Journal of NeuroVirology, 11: 571–581, 2005

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280500385310

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Glycoprotein-mediated induction of apoptosis limits the spread of attenuated rabies viruses in the central nervous system of mice

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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. *Journal of NeuroVirology* (2005) **11,** 571–581.

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

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This work is supported partially by Public Health Service grant AI-051560 from the National Institute of Allergy and Infectious Diseases (ZFF) and Canadian Institutes of Health Research grant MOP-64376 (ACJ). The authors express their gratitude to Dr. Charles E. Rupprecht at the CDC for supplying anti-N monoclonal antibody 802-2.

Received 23 June 2005; revised 5 September 2005; accepted 25 September 2005.

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 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

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*	*$ICLD_{50}$*	*Pathogenic index (IC)*	*No. of apoptotic cells (±SD)**	*Pathology score*	*Viral N antigen¶*	*Viral G antigen¶*
SHBRV	$10^{5.23}$	$10^{-4.5}$	0.19	5.25 ± 4.25	+	+ + +	+
N2C	$10^{5.11}$	$10^{-4.7}$	0.38	14.75 ± 3.81	++	+ + +	++
RSHBRV	$10^{6.72}$	$10^{-4.9}$	0.015	17.75 ± 4.75†	++	+ + +	+
RN2C	$10^{8.87}$	10^{-6}	0.0015	12.00 ± 3.44	++	+ + ++	++
B2C	$10^{7.94}$	10^{-5}	0.0011	44.25 ± 16.29†	+ + +	+ + ++	+ + ++
SN-10	$10^{10.18}$	10^{-7}	0.00066	20.50 ± 2.05†	++	+ + ++	++
RB2C	$10^{10.71}$	$10^{-5.8}$	0.000012	31.25 ± 23.75†	+ + +	+ + ++	+ + +
Control	−	−	−	2.75 ± 2.00	−	−	−

*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.
†Significantly different from that of controls ($P < .05$)
¶Pathological lesions were scored according to the severity. + + +, 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; −, 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. + + ++, strong immunostaining with almost all the neurons affected; + + +, strong immunostaining with more than 50% neurons affected, or slightly weak staining with almost all the neurons affected; ++, weak staining with about 25% neurons affected; +, weak staining with about 10% neurons affected; −, no detection of viral antigen.

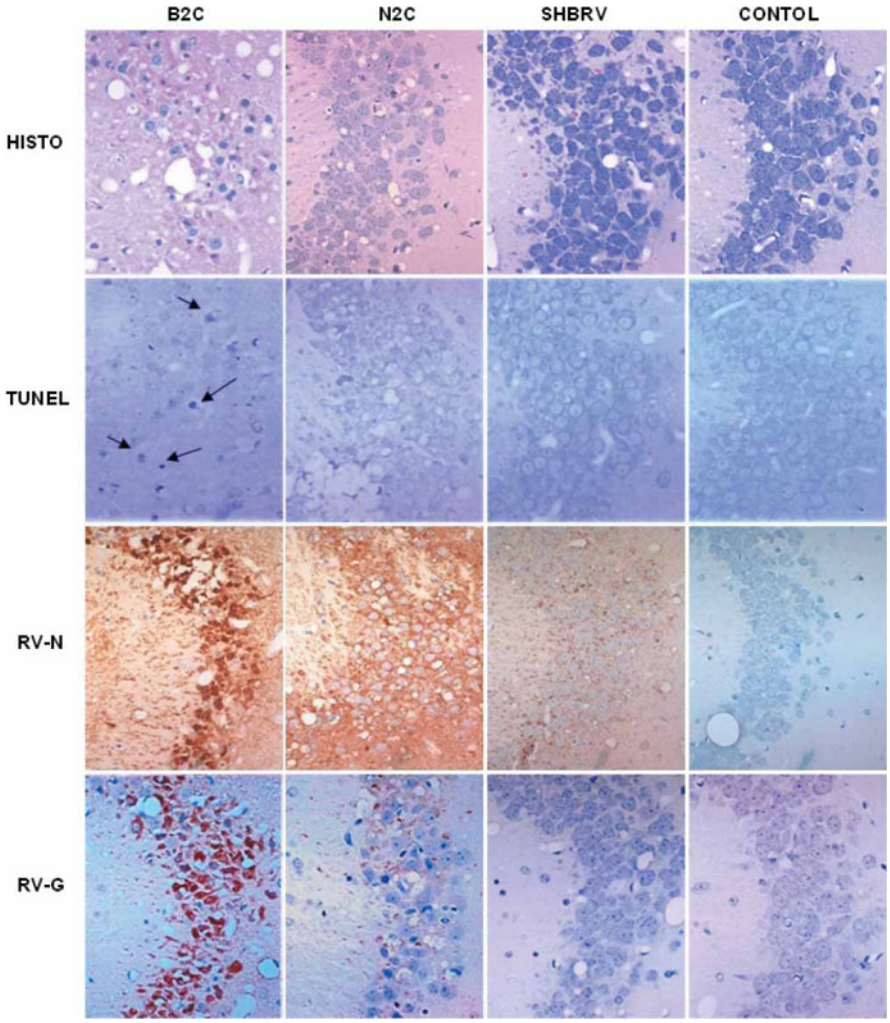

Figure 1 Detection of apoptosis, pathological changes, and viral antigens in mice infected with different RVs. Mice were infected with 10 $ICLD_{50}$ of each virus and brains were harvested for histopathology (HISTO) and detection of apoptosis using TUNEL assay (TUNEL; arrows indicate TUNEL-positive cells). Viral antigens (RV-N and RV-G) were detected using anti-G and anti-N antibodies as described in the text. (magnification 40×).

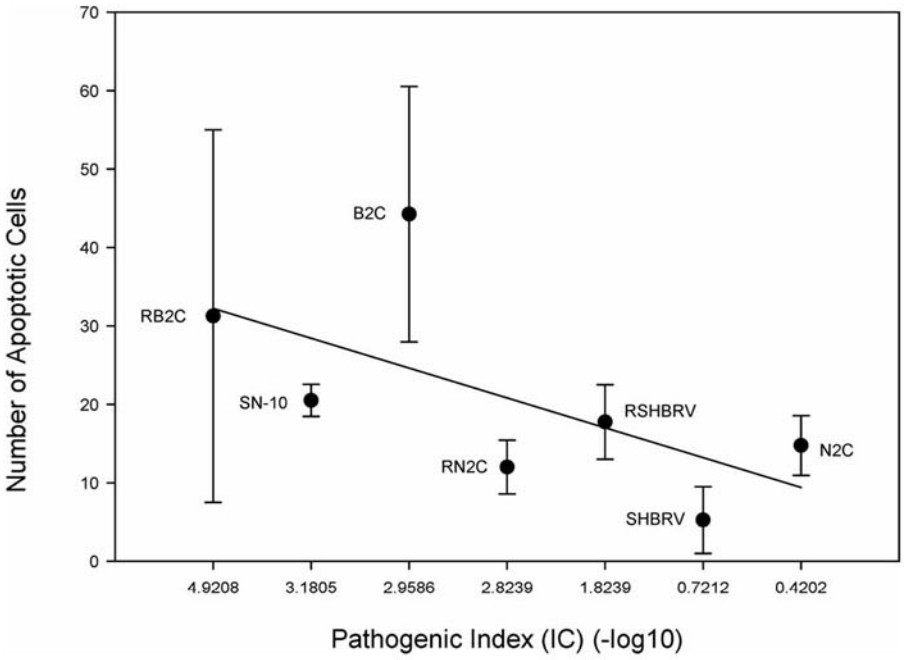

Figure 2 Inverse correlation between the induction of apoptosis and pathogenicity. Pathogenic index were plotted against the numbers of apoptotic cells observed in mice infected with different RVs using SigmaPlot. Vertical bars indicate the standard deviation.

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 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 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. 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 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

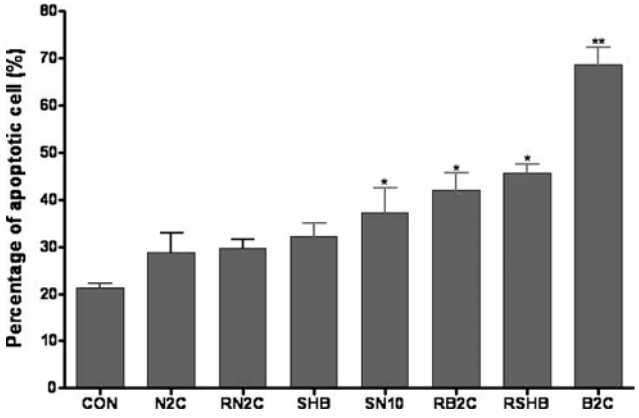

Figure 3 Induction of apoptosis by RVs in primary neurons. Primary neurons were infected with each of the viruses and apoptosis was detected using the TUNEL assay. The number of apoptotic neurons was assayed and analyzed using one way ANOVA at the $P < .05$ level.

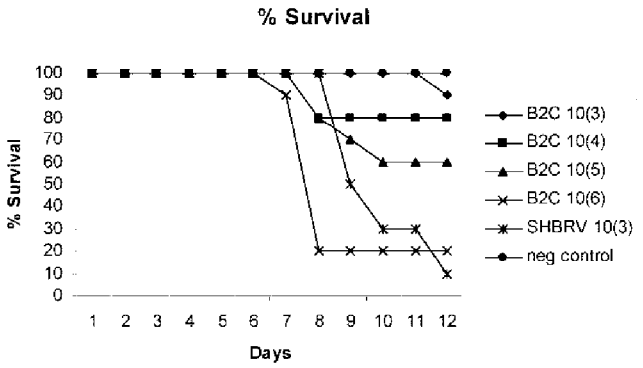

Figure 4 Survival rate in mice infected with different RVs at different doses. Mice (10 in each group) were infected with different doses of SHBRV or B2C and the development of rabies were recorded daily for 20 days. No animals died after 12 days p.i.

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*		
Virus	*Dose*	*5 Days p.i.*	*7 Days p.i.*	*9 Days p.i.*	*5 Days p.i.*	*7 Days p.i.*	*9 Days p.i.*
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		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.

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 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

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

		Brain region																	
		Spinal cord Days p.i.			*Medulla Days p.i.*			*Cerebellum Days p.i.*			*Thalamus/ hypothalamus Days p.i.*			*Hippocampus Days p.i.*			*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

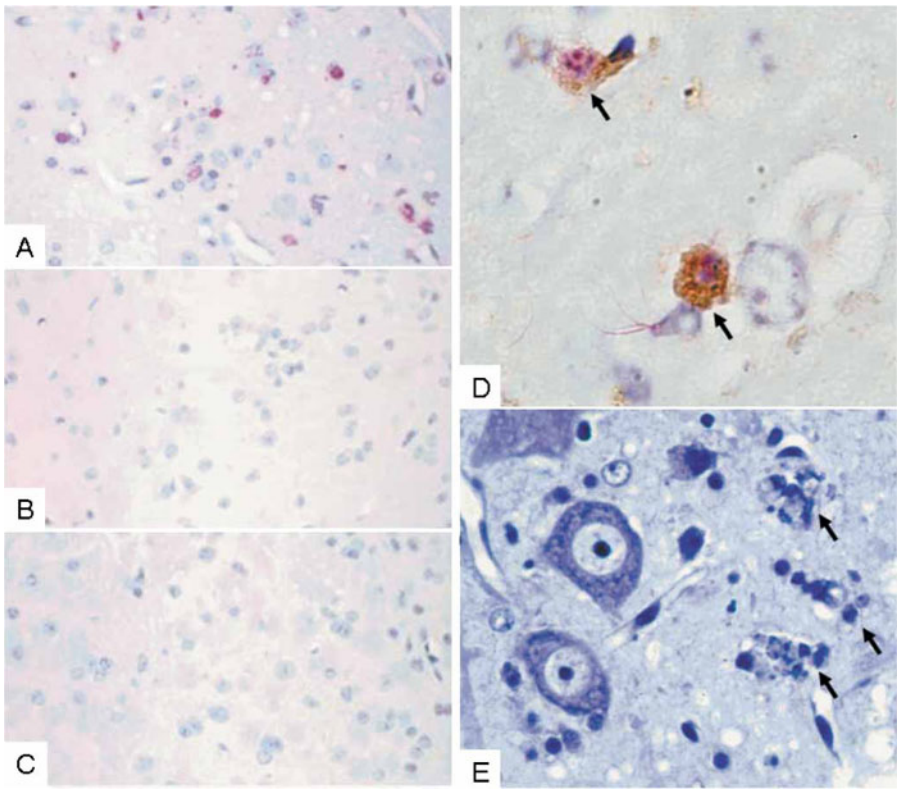

Figure 5 Induction of apoptosis in the spinal cords. Spinal cord tissues from mice infected with B2C (**A**) or SHBRV (**B**) by the IM route at day 7 p.i. were fixed for detection of apoptosis (*red*) using TUNEL assay (magnification 40×). Spinal cord from sham-infected mice was included as control (**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 100×). Ventral horn of a spinal cord from B2C-infected mouse stained with cresyl violet showing condensations of nuclear chromatin and neurophagia (*arrows*) (E; Magnification 100×).

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. (Table 2, Figure 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 5B). No apoptosis was detected in sham-infected mice (Figure 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 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 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 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. 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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