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Induction of Apoptosis by La Crosse Virus Infection and Role of Neuronal Differentiation and Human *bcl-2* Expression in Its Prevention

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La Crosse virus causes a highly cytopathic infection in cultured cells and in the murine central nervous system (CNS), with widespread neuronal destruction. In some viral infections of the CNS, apoptosis, or programmed cell death, has been proposed as a mechanism for cytopathology (Y. Shen and T. E. Shenk, Curr. Opin. Genet. Dev. 5:105–111, 1995). To determine whether apoptosis plays a role in La Crosse virus-induced cell death, we performed experiments with newborn mice and two neural tissue culture models. Newborn mice infected with La Crosse virus showed evidence of apoptosis with the terminal deoxynucleotidyl transferase-mediated nicked-end labeling (TUNEL) assay and, concomitantly, histopathological suggestion of neuronal dropout. Infection of tissue culture cells also resulted in DNA fragmentation, TUNEL reactivity, and morphological changes in the nuclei characteristic of apoptotic cells. As in one other system (S. Ubol, P. C. Tucker, D. E. Griffin, and J. M. Hardwick, Proc. Natl. Acad. Sci. USA 91:5202–5206, 1994), expression of the human proto-oncogene *bcl-2* was able to protect one neuronal cell line, N18-RE-105, from undergoing apoptosis after La Crosse virus infection and prolonged the survival of infected cells. Nevertheless, expression of *bcl-2* did not prevent eventual cytopathicity. However, a human neuronal cell line, NT2N, was resistant to both apoptosis and other types of cytopathicity after infection with La Crosse virus, reaffirming the complexity of cell death. Our results show that apoptosis is an important consequence of La Crosse virus infection in vivo and in vitro.

La Crosse virus is the principal cause of California encephalitis, a form of pediatric encephalitis that occurs in several geographic areas of the United States and Canada where its principal vector, the *Aedes triseriatus* mosquito, is indigenous (9). Transmission of the virus to small mammals and humans occurs via the bite of an infected mosquito (5). Although serological surveys have demonstrated that a significant proportion of the population in these regions has evidence of prior exposure to La Crosse virus, the total number of documented cases of encephalitis is only between 50 and 150 each year (9, 18). Presumably, most infections with La Crosse virus, particularly in adults, either are asymptomatic or result in nonspecific symptoms.

La Crosse virus is a member of the *Bunyavirus* genus of the *Bunyaviridae*, and like all members of the family has a trisegmented, primarily negative-sense RNA genome (9). A mouse model of La Crosse virus encephalitis mimics many features of the human disease, including age-dependent susceptibility to central nervous system (CNS) invasion (11, 16, 17, 28). For example, newborn mice are sensitive to subcutaneous inoculation with La Crosse virus of as little as 1 PFU and develop fatal encephalitis, whereas adult mice are resistant to high doses administered by the same route.

In contrast to peripheral inoculation, intracranial inoculation with La Crosse virus is uniformly fatal to mice of all ages. However, there are age-related differences in the distribution of viral antigens in the brains of infected mice: La Crosse virus infection of the brains of suckling mice results in widespread virus replication, while in adult mice viral antigen is more restricted (16). Interestingly, no areas of inflammatory cell infiltrates or lesions were found by Janssen et al. (16), suggesting that the damage resulting from La Crosse virus infection was not due to a host antiviral response.

Apoptosis, or programmed cell death, is a mechanism through which cells undergo chromosome condensation, DNA degradation, and morphological changes in the nuclear membrane (1, 1a, 36). Various stimuli, such as binding to Fas receptor or binding of tumor necrosis factor alpha to the tumor necrosis factor alpha receptor, can trigger apoptosis; proteins such as the *bcl-2* and *bax* proteins, NF-κB, and ICE-related proteases have been shown to play important roles in regulating programmed cell death. Apoptosis plays an important role in development, and its role in neuronal injury has recently been reviewed (1a). Apoptosis has also been implicated as a possible mechanism of cell and tissue damage in response to virus infections (7, 14, 20, 33, 34; reviewed in reference 29). Conversely, several viruses encode proteins that actively block various steps of the apoptotic pathway, in order to maintain an active virus infection or virus persistence (2–4, 13, 31, 32, 39).

In this study, we demonstrated that La Crosse virus infection induces apoptosis in the murine brain. To develop a system in which this phenomenon could be studied more directly, we used a panel of human and murine neuronal cell lines. La Crosse virus infection induced apoptosis in some neuronal lines, and the human proto-oncogene *bcl-2* could protect these cells at least partially from viral cytopathicity while having little or no effect on virus production. These results suggest a specific, cell-dependent mechanism involved in the tissue damage resulting from La Crosse virus infection of the CNS and of cultured cell lines.

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MATERIALS AND METHODS

Cells. All cell lines were maintained at 37°C in a 5% CO_2 incubator. BHK-21 cells were grown in minimum essential medium with Earle's salts (JRH Biosciences, Lenexa, Kans.) supplemented with 5% fetal bovine serum (Gibco BRL, Grand Island, N.Y.) and 5% horse serum (HyClone, Logan, Utah).

The human teratocarcinoma cell line uNT2 was cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 20 U of penicillin-streptomycin. Retinoic acid differentiation of uNT2 cells to the postmitotic, neuronal phenotype (NT2N) was performed as previously described (12, 22, 38). NT2N cells were cultured in Dulbecco's modified Eagle medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and 20 U of penicillin-streptomycin and were usually used within 2 weeks after retinoic acid differentiation had been completed.

The N18-RE-105 cell line is a mouse neuroblastoma-rat embryonic retinal neuron hybrid cell line (6, 24, 25). CMV-bcl-2 and CMV-neo plasmids (see below) were introduced into N18-RE-105 cells by the calcium phosphate precipitation technique. N18-RE-105 cells stably expressing human *bcl-2* (C6.15) or the neomycin resistance plasmid alone (C3.7) were grown in Dulbecco's modified Eagle medium (Gibco BRL) supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Gibco BRL), 20 U of penicillin-streptomycin (Gibco BRL), and 400 μg of G418 (Geneticin; Gibco BRL) per ml.

Plasmids. Plasmids encoding human *bcl-2* and neomycin resistance, or neomycin resistance only, were prepared by removing the 700-bp splenic focus-forming virus promoter in SFFVBcl-2nl and SFFVNeo (15) and replacing it with a 760-bp *Sau*3AI fragment containing the human cytomegalovirus major immediate early enhancer/promoter (30).

Virus replication. The derivation and characterization of the La Crosse/original virus have been described (16). To assay for virus replication, cells were plated on 12-well plates (Nunc, Naperville, Ill.) at approximately 10^6 cells per well and grown overnight at 37°C. The cells were then incubated with virus at a multiplicity of infection (MOI) of either 0.01 or 1.0 PFU per cell for 60 min at 37°C, washed extensively, and returned to 37°C. After infection, the entire supernatant was removed and plaque titers were determined in BHK-21 cells, as described previously (16), with a limit of detection of $10^{1.7}$ PFU/ml.

Western immunoblotting. Approximately 10^7 cells were resuspended in phosphate-buffered saline (PBS) containing 5 mM EDTA. The cells were pelleted at 200 × *g* for 10 min and resuspended in 0.1 M Tris HCl (pH 7.4)–0.1 M NaCl. An equal volume of 1% Triton X-100–0.5% Nonidet P-40 was added, and the mixture was incubated on ice for 30 min. Cellular debris was then pelleted at 13,000 × *g* for 10 min, and the supernatants were stored at −20°C until use. Equivalent amounts of protein lysates (as determined by protein assay [Bio-Rad Laboratories, Hercules, Calif.]) were loaded onto 12.5% polyacrylamide gels for analysis. Western blotting was performed as described in reference 28. Human *bcl-2* was detected with a hamster anti-human *bcl-2* antibody (used at 2 μg/ml; Pharmingen, San Diego, Calif.) followed by a goat anti-hamster immunoglobulin G antibody conjugated to horseradish peroxidase (used at 1:1,000; Caltag Laboratories, San Francisco, Calif.). Detection by chemiluminescence (ECL) was done by the manufacturer's protocol (Amersham Life Sciences, Buckinghamshire, England). Blots were exposed to Fuji RX medical X-ray film at room temperature for 1 to 10 min.

MTT conversion assay. At various times after infection at an MOI of 10, 60 μl of a 5-μg/ml MTT solution (Sigma Chemical Co., St. Louis, Mo.) was added in triplicate to either La Crosse virus- or mock-infected cells in 24-well plates containing 200 μl of medium. The cells were incubated at 37°C for 3 h, lysed by the addition of 150 μl of 10% sodium dodecyl sulfate (SDS) with 0.01 N HCl, and then incubated for an additional 3 h at 37°C (23). The samples were transferred to Titertek plates (ICN Biomedicals, Horsham, Pa.), and the optical density (at 570 nm) was measured with a Titertek Multiskan MCC/340 (ICN Biomedicals).

DNA fragmentation. Approximately 10^7 cells were infected with La Crosse virus at an MOI of 10 and then maintained at 37°C for 14 to 18 h. The medium was removed, and attached cells were resuspended with 0.05% trypsin and added back to the removed medium. Cells were pelleted at 500 × *g* for 10 min, resuspended in 10 mM Tris HCl (pH 7.4)–5 mM EDTA (TE buffer), and lysed by adding an equal volume of 1% SDS in water (14). The mixture was incubated at 4°C for 30 min and centrifuged at 13,000 × *g* for 15 min, and the chromatin pellet was then removed. The samples were extracted twice with phenol-chloroform-isoamyl alcohol (Amresco, Solon, Ohio), precipitated, and resuspended in TE buffer containing 2 μg of RNase H (Boehringer Mannheim, Indianapolis, Ind.) per ml. After a 30-min incubation at room temperature, the samples were analyzed for nucleosomal DNA laddering by electrophoresis on a 2% agarose gel in TE buffer.

TUNEL assay. To detect DNA strand breaks, the terminal deoxynucleotidyl transferase-mediated nicked-end labeling (TUNEL) assay was used, essentially as described in reference 37. To better detect morphological changes in the nucleus, cells were stained with Hoechst 33342 fluorochrome (bis-benzamide [Molecular Probes Inc., Eugene, Oreg.] diluted to 0.1 mg/ml in tissue culture media) for 1 h at 37°C before acid alcohol fixation as described above.

Mouse brain sections. Mouse brains were removed from moribund 4-day-old mice that had been inoculated at 1 day after birth with 700 PFU of La Crosse virus subcutaneously and from age-matched, mock-infected mice (16). The brains were cut into hemispheres, embedded in tissue freezing medium (Electron Microscopy Sciences, Fort Washington, Pa.), frozen at −70°C, and sectioned with a cryostat. Brain sections were treated for TUNEL analysis as described above.

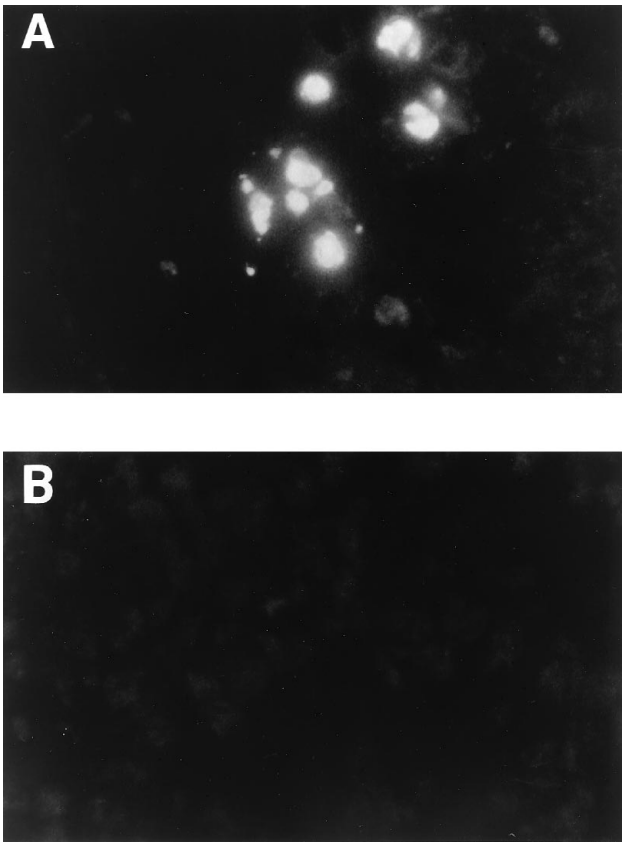

FIG. 1. TUNEL assay for apoptotic cells in La Crosse virus- or mock-infected mouse brain sections. Brains from 4-day-old mice that were infected with La Crosse virus at 1 day after birth or from age-matched control mice were harvested and processed for TUNEL analysis as described in Materials and Methods. Apoptotic cells were detected in La Crosse virus-infected (A) but not in mock-infected (B) mouse brain sections. Eleven fields were quantified, as discussed in Results. Magnification, ×1,000.

Immunofluorescence for viral antigen. After fixation, cells were blocked with 20% normal goat serum–10% normal rabbit serum in PBS for 30 min, incubated for 1 h with an anti-G1 monoclonal antibody cocktail (diluted 1:50 in blocking solution), washed four times in PBS, and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (diluted 1:50 in PBS with 20% normal goat serum–10% normal rabbit serum) for 30 min (10). Slides were washed four times with PBS and once with distilled H_2O and mounted with Vectashield.

RESULTS

La Crosse virus infection induces apoptosis in the mouse brain. To ascertain whether La Crosse virus infection can induce apoptosis in vivo, we infected 1-day-old mice by subcutaneous injection, harvested the brains on day 4, and performed a TUNEL assay for apoptosis on cryostat sections from those brains. Figure 1A shows end labeling of nuclear material, which we took as evidence of apoptotic cells in a La Crosse virus-infected brain. Under higher magnification, the TUNEL-positive cells showed nuclear membrane blebbing, a characteristic associated with apoptotic death. Quantification of 11 fields of the La Crosse virus-infected brain sections showed 4.8 ± 1.9 TUNEL-positive cells per field, whereas the brain sections from age-matched, uninfected mice had no positive cells (Fig. 1B). Apoptotic cells were distributed throughout the brain but

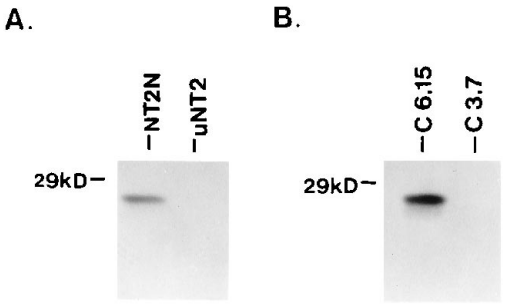

FIG. 2. Western analysis for expression of human *bcl-2*. (A) NT2N cells express *bcl-2*, but the undifferentiated uNT2 cells do not. (B) N18-RE-105 cells transfected with a plasmid expressing *bcl-2* (C6.15 cells) expressed the protein, but the cells transfected with a control plasmid (C3.7 cells) did not.

were particularly prominent in the hindbrain and midbrain. The regions of the infected mouse brain which were most positive for apoptosis also showed visible pathological changes resembling neuronal dropout as judged by hematoxylin-and-eosin staining, but the number of neurons was not formally quantified (data not shown) (16). These findings indicated that apoptotic neurons are present during La Crosse virus infections and that apoptosis is at least one mechanism for cell death in this model of encephalitis.

Expression of the antiapoptosis *bcl-2* protein in neuronal cell lines. To study apoptosis in further detail, we used the uNT2 cell line (12, 22, 35), which can be differentiated into a postmitotic neuronal phenotype (NT2N) by exposure to retinoic acid (see Materials and Methods), and N18-RE-105, a murine neuroblastoma-rat embryonic retinal neuron cell line that has been used to study a variety of neuron-specific functions, such as glutamate cytotoxicity and neurotoxin uptake (6, 24, 25). Since overexpression of the proto-oncogene *bcl-2* has been shown to inhibit apoptosis induced by a variety of stimuli (15, 21, 36), we used derivatives of N18-RE-105 cells that had been transfected with either a plasmid expressing human *bcl-2* (C6.15 cells) or a control plasmid expressing the neomycin resistance gene only (C3.7 cells).

Western blot analysis of the NT2N cells demonstrated a strong *bcl-2* signal, while the uNT2 cells did not demonstrate evidence of *bcl-2* expression (Fig. 2A). As expected, a strong *bcl-2* signal was demonstrated in the C6.15 cells and not in the C3.7 cells (Fig. 2B).

La Crosse virus infection of neuronal cell lines. Similar titers of infectious virus were produced in both C3.7 and C6.15 cells after infection with La Crosse virus at an MOI of 1 (Fig. 3A). However, at a lower MOI (0.01), the C6.15 cell line (which expresses *bcl-2*) produced slightly more virus than the control cell line, C3.7 (Fig. 3B). The MTT conversion assay, which measures the metabolic activity of the cultured cells (23), was then used to quantitate cell death (Fig. 4). Cells expressing *bcl-2* were more resistant to La Crosse virus-induced cell death than were control cells (Fig. 4A and B), and metabolic activity decreased to background levels at 72 h, as opposed to 24 h for the control cells. However, the *bcl-2*-expressing cells were eventually lysed.

The differentiated NT2N cells were susceptible to La Crosse virus infection at both high and low MOIs (Fig. 3C and D,

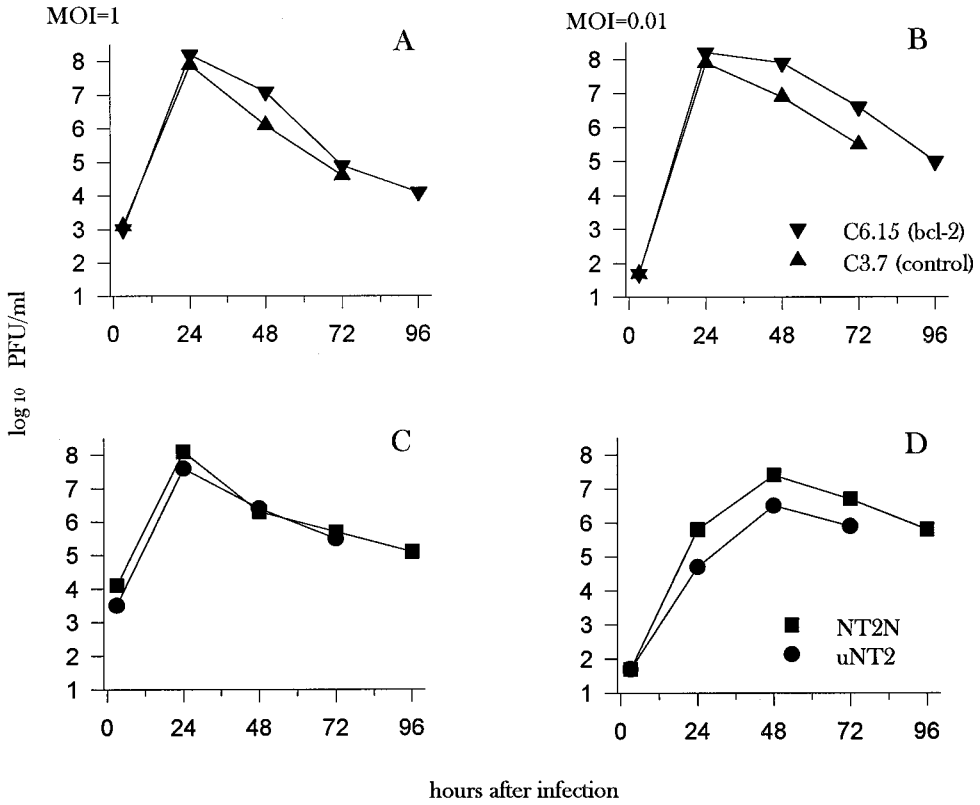

FIG. 3. La Crosse virus infection of neural cell lines. Cells were grown on 12-well plates and infected at an MOI of either 1 (A and C) or 0.01 (B and D) for 1 h. Then the cells were washed extensively, fresh medium was added, and the cultures were incubated at 37°C. Supernatant samples were taken at the indicated times and analyzed for infectious virus by plaque assay on BHK-21 cells.

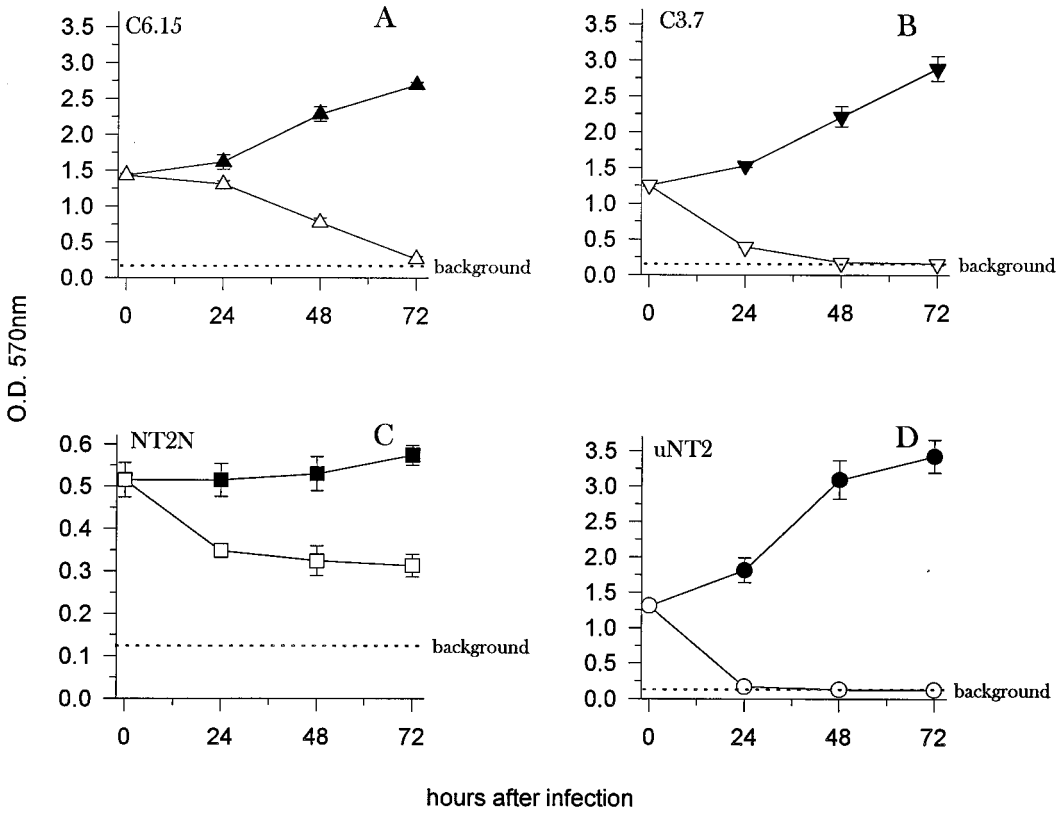

FIG. 4. MTT conversion by cell lines infected with La Crosse virus. At time zero, cells were infected at an MOI of 10, washed extensively, and incubated in fresh medium at 37°C. Cells transfected with a plasmid expressing *bcl-2* (A) are more resistant to La Crosse virus-induced cell death than control cells (B) but are eventually lysed by the infection. NT2N cells (C) are resistant to La Crosse virus-induced cell death, while uNT2 cells (D) are killed within 24 h of infection. Note that the scale for the MTT assay for NT2N cells is different. As these cells did not proliferate, there was no increase in MTT conversion with time. The background line reflects the optical density (O.D.) of acellular medium treated in parallel with the experimental wells. Open symbols, La Crosse virus infected; closed symbols, mock infected.

respectively), with peak titers (10^7 to 10^8 PFU/ml) in the range normally seen with La Crosse virus infection of BHK-21 cells, which we use as a standard (27). In comparison to their undifferentiated predecessor, uNT2 cells, there were only minimal differences in peak titers. However, the undifferentiated uNT2 cells showed the cytopathic effect (CPE) associated with La Crosse virus infection (cell rounding, detachment, and death), while the NT2N cells had no evidence of CPE. In various experiments, NT2N cultures had no CPE for up to 14 days after infection but still produced infectious virus (data not shown), indicating that a persistent infection was established over the life span of these cells.

The absence of CPE in La Crosse virus infection of the NT2N cells was clearly demonstrated by MTT conversion. Twenty-four hours after infection, the uNT2 cells had virtually no MTT-derived signal (Fig. 4D), whereas the NT2N cells were still able to convert MTT up to 72 h after infection, albeit at a lower rate than mock-infected NT2N cells (Fig. 4C). These data indicated that the differentiation process markedly reduced the susceptibility to La Crosse virus-induced cytopathicity, whereas it had little or no effect on the production of progeny virions.

La Crosse virus infection does not induce apoptosis in *bcl-2*-expressing neuronal cell lines. Since one of the mechanisms through which viruses mediate cell death is the active process of apoptosis (8, 26, 29), we determined the degree of DNA fragmentation present in infected cells by agarose gel electrophoresis. C6.15 cells showed no detectable DNA laddering at 16 or 24 h after infection with La Crosse virus (Fig. 5B), while the C3.7 (control) cells quite clearly demonstrated laddering, indicating cell death by apoptosis in the C3.7 cells. No DNA laddering was seen in mock-infected cells. Since the C6.15 cells eventually die, we assume that their cytopathicity occurs by

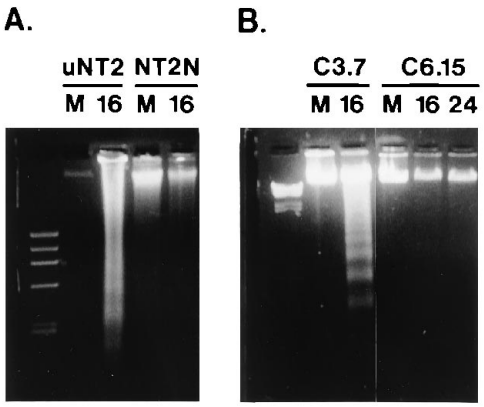

FIG. 5. DNA fragmentation induced by La Crosse virus infection. Chromosomal DNA was isolated from cells that were mock infected (M) or infected with La Crosse virus for the indicated times (in hours). (A) DNA fragmentation was detectable in uNT2 cultures but not in differentiated NT2N cells 16 h after infection with La Crosse virus (MOI, 10). The first lane shows *Hae*III-cut ϕX174 DNA as a DNA marker. (B) Expression of human *bcl-2* protects cells (C6.15) from La Crosse virus-induced DNA fragmentation, while control cells (C3.7) transfected with the neomycin resistance gene only are not protected. The first lane of panel B shows *Hin*dIII-digested λ DNA as a DNA marker.

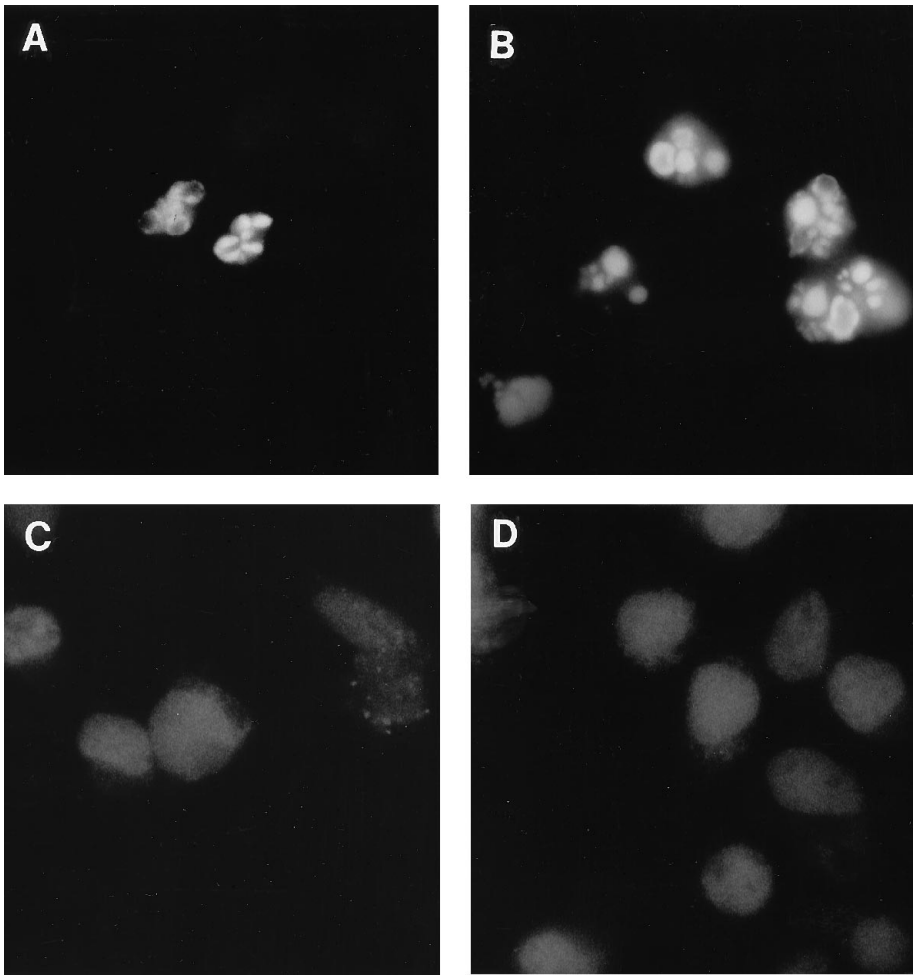

FIG. 6. TUNEL assay demonstrates the induction of apoptosis in cells infected with La Crosse virus. La Crosse virus-infected uNT2 (A) and C3.7 (B) cells show apoptotic nuclei as judged by increased fluorescence and nuclear membrane blebbing, while La Crosse virus-infected (C) and mock-infected (D) C6.15 cells appear normal. Mock-infected C3.7 and uNT2 cells (not shown) appeared similar to the cells in panel D. NT2N cells were not analyzed since no cell death was observed after infection and their growth characteristics make this morphological assay difficult to perform.

either a nonapoptotic mechanism or limited apoptosis that cannot be detected by our techniques.

In the uNT2-NT2N cell culture system, a pattern of DNA laddering was easily discerned in the DNA from the infected uNT2 cells but not in that obtained from the NT2N cells or from mock-infected cells of either cell type (Fig. 5A).

Quantification of apoptosis and virus infection. To verify and quantitate apoptotic cell death, we performed TUNEL assays (35, 37) for fragmented chromosomal DNA in La Crosse virus-infected and mock-infected cells. Figure 6 shows the detection of apoptotic nuclei which appear to have nuclear invaginations or blebbing in La Crosse virus-infected uNT2 (Fig. 6A) and C3.7 (Fig. 6B) cells; these changes were not detectable in La Crosse virus-infected C6.15 cells (Fig. 6C) or in mock-infected cells (for an example, see Fig. 6D). TUNEL assays were not performed on the NT2N cells, because they showed no cell death after La Crosse virus infection and because the nature of the cultures (large clumps of cells easily detachable from their culture dishes) made quantitation by TUNEL analysis very difficult. Hoechst staining of live cells also showed aberrant nuclear morphology (data not shown).

To quantitate the number of apoptotic and virus-infected cells, we performed TUNEL and immunofluorescence analyses for viral antigen on duplicate coverslips infected at an MOI of 1. Table 1 shows a significant number of apoptotic cells in La Crosse virus-infected uNT2 and C3.7 cells, while very few *bcl-2*-expressing C6.15 cells were apoptotic, despite the presence of a large number of infected cells. Taken together, the results indicate that *bcl-2* expression protects cells from La Crosse virus-induced apoptosis but not from eventual cell death from virus infection.

DISCUSSION

Infection with La Crosse virus induces a neuronal pathology characteristic of an acute encephalitis, both in the experimen-

TABLE 1. La Crosse virus-induced apoptosis[a]

Cell line	% Apoptotic	% Infected
uNT2	33.4	50.8
C6.15 (*bcl-2* expressing)	5.3	50.1
C3.7 (control)	32.4	33.5

[a] Values were calculated by the following formula: (number of antigen-positive or apoptotic cells/total number of cells) × 100. Results are representative of two or three experiments performed with each cell line. A total of 10 to 15 fields (100 to 200 cells per field) were counted for each condition.

tal animal (16) and in humans (18). Previous studies have assumed that the mechanism of cell death in the CNS is reflective of (i) the highly cytolytic nature of infection of cultured cells and (ii) the inhibition of host protein synthesis by the destruction of host mRNAs due to the "cap-snatching" function of the viral polymerase. The term "lethal parasitism" has been used by some to describe this model of cell and tissue damage (20). In this study, we have demonstrated that, both in vivo and in vitro, an active process (i.e., programmed cell death) is at least partly responsible for the deterioration of cellular function resulting from La Crosse virus infection. Although these results do not address the specific trigger that leads to the initiation of this process, they indicate that as with some other viral infections (7, 14, 21, 33, 34), cell death is a complex process that is heavily influenced by the different milieus in special cell types, since we found significant differences in the effect of La Crosse virus infection on different cell lines.

In addition to finding evidence of apoptotic death by several methods, we found that expression of the human proto-oncogene *bcl-2* protected some cells from La Crosse virus-induced apoptosis; however, *bcl-2* was unable to prevent the eventual cell death of some of the infected cells, i.e., the hybrid N18-RE-105 cells. In a human neuronal cell line (NT2N), La Crosse virus infection resulted in a nonlytic, productive infection that lasted for 10 to 15 days, or the life span of these postmitotic cells. The La Crosse virus-infected NT2N cells had a slightly lower metabolic rate (as judged by the lower conversion rate of MTT in comparison to that in mock-infected cells), but remarkably, virus production (and indirectly viral protein synthesis) was maintained at high levels. In contrast, the undifferentiated progenitor cells, uNT2 cells, were susceptible to La Crosse virus infection and became apoptotic. These data suggest that neuronal differentiation induces changes that lead to an increased resistance to La Crosse virus-induced apoptosis. A higher level of *bcl-2* may be one component of this increased resistance, as *bcl-2* has been shown to affect neural cell death occurring after different metabolic insults, including ischemia (reviewed in reference 1a). However, a lower metabolic rate in these noncycling cells could very well be equally important.

Why does La Crosse virus induce apoptosis in the murine brain, whereas it does not kill a postmitotic human neuron in culture? One possible explanation is that the level of *bcl-2*, or perhaps other proteins important in decreasing programmed cell death, is higher in the cultured cells than in the CNSs of newborn mice. Perhaps more likely, the brains of newborn mice contain replicating cells that may be more susceptible to apoptosis (1). Alternatively, the presence of cytokine-producing microglia and astrocytes may modulate the course of La Crosse virus infection in neurons within the CNS (5a). There may also be species-specific differences in susceptibility to La Crosse virus-induced apoptosis. In any case, these findings point out that the cellular biology of viral cytopathicity defies simple explanations.

Highly virulent strains of Sindbis virus induce apoptosis in the mouse brain, while avirulent strains induce apoptosis less efficiently (20, 21). While most virulent strains of Sindbis virus induce apoptosis regardless of the expression of *bcl-2*, at least one avirulent strain is unable to induce apoptosis in the presence of *bcl-2* (19), changing the infection from a lytic to a lifelong persistent infection in cell culture. However, *bcl-2* expression appears to reduce Sindbis virus replication (34), implying that *bcl-2* modulates Sindbis virus infection by at least two mechanisms: by preventing virus-induced apoptosis and by reducing the production of infectious virus. A similar inhibitory effect on virus replication was seen when influenza virus was grown on cell lines expressing *bcl-2* (14, 26). Apoptosis was blocked but progeny virus yields were lower, and *bcl-2* expression induced changes in the glycosylation of hemagglutinin glycoprotein. La Crosse virus replication does not seem to be inhibited by *bcl-2* expression; in fact, viral titers were slightly higher in the presence of *bcl-2*, perhaps because of increased survival of the infected cells.

La Crosse virus infection of mosquitoes and insect-derived cell lines results in a persistent, lifelong nonlytic infection (5, 9). While we have not studied the role of apoptosis in La Crosse virus infection of mosquito cell lines, our results would point to apoptosis and the presence of genes that prevent it as important areas of research for understanding virus replication in its insect host.

Baculovirus, adenovirus, Epstein-Barr virus, and poxvirus all encode proteins whose main function appears to be the prevention of apoptosis (2–4, 8, 13, 31, 32, 39). For these viruses, the selective advantage is clear: by preventing cell death the virus is able to either maintain infectious virus production or prevent the elimination of latently infected cells. La Crosse virus does not appear to have any genes similar to those encoding the proteins described above, but its short replication cycle allows virion production to proceed in spite of cytopathicity. Where CPE is low, or nonexistent, as in the *bcl-2*-expressing NT2N cells, virus production can persist for longer periods of time.

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REFERENCES

1. **Alvarez-Buylla, A., and C. Lois.** 1995. Neuronal stem cells in the brain of adult vertebrates. Stem Cells **13:**263–272.
1a. **Bredesen, D. E.** 1995. Neural apoptosis. Ann. Neurol. **38:**839–851.
2. **Brooks, M. A., A. N. Ali, P. C. Turner, and R. W. Moyer.** 1995. A rabbitpox virus serpin gene controls host range by inhibiting apoptosis in restrictive cells. J. Virol. **69:**7688–7698.
3. **Bump, N. J., M. Hackett, M. Hugunin, S. Seshagiri, K. Brady, P. Chen, C. Ferenz, S. Franklin, T. Ghayur, P. Li, P. Licari, J. Mankovich, L. Shi, A. H. Greenberg, L. K. Miller, and W. W. Wong.** 1995. Inhibition of ICE family proteases by baculovirus antiapoptotic protein p35. Science **269:**1885–1888.
4. **Clem, R. J., and L. K. Miller.** 1994. Control of programmed cell death by the baculovirus genes *p53* and *iap*. Mol. Cell. Biol. **14:**5212–5222.
5. **Cully, J. F., Jr., T. G. Streit, and P. B. Heard.** 1992. Transmission of La Crosse virus by four strains of *Aedes albopictus* to and from the eastern chipmunk (*Tamias striatus*). J. Am. Mosq. Control Assoc. **8:**237–240.
5a. **Dickson, D. W., S. C. Lee, L. A. Mattiace, S. C. Yen, and C. Brosnan.** 1993. Microglia and cytokines in neurological disease, with special reference to AIDS and Alzheimer's disease. Glia **7:**75–83.
6. **Eastman, C. L., T. R. Guilarte, and J. R. Lever.** 1992. Uptake of hydroxykynurenine measured in rat brain slices and in a neuronal cell line. Brain Res. **584:**110–116.
7. **Esolen, L. M., S. W. Park, J. M. Hardwick, and D. E. Griffin.** 1995. Apoptosis as a cause of death in measles virus-infected cells. J. Virol. **69:**3955–3958.
8. **Geiger, K. D., D. Gurushanthaiah, E. L. Howes, G. A. Lewandowski, J. C. Reed, F. E. Bloom, and N. E. Sarvetnick.** 1995. Cytokine-mediated survival from lethal herpes simplex virus infection: role of programmed neuronal death. Proc. Natl. Acad. Sci. USA **92:**3411–3415.
9. **Gonzalez-Scarano, F., and N. Nathanson.** 1996. Bunyaviridae. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia.
10. **Griot, C., A. Pekosz, R. Davidson, K. Stillmock, M. Hoek, D. Lukac, D. Schmeidler, I. Cobbinah, F. Gonzalez-Scarano, and N. Nathanson.** 1994. Replication in cultured C2C12 muscle cells correlates with the neuroinvasiveness of California serogroup bunyaviruses. Virology **201:**399–403.
11. **Griot, C., A. Pekosz, D. Lukac, S. S. Scherer, K. Stillmock, D. Schmeidler, M. J. Endres, F. Gonzalez-Scarano, and N. Nathanson.** 1993. Polygenic control of neuroinvasiveness in California serogroup bunyaviruses. J. Virol. **67:**3861–3867.

12. **Hardy, M., D. Younkin, C.-M. Tang, J. Pleasure, Q.-Y. Shi, M. Williams, and D. Pleasure.** 1994. Expression on non-NMDA glutamate receptor channel genes by clonal human neurons. J. Neurochem. **63:**482–489.
13. **Hay, B. A., D. A. Wassarman, and G. M. Rubin.** 1995. Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. Cell **83:**1253–1262.
14. **Hinshaw, V. S., C. W. Olsen, N. Dybdahl-Sissoko, and D. Evans.** 1994. Apoptosis: a mechanism of cell killing by influenza A and B viruses. J. Virol. **68:**3667–3673.
15. **Hockenbery, D. M., G. Nunez, C. Milliman, R. D. Schreiber, and S. J. Korsmeyer.** 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature (London) **348:**334–336.
16. **Janssen, R., F. Gonzalez-Scarano, and N. Nathanson.** 1984. Mechanisms of bunyavirus virulence. Lab. Invest. **50:**447–455.
17. **Janssen, R. S., N. Nathanson, M. J. Endres, and F. Gonzalez-Scarano.** 1986. Virulence of La Crosse virus is under polygenic control. J. Virol. **59:**1–7.
18. **Kalfayan, B.** 1983. Pathology of La Crosse virus infection in humans, p. 179–186. *In* C. H. Calisher and W. H. Thompson (ed.), California serogroup viruses. Alan R. Liss, Inc., New York.
19. **Levine, B., J. M. Hardwick, B. D. Trapp, T. O. Crawford, R. C. Bollinger, and D. E. Griffin.** 1991. Antibody-mediated clearance of alphavirus infection from neurons. Science **254:**856–860.
20. **Lewis, J., S. L. Wesselingh, D. E. Griffin, and J. M. Hardwick.** 1996. Alphavirus-induced apoptosis in mouse brains correlates with neurovirulence. J. Virol. **70:**1828–1835.
21. **Lin, K.-I., S.-H. Lee, R. Narayanan, J. M. Baraban, J. M. Hardwick, and R. R. Ratan.** 1995. Thiol agents and bcl-2 identify an alphavirus-induced apoptotic pathway that requires activation of the transcription factor NF-kappa B. J. Cell Biol. **131:**1149–1161.
22. **Llanes, C., R. G. Collman, R. Hrin, and D. L. Kolson.** 1995. Acetylcholinesterase expression in NTera 2 human neuronal cells: a model for developmental expression in the nervous system. J. Neurosci. Res. **42:**791–802.
23. **Mossman, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods **65:**55–63.
24. **Murphy, T. H., M. J. De Long, and J. T. Coyle.** 1991. Enhanced NAD(P)(H): quinone reductase activity prevents glutamate toxicity produced by oxidative stress. J. Neurochem. **56:**990–995.
25. **Murphy, T. H., R. L. Schnaar, J. T. Coyle, and A. Sastre.** 1988. Glutamate cytotoxicity in a neuronal cell line is blocked by membrane depolarization. Brain Res. **460:**155–160.
26. **Olsen, C. W., J. C. Kehren, N. R. Dybdahl-Sissoko, and V. S. Hinshaw.** 1996. *bcl-2* alters influenza virus yield, spread, and hemagglutinin glycosylation. J. Virol. **70:**663–666.
27. **Pekosz, A., C. Griot, N. Nathanson, and F. Gonzalez-Scarano.** 1995. Tropism of bunyaviruses: evidence for a G1 glycoprotein-mediated entry pathway common to the California serogroup. Virology **214:**339–348.
28. **Pekosz, A., C. Griot, K. Stillmock, N. Nathanson, and F. Gonzalez-Scarano.** 1995. Protection from La Crosse virus encephalitis with recombinant glycoproteins: role of neutralizing anti-G1 antibodies. J. Virol. **69:**3475–3481.
29. **Shen, Y., and T. E. Shenk.** 1995. Viruses and apoptosis. Curr. Opin. Genet. Dev. **5:**105–111.
30. **Stinski, M. F., and T. J. Roehr.** 1985. Activation of the major immediate early gene of human cytomegalovirus by *cis*-acting elements in the promoter-regulatory sequence and by virus-specific *trans*-acting components. J. Virol. **55:**431–441.
31. **Subramanian, T., B. Tarodi, and G. Chinnadurai.** 1995. p53-independent apoptotic and necrotic cell deaths induced by adenovirus infection: suppression by E1 B 19K and Bcl-2 proteins. Cell Growth Differ. **6:**131–137.
32. **Tarodi, B., T. Subramanian, and G. Chinnadurai.** 1994. Epstein-Barr virus BHRF1 protein protects against cell death induced by DNA-damaging agents and heterologous viral infection. Virology **201:**404–407.
33. **Tyler, K. L., M. K. T. Squier, S. E. Rodgers, B. E. Schneider, S. M. Oberhaus, T. A. Grdina, J. J. Cohen, and T. S. Dermody.** 1995. Differences in the capacity of reovirus strains to induce apoptosis are determined by the viral attachment protein σ1. J. Virol. **69:**6972–6979.
34. **Ubol, S., P. C. Tucker, D. E. Griffin, and J. M. Hardwick.** 1994. Neurovirulent strains of alphavirus induce apoptosis in *bcl-2* expressing cells: role of a single amino acid change in the E2 glycoprotein. Proc. Natl. Acad. Sci. USA **91:**5202–5206.
35. **Westmoreland, S. V., D. Kolson, and F. Gonzalez-Scarano.** 1995. Toxicity of TNFα and platelet activating factor for human NT2N neurons: a tissue culture model for human immunodeficiency virus dementia. J. Neurovirol. **2:**118–126.
36. **Wyllie, A. H.** 1995. The genetic regulation of apoptosis. Curr. Opin. Genet. Dev. **5:**97–104.
37. **Yasuda, T., J. Grinspan, J. Stern, B. Franceschini, P. Bannerman, and D. Pleasure.** 1995. Apoptosis occurs in the oligodendroglial lineage, and is prevented by basic fibroblast growth factor. J. Neurosci. Res. **40:**306–317.
38. **Younkin, D. P., C.-M. Tang, M. Hardy, U. R. Reddy, Q.-Y. Shi, S. J. Pleasure, V. M.-Y. Lee, and D. Pleasure.** 1993. Inducible expression of neuronal glutamate receptor channels in the NT2 human cell line. Proc. Natl. Acad. Sci. USA **90:**2174–2178.
39. **Zhu, H., Y. Shen, and T. Shenk.** 1995. Human cytomegalovirus IE1 and IE2 proteins block apoptosis. J. Virol. **69:**7960–7970.