Theiler’s Virus-Induced Intrinsic Apoptosis in M1-D Macrophages Is Bax Mediated and Restricts Virus Infectivity: a Mechanism for Persistence of a Cytolytic Virus

Kyung-No Son,1,2 Robert P. Becker,3 Patricia Kallio,1 and Howard L. Lipton1,2*

Departments of Neurology and Rehabilitation,1 Microbiology and Immunology,2 and Anatomy and Cell Biology,3 University of Illinois at Chicago, Chicago, Illinois 60612-7344

Received 30 October 2007/Accepted 13 February 2008

Theiler’s murine encephalomyelitis virus (TMEV), a member of the Cardiovirus genus in the family Picornaviridae, is a highly cytopathic virus that produces necrotic death in rodent cells except for macrophages, which undergo apoptosis. In the present study we have analyzed the kinetics of BeAn virus infection in M1-D cells, in order to temporally relate virus replication to the apoptotic signaling events. Apoptosis was associated with early exponential virus growth from 1 to 12 h postinfection (p.i.); however, ≥80% of peak infectivity was lost by 16 to 24 h p.i. The pan-caspase inhibitor qVD-OPh led to significantly higher virus yields, while zVAD-fmk completely inhibited virus replication until 10 h p.i., precluding its assessment in apoptosis. In contrast, while zVAD-fmk significantly inhibited BeAn virus replication in BHK-21 cells at 12 and 16 h p.i., virus replication at these time points was not altered by qVD-OPh. Bax translocation into mitochondria, efflux of cytochrome c into the cytoplasm, and activation of caspases 9 and 3 between −8 and 12 h p.i. (all hallmarks of the intrinsic apoptotic pathway) were transiently inhibited by expression of Bel-2, which is not expressed in M1-D cells. Thus, BeAn virus infection in M1-D macrophages, which restricts virus replication, provides a potential mechanism for modulating TMEV neuropvirulence during persistence in the mouse central nervous system.

* Corresponding author. Mailing address: Department of Microbiology-Immunology, MC 790, University of Illinois at Chicago, 835 South Wolcott, Chicago, IL 60612-7344. Phone: (312) 996-5754. Fax: (312) 355-3581. E-mail: hlipton@uic.edu.

1 Published ahead of print on 20 February 2008.

Published ahead of print on 20 February 2008.

Theiler’s murine encephalomyelitis virus (TMEV), a member of the Cardiovirus genus in the family Picornaviridae, is a highly cytopathic virus that produces necrotic death in rodent cells, including neurons and oligodendrocytes. The virus yield in one-step growth kinetics studies in rodent cells is on the order of 200 to 500 PFU/cell. An exception is infection of murine macrophages, which undergo programmed cell death or apoptosis and produce virus yields restricted to <10 PFU/ cell (10). The profile of TMEV-induced apoptosis and restricted virus yields has been observed during infection with low-neurovirulence BeAn in a number of murine macrophage lines, including J774A.1, M1-D, P388D1, PUS-1.8, RAW264.7, IC21, and ANA-1 (11, 12), and in primary peritoneally derived macrophages (15). Analyses of another low-neurovirulence TMEV, strain DA, have also revealed similar low virus yields during infection in macrophages (26, 27, 29, 37). Murine promyelomonocytic (M1) cells in particular provide a useful in vitro model to study the outcome of TMEV infection, since they can be induced to differentiate into mature macrophages with interleukin-6 and then into activated macrophages with gamma interferon (IFN-γ) (4, 35).

Limited data suggest that apoptosis following BeAn virus infection is associated with caspase activation (11). IFN-γ-activated M1-D cells to which BeAn virus has been adsorbed undergo apoptosis in the absence of detectable virus replication (13), whereas UV-inactivated BeAn virus adsorbed to the M1-D cell surface does not induce apoptosis (15). IFN-γ activation sensitizes these cells to death-inducing ligands, and virus infection causes increased IFN-α/β secretion, resulting in up-regulation of TRAIL and tumor necrosis factor alpha ligands that mediate apoptosis (13). Caspase-mediated apoptosis may be initiated through either of two broad pathways that are responsible for eventual cell death. In the extrinsic pathway, which begins outside the cell, ligands bind to specific death receptors, such as Fas or the tumor necrosis factor receptor, in a conventional manner to activate caspase-8, which in turn activates the executioner caspase, caspase-3 (21). When injury occurs within the cell, the intrinsic pathway is initiated at the mitochondrion by disruption of the mitochondrial transmembrane potential and release of cytochrome c into the cytosol, where it binds Apaf1 to activate caspase-9 and, in turn, activates caspase-3 (32). In certain settings, caspase-8 activation may directly induce loss of mitochondrial transmembrane potential through the activation of Bid. Mitochondrial homeostasis is regulated by both pro- and antiapoptotic Bel-2 family proteins (7). The precise signaling pathways leading to apoptosis in M1-D cells during BeAn infection have remained unclear.

While persistence of noncytopathic RNA viruses, such as lymphocytic choriomeningitis virus, Borra disease virus, and hepatitis C virus, is readily understood, persistence of cytopathic RNA viruses, such as picornaviruses, is enigmatic, since continuous cell-to-cell spread is required to perpetuate the infection. Clearly, an RNA virus lytic for the target cell population in which it persists provides no advantage for either the cellular reservoir or the host organism. Thus, either selection of attenuated genetic variants in the viral quasispecies is required or host factors associated with the target cell population itself restrict virus replication (23, 28). Since TMEV persisting in mice is not attenuated in neurovirulence (22), we examined the...
possibility that apoptosis of infected macrophages, the principal TMEV reservoir in the central nervous system (CNS) of persistently infected mice (1, 24, 30), and a population of continuously replenished blood-borne monocytes crossing the blood-brain barrier to infiltrate demyelinating lesions (22a) is the restricting element during persistent infection. Our analysis correlates the temporal kinetics of BeAn virus infection in M1-D cells with the hallmarks of the intrinsic apoptotic pathway in these cells.

MATERIALS AND METHODS

Cells and viruses. M1 cells, an immature myelomonocytic cell line derived from the SL mouse strain, were induced to differentiate into macrophages with supernatants from L929 and P388D1 cells as previously described (12). The resulting M1-D cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, and nonessential amino acids (complete medium). BHK-21 cells (purchased from the American Type Culture Collection) were maintained in Dulbecco’s minimum essential medium (Invitrogen, Carlsbad, CA) containing 2 mM l-glutamine, 0.5% tryptose phosphate, and 10% FBS. The origin and passage history of the BeAn virus stock have been described elsewhere (34). Virus titers of clarified lysates of infected cells were described elsewhere (34).

Infection (MOIs) for 45 min at 24°C, cell monolayers were washed twice with 0.5% Tween 20 in PBS and distilled water and viewed with a Zeiss digital confocal microscope. The Inouye kit (Calbiochem) for cytochrome c release from mitochondria that relies on selective permeabilization of cell membranes for release of cytosolic components leaving mitochondrial membranes intact was used following the manufacturer’s instructions. Cytochrome c was detected with a mouse monoclonal antibody and a fluorescein isothiocyanate-conjugated secondary antibody by flow cytometry.

Immuno blot analysis of cellular proteins. M1-D cells (monolayer cells and cells shed into the supernatant combined) were washed with PBS and lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-30, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) at the indicated times. Protein samples were electrophoresed on 12% NuPage bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes. Membranes were blocked with Tris-buffered saline containing 5% nonfat dry milk and 0.02% Tween 20 and incubated with primary antibody for 1 h and with 1:100 goat anti-rabbit-HRP or anti-mouse–HRP as the secondary antibodies for 1 h. A rabbit antibody to β-actin (Cell Signaling) was used as a loading control. Antibody dilutions were determined from initial experiments with M1-D cells induced to undergo apoptosis by treatment with 1 µg/ml actinomycin D. Quantification of the immunoblots was performed with the program Prism 4 (GraphPad Software, San Diego, CA).

Immunofluorescence staining. M1-D cells, grown and infected on glass coverslips, were fixed and permeabilized (see “TUNEL assay,” above). Infected cells were washed twice in PBS, incubated with 1:1,000 rabbit anti-BeAn serum (detects capsid proteins and immediate precursor), 1:100 rabbit anti-cytochrome c (Cell Signaling), or mouse anti-Bax monoclonal antibody for 30 min, washed once in PBS, and incubated with 1:200 goat anti-rabbit IgG (Abcam) or 1:200 goat anti-mouse IgG (BD Pharmingen) for 30 min. Coverslips were inverted on microscope slides onto gel mount (Biomedica, Foster City, CA) and viewed with a Zeiss digital confocal microscope.

Microscopy. M1-D cells were harvested and fixed with 3% glutaraldehyde in PBS. Cell were further fixed in aqueous 2% osmium tetroxide, stained with 0.5% aqueous uranyl acetate, dehydrated with a graded ethanol series, and embedded in epoxy resin LX112. Transverse sections (1 µm) were cut and further stained with toluidine blue O for light microscopy. For transmission electron microscopy, sections were cut at a 100-nm thickness, placed on Formvar-coated 200-mesh copper grids, stained further with uranyl acetate and lead citrate, and viewed under a JEOL model 1220 microscope (Tokyo, Japan) at 80 kV and with 1,600× to 150,000× magnification. Images were documented with a Gatan multiscan camera model 794.

Statistical analysis. A paired Student’s t test was used to compare groups, and differences were considered significant at a P level of <0.05.

RESULTS

Temporal kinetics of apoptosis in BeAn virus-infected M1-D cells. Death of M1-D cells infected with BeAn virus (MOI, 10) was apparent at 8 to 10 h postinfection (p.i.) and increased thereafter, as determined by cell survival assay (Fig. 1A). Phase microscopy revealed subtle signs of cytopathology at 8 h p.i., with increasing numbers of pyknotic and fragmented cells seen at 12 to 16 h p.i. (Fig. 1B). A TUNEL assay of infected cell monolayers showed stained cells as early as 6 h p.i. (Fig. 1B). Electron microscopy identified autophagy in monolayer cells at 12 and 16 h p.i., with typical features of double-layered membrane vacuoles and arcuate cytoplasmic clefts (Fig. 1C, cell b), whereas detached cells exhibited features of apoptosis, including condensed nuclei and nuclear chromatin and cytoplasmic blebbing (Fig. 1C, cell c). Some apoptotic cells contained autophagic vacuoles, and apoptotic fragments of cells were also observed in the supernatant fraction (Fig. 1C, cell d). Perinuclear collections of proliferative vesicles, indicative of sites of virus replication, were seen in some apoptotic cells (Fig. 1C, cell c) (36). Thus, both TUNEL and electron microscopic analyses indicated that M1-D cells undergo apoptosis as a result of BeAn virus infection.
Decrease in BeAn infectious virus after 12 h p.i. in association with apoptosis of infected M1-D cells. To correlate the observed temporal changes in apoptosis with the infection, virus antigens were detected by immunofluorescent staining and virus yields were measured by standard plaque assay (Fig. 2). Virus antigen expression was detected at 2 h p.i. (not seen in this image reproduction) with increasing numbers and intensity of staining between 4 and 12 h p.i. (Fig. 2A). Previously, BeAn virus yields in M1-D cells were only assessed at 24 h p.i. (14) and suggested that virus replication may have been restricted throughout the infection. Our present analysis revealed increasing virus yields to 12 h p.i., similar to that in BHK-21 cells (not shown), but with two- to threefold-lower amounts of virus than in BHK-21 cells at each time point. Unlike the infection in BHK-21 cells, virus yields in M1-D cells steadily declined after 12 h p.i. (Fig. 2B and C). A similar kinetics but with high virus yields to 24 h has also been seen in L929 cells which, unlike BHK-21 cells, are not deficient in IFN production (unpublished data). However, addition of polyclonal anti-IFN antibodies to the medium of BeAn virus-infected M1-D cells after virus adsorption did not increase virus yields after 12 h p.i. (not shown), suggesting that apoptosis, not IFN activity, restricted virus yields. Addition of zVAD-fmk to BeAn virus-infected BHK-21 cells also significantly reduced virus at 12 and 16 h p.i. but not at 20 h p.i., whereas addition of qVD-OPh did not increase virus yields as it did in M1-D cells (Fig. 2C).

Effects of pan-caspase inhibitors on BeAn virus infection. Based on previous analysis suggesting that BeAn virus-induced M1-D cell death is caspase mediated (12), we examined the effect of the pan-caspase inhibitor zVAD-fmk on infection. Although zVAD-fmk (20 μM) added to the medium inhibited virus replication until 12 h p.i., limited virus replication was observed at later times (Fig. 2B). zVAD-fmk has been reported to inhibit 2A of human rhinoviruses (5). Since cardiovirus 2A does not have proteolytic activity, it is likely that zVAD-fmk inhibits the only other cardiovirus protease, 3Cpro (21a). Thus, the prevention of caspase-3 and PARP cleavage at 12 h p.i. and protection of M1-D cells at 16 h p.i. (P < 0.001) by zVAD-fmk (Fig. 3A and B) was most likely due to its known antiviral activity rather than as a pan-caspase inhibitor. Because the antiviral effect of zVAD-fmk was transient, slight
cleavage of these products was seen at 16 h p.i. (Fig. 3A). In contrast, 10 μM qVD-OPh did not inhibit early virus replication but partially prevented caspase-3 cleavage at 12 and 16 h p.i. (Fig. 3A) (P < 0.001), protected cells from death until 16 h p.i. (Fig. 3B) (P = 0.005), and resulted in a significant increase (P < 0.01) in virus yields at 12 h and 16 h p.i. (Fig. 2B). The protection by qVD-OPh was no longer seen at 24 h p.i., and higher concentrations (20 and 50 μM) did not increase cell survival time (not shown). qVD-OPh did not have a similar protective effect in infected BHK-21 cells which undergo necrosis and not apoptosis (Fig. 2C). The antiviral effect of zVAD-fmk is due to the carboxy-terminal fluoromethylketone moiety, which binds the active proteolytic site, whereas qVD-OPh has a carboxy-terminal phenoxy group conjugated to Val and Asp residues (2). Thus, BeAn virus infection resulted in caspase-mediated apoptosis that was associated with loss of infectivity after 12 h p.i., providing a potential mechanism of attenuating this lytic picornavirus during its CNS replication in macrophages in the mouse.

Caspase proteolytic activity implicates the intrinsic apoptotic pathway. The temporal pattern of signal induction of the initiator and effector caspases and PARP was examined at 4-h intervals postinfection by immunoblotting of cell lysates. Cleavage of PARP was observed at 8 h p.i., caspase-9 and -3 at 12 h p.i., and caspase-8 to its fully active p18 form at 16 h p.i., followed by increasing cleavage of pro- to active caspase forms at subsequent times (Fig. 4A). Levels of pro- and active caspase forms decreased after 12 h p.i., due to increasing protein degradation and cell death (Fig. 4A, loading control β-actin). Figure 4B shows the results of densitometric analysis of immunoblot autoradiograms to quantitate the kinetic profile
from the ratio of the cleaved form to proenzyme form. Neither caspase-9 nor caspase-3 was cleaved at 0 h p.i. (data not shown). Caspase-9 does not require cleavage to become active; instead, caspase-9 is activated by dimerization (9, 33). Thus, it is likely that caspase-9 activation as well as caspase-3 cleavage and activation occurred earlier than 12 h p.i. and accounted for PARP cleavage and cytochrome c efflux into the cytoplasm at 8 h p.i. (see below). Moreover, the caspase-3 antibody used appears to be more sensitive in detecting the proform than the active cleavage product, possibly accounting for the delayed appearance of caspase-3 cleavage. As shown in Fig. 4A, caspase-8 was already partially cleaved to p41 at 0 h (after virus adsorption). Overall, the proteolytic profile is consistent with an intrinsic apoptotic pathway underlying death of BeAn virus-infected M1-D cells. Cleavage of Bid to its active form tBid only at 16 h p.i. provided further evidence against involvement of the extrinsic apoptotic pathway (Fig. 4C and D).

**Caspase-9 release from mitochondria.** Activation of the initiator caspase-9 results from freeing of proapoptogenic cytochrome c from mitochondria. Analysis of cytochrome c efflux into the cytosol as a surrogate indicator of mitochondrial outer membrane permeabilization in BeAn virus-infected M1-D cells by indirect immunofluorescence staining of infected cells revealed cytochrome c in mitochondria based on particulate staining until 6 h p.i. (4 h p.i. data not shown), whereas more diffuse staining throughout the cytoplasm was first seen at 8 h p.i. and at 10 and 12 h p.i. (Fig. 5A). An increase in cytosolic cytochrome c was first seen at 8 h p.i. as a distinct cap of staining around the nucleus (Fig. 5A). This pattern of cytochrome c efflux into the cytoplasm was also demonstrated by FACS analysis, which showed its release at 8 h p.i. (Fig. 5B). Moreover, immunoblotting of cytosolic and heavy membrane fractions separated from whole-cell extracts showed reactivity with antibodies to cytochrome c in the cytosol at 8 h, with increasing amounts released at 10 to 16 h p.i. (Fig. 5C and D). Thus, the kinetics of cytochrome c efflux into the cytoplasm from mitochondria paralleled that of caspase-9 activation, consistent with caspase-9 activation from dimerization prior to its cleavage observed at 12 h p.i.

**Infection results in Bax translocation to the mitochondria.** Analysis of the immediate upstream signals responsible for cytochrome c release in BeAn virus-infected M1-D cells revealed an increase in Bax, but not Bak, expression that was seen at 4 h p.i., increased thereafter, reaching fivefold greater levels at 10 h p.i. than in cells at the end of adsorption (0 h p.i.) (Fig. 6A). This result suggests that Bax expression is induced by the infection. Bax is located in the cytoplasm of viable cells and upon activation translocates to the mitochondria and is inserted into the mitochondrial outer membrane (8, 40). Immunoblotting to detect Bax translocation to the mitochondria in infected fractions using a mouse monoclonal antibody to Bax indicated decreased amounts of Bax in the cytosol during translocation into mitochondria in infected cells at 8 to 10 h p.i. (Fig. 6B and C), followed by a further decrease in Bax cytosolic levels at 12 to 16 h p.i. (Fig. 6B). Immunofluorescence staining of infected cells also revealed a shift from diffuse to particulate Bax cytoplasmic staining in a portion of cells at 8 h p.i. and in all cells at 10 h p.i. (Fig. 6D). Together the results indicate the essentially concomitant translocation of Bax and the release of cytochrome c from the mitochondria, correlating with the activation of caspase-9.

**Bax inhibition by RNAi abrogates cytochrome c release from mitochondria.** To confirm the requirement of Bax in mitochondrial permeability, Bax expression was knocked down by RNA interference (RNAi) in BeAn virus-infected M1-D cells. Immunoblot analysis of cells transfected with Bax siRNA (Cell Signaling) showed reduction of Bax to 25% the level in infected fractions using a mouse monoclonal antibody to Bax indicated decreased amounts of Bax in the cytosol during translocation into mitochondria in infected cells at 8 to 10 h p.i. (Fig. 6B and C), followed by a further decrease in Bax cytosolic levels at 12 to 16 h p.i. (Fig. 6B). Immunofluorescence staining of infected cells also revealed a shift from diffuse to particulate Bax cytoplasmic staining in a portion of cells at 8 h p.i. and in all cells at 10 h p.i. (Fig. 6D). Together the results indicate the essentially concomitant translocation of Bax and the release of cytochrome c from the mitochondria, correlating with the activation of caspase-9.

**Protection of infected M1-D cells by Bel-2 but not Bel-xL.** The antiapoptotic Bel-2 family proteins Bel-2 and Bel-xL play a central role in inhibiting the mitochondria-dependent cell death pathway. Moreover, it has been reported that Bel-2 but not Bel-xL is expressed in promyelomonocytic M1 cells, whereas differentiation of M1 cells into M1-D macrophages
results in loss of Bcl-2 and upregulation of Bcl-xL expression (11), as recapitulated in Fig. 8A. Analysis to test whether Bax is held in check by an antiapoptotic Bcl-2 family protein(s) using M1-D cells transfected with pcDNA3-Bcl-2 and pcDNA3 40 h before infection (transfection efficiencies of 35 to 40% were obtained in this macrophage cell line cotransfected with pEGFP-N1) showed that Bcl-2 resulted in inhibition of cleavage of caspase-9 and caspase-3 to their active forms at 12 h p.i. but not at 8 h p.i. (Fig. 8B and C). Cleavage of these caspases earlier than in untreated cells (Fig. 4) may be due to cell stress from transfection. Although caspase inhibition causes a shift to caspase-independent self-destructive processes and often does not prevent cell death, particularly in macrophages (20, 37a), 41 ± 0.81% (mean ± standard deviation) of M1-D cells transfected with pcDNA3-Bcl-2 before infection (transfection efficiencies of 35 to 40%) were obtained in this macrophage cell line cotransfected with pEGFP-N1) showed that Bcl-2 resulted in inhibition of cleavage of caspase-9 and caspase-3 to their active forms at 12 h p.i. but not at 8 h p.i. (Fig. 8B and C). Cleavage of these caspases earlier than in untreated cells (Fig. 4) may be due to cell stress from transfection. Although caspase inhibition causes a shift to caspase-independent self-destructive processes and often does not prevent cell death, particularly in macrophages (20, 37a), 41 ± 0.81% (mean ± standard deviation) of M1-D cells transfected with pcDNA3-Bcl-2 before infection survived to 16 h p.i., compared to 32 ± 2.56% of cells transfected with pcDNA3 (P = 0.03; n = 3) (Fig. 8D). However, this modest but significant (P = 0.03) protection of cell monolayers was lost at 20 h p.i. (not shown). In contrast, overexpression of Bcl-xL did not prevent caspase-9 and caspase-3 cleavage or protect from cell death (not shown). These data further support the conclusion that BeAn virus-induced apoptosis involves the intrinsic pathway mediated by Bax.

**DISCUSSION**

In the present study we examined the kinetics of BeAn virus replication in M1-D cells with respect to apoptotic signaling...
events in these cells. Previous evidence that BeAn virus-induced apoptosis involves caspase activation (11) led us to analyze the apoptotic pathway(s) involved. Electron microscopy, the gold standard for demonstrating apoptosis, revealed double membrane structures typical of autophagosomes before the appearance of the classic apoptotic features of cellular and nuclear shrinkage, chromatin condensation, blebbing, nuclear fragmentation, and fragmentation of apoptotic bodies in infected M1-D cells (16). Classifications of apoptosis include an autophagic type (type 2), and autophagy, a homeostatic process related to apoptosis, which provides a system to remove damaged organelles and aggregated proteins, functions as a cell survival mechanism (18). Blocking of autophagy leads to increased susceptibility to proapoptotic insults (31).

The many cellular stresses that can trigger autophagy include infections, and recent studies recognize that mammalian cells infected with positive-strand RNA viruses accumulate double membrane vesicles of autophagic origin which are believed to serve as a physical scaffold for virus replication complexes (viroplasm) (reviewed in references 17 and 38). The morphological events shown here in BeAn virus-infected M1-D cells might represent a failure of the autophagic system in cell survival, culminating in infected cells that progress to apoptosis.

Exponential virus replication typical of one-step growth kinetics of most picornaviruses was observed between 1 and 12 h p.i. in infected M1-D cells; however, 80% of peak infectivity was lost by 16 to 24 h p.i. (Fig. 2B). Previous studies of TMEV-induced apoptosis indicated that a higher MOI restricts final virus yields by 99% (10, 11, 14). In contrast, TMEV replication in rodent cell lines such as BHK-21 cells results in necrosis, with no reduction of peak infectivity titers at later times (Fig. 2C). These observations suggest that the loss of infectivity is due to apoptosis. Our preliminary data indicate that caspase activation associated with TMEV one-step growth kinetics in M1-D cells leads to cleavage of VP2 in 150S virions (from sucrose gradients) at 16 h p.i. but not earlier, resulting in high particle-to-PFU ratios.

The present study also shows that BeAn virus infection induces apoptosis through the intrinsic pathway. Hallmarks of activation of the intrinsic apoptotic pathway were observed at 8 to 12 h p.i. during exponential BeAn virus growth in M1-D cells. Bax translocation to the mitochondria outer membrane and release of cytochrome c into the cytoplasm occurred at 8 to 10 h p.i. (Fig. 5) when caspase-9 had probably dimerized and become activated (9, 33), with caspase-9 cleavage following later at 12 h p.i. (Fig. 4A and B). PARP cleavage and positive TUNEL staining at 8 h p.i. (Fig. 1C and 4A and B) suggest that at least some caspase-3 was cleaved to the active form before this was demonstrated on immunoblot assays. This interpretation of the temporal signaling events is also consistent with the linear cell death observed at 10 to 16 h p.i. (Fig. 1A). Partial cleavage of caspase-8 to p45 was seen in uninfected and mock-infected M1-D cells. Caspase-8 cleavage to the active p18 form and cleavage of Bid to tBid occurred at 16 h p.i. and most likely was due to activated caspase-3 “feeding back” to cleave

FIG. 7. Bax is required for cytochrome c release from mitochondria. A. Immunoblotting revealed that transfection of Bax siRNA in infected M1-D cells reduced Bax expression at 10 h p.i. to 25% of the level in cells transfected with an irrelevant siRNA and reduced cytochrome c release from mitochondria by 66%. B. Transfection of Bax siRNA in infected M1-D cells led to modest but significant (P = 0.01) cell survival at 16 h p.i.

FIG. 8. Expression of Bcl-2 protects against BeAn virus-induced apoptosis. A. Immunoblot showing that Bcl-xL, but not Bcl-2, is expressed in M1-D cells. B. Immunoblot showing inhibition of caspase-9 and -3 cleavage to their active forms at 12 h p.i. in cells transiently transfected with pcDNA3/Bcl-2 but not in cells transfected with pcDNA3. C. Densitometric analysis of the ratio of active to pro- or active forms of the caspases in panel B. D. Cell survival assay demonstrating significant protection (P = 0.03) from cell death by expression of Bcl-2 compared to vector only.
TMEV-INDUCED APOPTOSIS IN M1-D MACROPHAGES 4509

12. Jalchich, M. L., and H. L. Lipton. 1999. Restricted virus replication provide a potential mechanism for downstream cellular events during infection. TMEV infection in other rodent cell lines produces necrosis of population(s) and pathogenetic events in vivo. The fact that cells that bear little or no resemblance to the infected cell other animal viruses in which apoptosis has been induced in phages) relevant to the persistence of low-neurovirulence which sense cellular damage and engage their antiapoptotic proapoptotic BH3-only activators, e.g., Nova, Puma, and Bim, which sense cellular damage and engage their antiapoptotic regulators in macrophages, and its overexpression in M1-D cells may not only provide greater protection against Be/A virus-induced apoptosis but also may point to specific upstream proapoptotic BH3-only activators, e.g., Nova, Puma, and Bim, which sense cellular damage and engage their antiapoptotic relatives to overcome the block to Bax or Bak activation (39).

ACKNOWLEDGMENTS
We thank Prasad Kanteti for helpful discussions and Marina Hoffman for editorial assistance.

REFERENCES