RABIES VIRUS INFECTION-INDUCED APOPTOSIS

by

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(Under the Direction of Zhen Fang Fu)

ABSTRACT

Induction of apoptosis by rabies virus (RV) has been reported to be associated with the expression of the glycoprotein (G), but inversely correlated with pathogenicity. However, the importance of apoptosis in the cause of death of infected individuals is still not clear. The objective of this study was to understand the importance of apoptosis in RV infection and the role of rabies virus glycoprotein (RVG) in the induction of apoptosis. Furthermore, the apoptotic pathway by which RV induces apoptosis was delineated. To this end, wild-type (wt), laboratory-adapted (attenuated), as well as recombinant RVs with replacement of only the G gene were used to infect mice intracerebrally. Results indicate that attenuated RV and recombinant RVs expressing the G from attenuated viruses expressed a higher level of the G and induced more apoptosis in mice than recombinant RV expressing the G from wt or pathogenic RV, demonstrating that it is the G that determines the level of G expression and, consequently, the induction of apoptosis. Likewise, recombinant viruses expressing the G from wt or pathogenic RV are more pathogenic in mice than those expressing G from attenuated RV, confirming the inverse correlation between RV pathogenicity and the induction of apoptosis. To investigate the mechanism by which induction of apoptosis attenuates viral pathogenicity, mice were infected

with wt or attenuated virus intramuscularly. It was found that low doses of attenuated RV induced apoptosis in the spinal cord and failed to spread to the brain or produce neurological disease. On the other hand, apoptosis was not observed in the spinal cord of mice infected with the same doses of wt RV and the virus spread to various parts of the brain and induced fatal neurologic disease. To determine the pathways by which CVS-B2C induces apoptosis, cells were infected with CVS-B2C. Caspase activity and expression of several apoptotic proteins were analyzed. Caspase-8 and caspase-3 were activated in B2C infected cells. In addition, AIF was up-regulated and translocated to the nucleus. Overall, these results suggest that attenuated RVs, by inducing glycoprotein-mediated apoptosis, limit the spread of the virus in the CNS via caspase-dependent and caspase-independent pathways.

INDEX WORDS: Apoptosis, CNS, glycoprotein, pathogenesis, pathways, rabies virus.

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DEDICATION

I dedicate this work to my family for their unconditional love, patience, and support throughout these years.

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Special thanks to my Mom and Dad for helping me become the person that I am today; the values that you have passed on to me are priceless. I love you both very much.

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CHAPTER 1

INTRODUCTION

Rabies

The first statement reporting rabies was recorded in the pre-Mosaic Eshmuna Code of Babylon in the 23rd century B.C (Pearce, 2002). The disease was variously known as *lytta* or *lyssa*, coming from the belief that the disease was caused by a worm under the tongue (*lytta*), or hydrophobia, which describes the thirst and fear of water associated with the disease. The present English name, rabies, comes from the Latin *rabere*, meaning raging, furious, savage, or madness, whereas the Greek term hydrophobia is now specifically used for rabies in man (Woldehiwet, 2002).

Given the widespread distribution, public-health concerns, veterinary implications, and economic burdens, rabies is the most important viral zoonosis in the world (Meltzer and Rupprecht, 1998). Although there are some rabies-free countries and islands, such as Greece, Portugal, Chile, New Zealand and Barbados, rabies is widely distributed throughout the world and is present on all continents. Despite continued attempts at medical intervention, rabies retains the dubious distinction of being the infectious disease with the highest case–fatality ratio (Hemachudha et al., 2002) and the number of cases have been increasing, mainly because there are a large global rabies reservoirs, in both domestic and wildlife animals (Fu, 1997). Rabies accounts for over 55,000 deaths each year, and most of these deaths occur in Asia and Africa.

(Technical Report Series 824, WHO, 1992). Even by rudimentary surveillance, one person dies from the disease each 15 minutes, and more than 300 others are exposed. In 1998, more than 7 million people worldwide received postexposure treatment (WHO Fact Sheet N 99, Revised June 2001). The epidemiology of human rabies is an exact reflection of the epizootiology of the disease in animals. In most countries of Africa, Asia and Latin America, dogs continue to be the main hosts and are responsible for most of the human rabies deaths worldwide (Plotkin, 2000). In contrast, in North America, where dog rabies is controlled through animal vaccination and therefore the number of human cases has been reduced considerably, wildlife rabies emerges as a more challenging problem. For example, fox and skunk rabies has been endemic in the US for many years. Recently raccoon rabies has reached epidemic proportions along the east seaboard (Krebs et al., 1997). Furthermore, many human rabies cases in the U.S. have been caused by RV variants circulating in the insectivorous bat populations (Fu, 1997).

Despite the effort to control the disease in the U.S., an estimated 40,000 patients receive rabies postexposure prophylaxis each year (Gibbons et al., 2002) and cause an economic burden exceeding $1 billion annually (Dietzschold and Schnell, 2002). Taking these observations together, rabies remains one of the most feared zoonotic diseases and constitutes a serious public health problem around the world. It is of extreme importance that extensive research on rabies and RV continues, particularly in understanding the pathogenicity, which can constitute a foundation for more effective therapeutic intervention in clinical rabies.

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CHAPTER 2

LITERATURE REVIEW

Rabies virus: Rabies virus (RV) belongs to the genus *Lyssavirus* in the family *Rhabdoviridae* (Calisher et al., 1989); Wagner and Rose, 1996). RV is a negative-sense, non-segmented, single-stranded RNA virus measuring approximately 60 nm × 180 nm. It is composed of an internal protein core or nucleocapsid containing the nucleic acid, and an outer envelope, a lipid-containing bilayer covered with transmembrane glycoprotein (G) spikes.

Viral Proteins: The virus genome encodes five proteins associated with either the ribonucleoprotein (RNP) complex or the viral envelope. The L (transcriptase), N (nucleoprotein), and NS (transcriptase-associated, also called P protein) proteins comprise the RNP complex together with the viral RNA. These aggregate in the cytoplasm of virus-infected neurons and form Negri bodies, the characteristic histopathological finding of RV infection. The M (matrix) and G (glycoprotein) proteins are associated with the lipid envelope. The N protein of RV plays vital roles in regulating viral RNA transcription and replication by encapsidating de novo-synthesized viral genomic RNA (Wu et al., 2002). RV M protein is a multifunctional protein, playing a crucial role in virus assembly and budding. M protein is responsible for recruiting RNPs to the cell membrane, their condensation into tightly coiled ‘skeleton’-like structures and the budding of enveloped virus particles (Mebatsion et al., 1999). Recently, there has been evidence that M is a key factor in the regulation of RV polymerase functions that exerts opposite effects on transcription and replication, and thereby tips the balance toward replication (Finke et al., 2003).

Rabies virus G protein is a type I integral transmembrane protein with an ectodomain that can be divided into two immunologically autonomous halves, each providing a protective immune response (Bahloul et al., 1998). It forms the protrusions that cover the outer surface of the virion envelope associating into trimers (Langevin et al., 2002; Tuffereau et al., 2001). It is the only RV protein known to induce virus-neutralizing antibody (Baron, 1996). Furthermore, the G protein plays a crucial role in the process of rabies pathogenesis. It is believed that RV G protein, by binding to specific neural receptors such as nicotinic acetylcholine receptor (Broughan and Wunner, 1995), neural cell adhesion molecule CD56 (Thoulouze et al., 1998) and low affinity nerve growth factor receptor (Tuffereau et al., 2001), gains entry to the nervous system from the site of infection, thus contributing to the almost exclusive neurotropism (Dietzschold *et* al., 1996).

Rabies Virus Replication: Rabies virus infection initiates with the binding of viral glycoprotein to specific receptors on the target host cell surface (Flint et al., 2000; Tuffereau et al., 2001; Broughan and Wunner, 1995). Following adsorption, the virus penetrates the host cell membrane and uncoats its envelope, releases the viral cores into the cytoplasm, allowing infection to proceed. After viral entry and release of nucleocapsids into the cytoplasm, the negative-strand RNA genome is transcribed into mRNA by the RNA-directed RNA polymerase(L) encoded in the virion (Anderson et al., 1984). Transcription begins at the 3' end of the genome, producing a 48-nucleotide leader RNA followed by sequential synthesis of the individual mRNAs encoding the N, P, M, G and L proteins (Wagner and Rose, 1996). The transcriptional order follows the gene order. Then, the individual mRNAs are capped and polyadenylated. Once viral proteins are synthesized, viral transcription is switched to replication.

Replication is characterized by production of a full-length positive RNA strand complementary to the entire parental template, followed by production of full-length negative-strand RNAs. Unlike transcription, replication requires active, ongoing translation, particularly of viral N and P proteins (Wagner and Rose, 1996). After synthesis of N, P and L proteins, association of these proteins in the cytoplasm with newly replicated genomic RNA occurs to form the ribonucleoprotein core. The M protein associates with the core forming a coiled structure called skeleton, condenses the RNP core in the cytoplasm and binds cores to the membrane in preparation for budding (Wagner and Rose, 1996). The G protein is a trimeric, externally oriented, membrane-spanning protein. The G protein spikes bind the cell surface receptors and antibody binding sites and any variation in the gene encoding for this protein may affect the pathogenic and immunogenic properties of the virus (Wunner, 1991). It interacts with internal virion components, most likely the M or N proteins, and subsequent envelopment of the particles leads to the last phase of the infection cycle, which is budding, releasing the virus for new infections. (Wagner and Rose, 1996).

Pathogenesis: Rabies is an acute, progressive, incurable viral encephalitis. The most frequent way by which humans become infected with RV is through the bite of infected dogs and cats, wild carnivorous species like foxes, raccoons, skunks, jackals and wolves, and insectivorous and vampire bats. Cattle, horses, deer and other herbivores can become infected with rabies and although they could transmit the virus to other animals and man, this rarely occurs (WHO Fact Sheet N 99, Revised June 2001). The virus may enter the peripheral nervous system directly, replicate in muscle tissue after entering the host, or remain at or near the site of introduction for most of the incubation period. However, the precise sites of viral sequestration remain unknown,

since neither antigen nor virus can be found in any organ during this phase (Hemachudha, 1994). The virus may enter the peripheral nervous system via the neuromuscular junctions, and moves rapidly centripetally to the central nervous system, particularly to the nearest sensory or motor neuron in the dorsal root ganglion or anterior horn of the spinal cord where it replicates (Iwasaki, 1991). After replication, the virus may return to the site of the bite by orthograde axonal transport, or may travel along the corticospinal tract to the brain where it infects neurons in almost all brain regions (Fu, 1997). At the later stage of infection, RV can be transported centrifugally to many peripheral tissues and organs, such as respiratory tract, cornea, skin of the head and neck, adipose tissue, adrenal medulla, and renal parenchyma. Extremely high viral titers are directly reached by way of efferent secretory nerves to a primary exit portal, the acinar cells of the salivary glands, often exquisitely timed to host aberrant behaviors which enhance rabies shedding potential and its natural perpetuation (Rupprecht and Dietzschold, 1987). Patients infected with RV develop severe agitation, depression, hydrophobia, and paralysis followed by impaired consciousness and coma (Hemachudha, 1994). Patients eventually die of circulatory insufficiency, cardiac arrest, and respiratory failure (Tirawatnpong et al., 1989).

Although there is extensive RV antigenic involvement throughout the CNS, there is an overall paucity of gross and histopathologic lesions attributed to rabies. In general, gross examination of the brain shows mild congestion of the meningeal vessels and the spinal cord presents frequently focal congestion of parenchymal and meningeal vessels (Rupprecht and Dietzschold, 1987). Microscopic examination usually demonstrates slight perivascular cuffing, limited tissue necrosis, acidophilic intracytoplasmic neuronal inclusions, and rarely, neuronophagia. (Baron, 1996). These findings are often associated with fixed RV infection and

are normally uncommon in street viral infections (Rupprecht and Dietzschold, 1987). Electron microscopic examination of the brain stem of rabies- infected raccoons revealed accumulation of electron-dense material within neuronal perikarya (Hamir and Fischer, 1999). Light and electron microscopic examination indicated that the accumulated intracellular material had high lipid content. These lesions suggest a form of neuronal storage condition. Histopathologic and electron microscopic studies of the central nervous system of mice, dogs and cats infected with a RV strain isolated from a European bat showed neuronal cytoplasmic changes considered to be a form of spongiosis (Fekadu et al., 1988). Because of the overall low incidence of neuropathological findings under natural conditions (Iwasaki and Tobita, 2002), it has been suggested that the neurological disease in rabies must result from neuronal dysfunction rather than neuronal death (Tsiang, 1982). A variety of studies of RV infection in experimental animals and *in vitro* have provided evidence in neurotransmission involving acetylcholine, serotonin and γ-amino-n-butyric acid (GABA) (Jackson, 2002). The neuronal dysfunction perhaps could be explained by a defect in cholinergic synaptic neurotransmission as previously reported (Jackson, 1993). Dysfunction of ion channels has been demonstrated in infected culture cells (Iwata et al., 1999) and the induction of inducible nitric oxide synthase mRNA (Koprowski, 1993) and the increase in the levels of nitric oxide (Hooper et al., 1995) have been shown in rabies virus-infected rodents. Most recently, the structural alterations of neuronal processes in mice were investigated after mice were infected with RV. Silver staining of infected brain sections showed severe destruction and disorganization of neuronal processes in mice infected with pathogenic RV but not with attenuated RV, suggesting that pathogenic RV causes degeneration of neuronal processes possibly by interrupting cytoskeletal integrity (Li et al., 2005). Nevertheless, the significance of all of these findings is uncertain because no fundamental defect has yet been

found explaining neuronal dysfunction in natural rabies (Jackson, 2002). Despite all the effort and advances in the knowledge of rabies, the true causes of the beginning of the symptoms and the eventual mortality of rabies are still not well understood.

Apoptosis: In 1972, John Kerr, an Australian pathologist, described a novel form of cell death in rat liver. Some cells appeared to shrink and die without a swelling phase. For this kind of cell death John Kerr and co-workers introduced the term 'apoptosis' (Kerr et al., 1972). Apoptosis is the process whereby individual cells of multicellular organisms undergo systematic self-destruction in response to a wide variety of stimuli (Teodoro and Branton, 1997) and the main characteristics are cellular shrinkage, membrane condensation, membrane blebbing, and DNA fragmentation (Lam, 1996). Apoptosis is a genetically controlled pre-programmed event, which eliminates cells during development when they have become redundant, or which functions as an emergency response after for example radiation damage or aberrant growth induced by the activation of oncogenes (Teodoro and Branton, 1997).

Activation of proteolytic enzymes called caspases is a key step in the apoptotic program. Caspases are cysteine proteases classified into more than 10 different forms. Caspases exist in latent forms in almost all animal cells and become activated in response to apoptotic signals. The first caspases to become activated are so-called "initiator caspases". When triggered to assume active states, generally involving their proteolytic processing at conserved aspartic acid (Asp) residues, the zymogens pro-proteins are cleaved to generate the large (~20 kd) and small (~10 kd) subunits of the active enzymes. Once the initiator caspases are activated (such as caspase 8 and

9), they process and activate downstream effector caspases, such as caspases 3, 6 and 7 (Kumar and Vaux, 2002).

The process of apoptosis can be subdivided into three functionally distinct phases: the initiation phase, during which a number of signal transduction or damage pathways are activated in a stimulus-dependent fashion; the common effector phase, during which the balance between pro-apoptotic and anti-apoptotic signal favors the latter; and the degradation phase, during which morphological and biochemical changes occur in the cell, constituting hallmarks of apoptosis (Susin et al., 1999a).

There are at least two caspase-dependent pathways that lead to apoptosis, one involving death receptors and the other involving mitochondria-mediated apoptotic events (Fig. 1) (Benedict et al., 2002). The death receptor-mediated apoptotic events, also called the extrinsic pathway, are initiated by binding of death receptors such as fibroblast-associated (Fas), tumor necrosis factor receptor (TNF-R), and TNF-related apoptosis inducing ligand receptor (TRAIL-R) to their ligands. This binding initiates ligation of the receptors and transmission of the apoptotic signals through motifs called death domains (DDs), death effector domains (DED), and caspase recruitment domains (CARD). Death effector domains are found in the adaptor molecule and in procaspases 8 or 10 and play an important role in death receptor-induced apoptosis where the interactions between DEDs of the adaptor molecules and those of pro-caspases 8 or 10 enable formation of the death-inducing signaling complex (DISC) (Hussein et al., 2003). The formation

of DISC activates caspase-8 or caspase-10, which in turn activates directly pro-caspase-3, completing the initiation phase of the pathway.

Several apoptotic conditions, such as UV, anti-cancer drugs and viral infections, can lead to activation of the intrinsic pathway in which mitochondria intermembrane proteins are released and eventually activate executioner caspases. The release of mitochondria proteins seems to be related to members of the Bcl-2 superfamily (Daugas et al., 2000). Pro-apoptotic proteins, such as Bax and Bak, permeabilize mitochondrial membranes and anti-apoptotic proteins, such as Bcl-2 and Bcl-XL, stabilize the mitochondrial membrane barrier function (Daugas et al., 2000). Whether apoptosis occurs or not will depend on the level of expression of these proteins, the persistence of proapoptotic stimulus and the action of other proteins to inhibit or stimulate apoptosis, such as XIAP and Smac/DIABLO, respectively. Several mitochondrial proteins are involved in the mechanism of apoptosis. Cytochrome c is probably the most important pro-apoptotic mitochondrial protein. Once released into the cytoplasm, cytochrome c interacts with the adaptor protein Apaf-1, ATP and with pro-caspase-9 forming a complex called apoptosome. Cytochrome c induces the oligomerization of Apaf-1 in the presence of ATP and recruits pro-caspase-9, which then undergoes autoactivation and dissociates from the complex and becomes available to cleave and activate downstream caspases such as caspase-3 (Daugas et al., 2000; Hussein et al., 2003; Zou et al., 1999).

Recently, an alternative caspase-dependent pathway (the convergence of the intrinsic and extrinsic pathways) has been suggested. In this pathway, caspase-8 is activated in low levels and hardly any DISC is formed. In order to amplify the apoptotic signal caspase-8 cleaves BID which

translocates to the mitochondria. In the mitochondria BID most likely associates with BAD/BAK, activating the mitochondria to release cytochrome-c, Smac/Diablo, and Apaf-1 (Wesche-Soldato et al., 2005). As mentioned previously, Apaf-1 binds to pro-caspase-9 activating the intrinsic pathway (Zou et al., 1999).

The final phase of the caspase-dependent apoptotic pathways is cleavage of numerous substrates by caspase-3 (Nicholson and Thornberry, 1997) and other executioner caspases, such as caspase-6 (Orth et al., 1996) and caspase-7 (Talanian et al., 1997), leading to destruction of cell-cell interactions and nuclear structure, reorganization of the cytoskeleton, inhibition of DNA synthesis, repair and splicing, degradation of DNA, and disintegration of the entire cell contents into apoptotic bodies. One of the main substrates cleaved by executioner caspases is poly(ADP-ribose) polymerase-1 (PARP-1). Poly(ADP-ribose) polymerase-1 synthesizes poly (ADP-ribose) from β-nicotinamide adeninme dinucleotide (NAD^+) in response to DNA strand breaks and is involved in many genomic processes including DNA base excision repair, DNA replication and transcription (Lautier et al., 1993). Poly(ADP-ribose) polymerase-1 cleavage produces an 89 kDa C-terminal fragment (containing the catalytic domain), and 24 kDa N-terminal fragment with the DNA binding domain. The 89 kDa fragment retains the basal enzymatic activity due to the presence of the catalytic domain, although it cannot be stimulated by DNA strand breaks. After cleavage, PARP-1 loses the nick-sensor function and is inactive towards DNA damage, allowing the final steps of apoptosis to occur (Kaufmann et al., 1993; Soldani and Scovassi, 2002). Recently, an additional role for PARP related to apoptosis has been suggested. Yu et al. (2002) provided evidence that massive PARP-1 activation can trigger release of a mitochondrial

proapoptotic protein called apoptosis-inducing factor (AIF) that promotes programmed cell death through a caspase-independent pathway.

Apoptosis-inducing factor is a mitochondrial intermembrane flavoprotein with significant homology to bacterial and plant oxidoreductases (Susin et al., 1999a). Apoptosis-inducing factor is synthesized as a nonapoptogenic precursor in the cytoplasm and efficiently imported into the mitochondrial intermembrane space. Import of AIF to the mitochondria culminates into the proteolytic removal of the amino-terminal mitochondrial targeting sequence, attachment of a flavin adenine dinucleotide group, and refolding of the protein, which becomes potentially apoptogenic (Susin et al., 1999a). On induction of apoptosis, AIF translocates to the nucleus. Moreover, when added to purified nuclei, AIF induces partial chromatin condensation as well as large-scale (50 kbp) DNA fragmentation in a caspase-independent fashion. These nuclear changes resemble to some extent those observed in intact cells in which apoptosis is induced in conditions of caspase inhibition, suggesting that AIF may be responsible for at least some of the caspase-independent features of apoptosis (Deas et al., 1998).

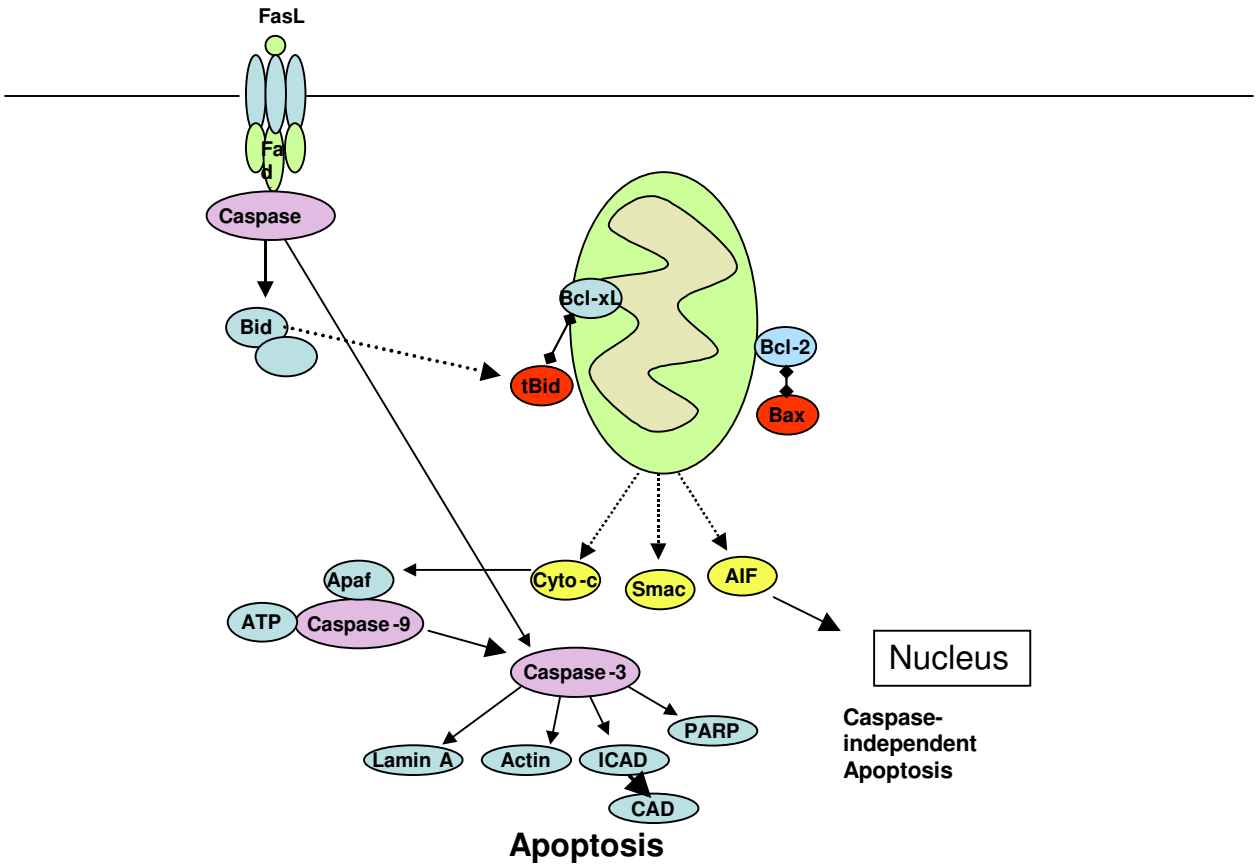

Figure 2.1. Apoptotic pathways.

Apoptosis in rabies: Recently, apoptosis has been suggested as playing an important role in experimental RV infection (Hussein et al., 2003; Jackson and Rossiter, 1997). Many in vitro studies have been conducted, basically using laboratory adapted RV strains to investigate the induction of apoptosis. Neuroblastoma cells were used to investigate the induction of apoptosis by a laboratory adapted strain (CVS-11) (Ubol et al., 1998). In this study investigators showed

that rabies virus-infected mouse neuroblastoma cells underwent chromatin condensation and DNA fragmentation within 48h post-infection. In another study, Jackson and Rossiter (1997) demonstrated that CVS infected cultured rat prostatic adenocarcinoma revealed morphological changes associated with apoptosis between 3 and 5 days after infection. Furthermore, primary neurons with the laboratory adapted RV strain CVS also underwent apoptosis. Infection of lymphocytes with the attenuated RV strain ERA (Evelyn Rotnycki Abelseth) also induced apoptosis (Thoulouze et al., 1997). To confirm the *in vitro* studies, adult and suckling mice were infected through intracerebral inoculation with CVS, resulting in production of fatal encephalitis associated with apoptotic cell death in brain neurons (Jackson and Rossiter, 1997). However, comparing the highly pathogenic strain CVS-N2C with a low pathogenic CVS-B2C, (Morimoto et al., 1999) reported that CVS-N2C induced much less apoptosis in neuronal cultures than CVS-B2C. Yan et al. (2001) obtained similar results investigating the induction of apoptosis in the brain of mice infected with CVS-24 or with the wild type strain SHBRV (Silver hair bat rabies virus), which is highly pathogenic; CVS-24 induced apoptosis, whereas SHBRV did not. These data suggest that the induction of apoptosis is inversely correlated with the pathogenicity. Therefore, apoptosis may contribute to protection rather than pathogenicity in RV-infected animals. Furthermore, (Thoulouze et al., 2003b) reported that the inverse correlation of the induction of apoptosis and the capacity of a virus strain to invade the brain suggests that blockage of apoptosis could be a strategy selected by neurotropic virus to favor its progression through the nervous system. Also, in addition to avoiding neuronal cell death, neurotropic viruses may induce apoptosis of T cells that migrate into the nervous system, which would limit inflammation and guarantee spread of the virus (Baloul and Lafon, 2003).

The role of rabies viral proteins in the induction of apoptosis has also been investigated. In general, apoptosis is thought to be triggered by one of two general stages of the viral life cycle. In some cases, the binding of the surface viral protein to its cellular receptor is sufficient for initiation of the apoptosis cascade, and in other cases, there is a requirement for de novo viral RNA or protein synthesis for the initiation of apoptosis (Licata and Harty, 2003). The importance of a receptor engagement in RV infection for rabies virus-induced apoptosis has been characterized. UV-inactivated RV particles were used to study the induction of apoptosis in lymphocytes. Apoptosis was not detected until 18h post-infection, suggesting that not only is necessary replication, but also the expression of viral proteins must be necessary to induce apoptosis (Thoulouze et al., 1997). Previously it has been suggested that there is a correlation between the G protein of RV and the induction of apoptosis. Morimoto et al. (1999) compared the expression of the G protein between the highly pathogenic strain CVS-N2C and the low pathogenic strain CVS-B2C in primary neuronal cultures. CVS-B2C induced much more apoptosis than CVS-N2C and expressed at least fourfold higher levels of G than CVS-N2C in infected neurons. This differential expression of G is not completely understood, but it appears to be largely determined by post-translational mechanisms that affect G protein stability. Pulse-chase experiments indicated that the G protein of CVS-B2C is degraded more slowly than that of CVS-N2C. On the other hand, the nucleoprotein (N protein) expression levels were similar in neurons infected with either variant. Thus, these data suggest that induction of apoptosis correlates with the level of G expression and that pathogenic RV strains prevent apoptosis by down-regulating the expression of the G protein. Yan et al. (2001) investigated the induction of apoptosis in the brain of mice infected with CVS-24 or with the wild type strain SHBRV, and the correlation with the expression of the G protein. Results showed that the expression of the

glycoprotein was more widespread and the immunostaining of G was generally stronger in CVS- than SHBRV-infected mice, whereas the expression of RV nucleoprotein (N) was similar in mice infected with either CVS or SHBRV. Therefore, a role for RV G protein in the process of apoptosis is suggested. Further studies also suggest that the G protein determines the distribution pattern of RV in the brain, adding to the importance of the G protein in the pathogenicity of the virus (Prehaud et al., 2003; Yan et al., 2001). Using a reverse genetic approach to investigate the role of RV glycoprotein in viral pathogenesis, (Morimoto et al., 2000) reported that to maintain pathogenicity, the interactions between various structural elements of RV must be highly conserved and the expression of viral proteins, in particular the G protein, must be strictly controlled. The emerging knowledge linking G protein expression to apoptosis, gave rise to studies using a recombinant virus expressing two identical G genes through reverse genetics. Results showed that the insertion of an additional gene did not affect virus replication, but flow cytometry experiments demonstrated increased staining of G protein on infected cells. In addition, the morphology of the infected cells indicated apoptosis induction and the cells stained positive for TUNEL (Faber et al., 2002).

Although it has been shown that some strains of RV induce apoptosis, the intracellular pathway of cell killing is not clear. Ubol et al (1998) studied the correlation between the induction of apoptosis and the anti-apoptotic protein Bax in mouse neuroblastoma cells. Bax was up-regulated while Bcl-2 remained unchanged. In addition, caspase-1 (Nedd-2) was upregulated and PARP, a DNA repair enzyme, was degraded after caspase up-regulation. Ubol and Kasisith (2000) suggested that the reactivation of Nedd-2 may also play a role in the induction of apoptosis in adult and suckling mice infected with a bat strain and a primary canine RV isolate.

In this study, expression of Nedd-2 correlated with the appearance of apoptotic nuclei within the infected brain, suggesting that reactivation of a developmentally down-regulated gene, Nedd-2, may be required for apoptotic elimination of cells damaged by infection. In a recent study, the characteristics of rabies virus-induced apoptosis was determined by comparing caspase activation and AIF translocation in Jurkat T cells infected with RV strain ERA. In that study, ERA infection induced both caspase-dependent and caspase-independent (AIF) apoptosis (Thoulouze et al., 2003b). In a more recent study, cDNA array analysis was used as a tool to screen for pro-apoptotic genes. In this study, two independent apoptotic mechanisms were proposed. The first is an immune-mediated death with activation of Fas-L receptor and TNF-receptor activated by caspase-1 and the pro-inflammatory cytokine, IL-1β. The other is a protease-mediated process, which involves lysosomal proteases and calcium-dependent neutral proteases. These two stimulating pathways were followed by Bad, Bak, Bid activation and subsequently the upregulation of cytochrome c and caspase-3 (Ubol et al., 2005).

Virus-induced apoptosis: Apoptosis has been shown to play an important role in the outcome of viral infection. Viruses such as Newcastle disease virus (NDV) (Kommers et al., 2003), dengue virus (Lam, 1996; Despres et al., 1998), Human Immunodeficiency virus (HIV) (Groux et al., 1992), influenza (Takizawa et al., 1993), and vesicular stomatitis virus (VSV) (Koyama, 1995) are known stimulators of apoptosis, especially at late stages of infection. Death by apoptosis offers several advantages for the virus. During apoptosis, the entire cellular contents, including progeny virions, are packaged into membrane-bound apoptotic bodies that are rapidly taken up by surrounding cells (Roulston et al., 1999). This process severely limits the inflammatory response and allows the infection to spread undetected by the host organism (Teodoro and

Branton, 1997). Virus particles enclosed within apoptotic vesicles are also protected from inactivation by host antibodies and proteases (Roulston et al., 1999). Apoptosis in Newcastle disease, for example, is an important mechanism in lymphoid depletion (Kommers et al., 2003). The induction of apoptotic cell death is a prominent cytopathic effect of dengue (DEN) viruses, attributing to the M protein its cytotoxic effects by activating a mitochondrial apoptotic pathway (Catteau et al., 2003). Vesicular stomatitis virus, the prototype rhabdovirus, induces apoptosis, which is related to the ability of the virus to inhibit host gene expression. Contrary to RV, VSV does not require viral replication to induce apoptosis. In addition, the M protein and not the G protein, plays a major role in the induction of apoptosis, by contributing to the cytopathogenesis through the ability to induce cell rounding (Kopecky and Lyles, 2003). Bad protein, one of the pro-apoptotic Bcl-2 family members, mediates apoptosis in the mammalian cells infected with Sindbis virus (Moriishi et al., 2002). Adenoviruses are small DNA tumor viruses that infect terminally differentiated epithelial cells. During the course of infection, a number of viral proteins stimulate apoptosis. For example, the 289R E1A product was found to induce apoptosis by a p53-independent pathway involving the transactivation of another early viral product identified as E4orf4, which can independently induce apoptosis (Marcellus et al., 1998). Herpes simplex virus 1 (HSV-1) induces apoptosis in peripheral blood mononuclear cells (PBMCs), CD4+ cells, and hepatocytes. In hepatocytes, apoptosis occurs in the absence of *de novo* protein synthesis, suggesting that a viral structural protein may be involved (Koyama and Adachi, 1997). Human Immunodeficiency virus 1 is the most intensely studied of the human retroviruses and infects mainly CD4+ T cells, macrophages, and some neuronal cells. Disease pathogenesis is characterized by a progressive decline in CD4+ T cells leading to a severe immunodeficiency syndrome known as AIDS. Several years ago, it was discovered that apoptosis might play a role

in the destruction of HIV-1 infected T cells. Since then, a multitude of viral proteins, such as Tat and gp120, have been shown to contribute to HIV-induced apoptosis (Banda et al., 1992; Purvis et al., 1995).

On the other hand, it has been reported that some viruses have developed several anti-apoptotic mechanisms to evade the defense response of the infected host (Riedl et al., 2001), because the induction of early cell death would severely limit virus production and reduce or eliminate spread of progeny virus in the host. Poxviruses, for example, inhibits distinct host proteins in cellular apoptotic pathways (Johnston and McFadden, 2003). Poxviruses encode proteins containing high homology to DED domains responsible for linking death receptors and the FADD adapter protein to procaspase-8 (Thome et al., 1997). Herpes simplex virus blocks apoptosis in hippocampal neurons by activation of the MEK/MAPK survival pathway (Perkins et al., 2002). Adenovirus E1B-19K encodes orthologs of the anti-apoptotic regulator Bcl-2 and this serves as an immune evasion strategy. Adenovirus E1B-19K is similar both in sequence and function to Bcl-2. Adenovirus E1B 55K encodes products that regulate p53. Adenovirus E1B-55K binds to p53 and blocks p53-mediated gene expression by tethering a transcriptional repression domain to p53. Furthermore, E1B 55K has the capability to act as a survival factor by preventing the loss of mitochondrial membrane potential induced by IL-3 deprivation (BenJilani et al., 2002). Like adenovirus, Simian virus 40 (SV40) and Human papilloma virus (HPV) also need to inactivate p53, but they use different mechanisms. Simian virus 40 LT binds to the DNA-binding domain of p53, preventing gene activation and leading to the accumulation of high levels of nonfunctional p53 complexes (Bargonetti et al., 1992). The HPV E6 protein also binds p53 and targets it for degradation by the ubiquitin pathway (Werness et al., 1990).

Research objectives

As described above, RV infection in humans and animals induces minimal necrosis, neurophagia, or inflammation in the brain (Baron, 1996). However, recent observations of apoptosis in cells or animals infected with RV raises the question if apoptosis plays a role in the cause of death of infected individuals (Jackson and Park, 1998; Jackson and Rossiter, 1997; Theerasurakarn and Ubol, 1998; Thoulouze et al., 1997). Furthermore, the induction of apoptosis by RV has been reported to be associated with the expression of the G protein, but inversely correlated with pathogenicity. Data from different laboratories using different virus strains have been controversial (Morimoto et al., 1999; Yan et al., 2001). Therefore, the main objective of this research was to determine the role of apoptosis in RV infection and to correlate the level of G expression with the induction of apoptosis in RVs-infected cells and animals. Specific objectives were:

1) To investigate the induction of apoptosis *in vivo* and *in vitro* by wild-type, laboratory-adapted and recombinant RVs with G replacement and correlate the induction of apoptosis with pathogenicity.

2) To investigate the mechanism (s) by which the induction of apoptosis protects animals from RV infection.

3) To investigate the correlation between the level of RV G expression and the induction of apoptosis.

4) To investigate the pathway (s) by which RV induces apoptosis.

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CHAPTER 3

GLYCOPROTEIN-MEDIATED INDUCTION OF APOPTOSIS LIMITS THE SPREAD OF ATTENUATED RABIES VIRUSES IN THE CENTRAL NERVOUS SYSTEM OF MICE[1]

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Abstract

Induction of apoptosis by rabies virus (RV) has been reported to be associated with the expression of the glycoprotein (G), but inversely correlated with pathogenicity. To further delineate the association between the expression of the G and the induction of apoptosis, recombinant RVs with replacement of only the G gene were used to infect mice by the intracerebral route. Recombinant viruses expressing the G from attenuated viruses expressed higher level of the G and induced more apoptosis in mice than recombinant RV expressing the G from wild-type (wt) or pathogenic RV, demonstrating that it is the G gene that determines the level of G expression and, consequently, the induction of apoptosis. Likewise, recombinant viruses expressing the G from wt or pathogenic RV are more pathogenic in mice than those expressing G from attenuated RV, confirming the inverse correlation between RV pathogenicity and the induction of apoptosis. To investigate the mechanism by which induction of apoptosis attenuates viral pathogenicity, mice were infected with wt or attenuated RV by the intramuscular route. It was found that low doses of attenuated RV induced apoptosis in the spinal cord and failed to spread to the brain or produce neurological disease. On the other hand, apoptosis was not observed in the spinal cord of mice infected with the same doses of wt RV and the virus spread to various parts of the brain and induced fatal neurologic disease. These results suggest that glycoprotein-mediated induction of apoptosis limits the spread of attenuated rabies viruses in the central nervous system (CNS) of mice.

Keywords: apoptosis; CNS; glycoprotein; pathogenesis; rabies virus; virus spread.

INTRODUCTION

Apoptosis, or programmed cell death, is the process whereby individual cells of multicellular organisms undergo systematic self-destruction in response to a wide variety of stimuli, and the main characteristics are cellular shrinkage, membrane condensation, membrane blebbing, and DNA fragmentation (Teodoro and Branton, 1997). Apoptosis plays an important physiological role in normal embryonic development and tissue homeostasis (Kerr and Harmon, 1991) and is also a common response of cells to virus infections (Barber, 2001; Roulston *et al*, 1999). Virus-induced apoptosis has been suggested both as pathological and protective responses of the host (Mori *et al*, 2004). In some circumstances, virus-induced apoptosis can contribute to pathogenesis, for example, destruction of many cells by apoptosis, particularly those nonreplenishable cells such as neurons, may result in diseases (Lewis *et al*, 1996). Indeed, the ability to induce apoptosis in neurons has been correlated with neurovirulence for alphavirus and flavivirus (Lewis *et al*, 1996; Despres *et al*, 1998). More typically, however, apoptosis represents an important host defense mechanism (Barber, 2001; Kerr and Harmon, 1991). Cells evolved to commit suicide upon viral infection to exterminate unwanted intracellular pathogens from tissues, organs or a whole organism (Mori *et al*, 2004). In addition, death by apoptosis instead of necrosis can significantly affect the efficiency of viral antigen capture by antigen-presenting cells and presentation to T cells, thus enhancing adaptive immune responses as well (Barber, 2001).

Both beneficial and detrimental effects of apoptosis have been suggested in rabies virus (RV) infections. In experimental animals infected intracerebrally (IC) with mouse-adapted

Challenge Virus Standard (CVS) virus, extensive apoptosis was observed in the central nervous system (CNS) (Jackson and Rossiter, 1997; Theerasurakarn and Ubol, 1998). These observations led to the hypothesis that apoptosis plays an important pathogenic role in experimental RV infections. However, Morimoto *et al* (1999) found that the ability of a RV to induce apoptosis in primary neuronal cultures correlated inversely with its pathogenicity in animals. In addition, extensive apoptosis was observed in mice infected with laboratory-adapted CVS-24, but not in mice infected with a street RV strain, SHBRV-18 (Yan *et al*, 2001). Recently, Thoulouze *et al* (2003) observed an inverse correlation between the induction of apoptosis and the capacity of a RV strain to invade the brain, suggesting that inhibition of apoptosis could be a strategy employed by neurotropic virus to favor its progression through the nervous system. Thus, induction of apoptosis is a host defense mechanism in RV infections. This hypothesis is further supported by the findings that recombinant RV expressing cytochrome *c* induced more apoptosis than parental virus and attenuated its pathogenicity (Pulmanausahakul *et al*, 2001).

It has also been reported that induction of apoptosis correlates with the level of G expression (Morimoto *et al*, 1999; Yan *et al*, 2001; Faber *et al*, 2002). In the present study, different recombinant RVs that differ only in the G gene were used to infect mice and the level of G expression was correlated with the induction of apoptosis in the brain. It was found that recombinant viruses expressing the G from attenuated viruses expressed higher levels of the G and induced more apoptosis than those expressing wild-type (wt) or pathogenic RV G, demonstrating that G gene determines the level of G expression, and consequently, the induction of apoptosis. Furthermore, attenuated RV induced apoptosis in the spinal cord and failed to spread to the brain, whereas little to no apoptosis was detected in the spinal cord of mice infected

with wt RV and the virus spread to various regions of the brain, suggesting that G-mediated induction of apoptosis limits the spread of attenuated rabies viruses in the CNS of mice.

RESULTS

Pathogenicity of recombinant RVs is largely determined by the G: In this study, four parental viruses (SN-10, B2C, N2C, and SHBRV) and three recombinant viruses (RB2C, RN2C, and RSHBRV) were used to determine the association between RV G and the pathogenicity. Among the four parental viruses, SHBRV is a wt RV isolated from a human patient (Rupprecht *et al*, 1997; Morimoto *et al*, 1996) and has been associated with most of the human rabies cases in the United States for the past 15 years (Centers for Disease Control and Prevention [CDC], 2003). The other three viruses are laboratory-adapted viruses. N2C and B2C were isolated from CVS-24 by passaging in neuroblastoma and BHK cell lines, respectively (Morimoto *et al*, 1998). SN-10 is a clone generated from the vaccine strain, SAD-B19, by reverse-genetics technology (Schnell *et al*, 1994). The three recombinant viruses (RB2C, RN2C, and RSHBRV) were obtained by reverse genetics using a SN-10 viral genomic backbone, replacing the G gene with G genes from B2C, N2C, or SHBRV (Morimoto *et al*, 2001).

To determine the pathogenicity, virus titers and $ICLD_{50}$ of the different virus stocks were measured in BHK cells and mice (by IC route of infection), respectively. The pathogenic index for a particular virus is the log $ICLD_{50}$/ml divided by the log virus titer/ml in BHK cells

(Morimoto *et al*, 1998, 2001). Virus titers, $ICLD_{50}$, and the pathogenic index for each of these viruses are summarized in Table 1. Among the seven viruses tested, SHBRV and N2C were the most pathogenic viruses, followed in order by RSHBRV, RN2C, B2C, SN-10, and RB2C. Overall, recombinant viruses were found to be less pathogenic than the parental viruses. However, recombinant viruses expressing the G from pathogenic viruses such as SHBRV and N2C were more pathogenic than attenuated strains such as B2C and SN-10. These results indicate that the G is largely the determinant for viral pathogenicity (Morimoto *et al*, 2000).

More apoptotic cells were observed in mice inoculated IC with attenuated than pathogenic viruses: To examine histopathological lesions, mice were infected with 10 $ICLD_{50}$ of each virus and brains were harvested at the time when mice developed paralysis. Brain sections from four mice infected with each virus were stained with hematoxylin and eosin (H&E). Brain sections from sham-infected mice were also included. Overall, pathological changes included apoptosis, necrosis, inflammation, vacuolation, and gliosis (Figure 3.1). The severity of histological lesions was scored for each virus and is summarized in Table 1. The most severe histopathological changes were observed in mice infected with B2C and RB2C, whereas mice infected with SN-10, N2C, RN2C, and RSHBRV had moderate histopathological changes. Mice infected with SHBRV had minimal histopathological changes. To quantify apoptosis, brain sections from four mice infected with each virus were examined for apoptosis by using the deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. Brain sections from sham-infected mice were also included. TUNEL-positive cells were counted in the cerebral cortex, hippocampus, thalamus, hypothalamus, brain stem, and cerebellum from each mouse and the average number from four animals was obtained for each virus and analyzed statistically by one-

way analysis of variance (ANOVA) and Student's *t* test. As summarized in Table 1, few apoptotic cells were observed in sham-infected animals. Apoptotic cells were observed in almost all the animals infected with each of the viruses. However, statistical analyses revealed that the number of apoptotic cells in mice infected with SHBRV was not significantly different from the number in sham-infected animals by either test. By one-way ANOVA, it was found that significantly more apoptotic cells ($P < .05$) were observed in mice infected with B2C, RB2C, SN-10, and RSHBRV than in mice infected with N2C or RN2C (Table 1). However, Student *t* test revealed that significantly more apoptotic cells ($P < .05$) were observed in mice infected with B2C, RB2C, SN-10, RSHBRV, N2C, and RN2C than in sham-infected mice (data not shown). Furthermore, B2C and RB2C were statistically different ($P < .05$) from all other groups. Overall, the recombinant viruses induced similar amount of apoptosis as the parental viruses from which the G was derived. Attenuated viruses (B2C, RB2C, and SN-10) induced more apoptosis than the pathogenic viruses (SHBRV, N2C, RN2C, and RSHBRV). Thus, induction of apoptosis inversely correlates with pathogenicity (Figure 3.2). These data suggest that apoptosis is part of the host defense responses that normally play a protective role in rabies virus infection by restricting viral spread to the brain.

Higher level of G expression was detected in mice infected with attenuated than pathogenic viruses: The above studies indicate that the RV G is a major determinant for the induction of apoptosis. To determine if the induction of apoptosis was associated with the level of G expression as reported previously (Morimoto *et al*, 1999; Yan *et al*, 2001; Faber *et al*, 2002), viral antigens (G and the nucleoprotein [N]) were examined by immunohistochemical analysis. The level of G and N expression was scored and the results are summarized in Table 1. RV G

was expressed abundantly in B2C- and RB2C-infected mice; a moderate level of G expression was detected in SN-10-, N2C-, and RN2C-infected animals, whereas the G expression was minimal in mice infected with SHBRV and RSHBRV. The level of G expression in the recombinant viruses is similar to that in the parental viruses from which the G is derived. On the other hand, the level of N expression was similar in animals infected with each of these viruses. N antigen was detected in almost all the neurons in the hippocampus, particularly in the CA3 region, although the antigen staining was less intense in mice infected with SHBRV than in mice infected with other laboratory adapted viruses (Figure 3.1). Brain extracts from SHBRV- or B2C-infected mice were also subjected to polyacrylamide gel electrophoresis (PAGE) and Western blot analysis, it was found that the level of G expression in SHBRV-infected mice was consistently threefold lower than in B2C-infected mice, whereas the level of N expression was similar in mice infected with either virus (Wang *et al*, 2005). These results indicate that the level of G expression is associated with a particular RV strain and may correlate with the induction of apoptosis.

More apoptotic cells were observed in primary neurons infected with attenuated than pathogenic viruses: To determine if the induction of apoptosis in mouse brain correlates with *in vitro* studies, primary neuronal cultures were prepared as described (Adamec *et al*, 2001; Li *et al*, 2005). At day 7 after plating, cultured neurons were infected with each of the seven viruses at a multiplicity of infection (moi) of 0.1 focus-forming units (ffu) per cell and the cells were fixed with 4% paraformaldehyde and stained with either fluorescein isothiocyanate (FITC)-conjugated RV antibodies or with the TUNEL assay at day 5 post infection (p.i.) as described (Morimoto *et al*, 1999). By day 5 p.i., neurons showed almost 100% infection with each of the viruses (data

not shown). To assay apoptosis, TUNEL assay was performed in triplicate for each virus. The percentage of apoptotic neurons was determined in six 40× fields and presented in Figure 3.3. Data were analyzed statistically by one-way ANOVA. Only neurons infected with CVS-B2C, RB2C, SN-10, or RSHBRV had significantly ($P < .05$) more apoptosis than uninfected neurons, whereas the number of TUNEL-positive neurons infected with SHBRV, CVS-N2C, or RN2C were not significantly more than uninfected neurons. Student's *t* test revealed significantly more apoptotic neurons in RN2C-infected cultures than sham-infected cultures and significantly more apoptotic cells ($P < .05$) were detected in B2C-infected neurons than in neurons infected with any other virus (data not shown). Overall, the induction of apoptosis in primary neurons correlates with that in mice by these viruses.

Attenuated and wt RV induced different clinical signs after intramuscular (IM) infection: To investigate the mechanism by which induction of apoptosis attenuates RV pathogenicity, mice were infected with RV by IM and virus spread was monitored in the spinal cord and the brain. Two viruses, SHBRV and B2C, were selected. Initially the $IMLD_{50}$ was determined for each virus by inoculation into both hind legs. Figure 3.4 shows the survival curve of mice when infected with either the SHBRV at 10^3 ffu or the CVS-B2C at 10^3, 10^4, 10^5, 10^6 ffu, respectively. At 10^3 ffu, SHBRV killed 90% of the infected mice. However, only 10% of the mice infected with B2C succumbed to rabies at this dose. The mortality rate increased with increasing doses of B2C. At the dose of 10^6 ffu, 80% of the mice infected with B2C succumbed to rabies. These data indicate that B2C is an attenuated virus and needs 3 logs more virus than SHBRV to kill a similar percentage of animals by IM.

Clinical signs were different in mice infected with these two viruses. In mice infected with B2C (10^6 ffu), first sign of disease, ruffled fur, was observed on day 4 p.i. On day 5 p.i., mice had paresis and on day 6 p.i. flaccid paralysis of one or two hind limbs. Hunchback was observed as the disease progressed, followed by paralysis of the fore limbs. The mice then became prostrated with loss of muscle mass (wasting) and finally death beginning at day 7 p.i. (Figure 4). In mice infected with SHBRV, on the other hand, no ruffled fur was observed. Paralysis was observed beginning on day 6 p.i. The paralysis in SHBRV-infected mice was different from that observed in B2C-infected mice. B2C-infected mice had flaccid paralysis whereas spastic paralysis was observed in SHBRV-infected mice. There was no voluntary joint movement, but involuntary spastic movement was common, and stimulation of the legs usually evoked a withdrawal reflex. As the disease progressed, the mice had hypersensitivity to noise and would jump vigorously or spin continuously until collapsing. In this situation, some would recover quickly while others would soon die. Mice infected with SHBRV began to die at day 8 p.i. (Figure 3.4).

Attenuated RV induced apoptosis in the spinal cord whereas wt RV did not: To monitor virus spread and the induction of apoptosis, mice were infected IM with SHBRV at 10^3 ffu or B2C at 10^3, 10^4, 10^5 and 10^6 ffu. At 3, 5, 7, and 9 days p.i., brains and spinal cords were collected after transcardial perfusion. The tissues (four mice in each group) were paraffin embedded and sectioned for detection of apoptosis and viral antigen expression. Because neither apoptosis nor viral antigen was observed in any mouse at day 3 p.i., no data are presented for this time point. The detection of apoptosis and antigen in the spinal cord and the brain are summarized in Tables 2 and 3, respectively. When mice were infected with 10^3 of B2C, RV

antigen was detected at day 5 p.i. in the spinal cord of 25% of the mice. However, RV antigen was detected in only a few neurons and their processes in the spinal cord and no RV antigen was detected in the brain. Furthermore, only a few TUNEL-positive cells were observed in the spinal cord of mice infected with 10^3 ffu of B2C. When mice were infected with 10^4 ffu of B2C, RV antigen was detected at day 5 p.i. in the spinal cord of 50% of the mice and the number of infected neurons increased when compared to mice infected with10^3 ffu of B2C. In addition, more apoptotic cells were observed in the spinal cord, particularly at day 5 p.i. By days 7 and 9 p.i., the number of apoptotic cells declined. In mice infected with 10^5 ffu of B2C, RV antigen was observed in 75% of the animals in the spinal cord and 50% of the mice in the brain. In the spinal cord, about 50% of the neurons were infected. In the brain, RV antigen was detected in many neurons in the medulla, but in only a few neurons in the cerebral cortex and a few Purkinje cells in the cerebellum. Moderate numbers of apoptotic cells were observed in the spinal cord as well as in the brain, particularly at day 7 p.i. When mice were infected with 10^6 of B2C, RV antigen was detected in all the infected animals in the spinal cord and most of the animals (75%) in the brain. Extensive RV antigen staining was detected in most of the neurons (80%) and their processes in the grey matter of the spinal cord at day 7 p.i. In the brain, RV antigen was also observed in most of the neurons in the medulla, 20% of the Purkinje cells in the cerebellum, and about 10% neurons in the cerebral cortex and thalamus/hypothalamus. Furthermore, apoptosis was detected in a moderate number of neurons at day 5 p.i. and extensive apoptosis at days 7 and 9 p.i. (Tables 2, Figure 3.5A).

When mice were infected with 10^3 ffu of SHBRV, on the other hand, RV antigen was detected in all animals in the spinal cord and most animals (75%) in the brain. In the spinal cord,

RV antigen was more prominent in the neuropil than in perikarya. In the brain, RV antigen was detected in about 50% of the neurons in major brain regions, including the medulla, cerebellum, hippocampus, and cerebral cortex at days 7 and 9 p.i. Despite the fact that viral antigen was detected in almost all the animals in the spinal cord, little apoptosis was detected in the spinal cord or in the brain of mice infected with 10^3 ffu of SHBRV (Table 2, Figure 3.5B). No apoptosis was detected in sham-infected mice (Figure 3.5C).

To confirm that infected neurons underwent apoptosis, double labeling was performed in the spinal cord for detection of viral antigen and apoptotic cells. As shown in Figure 3.5D, double-labeled neurons were detected in mice infected with B2C. Furthermore, condensations of nuclear chromatin with neuronophagia were observed in the spinal cords of mice infected with B2C (Figure 3.5E), but not in sham-infected mice or mice infected with SHBRV (data not shown).

DISCUSSION

Induction of apoptosis has been associated with RV G expression, particularly the level of G expression (Morimoto *et al*, 1999; Yan *et al*, 2001; Faber *et al*, 2002). Using a panel of recombinant RVs, we demonstrated that the induction of apoptosis is largely determined by the G. G gene also determines the level of G expression. Recombinant RVs expressed a similar level of the G and induced a similar level of apoptosis as the parental viruses from which the G was

derived. For example, parental N2C and recombinant RN2C expressed a low level of G and only a few apoptotic cells were detected. On the other hand, parental B2C and recombinant RB2C expressed high levels of the G and induced extensive apoptosis. The level of G expression is not due to the rate of viral replication because the level of N expression is similar in animals infected with each of these viruses and N antigen is detected in almost all the neurons, particularly in the hippocampal region. However, recombinant RSHBRV induced significantly more apoptosis than the parental SHBRV, although the level of G expression is similarly low in mice infected with either virus. Although RV G alone from CVS has been shown to induce apoptosis in cell culture (Préhaud *et al*, 2003) and recombinant RV expressing two copies of the G induced more apoptosis than RV expressing a single copy of the G (Faber *et al*, 2002), recently it has been reported that RV matrix (M) protein alone is also capable of inducing apoptosis in neuroblastoma cells (Kassis *et al*, 2004). In addition, the M proteins from other rhabdoviruses such as vesicular stomatitis virus (Kopecky *et al*, 2001; Kopecky and Lyles, 2003) and infectious hematopoietic necrosis virus (Chiou *et al*, 2000) have also been reported to induce apoptosis. It is possible that the M in the SN-10 backbone contributed to the induction of apoptosis in the RSHBRV-infected mice. However, both recombinant RB2C and RN2C induced less apoptosis (albeit not significantly) than the parental B2C and N2C, respectively, whereas recombinant RSHBRV induced significantly more apoptosis than the parental SHBRV. These results may indicate that both G and M can independently induce apoptosis (Préhaud *et al*, 2003; Kassis *et al*, 2004), and also the interaction between the G and M may contribute to the induction of apoptosis in RV infections.

It has also been found that the ability of a particular RV strain to induce apoptosis inversely correlates with its pathogenicity (Morimoto *et al*, 1999; Yan *et al*, 2001;

Pulmanausahakul *et al*, 2001; Faber *et al*, 2002). Overall the induction of apoptosis correlated inversely with pathogenicity among the seven parental and recombinant RVs tested in the present study (see Figure 3.2). Attenuated viruses such as B2C induced extensive apoptosis whereas wt SHBRV induced little apoptosis in adult mice. Yet, a few viral particles of the SHBRV killed infected animals whereas 1000 to 10,000 more viral particles were required by B2C to kill infected animals (see Table 1 and Figure 3.2). Therefore our study confirms the previous hypothesis that induction of apoptosis is a host defense mechanism in RV infections (Morimoto *et al*, 1999; Yan *et al*, 2001). However, the mechanisms by which induction of apoptosis protect RV-infected animals are not completely understood. It is possible that attenuated RV such as B2C, by inducing apoptosis, prevents virus spread within the CNS. To investigate such possibility, mice were infected with different doses of B2C by the IM route and the induction of apoptosis and virus spread were monitored in the CNS. It was found that lower doses of B2C resulted in mild RV infection limited to the spinal cord and infection of the spinal cord neurons induced apoptosis. This may explain why RV antigen was detected in only a few neurons in mice. Thus, we hypothesize that G-mediated induction of apoptosis limits the spread of attenuated rabies viruses in the CNS of mice. Although attenuated RV did not cause obvious neurological signs and death when given at lower doses, it is not known if induction of apoptosis in the spinal cord is associated with minor gait abnormalities or behavior changes.

In contrast, mice infected with low dose of SHBRV induced little apoptosis despite the fact that viral antigens were detected in most of the infected mice. Furthermore, a low dose of SHBRV resulted in spread to the brain and development of clinical rabies. Thus, our study suggests that induction of apoptosis is a host defense mechanism in RV infection that prevents

virus spread from the spinal cord to the brain. Nevertheless, mice infected with high doses of attenuated RV developed neurological diseases and died of rabies. Thus, induction of apoptosis can have protective functions on one hand, but may also play a role in pathogenesis on the other, particularly in animals experimentally infected with high doses of attenuated RVs by the IC route (Jackson and Rossiter, 1997; Theerasurakarn and Ubol, 1998).

In addition to the induction of apoptosis, other pathological changes, particularly inflammatory reactions, were also detected in animals infected with attenuated RVs than pathogenic RVs. In our previous studies, significantly more CD3-positive cells were detected in mice infected with attenuated B2C and SN-10 than pathogenic SHBRV and N2C (Li *et al*, 2005; Wang *et al*, 2005). Infiltration of T cells has been reported to play a major role not only in blocking RV spreading in (Camelo *et al*, 2001; Baloul and Lafon, 2003), but also in clearing RV from the CNS (Hooper *et al*, 1998). Recently, we (Wang *et al*, 2005) and others (Préhaud *et al*, 2005) have shown that attenuated RVs induce strong innate immune responses such as up-regulation of IFN-α/β and inflammatory cytokines and chemokines. Furthermore it has been shown that virus uptake by neurons determines neuroinvasiveness as well (Faber *et al*, 2004) because it takes a shorter time for SHBRV to infect 50% of the cells *in vitro* than attenuated SN-10. Thus multiple factors may be involved in determining RV neuroinvasiveness and thus pathogenicity.

In the present study, severe pathological changes including apoptosis, inflammatory reaction, and gliosis were observed in mice infected with high doses of attenuated RVs,

particularly B2C by either the IC or the IM route. On the other hand, only mild pathological changes were observed in mice infected with pathogenic RV such as SHBRV. The mild pathological changes observed in mice infected with pathogenic RVs resemble those observed in human patients who died of rabies (Murphy, 1977). Furthermore, mice infected with SHBRV developed clinical signs different from those in mice infected with B2C. Mice infected with B2C developed ruffled fur, weight loss, and flaccid paralysis. The presence of neuronophagia, inflammation, and gliosis, particularly in the spinal cord, correlates the clinical observations of a progressive, flaccid paralysis seen in mice infected with B2C and also reported in other virus infections, such as in humans infected with West Nile virus (Kelley *et al*, 2003). On the other hand, mice infected with SHBRV developed hypersensitivity to environmental stimulus and died suddenly without any obvious signs. In addition, mice infected with SHBRV developed spastic paralysis, which has previously been reported in experimental wt rabies virus infection of mice (Jackson *et al*, 1989). Based on these findings, we propose that pathogenic and attenuated RVs employ different mechanisms to induce neurological diseases. In mice infected with attenuated RV, particularly at high doses, apoptosis and possibly inflammation play a major role in the development of neurological diseases. The induction of apoptosis and inflammation has been associated with the level of G expression as shown in this study as well as reported by others (Morimoto *et al*, 1999; Yan *et al*, 2001; Préhaud *et al*, 2003; Faber *et al*, 2002, 2004; Wang *et al*, 2005). How pathogenic RVs induce neurological disease without causing severe pathological changes remains to be determined.

MATERIALS AND METHODS

Viruses, cells, and antibodies: Seven different RV strains were used in this study including four parental viruses (SN-10, B2C, N2C, and SHBRV) and three recombinant viruses (RB2C, RN2C, and RSHBRV). All these viruses were obtained from Dr. Bernhard Dietzschold, Thomas Jefferson University. Virus stocks were prepared as described (Morimoto *et al*, 1996, 1998; Schnell *et al*, 1994; Yan *et al*, 2002). Briefly, 1-day-old suckling mice were infected with 10 µl of viral samples by the IC route. When moribund, mice were sacrificed and brains removed. A 20% (*w*/*v*) suspension was prepared by homogenizing the brain in Dulbecco's modified Eagle's medium (DMEM). The homogenate was centrifuged to remove debris and the supernatant collected and stored at −80°C. Baby hamster kidney (BHK) cells were cultured in DMEM. Anti-RV N monoclonal antibody 802-2 (Hamir *et al*, 1995) was obtained from Dr. Charles Rupprecht, Centers for Disease Control and Prevention. Anti-RV G polyclonal antibody was prepared in rabbit as described (Fu *et al*, 1993).

Mouse primary neuronal cultures: Mouse primary neuronal cultures were prepared using standardized procedures as described (Adamec *et al*, 2001; Li *et al*, 2005; Wang *et al*, 2005). Swiss-Webster mice at gestation day 16 were euthanized and the embryos removed. Neocortex from these embryos were collected and digested with trypsin, separated neuronal cells were plated into culture wells treated with poly-D-lysine (50 µg/ml). The primary neurons were grown in Neurobasal medium supplemented with 2% B-27, 500 mM glutamine, 25 mM glutamate, 10% fetal bovine serum, and 1% horse serum in a humidified atmosphere of 5% CO_2–95% air at

37°C. Ara-C (cytosine furo-arabinoside) at a final concentration of 1 μM was added at 1 and 5 days after plating to prevent the proliferation of non-neuronal cells.

Animal infection and tissue collection: ICR mice (Harlan) at the age of 4 to 6 weeks were housed in temperature- and light-controlled quarters in the Animal Facility, College of Veterinary Medicine, University of Georgia. They had access to food and water *ad libitum*. Mice were infected with 10 $ICLD_{50}$ of each virus by the IC route. Alternatively, mice were infected with different doses of RVs by the IM route in the hind legs (both sides). Infected animals were observed twice daily for 20 days for the development of rabies. Sham-infected mice were included as controls. At the time of severe paralysis or at different time points after virus infection, mice were anesthetized with ketamine/xylazine at a dose of 0.2 ml and then perfused by intracardiac injection of phosphate-buffered saline (PBS) followed by 10% neutral-buffered formalin as described (Yan *et al*, 2001, 2002). Only brains were removed from mice infected by IC whereas both spinal cords and brains were collected from the IM infected mice. Tissues collected were placed in the same fixative (10% neutral-buffered formalin) for 1 week at 4°C. Tissues were paraffin embedded and coronal sections (4 μm) were obtained and placed on glass slides.

Determination of virus titers, LD_{50}, and pathogenic index: Virus titers were determined in BHK cells as described (Fu *et al*, 1996). Briefly, virus preparations were serially (10-fold) diluted in 96-well plates and cell suspension was added into each well. After 24 h of incubation at 37°C, infected cells were fixed in 80% acetone and viral antigen was detected with FITC-conjugated anti-RV antibodies (FujiRab, Malvin, PA). Infectious foci were counted under a

fluorescence microscope and calculated as focus-forming units per milliliter (ffu/ml). All titrations were performed in duplicate, and the average infectious foci were used to determine the virus titer. LD_{50} of individual viruses was determined by infecting 4- to 6-week-old ICR mice by either the IC or the IM route as described (Morimoto *et al*, 2001). Essentially, virus was serially (10-fold) diluted in DMEM and 10 µl of each dilution was used to infect each mouse. For each virus dilution, 10 mice were used. Infected animals were observed twice daily for 20 days for the development of rabies (paralysis and death). IC or IM LD_{50} were calculated as described by Reed and Muench (1938). RV pathogenic index was determined by the following formula: log $ICLD_{50}$/ml divided by the log virus titer/ml as described (Morimoto *et al*, 1998, 2001).

Histopathology and immunohistochemistry: Histopathology was performed by staining the paraffin embedded tissue sections with H&E or cresyl violet. Severity of the lesions was scored according to the degree of vacuolation, inflammation, and necrosis. Lesions are classified as, +++ (extensive pathology with more than 50% of the neurons affected); ++ (observable pathology with 25% to 50% of the neurons affected); + (mild pathological changes with 10% to 25% neurons affected); and - (no pathological changes).

For immunohistochemistry, paraffin-embedded brain and spinal cord tissue sections were heated at 70°C for 10 min, then dipped in Hemo-De for 3 × 5 min and dried until chalky white. Slides were incubated with proteinase K (20 µg/ml in 10 mM Tris · HCl pH 7.4 to 8.0) for 15 min at 37°C and rinsed for 3 × 5 min with PBS. For detection of apoptosis, the TUNEL assay was performed using the In Situ Cell death Detection kit, AP (Roche Scientific), as described previously (Yan *et al*, 2001). Briefly, TUNEL reaction mixture was added onto the slides

covered with cover slips and incubated for 60 min at 37°C. Converter-AP was then added to each slide (approximately 100 µl) and incubated again for 30 min at 37°C. Slides were rinsed 3 × 5 min with PBS and substrate (4-nitro blue tetrazolium chloride, NBT and 5-bromo-4-chlor-3indolyl-phosphate, BCIP) or Vulcan Fast Red (Biocare) was added. After color development, slides were counterstained with methyl green or hematoxylin and mounted with mounting medium. TUNEL-positive cells were counted and analyzed statistically by one-way ANOVA and Student's *t* test.

For viral antigen detection, tissue slides were incubated with either the anti-RV N monoclonal antibody (802-2) (Hamir *et al*, 1995) or with the anti-RV G polyclonal antibody as described previously (Yan *et al*, 2001). The secondary antibody used was biotinylated goat anti-mouse or goat anti-rabbit immunoglobulin G (IgG) from the VectaStain kits (Vectorlab). The avidin-biotin-peroxidase complex (ABC) then was used to localize the biotinylated antibody. Finally diaminobenzidine (DAB) was used as a substrate for color development. For double-labeling, spinal cord sections were detected first with anti-RV antibodies followed by TUNEL assay.

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Table 1: Virus titers, $ICLD_{50}$, IC pathogenic index, histopathological scores, the number of apoptotic cells, and expression of viral antigens in the CNS of mice infected with different RVs.

Viruses	Virus Titers	ICLD50	Pathogenic Index (IC)	# of Apoptotic Cells (±SD)*	Pathology Score¶	Viral N antigen¶	Viral G antigen¶
SHBRV	$10^{5.23}$	$10^{-4.5}$	0.19	5.25±2.63	+	+++	+
N2C	$10^{5.11}$	$10^{-4.7}$	0.38	14.75±3.81†	++	+++	++
RSHBRV	$10^{6.72}$	$10^{-4.9}$	0.015	17.75±2.99†	++	+++	+
RN2C	$10^{8.87}$	10^{-6}	0.0015	12.00±2.16†	++	++++	++
B2C	$10^{7.94}$	10^{-5}	0.0011	44.25±9.76††	+++	++++	++++
SN-10	$10^{10.18}$	10^{-7}	0.00066	20.50±1.29†	++	++++	++
RB2C	$10^{10.71}$	$10^{-5.8}$	0.000012	31.25±14.90††	+++	++++	+++
Control	-	-	-	2.25±1.26	-	-	-

*TUNEL-positive cells were counted in the cortex, hippocampus, thalamus, hypothalamus, brain stem and cerebellum from each mouse and the average number from four animals was obtained for each virus and analyzed statistically by one-way ANOVA and Student's t-Test.

†significantly different from that of controls ($P<0.05$)

†† significantly different from all other groups

Pathological lesions were scored according to the severity. +++ indicates extensive pathology with more than 50% of the neurons affected. ++ indicates observable pathology with 25-50% of the neurons affected. + indicates mild pathological changes with 10-25% neurons affected, while –

indicates no pathological changes. The level of antigen expression is scored according to the immunostaining intensity and the number of neurons affected in the hippocampal region. ++++ indicates strong immunostaining with almost all the neurons affected. +++ indicates strong immunostaining with more than 50% neurons affected, or slightly weak staining with almost all the neurons affected. ++ indicates weak staining with about 25% neurons affected. + indicates weak staining with about 10% neurons affected. – indicates no detection of viral antigen.

Table 2. The number of apoptotic cells observed in mice infected through IM route with B2C or SHBRV.

	The numbers of apoptotic cells (mean±SD)					
	spinal cord			brain		
Days p.i. / Virus/Dose	5	7	9	5	7	9
B2C 10^3	1.7±1.5	1.7±1.2	1.0±1.7	1.2±1.1	3.3±1.0	1.3±1.5
B2C 10^4	3.6±2.2	1.5±1.3	1.3±0.5	1.0±0.4	0.6±0.5	1.0±0.3
B2C 10^5	1.3±0.5	20±3.0	7.0±2.1	2.3±1.7	5.7±1.1	2.0±2.6
B2C 10^6	15±4.8	33±5.9	38±3.3	1.3±0.5	2.0±0.4	3.5±1.7
SHB 10^3	0.8±0.8	1.9±1.7	1.8±2.2	1.3±1.0	1.4±0.7	1.6±1.3
Control	0.8±0.5			1.3±0.5		

Mice (four in each group) were infected with different doses of RV and spinal cords as well as brains were harvested at different time points after infection for TUNEL assay for detection of apoptosis. Apoptotic cells were counted in six fields in the spinal cord or six fields in the medulla, cerebral cortex and cerebellum. The average number of apoptotic cells and the standard deviation are shown.

Table 3. Detection of RV N antigen in different regions of the CNS (numbers of mice shown positive staining/total animals tested).

Brain regions	Spinal cord			medulla			cerebellum			Thalamus/ hypothalamus			hippocampus			cortex		
Days p.i. / Virus/Dose	5	7	9	5	7	9	5	7	9	5	7	9	5	7	9	5	7	9
B2C 10^3	1/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
B2C 10^4	2/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
B2C 10^5	3/4	2/4	1/4	2/4	1/4	0/4	1/4	1/4	1/4	0/4	0/4	0/4	0/4	0/4	0/4	1/4	0/4	1/4
B2C 10^6	4/4	3/4	2/4	3/4	3/4	2/4	1/4	3/4	2/4	1/4	3/4	2/4	1/4	3/4	2/4	1/4	3/4	2/4
SHB 10^3	3/4	3/4	4/4	1/4	3/4	3/4	0/4	3/4	3/4	0/4	3/4	2/4	0/4	3/4	2/4	0/4	3/4	2/4

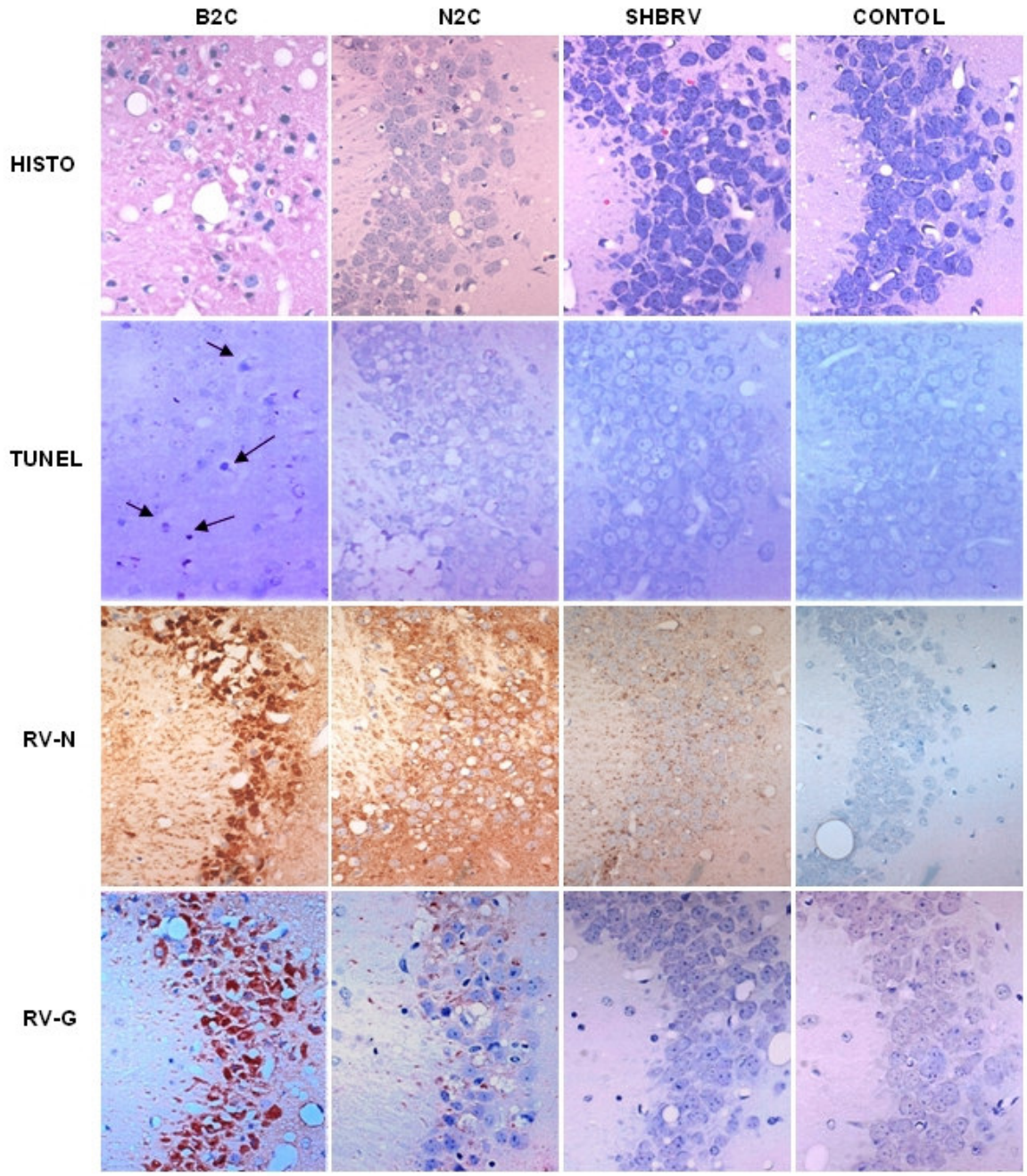

Figure 3.1. Detection of apoptosis, pathological changes, and viral antigens in mice infected with different RVs. Mice were infected with 10 ICLD50 of each virus and brains were harvested for histopathology (HISTO) and detection of apoptosis using TUNEL assay (TUNEL,

arrows indicate TUNEL-positive cells). Viral antigens (RV-N and RV-G) were detected using anti-G and anti-N antibodies as described in the text. (magnification 40X)

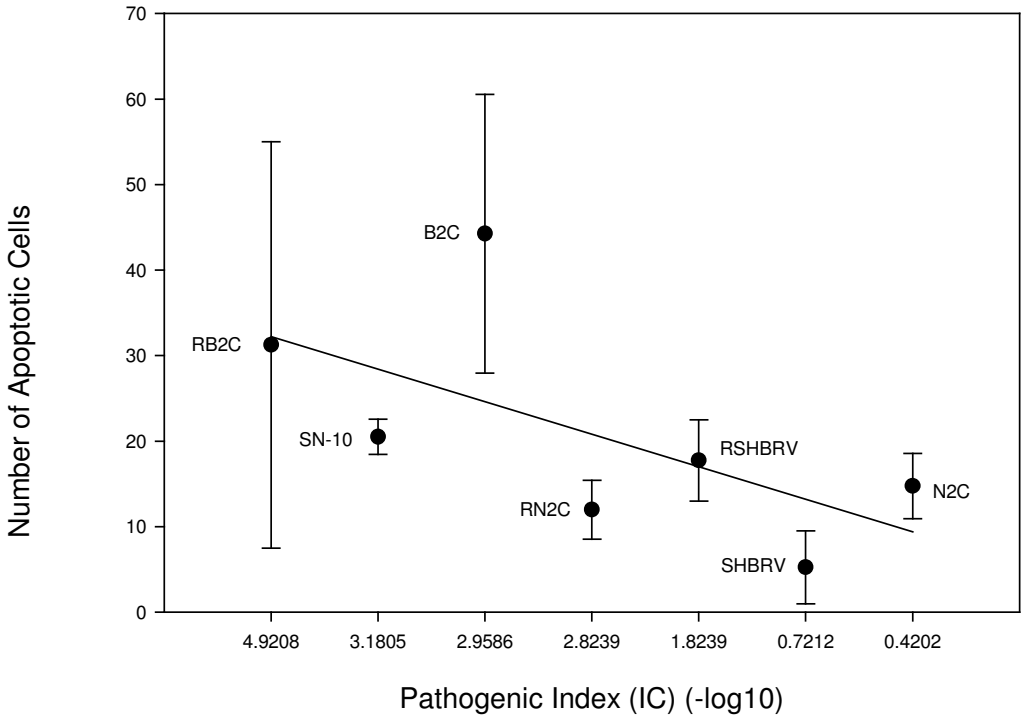

Figure 3.2. Inverse correlation between the induction of apoptosis and pathogenicity. Pathogenic index were plotted against the numbers of apoptotic cells observed in mice infected with different RVs using SigmaPlot. Vertical bars indicate the standard deviation.

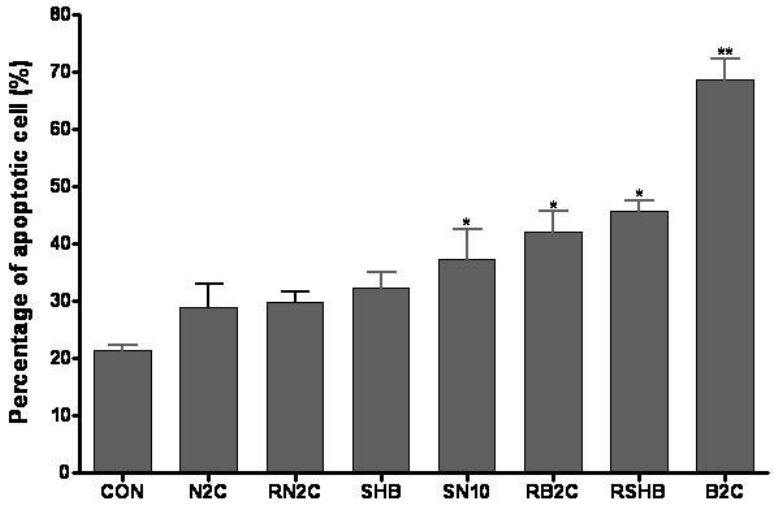

Figure 3.3. Induction of apoptosis by RVs in primary neurons. Primary neurons were infected with each of the viruses and apoptosis was detected using the TUNEL assay. The number of apoptotic neurons was assayed and analyzed using one way ANOVA at the $p<0.05$ level.

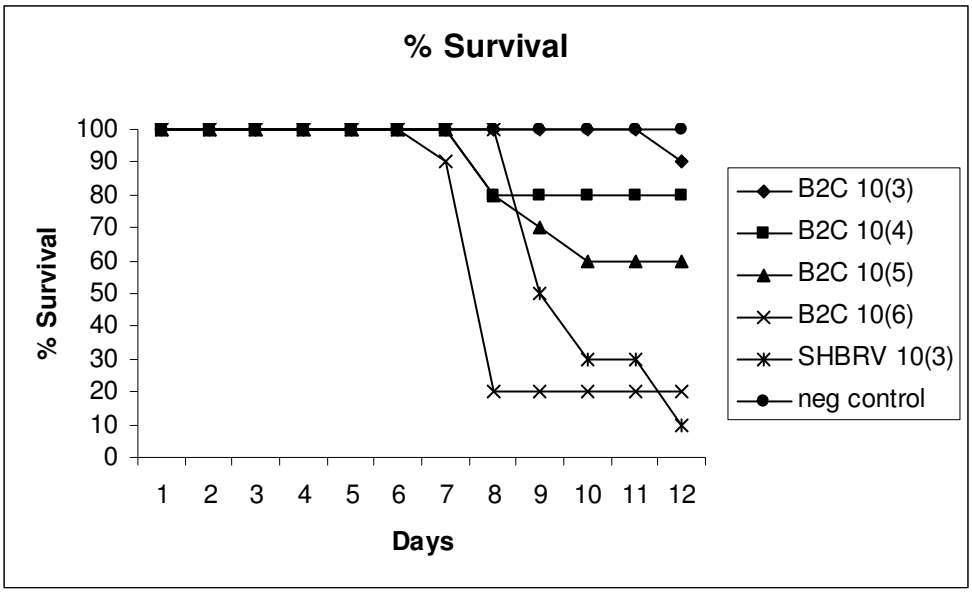

Figure 3.4. Survival rate in mice infected with different RVs at different doses. Mice (10 in each group) were infected with different doses of SHBRV or B2C and the development of rabies were recorded daily for 20 days. No animals died after 12 days p.i.

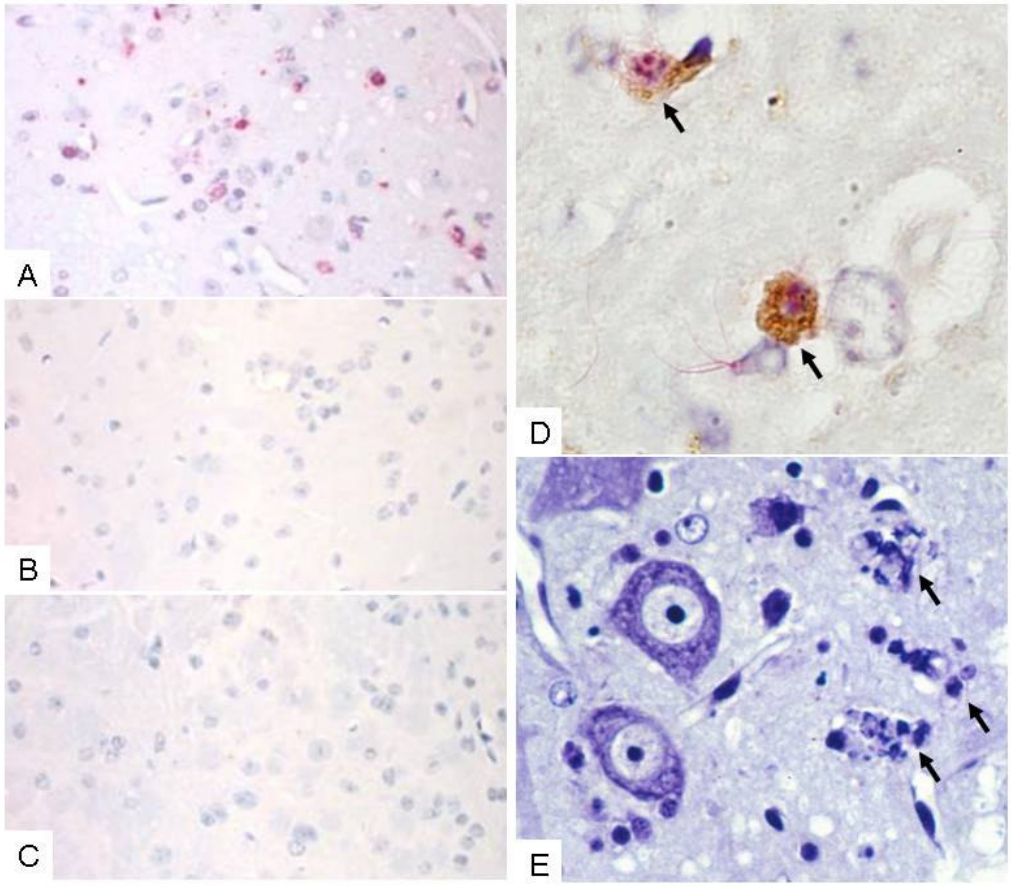

Figure 3.5. Induction of apoptosis in the spinal cords. Spinal cord tissues from mice infected with B2C (A) or SHBRV (B) by the IM route at day 7 p.i. were fixed for detection of apoptosis (red) using TUNEL assay (Magnification 40X). Spinal cord from sham-infected mice was included as control (C). Double labeling was performed to detected viral antigen (brown) and apoptosis (red) in the spinal cord of mice infected with B2C at day 7 p.i. (D). Both viral antigen and TUNEL-positive neurons are shown by arrows (Magnification 100X). Ventral horn of a spinal cord from B2C-infected mouse stained with cresyl violet showing condensations of nuclear chromatin and neurophagia (arrows) (E, Magnification 100X).

CHAPTER 4

RABIES VIRUS-INDUCED APOPTOSIS INVOLVES CASPASE-DEPENDENT AND CASPASE-INDEPENDENT PATHWAYS[1].

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Abstract

Previously it has been shown that the lab-attenuated rabies virus CVS-B2C, but not the wild-type virus SHBRV, induces apoptosis in mice and the induction of apoptosis is mediated by viral glycoprotein. Induction of apoptosis by CVS-B2C limits the spread of the virus in the CNS. In the present study, we characterized the pathways by which CVS-B2C induces apoptosis. Baby hamster kidney-derived cell line BSR cells were infected with CVS-B2C or SHBRV and harvested at different time points for detection of apoptosis by immunofluorescence and flow cytometry. Caspase activity and expression of several apoptotic proteins were analyzed by fluorometric assay and Western blotting. Activation of caspase-8 and caspase-3, but not of caspase-9, was observed in CVS-B2C-infected cells. In addition, the level of expression of Apaf-1 did not change. Furthermore, PARP was cleaved confirming activation of downstream caspases. These data suggest that CVS-B2C infection activates the extrinsic, but not the intrinsic, apoptotic pathway. In addition, AIF, a caspase-independent apoptotic protein was up-regulated and translocated from the cytoplasm to the nucleus post infection, suggesting that apoptosis induced by CVS-B2C also involves the activation of a caspase-independent pathway.

Key words: AIF, apoptosis, caspases, PARP, rabies virus

Introduction

Apoptosis has important biological roles in the development and homeostasis of cell populations, and in the pathogenesis and expression of many disease processes (Saikumar et al., 1999). An important regulatory event in the apoptotic process is the activation of caspases, a family of cysteine proteases. Caspases are synthesized as inactive precursors (zymogens) that are processed to large and small subunits to form the active enzymes (Nicholson and Thornberry, 1997). There are two well-known apoptotic pathways involving activation of caspases. The intrinsic, or mitochondria, pathway is associated with the release of mitochondria proteins, such as cytochrome-c. Cytochrome-c interacts with the adaptor protein Apaf-1, ATP and pro-caspase-9 to form the complex termed apoptosome, which activates caspase-9. The extrinsic pathway is characterized by binding of death receptors such as fibroblast-associated (Fas), tumor necrosis factor receptor (TNF-R), and TNF-related apoptosis inducing ligand receptor (TRAIL-R) to their ligands. This binding leads to interactions of adaptor molecules and pro-caspases 8, forming the death-inducing signaling complex (DISC), which activates caspase-8 (Hussein et al., 2003). Both pathways eventually culminate in the cleavage of the executioner caspase-3, which in turn cleaves a variety of target substrates, such as Poly(ADP-ribose) polymerase-1 (PARP-1). Poly(ADP-ribose) polymerase-1 is a nuclear enzyme that catalyses the transfer of the ADP-ribose moiety of NAD^+ to a specific subset of nuclear substrates in response to DNA damage (D'Amours et al., 2001). Although almost all caspases, including caspase-1, can cleave PARP *in vitro*, most likely caspase-3 and -7 are responsible for the *in vivo* processing of PARP to its apoptotic 24 and 89 kDa fragments (Nicholson and Thornberry, 1997).

Alternative apoptotic pathways have also been described, such as a pathway where intrinsic and extrinsic pathways converge (Gaddy and Lyles, 2005). In this pathway, low levels of activated caspase-8 cleave BID, a BH3 domain–containing proapoptotic Bcl2 family member. Cleaved BID (tBID) translocates to mitochondria and transduces apoptotic signals from cytoplasmic membrane to mitochondria. tBID induces the release of cytochrome c into the cytosol activating caspase-9 (Gaddy and Lyles, 2005)**.** Another alternative apoptotic pathway described is the caspase-independent pathway involving the apoptosis-inducing factor (AIF). Apoptosis-inducing factor is a flavoprotein found in the intramembrane space of the mitochondria (Joza et al., 2001). Apoptosis-inducing factor is released from the mitochondria during cell death and translocated to the nucleus to induce chromatin condensation as well as large-scale (~50 kbp) DNA fragmentation, causing the so-called caspase-independent cell death (Joza et al., 2001).

Many viruses have been shown to induce apoptosis, either as a mechanism for the release and dissemination of progeny virions or as a defense strategy of multicellular host organisms for the destruction of infected cells and therefore preventing the spread of the virus (Mori et al., 2004). Rabies virus (RV) is a negative-sense single-stranded RNA virus that replicates in the cytoplasm of infected cells. Previous reports have shown that laboratory-adapted and attenuated RV such as Evelyn Rotkitniki Abelseth (ERA) and challenge virus standard (CVS) induce apoptosis *in vivo* and *in vitro* (Jackson and Rossiter, 1997). However, the intracellular pathway by which RVs induce apoptosis is not well understood. In one study, Ubol et al. (1998) showed that the expression of Bax, but not Bcl-2, increased in RV-infected neuroblastoma cells. Furthermore, Bax-deficient mice had less prominent apoptotic cell death than wild-type mice (Jackson, 1999). In addition, caspase 1 (ICE) was upregulated with significant degradation of PARP (Ubol et al.,

1998). In another study (Ubol and Kasisith, 2000), a developmentally down-regulated apoptotic gene, Nedd-2, was found to be reactivated in mice infected with a bat RV or a primary canine RV isolate. Recently, Thoulouze et al. (2003) showed that apoptosis induced by ERA involves the activation of caspase-8 and disappearance of procaspase-9 and procaspase-3. In addition, AIF translocated from the cytoplasm to the nucleus, suggesting that caspase-independent pathway is also involved in RV-induced apoptosis. In the present study, we investigated the apoptotic pathway(s) by which apoptosis is induced by lab-attenuated CVS-B2C in comparison with wild-type SHBRV. It was found that CVS-B2C activated caspase-8 and caspase-3, but not caspase-9, indicating that the induction of apoptosis by CVS-B2C may involve an extrinsic apoptotic pathway. In addition, AIF was upregulated and translocated from the cytosol to the nucleus. Therefore, results suggest that CVS-B2C induces apoptosis through caspase-dependent and caspase-independent pathways.

Materials and methods

Antibodies, cells, viruses and other reagents

BSR cells, a cloned cell line derived from Baby hamster kidney (BHK-21) cells, were cultured at 37ºC and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 1% penicilin. Two RV strains (CVS-B2C and SHBRV) were used in this study. The CVS-B2C is an attenuated virus isolated from CVS-24 virus by passaging in BHK cells (Morimoto et al., 1998) and SHBRV is a wildtype RV isolated from a human patient (Rupprecht et al., 1995). Virus stocks were prepared in one-day-old suckling mice as described

(Morimoto et al., 1996; Schnell et al., 1994; Yan et al., 2001). When moribund, mice were euthanized and brains were removed. Brain homogenates (10%) were made in DMEM and stored at -80^0C. Virus titers were determined in BSR cells by using FITC-conjugated anti-RV antibodies (FujiRab, Malvin, PA) and expressed as fluorescent focus unit (FFU). Antibodies against AIF, Apaf-1 and PARP were obtained from Cell Signaling Technology (Danvers, MA). Anti-β Tubulin antibody was obtained from Upstate Cell Signaling Solutions (Lake Placid, NY).

TUNEL assay and flow cytometry

The TUNEL assay was performed to detect apoptotic cells by using In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). Briefly, cells were infected with CVS-B2C or SHBRV at an MOI of 1 and fixed at different time points with 4% paraformaldehyde. Fixed cells were rinsed with Phosphate Buffer Saline (PBS) and incubated with 50ul of TUNEL reaction mixture for 60 minutes at 37°C in the dark. Cells were rinsed once again and analyzed under fluorescence microscope. The TUNEL-positive cells were counted and analyzed statistically by one-way ANOVA and Student's t-Test.

Apoptosis was also detected by flow cytometry, 2 x 10^6 cells were harvested and fixed in 1% (w/v) paraformaldehyde for 15 min on ice. Cells were pelleted at 3000 rpm and washed with PBS. Ice-cold 70% (v/v) ethanol was added to the cells that were kept on ice for 30 min. Cells were resuspended in wash buffer and centrifuged for 10 min at 3000rpm. Cells were incubated with DNA-labeling solution for 60 min at 37°C. After washing, Alexa Fluor 488 dye-labeled anti-BrdU monoclonal antibody (Molecular Probes, Eugene, OR) was added and cells were

incubated for 30 minutes at room temperature (RT). Fluorescence was detected using CyAn Instrument (DakoCytomation, Ft. Collins, CO) and analyzed using FlowJo software (Tree Star, Inc., Stanford University).

Detection of caspase activities

Total caspase activity was determined using the Homogeneous Caspases Assay, fluorimetric (Roche Applied Science, Indianapolis, IN), which detects activated caspases 2, 3, 6, 7, 8, 9 and 10. The BSR cells cultured in 96-well plates were infected with CVS-B2C or treated with chemical inducers of apoptosis (positive controls). Sham-infected cells were included as negative controls. Each experiment was performed in triplicates. Each sample was incubated for 1 h at 37°C with the substrate solution, and fluorescence intensities were measured at excitation and emission wavelength of 470-500 and 500-560 nm, respectively. Individual caspase activities (caspase-3, -8, and -9) were also measured by using BD ApoAlert caspase fluorescent assay kits (Clontech Laboratories, Palo Alto, CA). In brief, approximately 10^6 cells were grown in 6-well plates and infected with CVS-B2C or SHBRV or sham-infected. Cells were harvested at 1000 rpm for 5 minutes and lysed in 50ul of chilled lysis buffer on ice for 10 minutes. Then cells were centrifuged at maximum speed for 10 min at 4°C and supernatants were collected. The supernatant (50 µl) was added to an equal volume of 2x reaction/dithiothreitol (DTT) buffer supplemented with caspase-3 substrate DEVD-AFC, caspase-8 substrate IETD-AFC, or caspase-9 substrate LEHD-AMC and incubated at 37°C for 2 h. Samples were transferred to a 96-well plate and fluorescence intensities were measured in a Synergy HT Fluoremeter (Bio-tek

Instruments, INC, Winooski, VT) at excitation and emission wavelength of 400 and 505, respectively.

Western blot analysis

For Western blotting, BSR cells were grown to about 80% confluency in 100mm petri dishes and infected with CVS-B2C. At the indicated time post infection, cells were lysed with RIPA buffer containing 1mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, and 1mM pepstatin. Whole cells lysates were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes. The membranes were blocked for 1 hr at room temperature in blocking buffer (1x TBS, 0.1% Tween-20 with 5% nonfat dry milk) and then incubated with antibodies against AIF, PARP or Apaf-1 overnight at 4°C according to the manufacturer's instructions. The membranes were washed three times with TBST and incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (Amersham Biosciences, Piscataway, NJ), depending on the primary antibody used. After washing, the membranes were incubated with the ECL Detection System (Amersham Biosciences, Piscataway, NJ).

AIF translocation

The BSR cells were grown in 24-well tissue culture plates containing sterile coverslips and infected with CVS-B2C at an MOI of 1. Immunocytochemistry protocol was carried out according to the manufacturer's instructions (Cell Signaling, Danvers, MA). Cells on the coverslips were

fixed at 2, 4, 6, 8, 12, 24 and 48 h with 4% paraformaldehyde for 10 min, washed with TBS, and permeabilized with 0.2% Triton X-100 for 5 min at RT. Then cells were washed for 5 min with TBS and blocked with blocking buffer (10% horse serum, 1%BSA, 0.02% NaN3, 1X PBS) for 45 min. Coverslips were incubated with AIF primary antibody (Cell Signaling, Danvers, MA) overnight at 4°C followed by Texas Red-labeled anti-rabbit secondary antibody (Vectorslab, Burlingame, CA) for 45 min. Finally, nuclei were stained with DAPI for 15 min and coverslips were mounted on slides using anti-fading mounting medium. AIF translocation was analyzed under a Zeiss inverted fluorescent microscope.

Results

More apoptotic cells were observed in cells infected with attenuated than in cells infected with pathogenic virus

It has been previously reported that RV strain CVS induces only limited apoptosis in the human neuroblastoma SK-N-SH cell line and in lymphoblastoid Jurkat T cells *in vitro* (Baloul and Lafon, 2003; Prehaud et al., 2003; Thoulouze et al., 1997; Thoulouze et al., 2003a). In contrast ERA, an attenuated strain of RV is a strong inducer of apoptosis (Thoulouze et al., 1997). To investigate the ability of attenuated CVS-B2C and wild-type SHBRV to induce apoptosis *in vitro*, BSR cells were infected with each virus and the number of cells undergoing apoptosis was detected using TUNEL assay. As shown in Fig. 4.1A, many apoptotic cells were detected in cells infected with CVS-B2C, but only a few in cells infected with SHBRV. On the other hand, 100% of the BSR cells were infected with each virus. TUNEL positive cells were

counted from four different fields and significantly more apoptotic cells ($p<0.001$) were observed in cells infected with CVS-B2C than in cells infected with SHBRV (Fig. 4.1B). However, the number of apoptotic cells infected with SHBRV was not significantly different from that in sham-infected cells. To further confirm these results, apoptotic cells were detected by using an anti-BrdU monoclonal antibody and analyzed by flow cytometry. At day 3 p.i., 24.7% of the cells infected with CVS-B2C underwent apoptosis, whereas SHBRV induced apoptosis in only 1.63% cells (Fig. 4.1C).

CVS-B2C activates caspase-3 and caspase-8

To determine the pathway(s) by which CVS-B2C induces apoptosis, we initially evaluated the overall activity of caspases by fluorometric assays. The results are expressed in relative fluorescence units (RFU) and the percentages of the increase in caspase activity relative to the negative control are indicated. As shown in Fig. 4.2A, infection with CVS-B2C caused a 23.9% increase in caspase activities over the negative controls. To further define whether CVS-B2C induces apoptosis via the intrinsic or the extrinsic pathway, the activities of specific caspases such as caspase-3, caspase-8 and caspase-9 were measured. In addition, the level of Apaf-1 expression was detected. As shown in Fig. 4.2B and 4.2C, infection with CVS-B2C resulted in a 13.84% and a 21% increase in caspase-3 and caspase-8 activities, respectively, when compared to sham-infected cells, suggesting that these two caspases are involved in CVS-B2C-induced apoptosis. On the other hand, caspase-9 activity increased only 4.4% in CVS-B2C-

infected when compared with sham-infected cells. In addition, Apaf-1 was not upregulated (Fig. 4.3) indicating that caspase-9 has, if any, minimal role in CVS-B2C-induced apoptosis.

PARP is cleaved in CVS-B2C infected cells

To further demonstrate the role of caspases in CVS-B2C-induced apoptosis, Western blotting was performed to investigate if PARP is cleaved. Poly(ADP-ribose) polymerase-1 is a well-known substrate for caspase-3 in the apoptotic events. Therefore, we used an antibody that detects endogenous levels of full-length PARP (116kDa), as well as the large fragment (89 kDa) of PARP resulting from caspase cleavage. As shown in Fig. 4.3, the 89 kDa cleaved fragment of PARP was detected in CVS-B2C-infected cells and in cells treated with etoposide, but not in sham-infected cells. This result confirms the importance of caspase-3 activation in CVS-B2C-induced apoptosis.

CVS-B2C upregulates expression of AIF and its nuclear translocation

To determine if a caspase-independent pathway was also involved in RV infection, endogenous levels of AIF were assessed by Western blotting. As shown in Fig. 4.3, CVS-B2C upregulated AIF expression, suggesting that CVS-B2C also activates a caspase-independent mechanism to induce apoptosis. To confirm the role of AIF in RV-induced apoptosis, cells infected with CVS-B2C were fixed at 2, 4, 8, 12, and 24 hr p.i. with 4% paraformaldehyde and subjected to immunocytochemistry using anti-AIF antibody. As shown in Fig. 4.4A, AIF was translocated from the cytoplasm to the nucleus. The percentage of cells with nuclear

translocation was quantified and statistically analyzed by Student t-test. There were only a few cells with translocated AIF at 2, 4, 8 and 12 hr p.i. (data not shown). As shown in Fig. 4.4B, significantly more cells with nuclear translocation were observed at 24 hr after infection. Thus, these data suggest AIF is involved in CVS-B2C-induced apoptosis.

Discussion

Virus-induced apoptosis has been well recognized throughout the years. Viruses such as Newcastle disease virus (NDV), dengue virus (Moriishi et al., 2002; Despres et al., 1998), HIV (Gougeon et al., 1991), influenza (Takizawa et al., 1993), and vesicular stomatitis virus (Koyama, 1995) are known stimulators of apoptosis. RV has also been reported to induce apoptosis, suggesting that apoptosis may play an important role in rabies pathogenesis (Jackson and Rossiter, 1997). However, subsequent studies have demonstrated that induction of apoptosis correlates inversely with viral pathogenicity, indicating that apoptosis plays a protective rather than pathogenic role in RV infections (Morimoto et al., 1999). We have consistently shown that attenuated CVS-B2C, but not wild-type SHBRV, induces apoptosis in mice (Sarmento et al., 2005; Yan et al., 2001). In the present study, we further demonstrate that CVS-B2C, but not SHBRV, induces apoptosis in BSR cells. Induction of apoptosis by CVS-B2C in BSR cells is in agreement with previous studies in primary neurons (Morimoto et al., 1999)

There are many pathways by which apoptosis is induced (Roulston et al., 1999). Caspases have a critical role in apoptosis. During apoptotic events, caspases cleave specific substrates activating downstream elements, which eventually culminate in cell death (Kumar and Vaux,

2002). To determine the pathway(s) by which CVS-B2C induces apoptosis, activation of caspases was measured. Caspase-8 and caspase-3 were activated in cells infected with CVS-B2C. Furthermore, our data also show that the functional 116Kda PARP was cleaved to inactive form of 89 Kda, further suggesting that caspase-dependent pathway is activated in CVS-B2C-induced apoptosis. During apoptotic cell death, precocious and transient stimulation of PARP causes poly(ADP-ribose) accumulation in early apoptotic cells. However, when PARP is cleaved by caspases, the poly(ADP-ribosylation) process is inactivated (Soldani and Scovassi, 2002).

Activation of caspase-8 and caspase-3 was, however, in moderate levels in CVS-B2C-infected cells. It has been reported that activation of caspase-8 in low levels can initiate a cross-talk between the extrinsic and intrinsic pathways via cleavage of Bid and translocation of the truncated Bid to mitochondria (Gaddy and Lyles, 2005). Thoulouze et al. (2003) showed that ERA strain indeed activates both caspase-8 and caspase-9, suggesting that both extrinsic and intrinsic pathways are involved. However, in our study, both caspase-8 and caspase-3, but not caspase-9, are activated. Furthermore, Apaf-1 was not upregulated. All these results indicate that apoptosis induced by CVS-B2C is via the extrinsic, but not the intrinsic, apoptotic pathway. The discrepancy between our study and the study by Thoulouze et al. (2003) could be due to the virus strains used. In our study, CVS-B2C was used while in the study by Thoulouze et al. (2003), ERA was used. Infection with different RV strains may trigger different apoptotic pathways.

The activation of caspase-3 and caspase-8 was moderate, yet the number of apoptotic cells was high in CVS-B2C-infected cells, which suggests that a caspase-independent pathway may also be involved. To this end, we determined the level of AIF expression as well as its

translocation in cells infected with CVS-B2C. Apoptosis-inducing factor is a novel flavoprotein that helps mediate caspase-independent apoptotic cell death (Susin et al., 1999b). Apoptosis-inducing factor translocates from mitochondria to the nuclei where it induces caspase-independent DNA fragmentation (Susin et al., 1999b). Indeed, AIF was upregulated in CVS-B2C infected cells at levels similar to the positive control etoposide. Furthermore, AIF translocated to the nucleus at 24 hr p.i., strongly suggesting a role for AIF in CVS-B2C-induced apoptosis. These results are similar to the results reported by Thoulouze et al. (2003).

The mechanism by which AIF is released from the mitochondria is controversial. One model suggests that pro-apoptotic proteins from the Bcl-2 superfamily permeabilize the mitochondria membrane, allowing the release of mitochondria proteins such as cytochrome-c Smac/Diablo and possibly AIF. Another model identifies the mitochondrial response to the pro-apoptotic Bcl-2 members as a selective process, where release of different mitochondria proteins is dependent on which pro-apoptotic Bcl-2 member is activated (Arnoult et al., 2003). In addition, Arnoult et al. (2003) proposes that the mitochondria release of AIF requires caspase-activated downstream of Bax/Bak-mediated permeabilization (Arnoult et al., 2003). In our experiment, AIF translocation and activation of caspases were detected at the same time, suggesting CVS-B2C, like ERA (Thoulouze et al., 2003a), induces apoptosis by both the caspase-dependent and caspase-independent pathways. Furthermore, Ubol et al. (1998) showed that Bax was upregulated in neuroblastoma cells infected with RV. Thus, it is possible that the release of AIF in RV-infected cells is mediated by Bax-mediated permeabilization.

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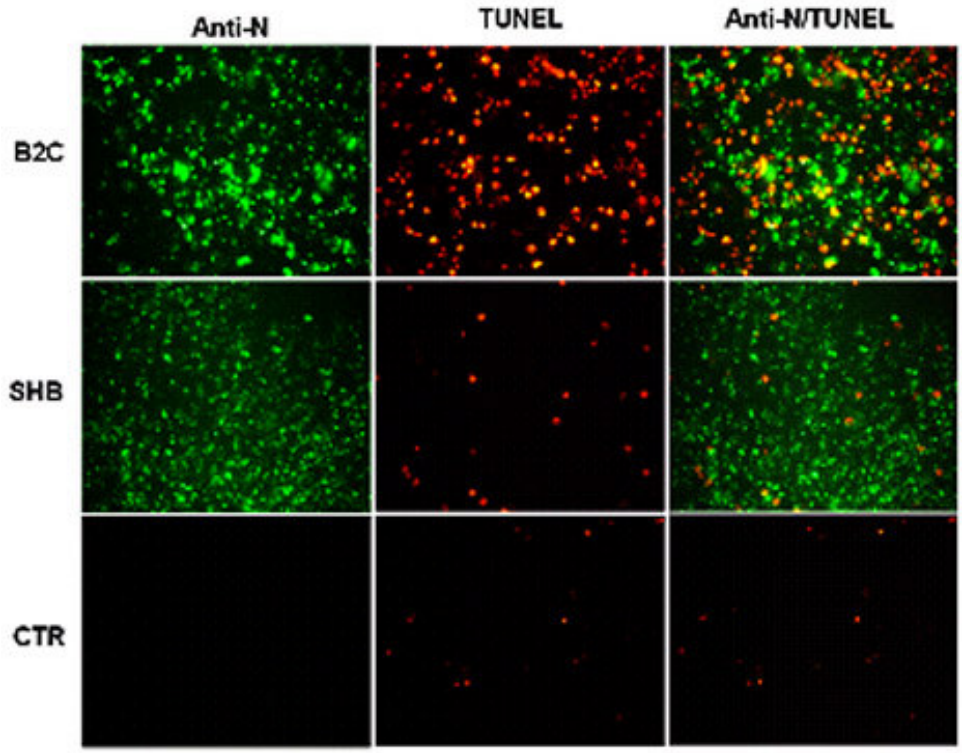
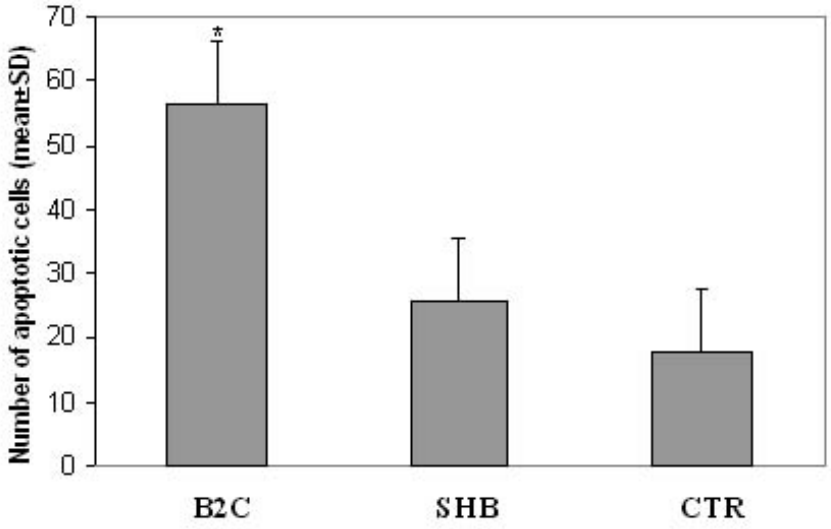
A


Anti-N
TUNEL
Anti-N/TUNEL
B2C
SHB
CTR

B


Number of apoptotic cells (mean±SD)
70
60
50
40
30
20
10
0
*
B2C
SHB
CTR

C

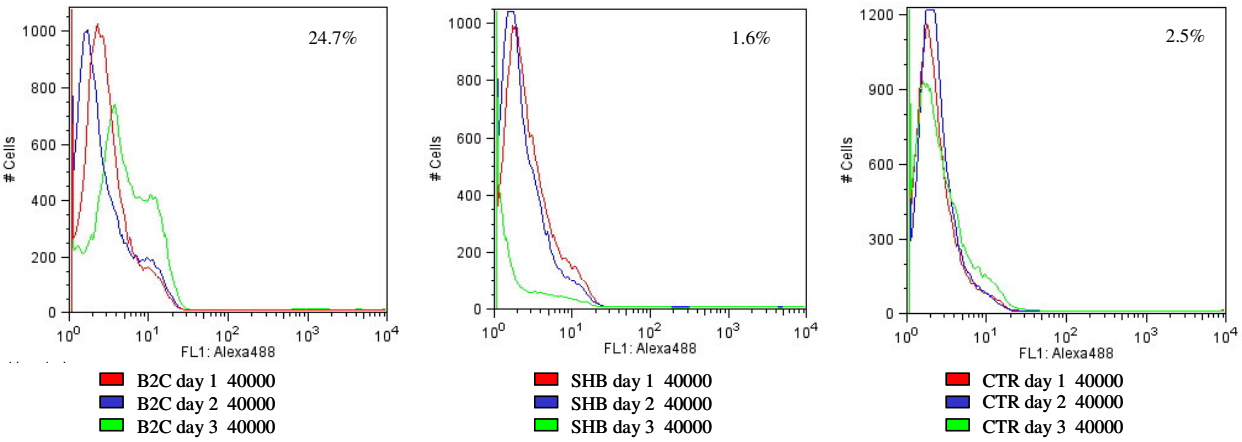


Fig. 4.1: Attenuated CVS-B2C, but not wild-type SHBRV, induces apoptosis in BSR cells. BSR cells were infected with CVS-B2C or SHBRV or sham-infected. At day 3 after infection, cells were fixed for detection of RV antigens with anti-RV N antibody and apoptosis using the TUNEL technique **(A).** The number of apoptotic cells were counted from 4 fields and expressed as mean ± SD **(B)**. The percentage of cells undergoing apoptosis was examined by flow cytometry with anti-APO-BrdU antibody. Fluorescence was analyzed in 40,000 gated cells **(C)**.

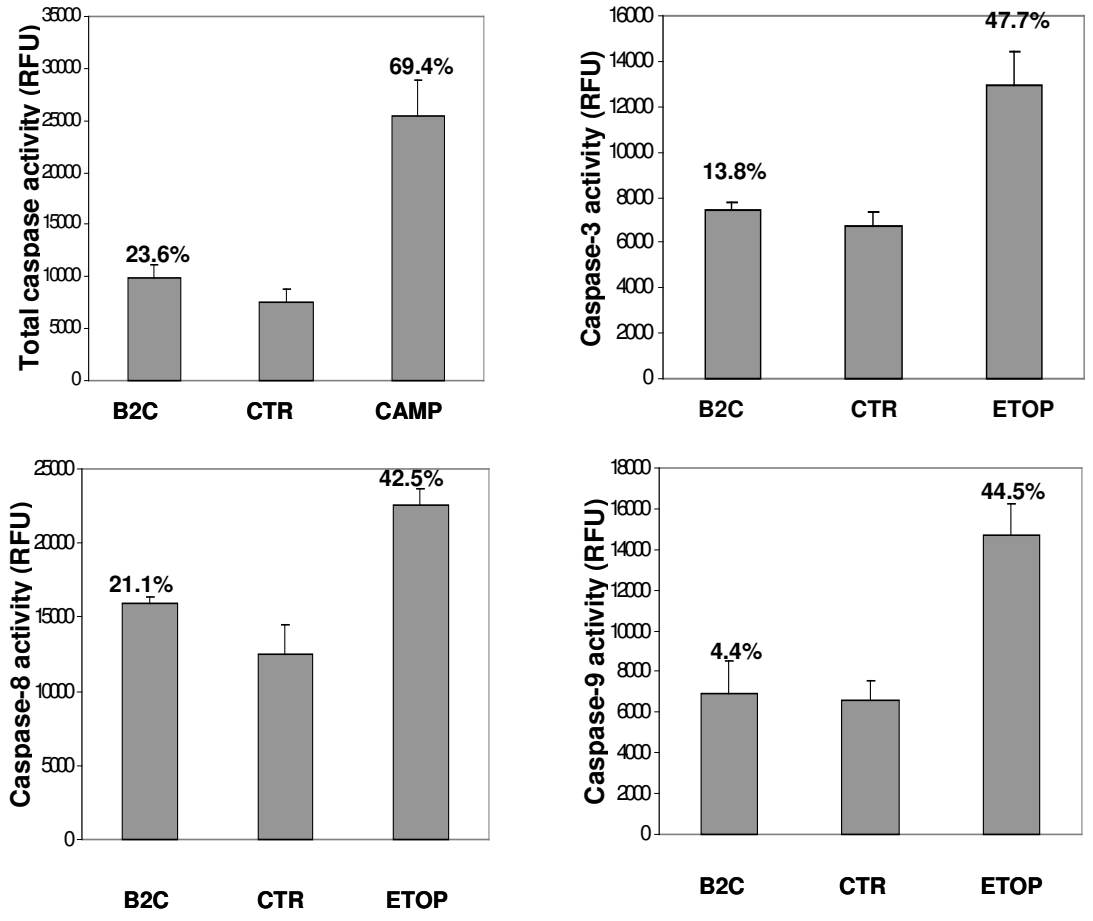


Fig. 4.2: CVS-B2C infection activated caspase-3 and caspase-8, but not caspase-9. BSR cells were infected with CVS-B2C or mock-infected. Alternatively, cells were treated with chemical inducers of apoptosis. CTR=mock-infected; Camp=Camptothecin; ETOP=Etoposide. Fluorometric detection of total caspase activity (**A**), caspase-3 (**B**), caspase-8 (**C**) and caspase-9 (**D**) was performed. Results are expressed in relative fluorescence units (RFU) and the percentages of the increase in caspase activity relative to the negative control are indicated.

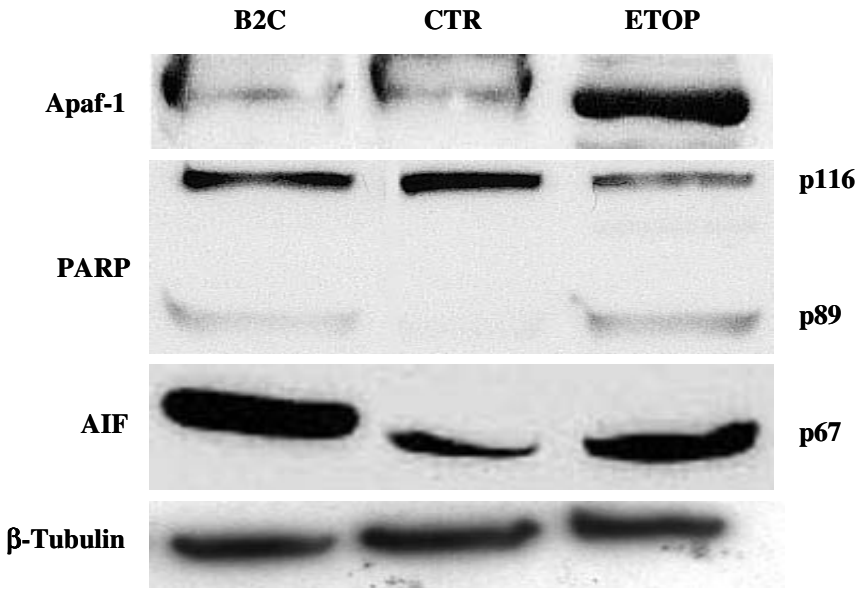
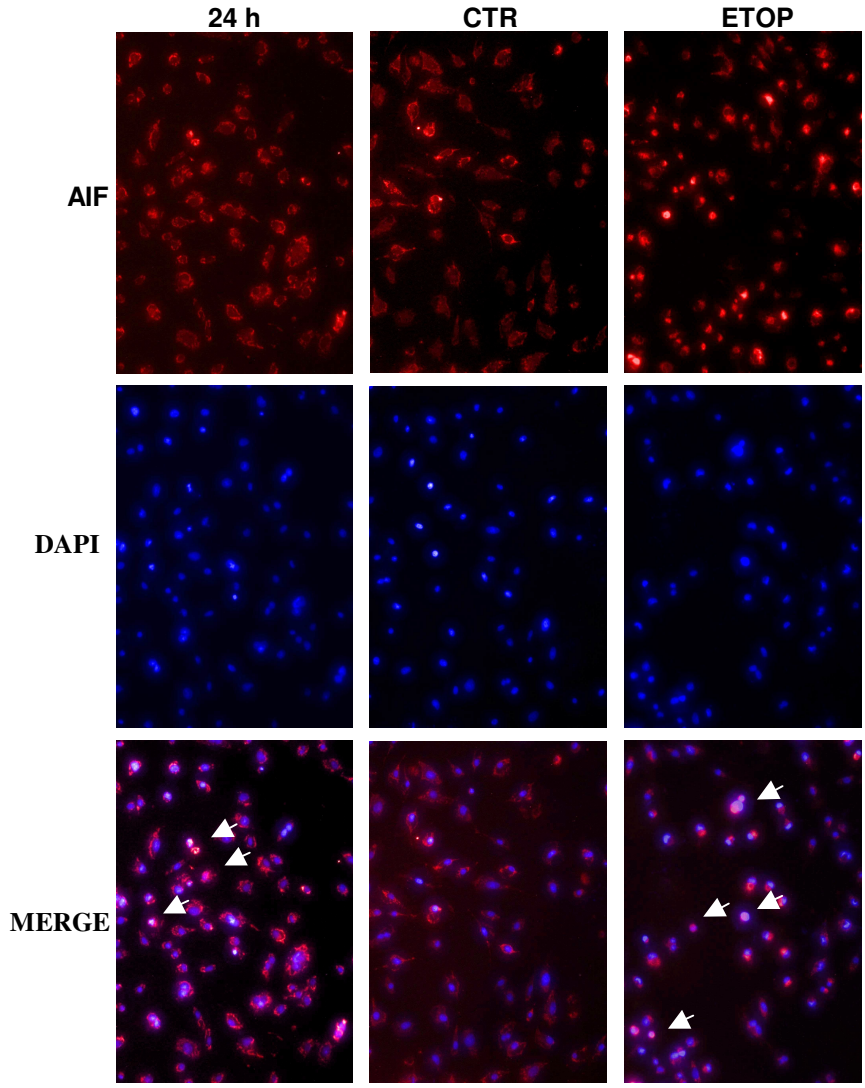


Fig. 4.3: Western blot analysis of apoptotic proteins in CVS-B2C infected cells. BSR cells were sham-infected or infected with CVS-B2C. Alternatively, BSR cells were treated with Etoposide for 12h at day 3 after infection. Proteins were extracted from these preparations and subjected to Western blotting with antibodies to Apaf-1, PARP and AIF. Tubulin was used for loading control.

A



B

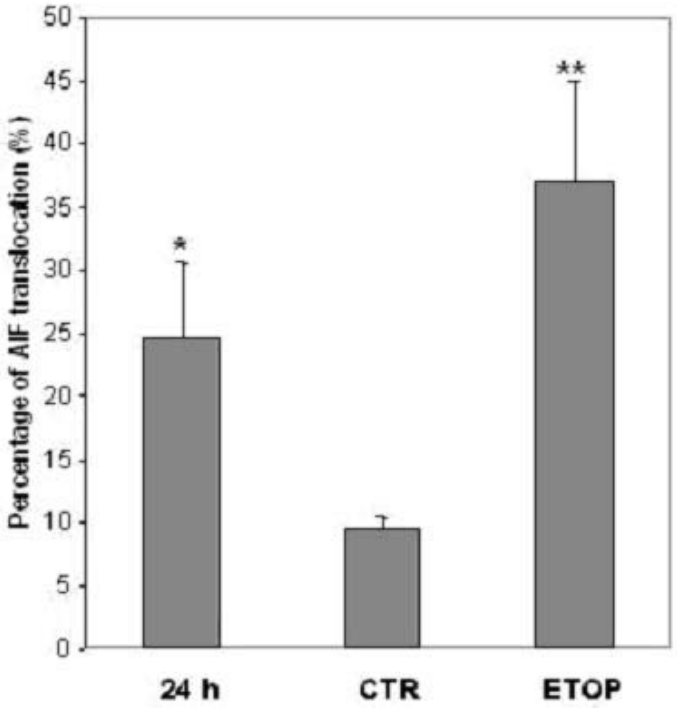


Figure 4.4: Nuclear translocation of AIF in BSR cells. BSR cells were infected with CVS-B2C and fixed at varied time points. AIF was detected by using anti-AIF polyclonal antibody and anti-rabbit secondary antibody conjugated with Texas-Red (red). DAPI was used for nuclear staining (blue). Cells were examined under an inverted fluorescent microscope (**A**). Arrows indicate cell with AIF nuclear translocation. The percentage of cells with nuclear translocation of AIF was quantified **(B).** *Significance at $p<0.01$, ** Significance at $p<0.001$.

CHAPTER 5

CONCLUSIONS AND DISCUSSION

Previous reports have shown that laboratory-adapted RV, such as CVS-B2C and ERA, induce apoptosis (Jackson and Rossiter, 1997; Morimoto et al., 1999; Thoulouze et al., 1997), whereas highly pathogenic strains, such as CVS-N2c and the wild-type (wt) SHBRV do not induce apoptosis (Morimoto et al., 1999; Yan et al., 2001). However, most of these studies were conducted *in vitro*, and *in vivo* studies were limited to few strains. Therefore, detailed investigations were necessary concerning different strains of viruses *in vivo* and *in vitro* to better understand how some rabies virus strains induce apoptosis while other strains prevent apoptosis, and if the induction of apoptosis plays a role in rabies pathogenesis. Using seven different strains of RVs with different pathogenicity to infect mice through IC route, we found that the induction of apoptosis is inversely correlated with the pathogenicity of the virus. Overall, attenuated viruses induced more apoptosis, whereas pathogenic viruses induced very little apoptosis. Primary neurons were also infected with these viruses, confirming the above findings. These data suggest that apoptosis may play a protective, rather than a pathogenic role in rabies virus infections.

Several reports suggest that the induction of apoptosis correlates with the level of expression of the G protein. In addition, Morimoto et al. (1999) suggest that pathogenic rabies virus strains prevent apoptosis by down-regulating the expression of the G protein, which enables the virus to spread effectively through synaptic junctions. Using a panel of recombinant rabies

viruses, we have found in the present study that the induction of apoptosis is largely determined by the level of RV G expression. Overall, detection of viral antigens demonstrated that G was expressed abundantly in attenuated virus-infected mice, whereas the G expression was almost undetectable in animals infected with pathogenic strains. Furthermore, the level of G expression was not due to the rate of viral replication because the level of N expression was similar in animals infected with each of these viruses.

It is clear from our studies that attenuated viruses induce extensive apoptosis while wt virus induces almost no apoptosis. Yet, a few viral particles of wt virus can kill infected animals while 1000 to 10000 more viral particles are required by attenuated virus to kill infected animals. Therefore, we hypothesized that the attenuated virus, by inducing apoptosis, prevents virus spread in the CNS. Thus, we infected mice with attenuated virus CVS-B2C or wt virus SHBRV through intramuscular (i.m.) route. We found that low doses of CVS-B2C virus induced apoptosis in the spinal cord and failed to spread to the brain or produce neurological disease. On the other hand, apoptosis was not observed in the spinal cord of mice infected with the same dose of SHBRV and the virus spread to various parts of the brain and induced fatal neurologic disease. These results suggest that attenuated RV limits viral spread in the CNS by induction of apoptosis. In addition to the induction of apoptosis, we observed that when mice were infected with high doses of attenuated viruses, there were severe histopathological changes, such as infiltration of inflammatory cells, necrosis and gliosis. Recently, Wang et al. (2005) showed that CVS-B2C activates the host innate immune responses, while SHBRV evades the host immune response. In this study, almost all the genes involved in the activation of the IFN-α/ß pathway and many of the inflammatory chemokines and cytokines were up-regulated in animals infected with CVS-B2C

by either the intracerebral or the intramuscular routes. However, many of these genes were not up-regulated in animals infected with pathogenic SHBRV (Wang et al., 2005). Therefore, we propose that apoptosis and inflammation play a role in preventing RV from spreading within the CNS.

In this study, we further demonstrated that the attenuated virus CVS-B2C, but not wt virus SHBRV, induces apoptosis in BSR cells. Induction of apoptosis by CVS-B2C in BSR cells is in agreement with previous studies in primary neurons (Morimoto et al., 1999). To date, several apoptotic pathways have been described in virus-infected cells (Azuma et al., 2006; Liu et al., 2006; Roumier et al., 2002). However, very little is known about the intracellular signals that regulate apoptosis in rabies virus-induced apoptosis. In this study, the activation of caspase-3, caspase-8 and caspase-9 was analyzed in CVS-B2C-infected BSR cells by Western blotting. Our data indicate that B2C-induced apoptosis triggers the activation of caspase-3 and caspase-8. In addition, Poly(ADP-ribose) polymerase (PARP), a molecule involved in DNA repair, was cleaved, confirming the role of caspases in CVS-B2C-induced apoptosis. In our study, caspase-9 was not activated. Furthermore, the expression of the adaptor protein, Apaf-1, was not up regulated. These data suggest that CVS-B2C triggers a caspase-dependent pathway, most likely through the extrinsic, but not the intrinsic pathway.

The activation of caspase-3 and caspase-8 were relatively low, although the number of apoptotic cells observed when cells were infected with CVS-B2C was high. This suggests that alternative mechanisms of programmed cell death that do not involve caspases are activated. The involvement of multiple apoptotic pathways by virus infection has been suggested (Gadaleta et

al., 2005; Genini et al., 2001). To investigate the existence of an alternative caspase-independent pathway, we determined the level of expression of AIF and its translocation from the cytoplasm to the nucleus. In this study, AIF was upregulated and translocated from the cytoplasm to nucleus at 24h p.i., suggesting a role for AIF in CVS-B2C-induced apoptosis. These results suggest that the induction of apoptosis by attenuated CVS-B2C is via a caspase-dependent and caspase-independent pathway.

Final remarks

There is a constant battle between viruses and host during an infection. The objectives of all viruses are to infect target cells, replicate large numbers of progeny virions, and spread these progeny to initiate new rounds of infection. On the other hand, organisms trigger both systemic and cell-based defenses to limit virus infection, including immune and inflammatory processes and the execution or suicide of infected cells. In the context of an infectious disease, the ultimate goal is to develop safer, more effective and more rapidly acting vaccines. Some of these features can be achieved by stimulating the immune response by introducing molecules that are immunogenic by themselves or that are able to potentiate immunogenicity. In the study described above, we have demonstrated that the level of RV G expression correlates with the induction of apoptosis and that apoptosis provides protection of the host from viral spread. In addition, caspase-dependent and caspase-independent pathways mediated the induction of apoptosis. Therefore, the knowledge we bring in this study can be useful for development of a vaccine, for example, by expressing pro-apoptotic proteins.

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