However, which NF-κB-regulated gene(s) in the target cells is/are responsible for UV-JEV cytotoxicity remains to be determined.

UV-JEV-induced cellular death occurs quickly after the virus encounters the receptors on the cell surface, much faster than the way infectious JEV can kill its infected targets (Liao et al., 1997). On the other hand, co-infection with infectious JEV was found to attenuate UV-JEV cytotoxicity on neuronal cells (Fig. 3). Competition between live and killed JEV for the cell-surface receptors that transmit the death signal could be a possible mechanism for this phenomenon. Alternatively, this observation suggests that replicating JEV may trigger not only cell-death but also cell-survival signals, as similar observations have been reported for other viruses (Roulston et al., 1999; Tschopp et al., 1998). Since non-replicating UV-JEV (Fig. 5) could induce stronger oxidative stress in neuronal cells than infectious JEV, co-infection of JEV (Fig. 3) may create a reducing state in infected cells to suppress UV-JEV cytotoxicity. In fact, it has been shown that influenza virus infection can robustly enhance expression of metallothioneins, which appeared to maintain the intracellular redox balance (Ghoshal et al., 2001). It would be of interest to know whether JEV replication can suppress UV-JEV-induced CPEs by modulation of the intracellular redox pathways.

Although cell proliferation and cell death are two seemingly opposing processes, accumulating evidence now indicates that the two are closely linked under normal circumstances (Evan & Littlewood, 1998). The previous reports of impaired generation of ROS (Pani et al., 2000) and lower activation of the stress-activated protein kinases (Lallemand et al., 1998) in confluent cells strongly indicate a link between redox changes and cell growth. Our data (Fig. 2), which show that only actively growing cells are vulnerable to killing by UV-JEV-triggered ROS, might also reflect a link between redox status and cell growth. The reason why only
the neuronal and not other cell types were killed by UV-JEV (Fig. 2) might reflect the fact that the oxygen metabolism rate in brain cells is much higher than that of average tissues (Maher & Schubert, 2000).

Overall, in this study, we show that UV-JEV can kill actively growing neuronal cells by triggering a ROS-dependent, partly NF-κB-mediated pathway. Although it involves mitochondrial injury, this killing is a cytochrome-c- and caspase-independent cell-death process. Taken together, our results seem to suggest that the interaction between the JEV virion and its cell-surface receptor probably triggers a killing process as a host defence mechanism to restrict virus replication, whereas replicating JEV can somewhat modify the cellular environment to overcome the cytotoxicity induced by this receptor engagement and make it suitable for timely productive replication before the target cells inevitably undergo apoptosis through various mechanisms.

**Fig. 7.** The NF-κB activation induced by UV-JEV was blocked by antioxidant NAC in N18 cells. (A) N18 cells transfected with a NF-κB–luciferase reporter plasmid were infected with various amounts of JEV or UV-JEV. At the indicated time points, levels of luciferase activity were determined in cell lysates. Values are shown as means ± SD for two independent wells. (B) The degradation of IκB-α in UV-JEV-infected N18 cells, with or without the addition of NAC (1 mM), was examined by Western blot analysis using an IκB-α-specific mAb. (C) Stable NF-κB–luciferase N18 transfected cells were infected with UV-JEV (m.o.i. 40) in the absence or presence of NAC (1 mM) for 2, 4 or 6 h, before NF-κB activation levels were determined by measuring luciferase activity. Values are shown as means ± SD for two independent experiments. White bars, mock-infected; grey bars, UV-JEV; black bars, UV-JEV + NAC.
ACKNOWLEDGEMENTS

We thank the indispensable help of Dr A. L. Huang at the Electron Microscopy Core Laboratory, IBMS, Academia Sinica. Y.-L. L. was supported by grants from the National Health Research Institute (NHRI-CN-CL8903P) and the National Science Council (NSC 90-2320-B-001-061), and Academia Sinica, Taiwan, Republic of China. C.-L. L. was supported by grants from the NSC (NSC 89-2323-B-016-001 and 89-2320-B-016-089) and the NHRI (NHRI-CN-CL8902P).

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Fig. 8. NF-κB inhibitors, lactacystin and SN50 reduced the cytotoxicity of UV-JEV in N18 cells. N18 cells were mock-infected or infected with UV-JEV (m.o.i. 40) in the absence or presence of (A) lactacystin (Lac.; 50 and 200 μM) or (B) SN50 (50 μg ml⁻¹). At 24 h.p.i., the percentage of cytotoxicity was determined by LDH assay as described in Methods. Values are shown as means ± SD for two independent wells.
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