Susceptibility of mouse primary cortical neuronal cells to coxsackievirus B

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Coxsackievirus B (CVB) is often associated with aseptic meningitis and encephalitis, but the six serotypes of CVB vary in their relative disease severity. To elucidate the detailed mechanisms of CVB-induced cytopathological effects, the morphological and biochemical characteristics caused by the CVB serotypes in mouse primary cortical neuronal cells were investigated. By 24 h post-infection, all CVB serotypes except CVB2 induced severe cytotoxic alterations, including a loss of neurites. Both fluorescence and transmission electron microscopy revealed CVB-induced morphological changes indicative of apoptosis, including heavily condensed nuclei, subsequent chromatin condensation into the periphery of the nuclei and oligonucleosomal DNA fragmentation. It was also found that infection with all six CVB serotypes led to productive virus replication, which was completed prior to an apoptotic signal. The caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone significantly inhibited nuclear changes associated with virus-induced apoptosis, but had less effect on virus-associated cytopathic effects and no effect on virus production. In contrast, the transcription inhibitor actinomycin D profoundly inhibited all three virus-induced events. Taken together, these findings demonstrate that all six CVB serotypes can efficiently replicate in mouse cortical neuronal cells and that productive replication of these CVBs, except for CVB2, induces multiple cytopathological effects, including apoptotic alterations.

INTRODUCTION

Many types of human viruses can infect and replicate in neuronal cells and are closely associated with various neuronal diseases, including rabies, encephalitis and meningitis (Griffin & Hardwick, 1999; Johnson, 1998a). Coxsackievirus B (CVB), a member of the non-polio enterovirus family, is a major cause of aseptic meningitis and encephalitis, even in immunocompetent populations (Johnson, 1998b; Zaoutis & Klein, 1998). The CVB genome consists of a single-stranded positive polyadenylated RNA. CVB has been classified into six serotypes, CVB1–6 (Rueckert, 1996), each of which is associated with different infection rates and disease severity (Muir, 1993; Nigrovic, 2001; Roivainen, 1999; Rotbart, 1995a). In particular, CVB3, -4 and -5 are often associated with neuronal disease, whereas the others are not (Rotbart, 1995a, b).

We previously found that CVB infection of permissive Vero cells led to productive virus replication, different types of cell death and eventual lysis of the infected cells, facilitating the dissemination of progeny virus (Ahn et al., 2003). In addition, we observed that CVB4 induced rapid death of rat primary neuronal cells, indicating the occurrence of apoptotic alterations by 24 h post-infection (p.i.) (Joo et al., 2002). Other studies have also suggested that neuronal cell damage resulting from viral infection can involve apoptosis (Griffin & Hardwick, 1999; White, 1996). Moreover, apoptotic cell death has been shown to be important in the pathogenesis of human viral diseases (Allsopp & Fazakerley, 2000; Allsopp et al., 1998). For example, infected neuronal cells show a number of morphological and biochemical changes indicative of apoptosis, including compaction of chromatin, oligonucleosomal DNA degradation and fragmentation into membrane-bound bodies (Schwartzman & Cidlowski, 1993). Caspases, a family of cysteine-dependent aspartate-directed proteases, catalyse the proteolysis of substrates involved in the cell death pathway (Cryns & Yuan, 1998; Earnshaw et al., 1999).

The detailed nature of CVB-induced cytopathogenesis and the preferential association of certain CVB serotypes with neural disease and disease severity are still poorly understood. To gain insight into the mechanism of cytopathic effects (CPE) resulting from the infection of neuronal cells with CVB, we investigated various cellular alterations resulting from the infection of primary mouse cortical neuronal cells with each of the six CVB serotypes. In

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addition to determining differential virus replication and the morphological and biochemical alterations resulting from infection, we assayed the effects of the caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD.fmkn) and the macromolecule synthesis inhibitor actinomycin D (Act D) on infected cells.

**METHODS**

**Cell cultures and viruses.** Vero cells were purchased from the ATCC and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 IU ml−1) and streptomycin (50 μg ml−1) (Gibco-BRL). Primary cultures of mouse cortical neurons were prepared from 16-day-old BALB/c embryos, as described previously (Joo et al., 2002). Briefly, cerebral cortices were dissected in calcium- and magnesium-free buffered Hank’s balanced salt solution and incubated with 0.125% trypsin for 10 min at 37°C. After inactivating the trypsin with neurobasal medium containing 20% FBS, further dissociation of cortical tissue was completed with multiple triturations using Pasteur pipettes of decreasing pore size. The dissociated cells were diluted in neurobasal medium supplemented with B27 components, plated on cell culture plates coated with poly-D-lysine (50 μg ml−1; Sigma) and maintained at 37°C in a 5% CO2 incubator.

CVB serotypes CVB1 (VR-687), CVB2 (VR-29), CVB3 (VR-30), CVB4 (VR-184), CVB5 (VR-1036) and CVB6 (VR-1037) were purchased from the ATCC and propagated and titrated by conventional methods in Vero cells as described previously (Minor, 1996). For one-step virus growth, the neuronal cells were infected at an m.o.i. of 1 for 1 h. The virus inocula were thoroughly washed several times and the media and cells were harvested simultaneously. Production of progeny virus was estimated by plaque assay in Vero cells.

Z-VAD.fmkn (Enzyme System Products) was maintained as a stock solution of 50 mM in DMSO and added to the cells at a final concentration of 100 μM. Act D, maintained as a stock solution of 10 mg ml−1 in DMSO, was added to the cells at a final concentration of 0-1 μg ml−1.

**Low molecular mass DNA fragmentation analysis.** Degradation of oligonucleosomal DNA from the nucleus and cytoplasm was analysed as described previously (Saecki et al., 1997) with minor modifications. Briefly, cells in a 10 cm dish were harvested and treated with 350 μl lysis buffer (10 mM Tris/HCl, pH 8-0, 0.6% SDS, 0.1% EDTA) for 10 min at room temperature. To each lysate, 21 μl 5 M NaCl was added and the samples were incubated at 4°C for at least 8 h and centrifuged at 15000 r.p.m. at 4°C for 20 min. Each supernatant was treated with 10 μg heat-inactivated RNase A ml−1 at 45°C for 90 min followed by 200 μg proteinase K ml−1 at 55°C for 60 min. Nuclear DNA was recovered by phenol extraction and ethanol precipitation. One-third of each DNA sample was used on a 1-5% agarose gel in TAE solution and the DNA fragmentation pattern was examined by ethidium bromide staining.

**Light, fluorescence and transmission electron microscopy.** Cytopathic effects were examined by light microscopy (LM) and the pattern of nuclear condensation was examined by fluorescence microscopy (FM). Cells in 48-well plates were incubated for 30 min with the membrane-permeable fluorescent dye Hoechst 3342 (Molecular Probe; maintained as a stock solution of 10 mg ml−1 in dH2O), at a final concentration of 5 μg ml−1.

For transmission electron microscopy (TEM), neuronal cells were cultured on glass plates pre-coated with poly-l-lysine and fixed overnight in 4% glutaraldehyde at 4°C. The cells were washed three times with 0.2 M cacodylate buffer (pH 7.2), post-fixed with 2% osmium tetroxide for 1 h at room temperature and again washed three times in cacodylate buffer. The cells were then embedded in Epon-Araldite epoxy mixture No. 1 at 70°C for 2 days. Ultrathin sections were prepared using a Sorvall MT3000 microtome and collected on 150-mesh copper grids. Sections were stained with 1% uranyl acetate and/or lead citrate and photographed in a JEOL 100CX transmission electron microscope.

**RESULTS**

We first examined CPE in neuronal cells following infection with the six CVB serotypes. On day 3 of primary culture, mouse cortical neuronal cells were infected at an m.o.i. of 1. By LM, mock-infected cells were characterized by round phase-bright cell bodies with smooth contours and many neurites. In contrast, by 24 h p.i., cells infected with most of the CVB serotypes experienced significant morphological alterations, including severe loss of neurites and/or cytoplasmic blebbing. However, cells infected with CVB2 did not show any obvious morphological changes (Fig. 1). To quantify the degree of cell damage, we estimated cell death using the lactate dehydrogenase assay, which measures cytoplasmic enzyme activity released into the culture medium (Mosmann, 1983). As expected, we consistently observed significant loss of living cells by 24 h p.i. (53.0±20.3% dead cells) after infection with CVB1, -3, -4, -5 or -6, which closely reflected the degree of morphological aberration (data not shown). In contrast, CVB2-infected cells experienced only 25.0±19.0% cell death, similar to that of mock-infected cells.

To determine the involvement of apoptosis in the death of CVB-infected cells, we analysed their nuclear morphology after incubation with the membrane-permeable DNA-binding dye Hoechst 33342 at a concentration of 1 μg ml−1. By FM, we found that the nuclei of CVB-infected cells, except those infected with CVB2, were heavily condensed and ultimate chromatin distortion occurred within 24 h p.i. (Fig. 2A). To confirm that these nuclear changes were due to apoptosis, we analysed the appearance of low molecular mass DNA fragments following CVB infection. As expected, no sign of DNA laddering occurred in mock-infected primary neuronal cells. In cultures infected with CVB1, -3, -4, -5 or -6, distinct DNA laddering, manifesting as mono- (180–200 bp), di- and multineucleosome fragments, was clearly observed as early as 12 h and became dramatically detectable by 24 h p.i. (Fig. 2B), indicating that these CVB-infected neuronal cells had undergone apoptotic cell death.

CVB replication in neuronal cells was investigated by a one-step growth curve assay, in which cells were infected at an m.o.i. of 1 for 1 h. Progeny virus production was visualized by TEM and quantified by plaque assay. For CVB5, progeny viruses were first detectable at 3 h p.i. and continued to be amplified until reaching a plateau at about 6 h p.i. (data not shown). A similar time course was observed for all the other CVB serotypes, including CVB2. Under TEM, we
Fig. 1. Cytotoxicity in primary cortical neuronal cells following CVB infection. Cells were infected with CVB serotypes 1–6 at an m.o.i. of 1 for 24 h. Photographs of the infected cells were taken by LM at a magnification of 200×. The infected cells experienced profound morphological alterations, including the breakdown of damaged neurites, except for those infected with CVB2. MI, mock-infected cells.
Fig. 2. Alterations in nuclear morphology of infected neuronal cells. Cells were infected with CVB serotypes 1–6 at an m.o.i. of 1 for 24 h. (A) Nuclear changes of infected cells monitored with the membrane-permeable DNA probe Hoechst 33342 were assessed by FM. Infection with five of the CVB serotypes induced significant nuclear condensation (arrows), whereas CVB2-infected cells did not show any nuclear changes. (B) DNA fragmentation analysis by ethidium bromide staining following agarose gel electrophoresis. The appearance of low molecular mass nuclear DNA was identified in all infected cells except those infected with CVB2.
Fig. 3. Progeny virus production in infected neuronal cells. (A) TEM of CVB5-infected cells at an m.o.i. of 1. Neuronal cells were cultured on glass plates pre-coated with poly-L-lysine. Within 12–24 h p.i., mature progeny virus particles were observed as crystalline shapes in the cytoplasm. (B) Total infectious progeny virus production from cells infected with each CVB serotype. Culture media and cells were collected and plaque assays were performed. All infected cells productively generated progeny viruses. MI, mock-infected cells.

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<tr>
<th>Virus</th>
<th>Titre ($\times 10^6$ p.f.u. ml$^{-1}$)</th>
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<tr>
<td>CVB1</td>
<td>4.6 ± 3.1</td>
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<tr>
<td>CVB2</td>
<td>0.7 ± 0.3</td>
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<tr>
<td>CVB3</td>
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<td>CVB4</td>
<td>1.5 ± 1.3</td>
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<tr>
<td>CVB5</td>
<td>37.7 ± 23.5</td>
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<td>CVB6</td>
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Fig. 4. Effect of Z-VAD.fmk and Act D on CVB infection. Cells were infected with CVB serotypes 1–6 at an m.o.i. of 1 in the absence or presence of Z-VAD.fmk or Act D. (A) Lack of nuclear condensation in infected cells. (B) Effect on the ultrastructure of infected cells. (C, D) Infectious virus production (C) and the single growth curve of CVB5 in infected cells (D) in the absence/presence of Z-VAD.fmk or Act D. Act D particularly inhibited the ultrastructural alterations induced by virus infection and progeny virus production. MT, Mock-treated cells.
consistently observed active production of groups of CVB progeny virus as ladder- or honeycomb-shaped crystalline structures, exclusively in the cytoplasm, as well as vesicular structures corresponding to virus replication complexes (Fig. 3A) (Roivainen et al., 2000). Virus production at 24 h p.i. was estimated as $4.6 \pm 3.1 \times 10^6$, $0.7 \pm 0.3 \times 10^5$, $1.7 \pm 2.1 \times 10^6$, $1.5 \pm 1.3 \times 10^5$, $37.7 \pm 23.5 \times 10^8$, and $1.7 \pm 1.8 \times 10^6$ p.f.u. ml$^{-1}$ for the six CVB serotypes 1–6, respectively (Fig. 3B).

To evaluate the effects of caspase inhibition or inhibition of RNA transcription on virus production and CPE, we infected the cells in the presence or absence of Z-VAD.fmk or Act D. At a concentration of 100 μM, the irreversible and cell-permeable pan-caspase inhibitor Z-VAD.fmk has been shown to inhibit caspase activity completely in cultured mammalian cells with no sign of cytotoxicity (Slee et al., 1996). In addition, a low concentration of Act D (0.1 μg ml$^{-1}$) has been used as an inhibitor of apoptosis (Joo et al., 2002). We found that treatment of primary neuronal cells with Z-VAD.fmk or Act D significantly inhibited CVB-induced nuclear condensation (Fig. 4A) and ultrastructural changes (Fig. 4B). We found, however, by LM that infected cells still showed signs of cytotoxicity in the presence of Z-VAD.fmk, but not Act D. In the absence of Z-VAD.fmk or Act D, TEM showed that the chromatin of the infected cells was heavily condensed and collapsed into the peripheral of the nuclei, reflecting the nuclear pattern shown by Hoechst staining. In addition, the mitochondria of infected cells were heavily stained and the cytoplasmic suborganelles were disorganized, with no intact dendrites. There were no obvious signs of disruption of either nuclear envelopes or plasma membranes. These ultrastructural abnormalities were significantly reduced when the cells were treated with Z-VAD.fmk or Act D. Act D-treated cells showed the most dramatic inhibition of ultrastructural changes, which was almost indistinguishable from mock-infected cells. Z-VAD.fmk-treated cells also experienced significantly reduced morphological changes. However, most cytoplasmic suborganelles and neurites were distorted. While virus production was not altered by Z-VAD.fmk treatment ($7.6 \times 10^6$ p.f.u. ml$^{-1}$), Act D treatment profoundly reduced all virus production by CVB (Fig. 4C and D). The amount of CVB5 progeny virus produced in the presence of Act D was only $1.6 \times 10^4$ p.f.u. ml$^{-1}$, compared with that of CVB5-infected cells ($9.8 \times 10^6$ p.f.u. ml$^{-1}$). Taken together, these results suggest that the caspase inhibitor Z-VAD.fmk reduced apoptotic cell death, but not virus production or cytotoxicity, whereas the macromolecule synthesis blocker Act D was able to block all of these activities in infected neuronal cells.

**DISCUSSION**

We have shown here that five of the six CVB serotypes induced severe cytotoxic effects, along with productive virus replication, in mouse primary neuronal cells. The pattern of virus growth was similar to that observed in permissive Vero cells, although the titre of infectious virus was slightly lower than that observed in infected Vero cells (Ahn et al., 2003). CVB-infected neuronal cells showed apoptotic characteristics, identified by nuclear staining patterns, DNA fragmentation and ultrastructural analysis. Apoptosis has been identified as an important host defence mechanism, both for eliminating infected cells and for interfering with virus replication (Hay & Kannourakis, 2002; Roulston et al., 1999). Many RNA viruses, such as vesicular stomatitis virus, escape these detrimental effects by completing virus replication prior to the onset of apoptosis (Gadaleta et al., 2002; Koyama et al., 2000; Kurokawa et al., 1999). Similar to other RNA viruses, our data indicated that, in neuronal cells, active CVB replication preceded CVB-induced apoptotic events.

Virus-induced apoptosis, however, may have detrimental effects on the host. For example, in susceptible cells, poliovirus (PV)-induced apoptotic effects may be responsible for virus pathogenesis, which is directly associated with viral load (Couderc et al., 2002; Girard et al., 1999). Using cells from transgenic mice expressing the PV receptor, it has been shown that, following PV treatment, virus replication and apoptotic cell death are co-localized in both primary neuronal cells and neurons of spinal cords from paralysed mice.

Since both TEM and Hoechst staining of CVB-infected cells in the presence of Z-VAD.fmk showed substantial inhibition of DNA condensation and nuclear fragmentation, we hypothesized that caspsases may be involved in CVB-induced apoptosis in primary cortical neuronal cells. When cells were treated with the caspase inhibitor Z-VAD.fmk, we observed no inhibition of virus production or cell death (data not shown). Previous studies suggested that Z-VAD.fmk treatment also did not inhibit cytopathic changes in CVB-infected Vero cells or Hela cells, although caspase activation and cleavage of substrates were substantially inhibited (Ahn et al., 2003; Carthy et al., 1998). Using PV-infected Hela cells, it was shown that 100 μM Z-VAD.fmk affected apoptosis, but had no effect on virus growth or cellular pathological changes, suggesting that these cells undergo two types of cell death (Agol et al., 1998, 2000). The findings presented here suggest that interference with the caspase-dependent apoptotic phenotype using Z-VAD.fmk did not completely prohibit the cytotoxic effects of CVB on infected neuronal cells and are in good agreement with previous results.

In contrast to Z-VAD.fmk, Act D treatment dramatically prevented both cytopathic effects and virus replication. Act D has been widely utilized as an RNA synthesis inhibitor, which acts by incorporating into double-stranded DNA via deoxyguanosine residues. Act D can also be incorporated into double-stranded RNA, such as yeast rRNA (D’Arcy, 1983). In this regard, the findings presented here suggest that novel host RNA synthesis is essential for active virus
production and that productive virus infection might be necessary to induce CPE.

Several epidemiological studies have shown that, in patients with neural disease, the most frequently observed CVB serotypes are CVB3, -4 and -5, with CVB1, -2 and -6 observed less frequently (Draganescu et al., 1980; Rotbart, 1995b; Rotbart & Romero, 1995; Xie & Xiang, 2000). Our results, showing that all six CVB serotypes can actively grow in mouse primary neuronal cells, seem to be at odds with these clinical findings. The clinical results may simply reflect the incidence rate of each serotype in the population. It may also indicate, however, that cytopathogenesis ex vivo cannot fully explain CVB pathogenicity in vivo. Our results with CVB2, which did not show any obvious cytoxicity, even during active virus production, may indicate that productive virus replication is not sufficient for CPE and that an unidentified viral or cellular factor is needed to trigger cell toxicity.

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REFERENCES


