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Effect of Enforced Expression of Human *bcl-2* on Japanese Encephalitis Virus-Induced Apoptosis in Cultured Cells

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Infection by Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, causes acute encephalitis in humans and induces severe cytopathic effects in different types of cultured cells. This study attempted to determine whether apoptosis contributes to virus-induced cell death in a culture system by characterizing JEV lytic infection in baby hamster kidney BHK-21 cells, murine neuroblastoma N18 cells, and human neuronal progenitor NT2 cells. According to our results, the replication of JEV, and not the UV-inactivated virions per se, triggered apoptosis in these cell lines, as evidenced by nuclear condensation, DNA fragmentation ladder, and in situ end labeling of DNA strand breaks with terminal transferase (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling assay). Different strains of JEV, regardless of whether they are neurovirulent to mice, could induce apoptosis of the infected cells. In addition, enforced expression of the human protooncogene *bcl-2* in BHK-21 cells, which did not influence virus production, appeared to delay the process of JEV-induced apoptosis, despite the fact that most infected cells were inevitably killed after prolonged cultures. However, Bcl-2 proteins expressed in N18 cells failed to block JEV-induced apoptosis, although they did prevent Sindbis virus-induced apoptosis from occurring in the same cells. This finding suggests that these two viruses may utilize similar but not identical mechanisms to kill their infected cells. The results presented here thus demonstrate that apoptosis can be a general mechanism for JEV-induced cell death and that enforced *bcl-2* expression may be inadequate in protecting all cell types from JEV-induced apoptosis in cell cultures.

Japanese encephalitis virus (JEV), a member of the *Flaviviridae*, is a mosquito-borne positive-sense RNA virus. JEV infection can cause acute encephalitis in humans, frequently resulting in a high mortality rate, which is particularly prevalent in some East Asian countries (7, 54, 55). The natural life cycle of JEV involves complex relationships among insect vectors, vertebrate reservoirs, and human subjects, illustrating the important characteristic of a broad host range for JEV infection (reviewed in 8). Similar to other mosquito-borne viruses in this family, JEV is transmitted to humans through persistently infected mosquito vectors. Vertebrate animals, such as swine and avians, may serve as viremia-amplifying reservoirs deemed necessary for maintaining JEV in transmission cycle. However, humans are a dead-end host for JEV because only an insignificant viremic phase can occur during the infection periods, thereby hindering the transmission route from humans to mosquitoes (6).

In humans, the primary sites for JEV multiplication are likely either in myeloid and lymphoid cells or in vascular endothelial cells (33). However, how JEV penetrates the blood-brain barrier to infect the central nervous system (CNS) remains unclear. Several lines of evidence suggest that the principal target cells for JEV in the CNS are neurons, and as a result of infection, massive destruction of neurons should be responsible for the disease manifestation of encephalitis. In fact, in a fatal human case, JEV antigens have been localized mainly in neurons but not in the neighboring glial cells (28), and the major pathological changes were primarily observed in the brain's gray matter, where the neurons in the thalamus and brain stem appeared to be particularly vulnerable to JEV infection (reviewed in references 24 and 36). Additionally, a wide variety of primary and continuous cell cultures from different origins, e.g., monkey, hamster, porcine, chicken, and mosquito, can support the productive growth of JEV. Among them, due to apparent cytopathic effects (CPE) induced by JEV infection, Vero, LLC-MK2 (monkey kidneys), and BHK-21 (baby hamster kidney) cells are frequently used for virus titration by plaque assays (49). Nevertheless, why JEV infection is selectively cytolytic to certain cells but is nonlytic to others and by what exact mechanism JEV induces the death of infected cells remain largely unknown.

Cell death can be categorized by mechanism into necrosis, apoptosis (2, 56), and a combined type of the two (14). Recently, apoptosis has become widely recognized as a physiological suicide process with common features in many different cell types. One of the biological reasons for cells to undergo apoptosis is to exclude superfluous or harmful cells so that multicellular organisms can tightly control the homeostasis of cell numbers during embryogenesis, immune system development, aging, and tumor regression (reviewed in references 16 and 42). On the other hand, diverse stimuli, e.g., toxic substances, hormones, cytokines, ionizing irradiation, and microbial infections, may trigger normal cells to undergo apoptosis, frequently resulting in specific disease situations. Numerous DNA (21, 27, 48) and RNA (17, 23, 26, 30, 31, 41, 50–53) viruses have been demonstrated to induce apoptosis of the

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infected cells. Conceivably, to restrict virus replication and spread, the host may benefit by undergoing apoptosis to eliminate any infected cells, especially during the early stage of virus infection. Conversely, to overcome this kind of cellular defense system, some DNA viruses (5, 13, 20, 25, 37, 44) have evolved antiapoptotic gene products that prolong the infected cell's life span, thereby facilitating viral replication and spread. In contrast, no RNA viruses have been found to encode genes that inhibit the cellular suicide pathway, presumably due to scant capacity of the viral genomes, which are unable to accommodate such genes.

Human *bcl-2* was the first cellular gene recognized to be capable of blocking certain forms of apoptosis, which is functionally similar to viral versions of apoptotic repressors. A number of Bcl-2 homologs were subsequently also identified in humans, mice, birds, and nematodes, as well as in particular viral systems (reviewed in reference 45). Thus, the evolutionary conservation of the Bcl-2 homologs, both structurally and functionally, reiterates the vital role of apoptosis in all complex organisms. Studies involving Sindbis virus (31) and influenza virus (23, 39) have indicated that constitutive *bcl-2* expression could prevent the infected cells from undergoing apoptosis and consequently cause the cells to be persistently infected. These results suggest that *bcl-2* plays a prominent role in determining whether a cytopathic RNA virus can trigger apoptosis of the infected cells. In this study, we demonstrate that infection by JEV, irrespective of whether it is neurovirulent, could induce apoptosis in several neuronal and nonneuronal cell lines and that enforced *bcl-2* expression in nonneuronal BHK-21 cells, but not in murine neuroblastoma N18 cells, delayed the apoptotic process. Our results not only suggest that apoptosis is a general mechanism by which JEV kills its infected cells but also reinforce the notion that the ability of *bcl-2* to prevent virus-induced apoptosis is not universal.

MATERIALS AND METHODS

Viruses and cell lines. A Taiwanese local JEV strain, NT109, isolated from infected *Culex tritaeniorhynchus* mosquitoes in 1985, and a pair of virulent (RP-9) and attenuated (RP-2ms) JEV mutant strains, generated from NT109 by gamma irradiation (10), were employed throughout this study. In some experiments Sindbis virus (Ar-339; American Type Culture Collection) was used to infect the target cells. N18, a mouse neuroblastoma cell line (1), was grown in RPMI 1640 medium containing 10% fetal calf serum (FCS) (GIBCO). NT-2 (ATCC CRL-1973), a human neuronal precursor cell line, was cultured in Opti-MEM (GIBCO) supplemented with 10% FCS. The propagation of virus was carried out in BHK-21 cells with RPMI 1640 medium containing 2% FCS. Virus titers were determined by plaque formation assay on BHK-21 cells.

Virus infection and titration. To infect cells with JEV, monolayers of the indicated cell lines grown in 6- or 12-well plates were initially adsorbed with JEV at multiplicity of infection (MOI) of 5 for 1 hr at 37°C. After adsorption, the unbound viruses were removed by three gentle washings with serum-free RPMI 1640 medium. Fresh medium containing 2% FCS was added to each plate for further incubation at 37°C. At the end of infection, the culture media were harvested for plaque formation assay to determine virus titers. Briefly, a virus dilution was added to 80% confluent BHK-21 cells and incubated at 37°C for 1 h. After adsorption, the cells were washed and overlaid with 1% agarose (SeaPlaque; FMC BioProducts) containing RPMI 1640 with 1% FCS. After incubation for 4 days, the resulting cells were fixed with 10% formaldehyde and stained with 0.5% crystal violet for plaque counting. Virus titers were denoted as PFU per milliliter.

Viral one-step growth curve. BHK-21 and N18 cells (2×10^6) were infected with viruses at an MOI of 5. After 1 h of adsorption at 37°C, the unbound virus particles were removed from cells by three washes with phosphate-buffered saline. The infected cells were then grown in RPMI 1640 medium supplemented with 2% FCS at 37°C. At various times postinfection (p.i.), the culture supernatants of infected cells were collected and clarified by centrifugation. Next, the virus titers in supernatants were determined by plaque assay on BHK-21 cells. The burst size (PFU per cell) of viruses was determined as the total numbers (PFU) of viable viruses released in supernatants divided by the number of infected cells.

PI staining of cells. The cell monolayers were fixed with 75% ethanol at 4°C for 1 h, treated with RNase (0.5 mg/ml) and propidium iodide (PI) (50 μg/ml) for 15 min at room temperature (RT), and visualized by fluorescence microscopy (Fluovert, FU; Leitz).

TUNEL assay. Apoptosis-induced DNA strand breaks were end labeled with dUTP by use of terminal deoxynucleotidyltransferase (TdT) with a commercial kit (In Situ Cell Death Detection kit, alkaline phosphatase; Boehringer Mannheim) according to the manufacturer's instructions. Briefly, the cells were fixed with paraformaldehyde solution (4% in phosphate-buffered saline, pH 7.4) for 30 min at RT and permeabilized in 0.1% Triton X-100–0.1% sodium citrate for 2 min on ice. (The TdT-mediated dUTP-biotin nick end labeling (TUNEL) reaction was performed by using fluorescein isothiocyanate-dUTP at 37°C for 60 min; the labeling was then analyzed under a Leitz fluorescence microscope. In some experiments, the signals were further converted by alkaline phosphotase conjugated with antifluorescein antibody in the presence of a chromogenic substrate and then analyzed under a Leitz light microscope.

Electron microscopy. The JEV- or mock-infected cells were collected as cell pellets at 48 h p.i., fixed in 2.5% glutaraldehyde containing 0.1 M cacodylate buffer (pH 7.4) for 60 min, and then washed overnight in the same buffer. The cell pellets were then stained en block with uranyl acetate, postfixed with osmium, dehydrated with graded ethanol, and then embedded in Eponate-12 resin. Thin sections were double stained with uranyl acetate and lead citrate and examined under a Zeiss 900 electron microscope.

Cellular DNA fragmentation ELISA. The levels of apoptotic cell death were measured by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) by using a commercial kit (Cellular DNA Fragmentation ELISA; Boehringer Mannheim). Cells were labeled with bromodeoxyuridine (BrdU) overnight prior to virus infection. At various time points p.i., cells were permeabilized to release the cytoplasmic DNA fragments into the supernatant. The amounts of BrdU-labeled DNA released were measured with an ELISA reader (Microplate reader; Molecular Devices) by using antibodies against DNA and BrdU.

Establishment of cell clones permanently expressing Bcl-2. BHK-21 and N18 cells were transfected by use of Lipofectamine (Bethesda Research Laboratories) with human *bcl-2* expression plasmid pZipBcl-2 or a vector control, pZipneo (31). The transfected cells were selected and cloned in the presence of Geneticin (GIBCO). The expression of *bcl-2* in cell clones was assessed by Western blotting and indirect immunofluorescence assay, using an antibody specific for the human Bcl-2 protein (Santa Cruz).

LDH assay. Cell viability was assessed by the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) by using a commercial kit (Cytotoxicity Detection Kit; Boehringer Mannheim) according to the manufacturer's instructions. Briefly, the culture supernatants from cell samples were clarified by centrifugation, mixed with reaction mixture {diaphorase-$NADH^+$, tetrazolium salt INT [2-(4-iodophenyl)-6-(4-nitrophenyl)-3-phenyltetrazolium chloride]-sodium lactate}, and incubated at RT for about 30 min, and the results were then read with an ELISA reader (Microplate reader; Molecular Devices) at 490 nm.

DNA fragmentation assay. Low-molecular-weight DNA was extracted from apoptotic cells according to a published method (43). Briefly, cell suspensions in Hanks buffered salt solution were incubated with 70% ethanol for 24 h at −20°C. The resulting cells were centrifuged to removed ethanol, and the cell pellets were resuspended and incubated in 40 μl of PC buffer (192 mM Na_2HPO_4, 4 mM citric acid, pH 7.8) at RT for 30 min. After centrifugation at $1,000 \times g$ for 5 min, the supernatants were collected and vacuum concentrated in new microcentrifuge tubes with a SpeedVac for 15 min. Three microliters of Nonidet P-40 solution (0.25%) and 3 μl of RNase A solution (1 mg/ml) were then added and incubated at 37°C for 30 min. After incubation, 3 μl of proteinase K solution (1 mg/ml) was added and further incubated at 37°C for 30 min. The resulting DNA-containing extracts were then analyzed by 2% agarose gel electrophoresis in 1× Tris-borate-EDTA buffer with ethidium bromide staining.

RESULTS

One-step growth curves of JEV in cell lines. Our previous studies have demonstrated that JEV infection appears to be cytolytic in nonneuronal BHK-21 cells as well as in murine neuroblastoma N18 cells (9). To ascertain whether these cells could support JEV productive replication, a neurovirulent JEV strain, RP-9 (10), was utilized to infect BHK-21 and N18 cells; the kinetics of JEV replication (at an MOI of 5) in the two cells were determined by one-step growth curves. For both types of RP-9-infected cells, new virus was first detected in supernatants between 8 and 12 h p.i., and the virus titers peaked at approximately 24 h p.i. (10) (see Fig. 4B and 6B). Starting from 30 h p.i., CPE in the infected cells became apparent (not shown), concurrent with the periods in which the infected cells actively produced large quantities of the virus released in culture media. At 32 p.i., the burst sizes in JEV-infected BHK-21 and N18 cells were 26 and 22.5 PFU/cell,

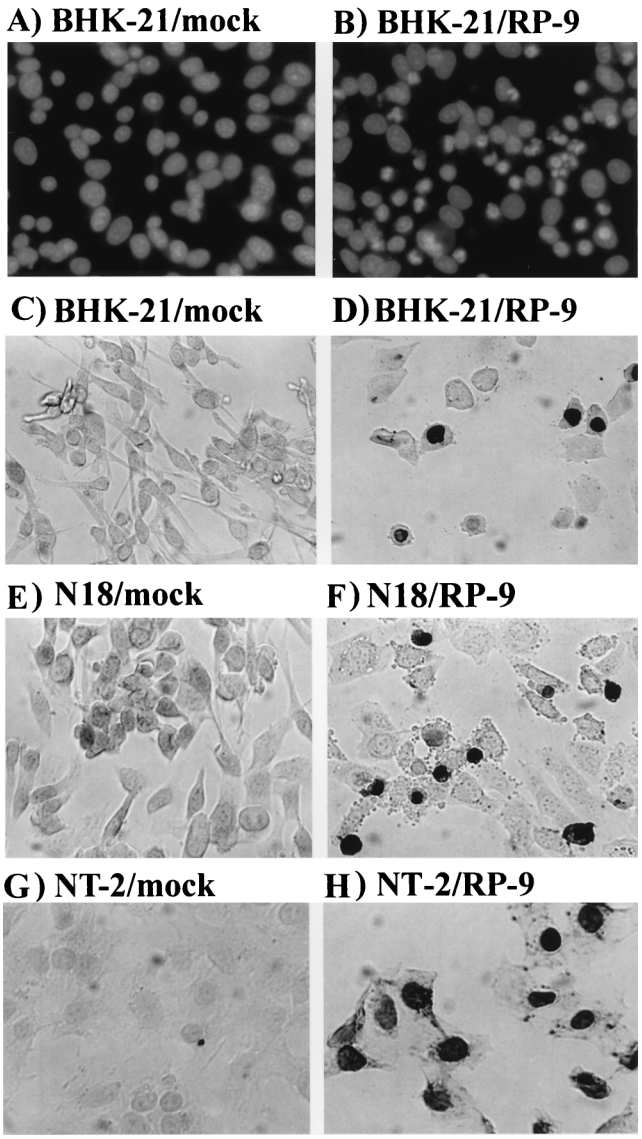

FIG. 1. Morphological analysis of cell death of JEV-infected cells. (A and B) PI staining of BHK-21 cells which were either mock infected (A) or infected with JEV RP-9 at an MOI of 5 (B). At 40 h postinfection, the cells were stained with PI and examined by fluorescence microscopy (magnification, ×300). (C through H) After proper incubation periods, the mock- or JEV-infected cells were end labeled with fluorescein-dUTP by use of TdT and by using alkaline phosphatase-conjugated antifluorescein antibodies and a colorimetric substrate. DNA breaks in nuclei were stained and examined under light microscopy as described in Materials and Methods (magnification, ×190).

respectively. These data indicate that both BHK-21 and N18 cells could support JEV productive replication, and as a result, such an infection led to severe CPE for both cells.

JEV replication induced apoptosis in culture cells. To determine whether apoptosis contributed to JEV-induced cell death, the RP-9-infected BHK-21 and N18 cells were analyzed by several morphological and biochemical assays for the presence of apoptotic characteristics. To visualize the morphology of nuclei by fluorescence microscopy, the JEV- or the mock-infected cells were stained with PI. Many BHK-21 cells (Fig. 1B) and N18 cells (data not shown) infected with JEV, but not mock-infected cells (Fig. 1A), exhibited chromatin condensation and nucleus fragmentation, a diagnostic feature of apoptosis. To distinguish the mechanism by which apoptosis is merely induced extracellularly by binding virus particles to the cell surface from the mechanism triggered intracellularly by virus replication, in some experiments we infected cells with RP-9 virions either pretreated or not with UV irradiation. Additionally, to further inspect whether chromosomal DNA breaks could be generated during JEV infection, the infected cells were labeled and analyzed by TUNEL assay and then microscopically examined for the presence of DNA fragmentation in the nuclei. After appropriate incubation periods, the infected cells were end labeled with fluorescein isothiocyanate-dUTP by using TdT and by using alkaline phosphatase-conjugated antifluorescein antibodies, DNA breaks in nuclei were stained by a colorimetric method. Figure 1D shows the appearance by microscopy of apoptotic nuclei detected in the JEV-infected BHK-21 cells, which under a higher magnification appeared to have nuclear membrane blebbing (not shown). In addition, Fig. 1D reveals that the JEV-infected BHK-21 cells rounded up and detached from the monolayer at 48 h p.i., a characteristic feature of JEV-induced CPE in culture systems. However, such morphological changes in nuclei were not detected in the BHK-21 cells either mock infected (Fig. 1C) or infected with UV-inactivated virions at an MOI of 5 (data not shown). Similarly, TUNEL-positive cells could be readily observed among the JEV-infected N18 cells (Fig. 1F) but not in either the mock-infected cells, (Fig. 1E) or cells infected with UV-inactivated virions at an MOI of 5 (data not shown). Moreover, the use of a higher titer (MOI of 50) of inactivated virions still failed to induce apoptosis from the infected cells, as judged by PI and TUNEL staining assays (data not shown). These data illustrate that it was JEV replication in the cytoplasm, rather than binding by virions per se, that induced apoptosis in the infected cells. Next, whether JEV infection could also induce apoptosis in other neuronal cells was explored by studying a human neuronal progenitor cell line, NT2, by TUNEL analysis. At 32 h p.i. a typical positive staining by TUNEL was seen only in the nuclei of JEV-infected NT2 cells (Fig. 1H) and not in those of mock-infected NT2 cells (Fig. 1G). This finding suggests that JEV could induce apoptosis in NT2 cells. This conclusion was further substantiated by electron microscopy (Fig. 2). JEV appeared to induce characteristic condensation and margination of chromatin in the nuclei of infected NT2 cells (Fig. 2B), whereas the nuclei of normal cells displayed a homogenous distribution of chromatin (Fig. 2A). An abnormal nuclear envelope and scattered dense granular particles were also observed in the nucleus (Fig. 2B). Together, the results described above indicate that JEV infection could induced apoptosis in neuronal cells of different origins.

Another well-defined biochemical hallmark of apoptosis is internucleosomal DNA fragmentation (ladder formation) generated during the apoptotic process, which can be visualized by agarose gel electrophoresis with ethidium bromide staining. Previously, we have demonstrated that when injected into mice intracerebrally, RP-2ms was much less virulent than RP-9, with 50% lethal doses of $>10^7$ and 0.4 PFU, respectively (10). We therefore investigated whether JEV infection could also induce cellular DNA ladder formation (apoptosis) and whether strain variations in virulence would affect JEV's ability to trigger apoptotic cell death in cells in culture. To accomplish this task, a wild-type Taiwanese isolate, NT109, and an attenuated strain, RP-2ms (10), as well as the neurovirulent strain RP-9 used in the experiments described above, were employed to infect BHK-21 and N18 cells. At 32 h p.i., low-molecular-weight DNA was isolated from the mock- or JEV-infected cells and then examined by agarose gel electrophoresis. As ex-

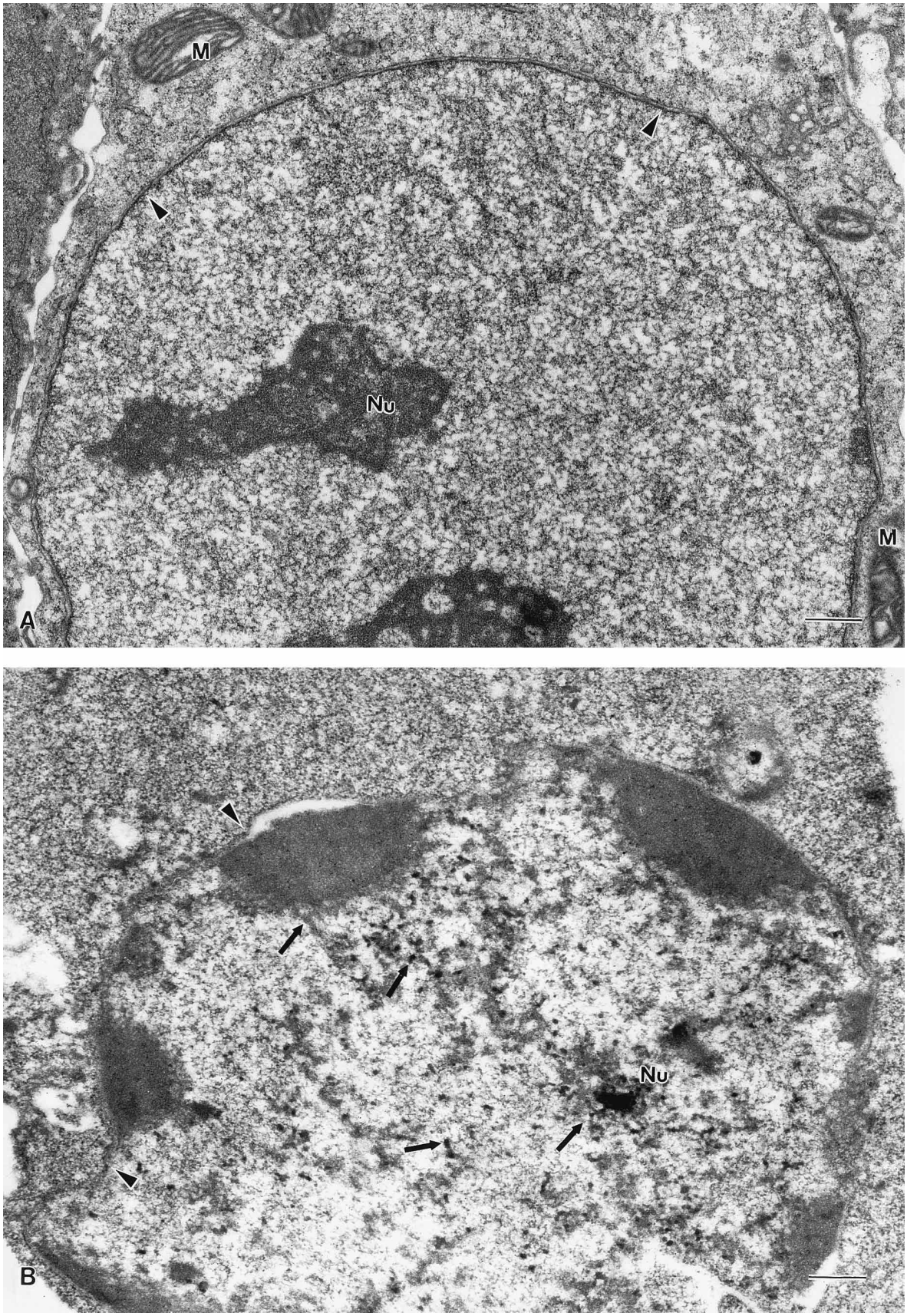

FIG. 2. Electron microscopy of fine structural changes in JEV-infected NT2 cells. NT2 cells were mock infected (A) or infected with JEV at an MOI of 5 (B) and incubated for 48 h. (A) Morphology of the mock-infected NT2 cells. Chromatin materials are homogeneously distributed in the nucleus. Normal appearances of nucleoli (Nu), the round nuclear envelope (arrowheads), and mitochondria (M) are observed. Magnification, ×15,700; bar, 0.6 μm. (B) Morphology of the JEV-infected NT2 cells. JEV appears to induce condensation and marginalization of chromatin in the nucleus of NT2 cells. An abnormal nuclear envelope (arrowheads) is found, and dense granular particles (arrows) are irregularly dispersed in the nucleus. Magnification, ×25,200; bar, 0.4 μm.

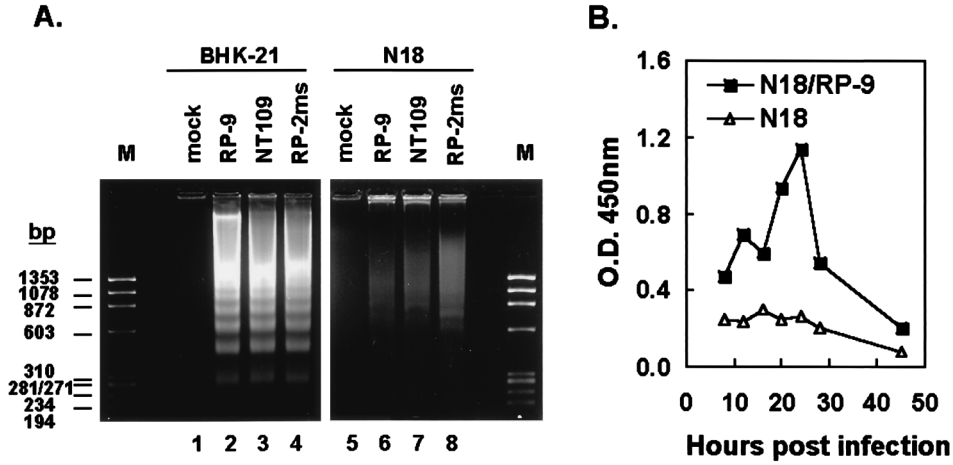

FIG. 3. Biochemical analysis of DNA fragmentation in JEV-infected cells. (A) Agarose gel electrophoresis of DNA extracted from BHK-21 (lanes 1 to 4) and N18 (lanes 5 to 8) cells that were mock infected (lanes 1 and 5) or infected with different strains of JEV (MOI of 5) as indicated. Low-molecular-weight DNA was isolated from the infected cells at 32 h p.i. Lanes M, *Hae*III-cut ϕX174 DNA as DNA markers. (B) Kinetics of DNA fragmentation, determined by ELISA, in N18 cells infected with JEV RP-9 or not infected. Prior to virus infection, cells were labeled with BrdU overnight. At the indicated times p.i., cells were permeabilized to release the cytoplasmic DNA fragments into the supernatant. The amounts of BrdU-labeled DNA released were measured by ELISA at 450 nm with antibodies against DNA and BrdU (see Materials and Methods). O.D., optical density.

pected, the DNA samples derived from BHK-21 cells infected with RP-9 (at an MOI of 5) exhibited a characteristic internucleosome-size laddering; similarly, DNA samples from the cells infected with the other two strains of JEV also displayed ladder formation (Fig. 3A, lanes 2 to 4), whereas this DNA pattern was not observed in the DNA sample from mock-infected cells (Fig. 3A, lane 1). This phenomenon indicates that the infected cells had undergone apoptosis during the 36-h infection periods. Similarly, although showing a lesser extent of DNA fragmentation than that in the infected BHK-21 cells, all three JEV strains could also induce DNA ladder formation in the infected N18 cells (Fig. 3A, lanes 6 to 8), whereas no detectable DNA degradation was observed in the mock-infected cells (Fig. 3A, lane 5). Such results clearly demonstrate that JEV-induced apoptosis in cell cultures is not a virus strain-specific killing event. To further characterize the kinetics of JEV-induced apoptosis in cell culture, we performed DNA fragmentation ELISA (see Materials and Methods) with the RP-9-infected N18 cells as a model. This quantitative analysis allows us to measure the amounts of free DNA fragments released in the cytoplasm of apoptotic cells at different time points after infection. As the data in Fig. 3B show, the appearance of cytoplasmic DNA fragments in infected N18 cells could be detected as early as 8 h p.i., and the amounts of DNA fragments progressively increased with time, peaking at 24 h p.i. but declining thereafter to a level comparable to that in uninfected cells. What exactly caused the decreased quantities of DNA fragments from the infected N18 cells after 28 h p.i. (Fig. 3B) remains unclear; one possible explanation is that the DNA fragments were further digested by an activated, as-yet-unidentified DNase in the cytoplasm during apoptosis. Cumulatively, JEV, irrespective of its virulence, could trigger the infected neuronal as well as nonneuronal cells to undergo apoptosis. Thus, apoptosis likely represents a general mechanism for JEV to kill its infected host cells in a culture system.

Effect of *bcl-2* expression on JEV-induced apoptosis in BHK-21 and N18 cells. To examine whether the expression of human *bcl-2*, a proto-oncogene, could inhibit JEV-induced apoptosis in cell culture, we established several permanent BHK-21 and N18 cell lines stably expressing *bcl-2* by transfection of pZipBcl-2 or pZipneo (as a negative control). By using mouse antiserum specific to the human Bcl-2 protein, *bcl-2* expression in these cell lines was confirmed by indirect immunofluorescence assay and Western blot analyses. JEV was then used to infect these cells to investigate the effect of Bcl-2 protein on apoptosis induced in cell cultures. Figure 4 presents representative data derived from one such BHK-21 cell clone, B2-5, demonstrating how *bcl-2* expression affected JEV-induced apoptosis in a nonneuronal cell line. Morphologically, at 28 h p.i. CPE resulting from RP-9 infection was evident in the cells without *bcl-2* expression; however, the RP-9-infected B2-5 cells seemed to be more resistant to virus-induced cytopathic damage than its infected counterparts (data not shown). The B2-5 cells were, as confirmed by Western blot assay (Fig. 4A), able to synthesize human Bcl-2 protein. The inability of JEV to trigger apoptosis in B2-5 cells could not be attributed to an alteration of cell susceptibility to the virus infection, since viral growth curves appeared to be similar among the parental BHK-21 cells, the BHK-21 cells containing pZipneo, and the B2-5 cells (Fig. 4B). Next, we quantified the extent of cellular damage caused by JEV infection by measuring the amounts of LDH, a cytoplasmic enzyme, released into culture media from the tested cells. As Fig. 4C indicates, up to 48 h p.i., the B2-5 cells infected with RP-9 could still maintain their cellular integrity, whereas the release of LDH from the infected parental BHK-21 cells and BHK-21 cells containing pZipneo appeared to increase with time. In addition, when analyzed by DNA ladder formation, the RP-9-infected B2-5 cells exhibited a deferred pattern of apoptosis, at least for 42 h p.i. (Fig. 5A, lanes 6 and 12). However, most infected B2-5 cells (about 80%) were eventually killed by apoptosis after longer culture periods, as evidenced by the finding that the virus could trigger DNA ladder formation starting from approximately 48 h p.i. (Fig. 5A, lane 18). To ensure that the phenomena in Fig. 4 and 5 were not uniquely associated with the B2-5 cell clone, four other independent clones of BHK-21 cells expressing Bcl-2 were also studied in a series of parallel experiments, from which the comparable results could be reproducibly obtained (data not shown). Taken together, these results illustrate that *bcl-2* expression could delay JEV-induced apoptosis in nonneuronal BHK-21 cells. The antiapoptotic effect of Bcl-2 in this case apparently was not by inhibition of virus replication and

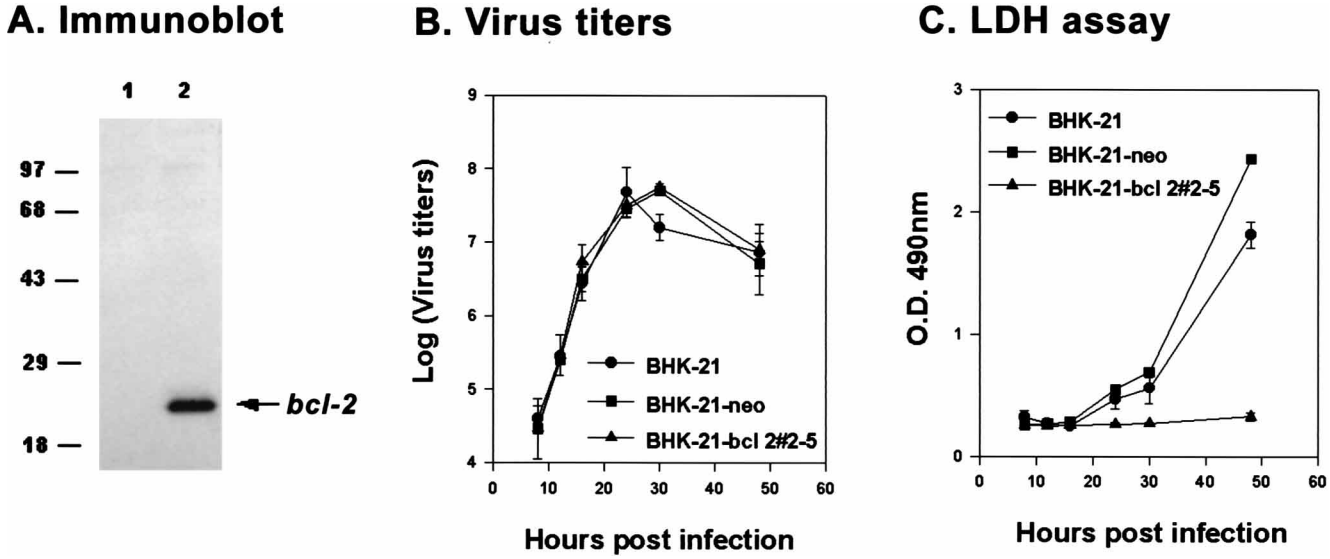

FIG. 4. Effect of *bcl-2* expression of JEV-induced cell death in BHK-21 cells. (A) Western analysis of the expression of human *bcl-2*. B2-5 cells (lane 2) expressed the 26-kDa Bcl-2 protein (arrow), but the BHK-21 cells containing pZipneo (lane 1) did not. Numbers on the left are molecular masses in kilodaltons. (B) One-step growth curves of JEV RP-9 in different BHK-21 cells. The cells were infected with RP-9 at an MOI of 5, and virus titers in culture supernatants were determined by plaque assay at the indicated times. (C) LDH release assay. The amounts of LDH released from RP-9-infected cells at different times were measured by quantitating the formazan dye formed in ELISA plates read at 490 nm (see Materials and Methods). Values for virus titers and optical densities (O.D.) at 490 nm are shown as means ± standard errors of the means for representative experiments performed in triplicate.

was likely mediated by a not-yet-identified mechanism(s) that renders the cells more resistant to apoptotic pressure at the early phase of infection.

Next, we investigated the effect of *bcl-2* expression on the JEV-infected neuroblastoma N18 cells. For this purpose, several independent cell clones stably expressing Bcl-2 proteins were established, and the resulting cells were then infected with RP-9 to perform experiments similar to those done with B2-5 cells for Fig. 4 and 5. Figure 6 summarizes representative data obtained from one such clone, N18-bcl-2#1. Morphologically, at 28 h p.i. severe CPE were found in both the parental N18 and the N18-neo cells infected with RP-9; meanwhile, moderate CPE could be observed in the N18-bcl-2#1 cells infected with RP-9 (data not shown). Bcl-2 protein was shown to be constitutively expressed in N18-bcl-2#1 cells (Fig. 6A). During 24 to 48 h p.i., virus production decreased by about 10-fold in the RP-9-infected N18-bcl-2#1 cells as compared to that in the parental N18 and N18-neo cells infected with RP-9 (Fig. 6B). Exactly what caused the virus yields in N18-bcl-2#1 cells but not those in B2-5 cells (Fig. 4B), to be reduced remains unknown. Nevertheless, this result implied that overexpression of *bcl-2* might somewhat impair JEV replication in neuronal cells. As evaluated by an LDH release assay, we found that the N18-bcl-2#1 cells appeared to preserve cellular integrity for only 32 h after RP-9 infection and that the cells became permeable thereafter, although to lesser extent than the infected parental N18 and the N18-neo cells (Fig. 6C). These data contrasted with those for RP-9-infected B2-5 cells (Fig. 4C). Moreover, as assessed from DNA ladder formation, apoptosis could be detected as early as 32 h p.i. in the N18-bcl-2#1 cells, as well as in the controls of N18 and N18-neo cells infected with RP-9 (Fig. 5B, lanes 4 to 6); this became more obvious at 42 h p.i. (Fig. 5B, lanes 10 to 12) but subsequently turned into a smear pattern at 48 h p.i. (Fig. 5B, lanes 16 to 18), a unique feature of "secondary apoptosis" (3). The fact that at 32 h postinfection, while we could see a delay pattern of LDH release from JEV-infected N18-bcl-2 cells, the

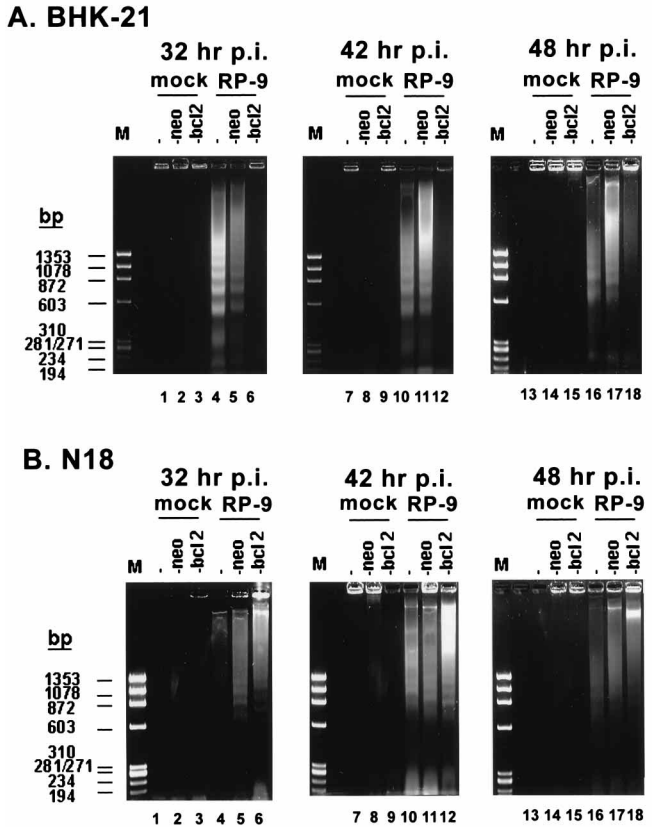

FIG. 5. DNA fragmentation analyzed by agarose gel electrophoresis. BHK-21 cells (A) or N18 cells (B) were either mock infected or infected with RP-9 at an MOI of 5, and DNA was extracted at 32, 42, and 48 h p.i. and analyzed by 2% agarose gel electrophoresis in the presence of ethidium bromide. Lanes M, same DNA markers as described for Fig. 3.

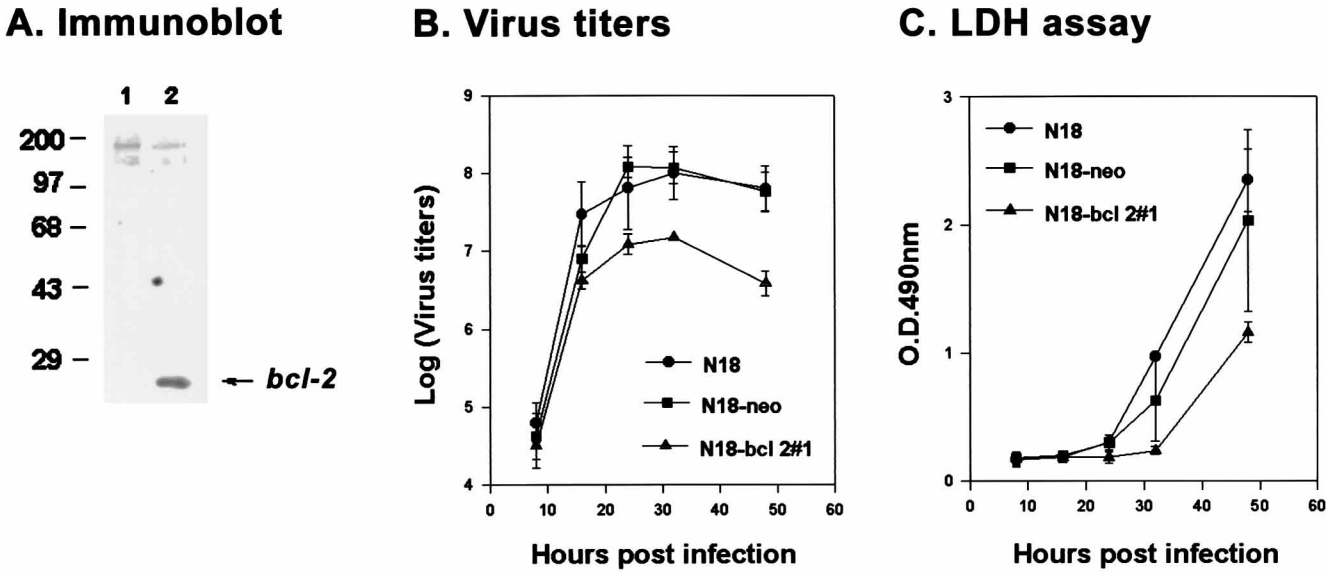

FIG. 6. Effect of *bcl-2* on JEV-induced cell death in N18 cells. (A) Western analysis of the expression of human *bcl-2*. N18-bcl-2#1 cells (lane 2) expressed the 26-kDa Bcl-2 protein (arrow), but N18-neo cells (lane 1) did not. Numbers on the left are molecular masses in kilodaltons. (B) One-step growth curves of JEV RP-9 in different N18 cells. The cells were infected with RP-9 at an MOI of 5, and virus titers in culture supernatants were determined by plaque assay at the indicated times. (C) LDH release assay. LDH released from RP-9-infected cells were measured with an ELISA plate reader at 490 nm at the indicated times. Values for virus titers and optical densities (O.D.) at 490 nm are shown as means ± standard errors of the means for representative experiments performed in triplicate.

infected cells had already undergone apoptosis strongly suggests that *bcl-2* expression is unable to protect against JEV-induced in N18 cells. In a similar experiment, two additional independent clones of N18 cells stably expressing Bcl-2 were also studied, and the results revealed that they were both vulnerable to JEV-induced apoptosis (data not shown), consistent with the properties of N18-bcl-2#1 cells observed in Fig. 6. When comparing the levels of Bcl-2 protein made by N18 and BHK cell clones, our results revealed that the *bcl-2* expression levels were comparable among the clones tested (data not shown), indicating that the difference in the degrees of *bcl-2* protective ability observed in JEV-infected N18 and BHK cells was not due to variation of Bcl-2 expression levels. Most likely, cell type specificity might account for the differences in *bcl-2* protection seen between the two types of JEV-infected cells. Moreover, infection with the attenuated strain RP-2ms could also provoke N18-bcl-2#1 cells to undergo apoptosis (data not shown). Collectively, these data illustrate that the infection by JEV, irrespective of its virulence, could induce apoptosis of neuronal N18 cells even with overexpression of *bcl-2.*

Effect of *bcl-2* overexpression on Sindbis virus-induced apoptosis in N18 cells. Levine et al. (31) demonstrated that Sinbis virus can induce apoptosis in N18 cells. Therefore, this study investigated whether the expression of antiapoptotic Bcl-2 proteins would influence Sindbis virus-induced apoptosis in N18 cells. Production of Sindbis virus (Ar-339; American Type Culture Collection) was apparently not influenced by whether the infected N18 cells expressed Bcl-2; the production in all cases reached approximately 10^9 PFU/ml at 17 h p.i. However, only N18-bcl-2#1 cells could maintain cellular integrity, while the cells without enforced *bcl-2* expression had altered membrane permeability, thereby causing substantial amounts of cytoplasmic LDH to be released after Sindbis virus infection (data not shown). In close correlation with the results for LDH release, the appearance of apoptosis could be observed, by DNA ladder formation and PI and TUNEL staining assays, in the Sindbis virus-infected parental N18 and N18-neo cells, but not in the N18-bcl-2#1 cells, at 17 h p.i. (data not shown). These results clearly indicate that the Bcl-2 proteins expressed in N18 cells appeared to function normally as an antiapoptotic agent. However, the difference in the ability of *bcl-2* to effectively suppress apoptosis triggered by JEV and Sindbis virus implies that these two viruses utilize similar but apparently not identical apoptotic pathways to kill their infected cells.

DISCUSSION

This study has already established that apoptosis can be a common lethal mechanism used by JEV to kill the infected cells in a culture system. JEV replication in cytoplasm appears to be a prerequisite for triggering the apoptotic process of the infected cells, implying that the death stimulus should stem from intracellular interactions between the viral and the cellular components. However, determination of which viral proteins and cellular counterparts (see below) are involved in the induction of apoptosis requires further study. Nevertheless, our results clearly demonstrate that JEV is a bona fide apoptotic inducer for some neuronal and nonneuronal cells in a culture system. The neurovirulent JEV strains NT109 and RP-9 can trigger apoptotic cell death in cultured cell lines (Fig. 3A), raising the possibility that apoptosis of infected neurons as well as other target cells in the CNS may contribute to the subsequent disease and mortality of infected mice. Indeed, with the TUNEL assay, our preliminary results showed that JEV could induce apoptosis in the CNS of infected mice (unpublished observations). Interestingly, an avirulent strain of JEV, RP-2ms, can induce apoptosis as efficiently as its neurovirulent counterparts, NT109 and RP-9 (Fig. 3A), suggesting that, despite varying in virulence, distinct JEV strains can trigger apoptosis probably through a common pathway in cell cultures. These data correspond to the results from a previous study involving Sindbis virus, in which the avirulent strain AR-339 could kill most cultured cell lines by apoptosis (53). These observations, therefore, imply that judging the viral virulence of a JEV strain only on the basis of whether it can kill target

cells by apoptosis in a culture system is inappropriate. Intriguingly, JEV RP-2ms is highly attenuated in mice even when administered intracerebrally (10, 32) and yet is apoptotic to neuronal N18 cells (Fig. 3A) and NT2 cells (not shown) in a culture system. One possible explanation is that the RP-2ms-infected mice were protected by their local immunity in the CNS, where cytokines and/or nitric oxide-related compounds produced by microglial cells and astrocytes (15, 19) might modulate the infection course of RP-2ms in neurons. Alternatively, perhaps owing to different but uncharacterized conditions of in vivo and in vitro growth, the cultured neuronal cells are far more susceptible to be killed by JEV infection than the neurons in the CNS.

In cell cultures, overexpression of human *bcl-2* has been shown to either inhibit or defer the apoptotic process induced by infection with Sindbis virus (31, 53), influenza A and B viruses (23), and La Crosse virus (41). Adding to this growing list, our results demonstrate that *bcl-2* also appears to delay JEV-induced apoptosis in BHK-21 cells (Fig. 4 and 5). However, enforced *bcl-2* expression did not completely protect BHK-21 cells from the eventual death caused by JEV-associated apoptosis (Fig. 5A), since only about 20% of the infected cells could survive after prolonged culture. Pekosz et al. (41) observed a similar phenomenon in which *bcl-2* could only to some extent prevent the apoptotic death of hybrid N18-RE-105 cells infected by La Crosse virus. How Bcl-2 protects against apoptosis remains unclear, and most components of the apoptotic pathway(s) are unexplored. However, to be an efficient antiapoptotic agent, Bcl-2 presumably regulates an important step downstream of all of the putative death triggers targeted by the viruses mentioned above. Otherwise, enforced expression of *bcl-2* may possibly block apoptosis by restricting virus replication in infected cells, as suggested by earlier studies involving Sindbis virus (53) and influenza viruses (23, 39). In fact, *bcl-2* expression in BHK-21 cells does not limit JEV replication (Fig. 4B), suggesting that Bcl-2 proteins play an antiapoptotic, rather than an antiviral, role in this system. As a result, the 20% of surviving BHK cells expressing *bcl-2* following JEV infection became persistently infected during long-term culture (unpublished observations). A similar observation for a reovirus system has been described (47), in which *bcl-2* expression can inhibit virus-induced apoptosis without affecting virus replication.

Despite its apparent importance in regulating the apoptotic process, *bcl-2* cannot prevent apoptosis in all systems (reviewed in reference 4). In correlation with this notion, some virus-associated apoptotic processes did defy the control by *bcl-2*. Ubol et al. (53) noted that a neurovirulent strain of Sindbis virus still induces apoptosis in *bcl-2*-expressing rat-prostate carcinoma AT-3 cells. In addition, Suarez et al. (50) observed that overexpression of Bcl-2 failed to inhibit the apoptosis of N2A neuroblastoma cells induced by infection by porcine reproductive and respiratory syndrome virus. Similarly, our results also indicate that all three different strains of JEV tested here can trigger apoptosis of neuroblastoma N18 cells regardless of enforced *bcl-2* expression (Fig. 5B). The inability of *bcl-2* to block JEV-induced apoptosis cannot be attributed to the presence of an aberrant apoptotic pathways inherited by N18 cells, because the same Bcl-2-expressing N18 cells are resistant to Sindbis virus-associated apoptosis. While the cause for such a discrepancy between the viruses remains unclear, these results suggest that JEV and Sindbis virus may utilize similar but not identical mechanisms of apoptosis to kill N18 cells. If both JEV and Sindbis virus can induce the same apoptotic pathway in N18 cells, we can infer that a specific, as-yet-unidentified JEV protein(s) might interact with the lethal trigger(s) in the lower part of the apoptotic cascade, where Bcl-2 proteins cannot intervene in the death process. However, if the two viruses could induce two distinct apoptotic pathways in N18 cells, this might readily explain why *bcl-2* was effective only in Sindbis virus-induced apoptosis and not in JEV-induced apoptosis. By employing this system, we can unequivocally demonstrate the presence of both *bcl-2*-sensitive and *bcl-2*-insensitive apoptoses signaled by different virus infections in the same cells. Therefore, in the future, the molecular differences between the two types of apoptotic processes can be distinguished.

Interestingly, the intracellular localizations of Bcl-2 (35, 38) and some JEV proteins (reviewed in reference 46) largely overlap in the endoplasmic reticulum and perinuclear membrane. In addition, several recent studies have shown that Bcl-2 can form heterodimers with Bax or Bak (both are apoptotic inducers); the ratio of such complexes has also been implicated in dictating whether the cells should undergo apoptosis (12, 18, 29, 34, 40). Thus, identifying the interactions between viral and Bcl-2 proteins and their roles in JEV-induced apoptosis will be of interest.

Intriguingly, while this study demonstrates that JEV is an apoptotic inducer for N18 cells, our previous work demonstrated that JEV could also persistently infect N18 cells and that the persistency was strongly related to the abnormal expression of JEV nonstructural protein 1 (NS1) in the host cells (9). In response to JEV infection, a switch from the apoptotic process to viral persistency in N18 cells might have occurred under tremendous selection pressure. Several interesting but puzzling issues remain, including (i) what viral and/or cellular factor(s) can determine the fate of the infected cells, in terms of either undergoing apoptosis or persisting; (ii) whether overexpression of truncated JEV NS1 (9) alone in target cells influences JEV-associated apoptosis; (iii) what makes the difference in virus production between persistent JEV infection in mosquito cells (high virus titers) and that in mammalian cells (low virus titers); (iv) whether inhibitors of the interleukin-1β-converting enzyme family proteases (22) can block JEV-induced apoptosis; (v) whether enforced expression of a known JEV protease (nonstructural protein 3) alone in target cells can trigger apoptosis; and (vi) whether other members of the *bcl-2* family, such as *bcl-*X_L, which has been shown to inhibit apoptosis induced by Sindbis virus (11), can block JEV-induced apoptosis. Resolving these issues would facilitate further understanding of the complexity of virus-cell interaction in JEV pathogenesis.

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