Rubella Virus-Induced Apoptosis Varies among Cell Lines and Is Modulated by Bcl-X<sub>L</sub> and Caspase Inhibitors

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Rubella virus (RV) causes multisystem birth defects in the fetuses of infected women. To investigate the cellular basis of this pathology, we examined the cytopathic effect of RV in three permissive cell lines: Vero 76, RK13, and BHK21. Electron microscopy and the TUNEL assay showed that the cytopathic effect resulted from RV-induced programmed cell death (apoptosis) in all three cell lines, but the extent of apoptosis varied among these cells. At 48 h postinfection, the RK13 cell line showed the greatest number of apoptotic cells, the Vero 76 cell line was ~3-fold less, and BHK21 had very few. An increased multiplicity of infection and longer time postinfection were required for the BHK21 cell line to reach the level of apoptotic cells in Vero 76 at 48 h. Purified RV induced apoptosis in a dose-dependent fashion, but not UV-inactivated RV or virus-depleted culture supernatant. Specific inhibitors of the apoptosis-specific proteases caspases reduced RV-induced apoptosis and led to higher levels of RV components in infected cells. To address the role of regulatory proteins in RV-induced apoptosis, the antiapoptotic gene Bcl-2 or Bcl-X<sub>L</sub> was transfected into RK13 cells. Although a high level of Bcl-2 family proteins was expressed, no protection was observed from apoptosis induced by RV, Sindbis virus, or staurosporine in RK13 cells. In BHK21 cells, however, increased expression of Bcl-X<sub>L</sub> protected cells from apoptosis. The observed variability in apoptotic response to RV of these cell lines demonstrates that programmed cell death is dependent on the unique properties of each cell and may be indicative of how selective organ damage occurs in a congenital rubella syndrome fetus.

INTRODUCTION

Rubella virus (RV) is an enveloped, positive sense RNA virus of the family Togaviridae. Infection during the first trimester of pregnancy can cause fetal defects that vary in severity from malformed digits to fetal death; this is termed congenital rubella syndrome (CRS) (Wolinsky, 1996). The continued importance of understanding this phenomenon is underscored by reports of CRS in infants born to mothers who had been vaccinated against RV (Barfield et al., 1997; Hornstein et al., 1988; Robinson et al., 1994). Although animal models have not been used extensively, congenital rubella effects have been demonstrated in rats (Avila et al., 1972; Collier et al., 1968), rabbits (Kono et al., 1969; London et al., 1970), and ferrets (Rorke et al., 1968). The selective organ damage characteristic of CRS (Wolinsky, 1996) may indicate cell type differences in response to RV infection in the developing fetus. Host cell–virus interactions have been shown to be important for RV replication (Atreya et al., 1998; Duncan and Nakhasi, 1997; Nakhasi et al., 1994, 1988). Thus, a basis for CRS pathology could be revealed at the cellular level. Tissue culture cell lines have been identified that support RV infection and show a variable level of cytopathic effect (CPE) (Frey, 1994). In light of recent findings on the cytopathogenesis of other RNA viruses (Esolen et al., 1995; Levine, 1993; Liao et al., 1997; Scallan et al., 1997; Tolskaya et al., 1995), the morphological changes and cell death due to RV infection, described as CPE, may be the result of programmed cell death (PCD), also known as apoptosis.

Apoptosis is a form of cell death distinct from necrosis that results from the triggering of a specific, genetically determined series of events leading to self-destruction and disposal of unwanted cells. This process of disposal is essential in development, for the resolution of the immune response, and the elimination of infected cells (Vaux and Strasser, 1996; White, 1996). Apoptotic cell death is characterized by internucleosomal DNA cleavage, chromatin condensation, nuclear fragmentation, and plasma membrane changes, including loss of microvilli and movement of phosphatidylserine to the outer layer (Schwartzman and Cidlowski, 1993; White, 1996). A central role in the regulation of apoptosis is played by the Bcl-2 family of proteins. The cellular oncogene Bcl-2 was shown to lead to tumors because it is a suppressor of apoptosis (Nunez et al., 1990; Vaux et al., 1988). The closely related Bcl-X<sub>L</sub> is also antiapoptotic; however, other related proteins, such as Bax and Bak, facilitate the cell death program (Kroemer, 1997; Reed, 1997). These
regulatory proteins are components in a pathway that culminates in the activation of members of a family of proteases called caspases, which begin the breakdown of the cell (Nicholson and Thornberry, 1997). Caspases are an essential element in the cell death program because their inhibition by virally encoded proteins (Bump et al., 1995; Tewari et al., 1995) or synthetic inhibitors (Cain et al., 1996; Schlegel et al., 1996) can effectively block apoptosis.

Viruses engage the mechanisms of PCD in various ways (Razvi and Welsh, 1995; Teodorro and Branton, 1997). Most viruses induce apoptosis, which can be blocked by the constitutive expression of Bcl-2 (Levine, 1993; Scallan et al., 1997). Some viruses have evolved proteins that block PCD (Bertin et al., 1997; Leopardi and Roizman, 1996), allowing them to replicate longer before killing the cell.

A clearer understanding of the precise mechanisms of cell death has already made an impact on the understanding and therapeutic strategies in neoplastic and inflammatory diseases (Blank et al., 1997; Rudin and Thompson, 1997). Similar insights into viral pathogenesis and treatment are arising from the study of virus-induced apoptosis. In this study, we demonstrate that RV kills cells by initiating programmed cell death and that the threshold level of virus required to induce apoptosis varies substantially among cell types. This system has the potential to reveal critical cellular components required for apoptosis and will focus the approach to understanding the mechanism of apoptosis induction by RV.

RESULTS

Variation in RV CPE among cell lines does not correlate with virus infection

Three cell lines that are permissive for RV, RK13 (rabbit kidney epithelial cells), Vero 76 (monkey kidney epithelial cells), and BHK21 (hamster kidney fibroblast cells), were infected with the same virus stock (according to titration on Vero 76 cell at an m.o.i. of 5 PFUs/cell). After 48 h of infection, the number of cells that have rounded up and are becoming detached from the plate, representing the CPE of RV, varies among these cell lines (Fig. 1). These differences were quantified by counting the number of detached cells released into the media from the same surface area of cultured cells and expressed as a percent of the total number of cells (Fig. 1). The average number of detached RK13 cells on RV infection represented 75% of cells in the culture vessel. Detached cells for infected Vero 76 were about 3 times fewer but still a substantial 25% of the total. The numbers of detached cells in both infected and mock-treated BHK21 were nearly the same and represented only 2% of the total cells, substantially less than either of the other cell lines.

To assess the relationship between the amount of viral components in infected cells and the quantity of CPE, we performed capsid immunoblots of cell lysate from these samples. The level of this protein has been shown to correlate with the level of other viral components as well as RV titers (PFUs) released from Vero 76 cells (Nakhasi et al., 1989). Surprisingly, 48 h after infection, the level of virus protein synthesis in these cell types did not correlate with the amount of CPE observed (Fig. 1). RK13 showed the highest number of detached cells yet had the lowest level of RV capsid protein (see the insets of RV capsid immunoblots at the borders of Fig. 1). BHK21 cells contained as much or more capsid protein than Vero 76 yet showed very little CPE. The variation in capsid level seen here correlates with the virus titers previously reported for these cell lines (Frey, 1994). The faint band visible in mock-infected Vero 76 and RK13 is due to antibody cross-reaction with a cellular protein because this band and the capsid band can be differentiated on a lower percent acrylamide gel (data not shown). The variation among cell lines suggested that differences in the cellular response, not the virus, accounted for the quantity of cell death.

One form of cell response to viruses is programmed cell death, or apoptosis, shown to be induced by other related viruses, such as Sindbis (Levine, 1993) and polio (Tolskaya et al., 1995). Therefore, we examined the RV-infected cells for evidence of apoptosis.

CPE caused by RV is the induction of apoptosis

At 48 h after RV infection, detached and adherent cells were collected separately for each cell line and examined by electron microscopy (Fig. 2). Immunofluorescent assays on adherent Vero 76 cells using anti-RV E1 glycoprotein antibodies (R. Duncan, unpublished data) as well as the high magnification inset showing virus particles (Fig. 2E) indicated that as many as 50% of the adherent cells are infected; however, these cells rarely showed apoptotic morphology (Figs. 2A, 2C, and 2E). In contrast, the detached cells had condensed chromatin, fragmented nuclei, loss of microvilli on their surfaces, and vacuolization, all features of apoptotic cells (Figs. 2B, 2D, and 2F). Significant, the detached, BHK21 cells are equally apoptotic in appearance (compare Fig. 2F with Figs. 2B and 2D), indicating RV can also induce programmed cell death in this cell type, but there are fewer detached cells than in Vero 76 and RK13 cell infections under these conditions.

To determine whether increasing the level of RV could alter the induction of apoptosis in BHK21 cells, infection was performed as above with a higher concentration of virus and allowed to proceed for a longer time. Cells were infected at 10× the previous level of RV (~50 PFUs/cell). Significantly higher levels of virus infection were achieved as indicated by the level of capsid protein in cell lysates (data not shown). Five days after infection, the mock-infected culture had <1% detached cells, whereas the RV-infected culture had 14.6% and 23.1%...
detached cells in two experiments. Thus under these conditions, the level of shed cells was similar to that seen in Vero 76 cells at 48 h (see Fig. 1).

To better understand the process of RV-induced cell death, we examined infected cells in an earlier stage of apoptosis, before they became detached. Vero 76 cells were RV- or mock-infected in chamber slides, and at 72 h postinfection, detached cells were washed away and adherent cells were fixed. The fixed cells were incubated with terminal deoxytransferase in the presence of FITC-labeled dUTP (TUNEL assay). Fluorescent micrographs showed incorporation of dUTP indicating the nicked DNA diagnostic of apoptosis (Figs. 3C and 3D). Comparison of

RV replication is required for induction of apoptosis

The observed apoptosis induction could be due to a substance other than virus secreted into the medium by
infected cells. To eliminate this possibility, RV was pelleted by ultracentrifugation and resuspended in culture medium from uninfected cells. Vero 76 cells were treated with virus resuspended at various multiples of its original concentration or with the virus-depleted supernatant. Counting of the detached, apoptotic cells 48 h after treatment indicated that the virus, not the supernatant, induced apoptosis. The number of detached cells increased with the concentration of virus, and the number of detached cells in the group treated with RV-depleted supernatant was 75-fold less than that with standard infection, nearly as low as with mock infection (Table 1). The level of apoptosis correlated with the amount of virus protein, as indicated by the quantity of RV capsid on immunoblots from these samples (data not shown).

To answer the question of whether active replication is required for apoptosis, virus stock was irradiated with UV light for varying times in a dose range that should render the RNA incapable of replication (Lai and Joklik, 1973) and then used in the standard infection protocol with Vero 76 cells. With as little as 10 s of irradiation, the number of detached cells was reduced 100-fold. The 50-s-treated RV stock caused no more apoptosis than mock infection (Table 1). The capsid level from lysates of these cells indicated that viral protein synthesis was absent, suggesting no replication. The suggestion that active replication was required, not just exposure to virus particles, was further supported by the lack of response to 10× concentrated, UV-irradiated RV (data not shown).

**FIG. 2.** Detached cells show apoptotic morphology by electron microscopy. Thin-section electron micrographs of RV-infected cells of all three cell lines. Detached cells (B, D, and F) were collected with the medium, after lightly tapping the container, and were pelleted and fixed for electron microscopy. The adherent cells (A, C, and E) were trypsinized, washed, pelleted, and fixed for electron microscopy. Vero 76 cells are shown in A and B, RK13 in C and D, and BHK21 in E and F. The bar in B is 5 μm for all low magnification. The inset in E shows an example of RV particles from BHK21 cells (bar = 200 nm).

**FIG. 3.** Rounded-up cells resulting from RV infection have nicked DNA characteristic of apoptosis in the TUNEL assay. Phase-contrast micrographs (A and B) or fluorescent micrographs (C and D) of the same field of Vero 76 cells 48 h after RV infection (B and D) or mock treatment (A and C). Green nuclei resulting from the incorporation of FITC-labeled dUTP into nicked DNA are found only in rounded cells, which can be identified in the phase photograph.
Inhibition of caspases reduced RV-induced cell death

In most cells studied, the effector phase of apoptosis involves the activation of a class of cysteine proteases called caspases (Nicholson and Thornberry, 1997). The subgroup of caspases most directly involved in cleavage of apoptotic substrates have a tetrapeptide cleavage site preference for the sequence DExD. To assess the role of caspases in RV-induced apoptosis, infected cells were cultured in the presence of a membrane-permeable derivative of zDEVD-fmk (n-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone), an irreversible caspase inhibitor (Nicholson and Thornberry, 1997). The number of detached cells was counted 48 h postinfection as a measure of the extent of apoptosis (Fig. 4). Caspase inhibitor resulted in a 35% decrease in the level of apoptosis in RK13 cells (statistically significant at \( P < .005 \)). zDEVD-fmk treatment also reduced the number (an average of 65% in two experiments) of apoptotic Vero 76

**TABLE 1**

<table>
<thead>
<tr>
<th>Preparation used for Vero 76 cell treatment</th>
<th>Total number of detached cells per 75-cm² flask</th>
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</thead>
<tbody>
<tr>
<td>Standard RV stock (m.o.i. 5)</td>
<td>( 1.74 \times 10^6 )</td>
</tr>
<tr>
<td>Culture supernatant from uninfected cells</td>
<td>( 7.35 \times 10^3 )</td>
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<tr>
<td>UV-treated RV stock</td>
<td></td>
</tr>
<tr>
<td>0 s</td>
<td>( 1.22 \times 10^6 )</td>
</tr>
<tr>
<td>10 s</td>
<td>( 1.39 \times 10^4 )</td>
</tr>
<tr>
<td>50 s</td>
<td>( 7.07 \times 10^3 )</td>
</tr>
<tr>
<td>Ultracentrifuged and reconstituted RV</td>
<td></td>
</tr>
<tr>
<td>10× (m.o.i. 5)</td>
<td>( 2.93 \times 10^6 )</td>
</tr>
<tr>
<td>1× (m.o.i. 5)</td>
<td>( 1.29 \times 10^6 )</td>
</tr>
<tr>
<td>0.1×</td>
<td>( 2.70 \times 10^5 )</td>
</tr>
<tr>
<td>RV-depleted virus stock</td>
<td>( 2.29 \times 10^4 )</td>
</tr>
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a RV stock was culture supernatant from Vero 76 cells harvested 8 days postinfection.

**FIG. 4.** Inhibition of caspase activity restricts RV-induced apoptosis. Addition of the caspase inhibitor DEVD-fmk to cells after RV infection reduces apoptosis. Caspase inhibitor was added to the culture medium at a concentration of 20 μM after 2 h of virus attachment (RV) or 2 h of mock treatment (M), and detached cells were counted 48 h later. The number of detached cells for DEVD-fmk inhibitor-treated samples are indicated by the striped bars. Lightly shaded bars represent detached cell counts from carrier (DMSO)-treated samples of each cell type indicated, infected (RV) or mock-treated (M). BHK21 cells were infected with 10× more concentrated RV stock than Vero 76 and RK13, and cell counts were taken at 96 h postinfection. The dark bars represent detached cell counts from BOC-D-fmk-treated cells.
Bcl-XL could interfere in their activation and reduce the apoptosis suggested that forced expression of Bcl-2 or Bcl-XL in Vero 76 and RK13, the reduction in apoptotic cells occurred with no reduction in the synthesis of RV components as indicated by a capsid immunoblot (Fig. 4).

Role of antiapoptotic cellular proteins in RV-induced cell death

In the current model of caspase activation during apoptosis, the cellular proteins Bax and Bcl-2 or Bcl-XL are involved in the release or retention, respectively, of factors from the mitochondrion that participate in cleavage of the caspase proenzymes and their assembly into active heterotetramers (Kluck, 1997; Reed, 1997; Vaux, 1997; Yang et al., 1997). When Bcl-2 or Bcl-XL is in excess, apoptosis is delayed or prevented (Yang and Korsmeyer, 1996). The observed role of caspases in RV-induced apoptosis suggested that forced expression of Bcl-2 or Bcl-XL could interfere in their activation and reduce the amount of cell death. Considering their sensitivity to RV-induced apoptosis, RK13 cells were chosen to study the effect of Bcl-2 and Bcl-XL.

Cells were cotransfected with a plasmid expressing one of the following proteins: Bcl-2, Bcl-XL, or β-galactosidase as a control and a plasmid expressing green fluorescent protein (GFP). All plasmids were driven by the same enhancer–promoter so the proteins should be expressed with similar kinetics. The GFP plasmid and each coexpression plasmid were transfected in a 1:1 ratio. At 24 h after transfection, cells were infected with RV or mock-treated. Assuming that green fluorescent cells were also expressing the antiapoptotic or control protein, by following the fate of green cells, we determined the effect of the expressed protein on RV-induced cell death. To ensure that the proteins of interest were expressed over the time course of the experiment, immunoblots of cell lysates were performed for Bcl-2 and Bcl-XL samples, and β-galactosidase assays were performed for the corresponding samples (Fig. 5). For each expressed protein, the number of live green cells in the infected wells was divided by the mean number of live green cells in the uninfected wells for each time point. The mean of four independent transfections showed that the number of live green cells in RV-infected wells was almost equal to that in mock-treated wells (~100% of control) at the time of infection and 24 h later, but there was a 70% loss of cells at 48 h due to RV infection. Surprisingly, the RV-induced cell loss was the same in β-galactosidase, Bcl-2, and Bcl-XL-transfected cells, indicating no protection by Bcl-2 or Bcl-XL. Because the expected levels of expressed proteins were achieved without altering RV-induced cell death, other apoptosis-inducing agents were used to treat similarly transfected RK13 cells to confirm the functionality of these proteins in this cell line. When challenged with Sindbis virus or staurosporine, known to be blocked by Bcl-2 and Bcl-XL in other cell lines, the same lack of protection was observed (data not shown).

Two explanations could account for the lack of apoptosis protection in RK13 cells: (1) the plasmid constructs are not producing functional proteins, although they are immunologically recognizable, or (2) RK13 cells could have some inherent deficiency that does not permit Bcl-2 and Bcl-XL to function in their antiapoptotic capacity. To distinguish between these two hypotheses, we repeated the transfection experiments in BHK21 cells. Clear protection from staurosporine-induced apoptosis was observed in these cells (data not shown). Workers in other laboratories have previously demonstrated Bcl-2 and Bcl-XL protection from Sindbis virus in this cell line (Cheng et al., 1998), so we proceeded to test rubella. BHK21 cells were transfected with either β-galactosidase, Bcl-2, or Bcl-XL expression plasmids, as above, infected with 10X concentrated RV stock, and the live green cells were counted for 5 days. The transiently expressed proteins could still be measured on an immunoblot of whole cell lysate out to the fifth day posttrans-
fection (Fig. 6A). From the day of infection through the third day, the number of live cells in the infected and uninfected wells was about the same (Fig. 6B). At 96 h postinfection, the time when RV-induced apoptosis begins to appear in this cell line (see above), a reduced number of cells in RV-infected wells was observed. By 120 h, there was a 75% cell loss due to RV in the β-galactosidase-transfected cells in contrast to only a 56.5% loss with Bcl-2 and 52.8% with Bcl-XL. Although both proteins seem to afford partial protection from RV-induced apoptosis, only the effect of Bcl-XL was significantly different from the effect of β-galactosidase (P < .02). Significantly, the protection occurred with no reduction in the level of RV infection as determined by capsid protein level (Fig. 6A).

**DISCUSSION**

We have shown that RV infection induces apoptosis in cultured cells. The rounded, detaching cells that result from RV infection were shown to have nicked DNA in the nucleus by TUNEL assay, condensed chromatin, fragmented nuclei, and loss of surface microvilli by electron microscopy, all characteristics of apoptosis. Active virus replication was required for initiation of the cell death program. Apoptosis did not result from stimulation with other substances secreted into the culture medium such as cytokines, which could have triggered cell surface pathways, or from exposure to virus particles, which were rendered incapable of replication by UV irradiation.

An additional clue to the mechanism of RV-induced apoptosis is the correlation between the amount of virus and the amount of cell death within a single cell type. By adjusting the m.o.i., the level of apoptosis at a given time point can be increased or decreased as long as no caspase inhibitor is added (see Table 1 and the adjustment for induction of apoptosis in BHK21 cells, above). This quantitative relationship suggests that viral RNA or protein synthesis in a cell is building up a quantity of a viral and/or cellular factor, which is the signal for the cell to initiate apoptosis. The accumulation of a viral protein component as a signal for apoptosis was described for dengue virus (Despres *et al.*, 1996), and recently, a possible signal for Sindbis-induced apoptosis was shown to be the transmembrane region of its envelope glycoproteins E1 and E2 (Joe *et al.*, 1998). RV has similar E1 and E2 envelope proteins, which also may be the trigger for RV induction of apoptosis. Alternatively, RV strain differences in cytopathogenicity that map to the nonstructural proteins (Pugachev *et al.*, 1997) suggest these viral components may play a role in apoptosis induction as well. Additionally, viral capsid protein and viral RNA must be examined as components that may be the signal for RV-induced apoptosis.

We have also shown that the apoptotic response to RV varies considerably among the cell lines examined. RK13 cells rapidly become apoptotic at a relatively low level of virus infection, which is consistent with the high sensitivity to RV infection previously observed in this cell line (Fogel and Plotkin, 1969; Umino *et al.*, 1990). At times postinfection when apoptosis is maximal in RK13 cells,
about one third the number of apoptotic cells were observed in Vero 76 cultures, although the synthesis of RV components had reached a much higher level. To achieve the same number of apoptotic cells in the BHK21 cell line, longer times postinfection or a higher initial m.o.i. was required. Thus each cell line has a different threshold for the triggering of apoptosis, and that threshold determines its maximal level of RV load. The levels of virus load in these cell lines, measured in our study by capsid protein, are consistent with reports of low RV titer with infected RK13 cells, higher titers in Vero, and highest titers in BHK21 cells (Frey, 1994).

The stimulation of caspase activity is important in RV cytotoxicity as indicated by the increased cell survival when specific caspase inhibitors zDEVD-fmk and BOC-D-fmk were added to cells after infection. Significantly, the inhibitors did not reduce virus protein synthesis, indicative of replication; in fact, a slightly higher level of virus protein was observed in RK13 cells, indicating that RV could replicate to a higher level in these cells if apoptosis is blocked. This suggests that apoptosis is restricting the level of replication of RV and is consistent with the hypothesis proposed by others that apoptosis is a cellular response to limit viral spread (Razvi and Welsh, 1995; Teodoro and Branton, 1997).

Determining which caspases are activated by RV infection, of the >10 identified, will further elucidate the mechanism of apoptosis induction. Caspases have been grouped according to their cleavage site specificity (Nicholson et al., 1995). The major group of effector caspases, exemplified by caspase-3, prefer the cleavage site DXexD and thus would be blocked by the inhibitor z-DEVD-fmk used in this study. In all three cell lines, partial protection from RV-induced apoptosis afforded by zDEVD-fmk and the use of a broader-spectrum inhibitor in BHK21 cells to achieve a greater reduction of apoptosis suggests other caspases are involved, in addition to caspase-3. The similarity in response to caspase inhibitors among these cell lines suggests that the type of caspase activated is not the key to the observed differences in apoptotic response but, more likely, the threshold level of viral components required to initiate caspase activation.

Forced expression of the antiapoptotic proteins Bcl-2 and Bcl-X<sub>L</sub> protected BHK21 cells from RV-induced apoptosis, yet the same proteins did not protect another cell type, RK13. Similarly, Japanese encephalitis virus induces apoptosis, which can be delayed by Bcl-2 in BHK21 cells but not in another cell type (Liao et al., 1997). The steps in apoptosis, although not completely defined, typically begin with an inducing signal and lead to the activation of caspases, which are responsible for the final destruction of the cell. Our data suggest that in this sequence of events, the signal initiated by RV leading to caspase activation is upstream of Bcl-2 and Bcl-X<sub>L</sub> in BHK21 cells as commonly observed for other viruses (Teodoro and Branton, 1997). Although Bcl-2 family proteins do not protect RK13 cells, we do not favor a hypothesis that RV initiates the caspase cascade by another mechanism downstream of Bcl-2 in these cells. Rather, we suggest that RV engages a similar trigger in both cell types but that the transfected proteins can not function properly in RK13 cells. This hypothesis is favored because Bcl-2 family proteins fail to protect RK13 cells from other inducing agents, Sindbis virus and staurosporine, that have been blocked by Bcl-2 and Bcl-X<sub>L</sub> in multiple cell types (Cheng et al., 1996; Jacobsen et al., 1996). The failure of the Bcl-2 family proteins to function could be due to the absence of a critical cellular component in RK13 cells, which may also account for their higher sensitivity to RV. Identification of this factor or factors could reveal a key step in the mechanism of RV-induced apoptosis. Alternatively, apoptosis-associated caspase cleavage of Bcl-2 and Bcl-X<sub>L</sub> could differ between RK13 and BHK21 cells.

Recent studies of Sindbis virus and Semliki forest virus have shown that infection results in caspase-mediated cleavage of Bcl-2 and Bcl-X<sub>L</sub> in cell lines in which the antiapoptotic effect of these proteins was reduced (Clem et al., 1998; Grandgirard et al., 1998). In our study, immunoblots of Bcl-2-transfected, RV-infected RK13 cells showed no cleavage of 26-kDa Bcl-2 (data not shown). Thus caspase-mediated cleavage of Bcl-2 or Bcl-X<sub>L</sub> is not likely to account for the failure of protection in this cell line. This study demonstrates that the ability of RV to induce apoptosis varies considerably with cell type. In addition, RV can infect a wide variety of cell types with no obvious CPE, although the level of replication remains low (Frey, 1994). This lack of cytolysis has been cited as an explanation for the observed lack of cytosis in postmortem examinations of RV-infected fetuses (Cooper et al., 1995). However, earlier studies have reported that CRS fetuses are characterized by malformed organs with decreased cell numbers (Naeye and Blanc, 1965). Cell destruction has also been disfavored as an explanation for low cell numbers because of the absence of inflammation (Cooper et al., 1995). The studies from which these interpretations were drawn were done before the understanding of the apoptotic process. However, in one study during the same period, after microscopic observation of cells from a CRS fetus, the authors described some cells with “disorganization of the nucleus, which showed an eosinophilic center and margination of basichromatin on the nuclear membrane” (Selzer, 1963), suggesting a description of apoptotic cells. Based on reinterpretation of such earlier studies and our results, it is possible that particular cell types in the fetus are susceptible to RV-induced apoptosis. Efficient phagocytosis of the apoptotic bodies would reduce the cell number while avoiding inflammation, and therefore cell death.
could be responsible for the defects due to congenital RV infection.

MATERIALS AND METHODS

Cell culture and transfection

Vero 76 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS (HyClone Laboratories, Inc.). RK13 and BHK21 cells were grown in minimal essential medium with Earle’s salts supplemented with 10% FCS. The M33 strain of RV in Vero 76 cell culture supernatant was titered by plaque assay at $5 \times 10^6$ PFUs/ml. Mock treatment consisted of incubating cells with cell culture supernatant from uninfected Vero 76 cells. Cell counts were performed microscopically with a standard hemacytometer for detached or trypsinized cells.

Cells on 35-mm culture plates were transiently transfected with 100 ng of the pEGFP plasmid (Clontech Laboratories, Inc.) plus 100 ng of an expression plasmid (described below) with Lipofectamine Plus (Life Technologies) according to the manufacturers’ instructions. Live, green fluorescent cells were counted microscopically, and their viability assessed by morphology; the average number of live green cells per field was determined by counting at least three fields.

Immunoblot analysis

Protein samples were prepared as a whole-cell, RIPA lysate. Protein concentration was determined according to the Bradford method (Bio-Rad Laboratories) and 30 μg of total protein separated on 15% acrylamide SDS–PAGE. After electrotransfer, blots were developed with monoclonal anti-RV capsid antibody (Chemicon International, Inc.), anti-human Bcl-2 (05–341; Upstate Biotechnology), polyclonal anti-human Bcl-XL (S-18; Santa Cruz Biotechnology, Inc.), or monoclonal anti-human Bcl-XL (clone 2H12, a generous gift from Y. T. Hsu, NINDS, Bethesda, MD) as primary antibodies, horseradish peroxidase-coupled anti-Ig as secondary antibodies, and visualized by enhanced chemiluminescence (Amersham).

Electron microscopy

Cells ($3 \times 10^5$) were fixed in 75% methanol/2.5% acetic acid, rinsed twice with PBS, layered with TUNEL reaction mixture (Boehringer Mannheim) containing FITC-labeled dUTP and terminal deoxynucleotidyl transferase, and incubated for 1 h at 37°C in a moist chamber. Cells were visualized on a fluorescent microscope, and images captured with a digital video camera and processed with Adobe Photoshop software.

Virus purification

Culture supernatant from RV-infected cells was centrifuged at 40,000 rpm in a Beckman 60ti rotor for 2 h. The pellet was resuspended in the same culture supernatant from uninfected Vero 76 cells that was used for mock infection.

Caspase inhibition

Cell-permeable derivatives of z-DEVD-fmk and BOC-D-fmk were obtained from Enzyme System Products (Livermore, CA). The peptides were dissolved in DMSO to a concentration of 10 mM. Inhibitors or DMSO as control was added to the cell culture medium to a final concentration of 20 μM, and replenished each day the cells remained in culture.

Plasmid constructs

The pEGFP plasmid for expression of GFP was obtained from Clontech Laboratories, Inc. The pD/Bcl-2 plasmid was constructed by cutting the EcoRI fragment containing the Bcl-2 cDNA from pZip/Bcl-2 (a kind gift of Dr. John Reed) and inserting it into the EcoRI site in pcDNA3 (Invitrogen, Inc.). The pD3/Bcl-XL plasmid containing the complete cDNA for Bcl-XL in the pcDNA3 vector was a gift of Dr. Y.-T. Hsu (NINDS, Bethesda, MD). The β-galactosidase plasmid containing the complete cDNA in the pcDNA vector was kindly provided Dr. Richard Nordan (CBER, Bethesda, MD).

β-Galactosidase activity

Cells ($1 \times 10^6$) were lysed in 100 μl reporter lysis buffer (Promega) and 10 μl of lyase incubated with 50 μl Galacton chemiluminescent substrate (Tropix, Inc., Bedford, MA) solution (substrate diluted 1:100 into 0.1 M NaPO₄, pH 8.0, 1 mM MgCl₂) for 15 min at room temperature. Subsequently, 50 μl of Emerald enhancer (Tropix) solution (dilute Emerald 1:10 in 0.2 N NaOH) was added, and the luminescence was immediately measured in a Turner TD-20e luminometer for 15 s. β-Galactosidase activity is reported as light units/μl/min.

Statistical tests

The significance of the difference between two mean values was evaluated with the Student’s t test calculated...
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