Attenuated Rabies Virus Activates, while Pathogenic Rabies Virus Evades, the Host Innate Immune Responses in the Central Nervous System

Zhi W. Wang,¹ Luciana Sarmento,¹ Yuhuan Wang,² Xia-qing Li,¹ Vikas Dhingra,¹
Tesfai Tseggai,¹ Baoming Jiang,² and Zhen F. Fu^{1,3*}

Departments of Pathology¹ and Infectious Diseases,³ College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602, and Centers for Disease Control and Prevention, Atlanta, Georgia 30333²

Received 25 April 2005/Accepted 1 July 2005

Rabies virus (RV) induces encephalomyelitis in humans and animals. However, the pathogenic mechanism of rabies is not fully understood. To investigate the host responses to RV infection, we examined and compared the pathology, particularly the inflammatory responses, and the gene expression profiles in the brains of mice infected with wild-type (wt) virus silver-haired bat RV (SHBRV) or laboratory-adapted virus B2C, using a mouse genomic array (Affymetrix). Extensive inflammatory responses were observed in animals infected with the attenuated RV, but little or no inflammatory responses were found in mice infected with wt RV. Furthermore, attenuated RV induced the expression of the genes involved in the innate immune and antiviral responses, especially those related to the alpha/beta interferon (IFN- α/β) signaling pathways and inflammatory chemokines. For the IFN- α/β signaling pathways, many of the interferon regulatory genes, such as the signal transduction activation transducers and interferon regulatory factors, as well as the effector genes, for example, 2'-5'-oligoadenylate synthetase and myxovirus proteins, are highly induced in mice infected with attenuated RV. However, many of these genes were not up-regulated in mice infected with wt SHBRV. The data obtained by microarray analysis were confirmed by real-time PCR. Together, these data suggest that attenuated RV activates, while pathogenic RV evades, the host innate immune and antiviral responses.

Rabies virus (RV) is a nonsegmented negative-stranded RNA virus of the *Rhabdoviridae* family and induces a fatal neurological disease in humans and animals (15). Although significant advances have been made in rabies prevention and control, the disease remains a major threat to public health and continues to cause numerous human deaths around the world. The dog remains the most important reservoir in Asia, Africa, and Latin America, where most human rabies cases occur (19). In the United States, dog rabies has been largely brought under control through pet vaccination programs, and there have been only a few incidents where large carnivores have transmitted rabies directly to humans (11, 26). Most of the human cases in the past decade have been associated with RV found in bats, particularly silver-haired bats (11, 18, 39, 47). Furthermore, most of the cases occurred without a history of exposure (11), suggesting that the silver-haired bat RV (SHBRV) is highly pathogenic and neuroinvasive (18, 47).

RV invades the nervous system by binding to neural receptors, such as acetylcholine receptor (31), neural cell adhesion molecule (52), or nerve growth factor receptor (NTR75) (53). Then, RV is transported to the central nervous system (CNS) by retrograde transportation, possibly by binding to cytoplasmic dynein (29, 46). Despite extensive investigation in the past 100 years, the pathogenic mechanisms by which street (wild-type [wt]) RV infection results in neurological diseases and death in humans are not well understood. This is because there

is very little neuronal pathology or damage in the CNS of rabies patients on which to base relevant mechanisms (40). Inflammatory reactions are mild, with relatively little neuronal destruction (34, 40). Laboratory-attenuated RV, on the other hand, induces extensive inflammation and neuronal degeneration in experimental animals (34, 54). However, it is not known how the attenuated and pathogenic RVs induce different host responses.

In the present study, we used an oligonucleotide microarray (Affymetrix mouse expression set MOE430A) and real-time PCR to identify candidate genes that are differentially expressed in the CNS of mice infected with the pathogenic SHBRV or the attenuated B2C. It was found that the attenuated RV is a potent activator of the host innate immune system, particularly the alpha/beta interferon (IFN- α/β) signaling pathway and inflammatory reaction, whereas the pathogenic SHBRV is a poor inducer of the innate immune responses. Thus, evasion of the innate immune responses may be one of the mechanisms by which wt SHBRV contributes to its pathogenicity and neuroinvasiveness.

MATERIALS AND METHODS

Animals, viruses, and antibodies. Female 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. Two RV strains were selected for this study. One is SHBRV, a wt RV isolated from a human patient (39), and the other is CVS-B2C, a laboratory-adapted, attenuated virus isolated from CVS-24 virus by passage in BHK cells (36). Virus stocks were prepared as described previously (54). Briefly, 1-day-old suckling mice were infected with 10 μ l of viral samples by the intracerebral (i.c.) route. When moribund, mice were euthanized and brains were removed. A 10% (wt/vol) suspension was prepared by homog-

* Corresponding author. Mailing address: Department of Pathology, College of Veterinary Medicine, University of Georgia, 501 D.W. Brooks Drive, Athens, GA 30602. Phone: (706) 542-7021. Fax: (706) 542-5828. E-mail: zhenfu@vet.uga.edu.

enizing the brain in Dulbecco's modified Eagle's medium. The homogenate was centrifuged to remove debris, and the supernatant was collected and stored at -80°C . Anti-RV nucleoprotein (N) monoclonal antibody 802-2 (24) was obtained from Charles Rupprecht, Centers for Disease Control and Prevention. Anti-RV glycoprotein (G) polyclonal antibody was prepared in rabbits as described elsewhere (20) and has been shown to have similar affinity to the G from wt SHBRV and laboratory-adapted CVS-N2C (54). Antibodies to STAT1, STAT2, and STAT3 were obtained from Chemicon International, Inc. Anti-CD3 polyclonal antibody was purchased from Abcam, England.

Mouse primary neuronal cultures. Mouse primary neuronal cultures were prepared using standardized procedures as described elsewhere (2, 32). Swiss-Webster mice at gestation day 16 were euthanized, and the embryos were removed. The neocortex from these embryos was collected and digested with trypsin. Separated neuronal cells were then plated into culture wells treated with poly-D-lysine (50 $\mu\text{g}/\text{ml}$). The primary neurons were grown in minimal essential medium (MEM) in a humidified atmosphere of 5% CO_2 -95% air at 37°C . Ara-c (cytosine furo-arabinoside) at 1 μM final concentration was added at 3 to 5 days after plating to prevent the proliferation of nonneuronal cells.

Animal infection and tissue collection. Mice were infected with 10 i.c. 50% lethal doses (LD_{50}) of either virus (B2C or SHBRV) by the i.c. route. Alternatively, mice were infected by the intramuscular (i.m.) route in the hind legs (both sides) with 10 i.m. LD_{50} . 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, mice were sacrificed and brains removed and flash-frozen on dry ice before being stored at -80°C . For histopathology and immunohistochemistry, animals 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 previously (54). Brain tissues were removed and paraffin embedded for coronal sections (4 μm).

Total RNA extraction. Mouse brain (400 to 500 mg each) was homogenized in 3 ml TRIzol (Invitrogen-Life Technologies). Total RNA was extracted and purified using an RNeasy Mini kit (QIAGEN) following the manufacturer's specifications.

Microarray hybridization and analysis. cRNA used for microarray hybridization was prepared following the Affymetrix eukaryotic sample and array processing protocol and then hybridized to an Affymetrix mouse expression microarray (mouse expression set MOE430A). Eight micrograms total RNA was used in the first-strand cDNA synthesis, together with T7-(dT)₂₄ primer and Superscript II reverse transcriptase (Invitrogen-Life Technology). Second-strand cDNA was synthesized using *Escherichia coli* DNA ligase, DNA polymerase I, RNase H, and T4 DNA polymerase (Invitrogen-Life Technologies) and then purified using the GeneChip sample cleanup module (Affymetrix). Biotin-labeled cRNA was prepared by using the Enzo RNA transcript labeling kit (Affymetrix) and then purified by using the GeneChip sample cleanup module. cRNA was fragmented and spiked with bacterial control genes (*bioB*, *bioC*, *bioD*, and *cre*) before overnight hybridization to Affymetrix mouse MOE430A. The hybridized microarrays were washed by using a GeneChip fluidics station and then stained with R-phycoerythrin-streptavidin using the antibody amplification washing and staining protocol. A GeneArray scanner was used to scan the hybridized gene chip. GeneChip operating software was used to collect data, and the statistical expression algorithm was used to obtain the signal values. Signals were scaled to a target intensity of 500 for normalization. Genes that were differentially expressed (at least twofold) were used in a hierarchical analysis by dChip developed by the Wong Lab, Department of Biostatistics, Harvard School of Public Health (<http://biosun1.harvard.edu/complab/dchip/install.htm>). Analysis of gene pathways was carried out by using gene ontology from the GO Consortium (<http://www.geneontology.org/GO.consortiumlist.shtml>).

Real-time SYBR Green PCR. To confirm the data generated from the microarray, real-time PCR was performed on the RNA samples using gene-specific primers in a Stratagene Mx3000P instrument. PCR was performed in one step in a 25- μl volume, with 100 ng sample RNA. Each reaction was carried out in duplicate. The reverse transcriptase and DNA polymerase were from the Brilliant SYBR green QRT-PCR master mix kit (Stratagene). cDNA synthesis was performed at 50°C for 30 min. During quantitative analysis, standard curves of three points were used to calculate the amplification efficiency for each pair of primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference gene.

Histopathology, immunohistochemistry, and Western blotting. Histopathology was performed by staining the paraffin-embedded sections with hematoxylin and eosin. For immunohistochemistry, paraffin-embedded brain sections were heated at 70°C for 10 min and then dipped in CitriSolv (Fisher Scientific) three times for 5 min and dried until chalky white. Slides were incubated with proteinase K (20 $\mu\text{g}/\text{ml}$) in 10 mM Tris-HCl (pH 7.4 to 8.0) for 15 min at 37°C and

rinsed three times with PBS. The primary antibody used was either the monoclonal antibody 802-2 directed against RV N (24), the rabbit polyclonal anti-RV G antibody (20), or anti-CD3 polyclonal antibody. The secondary antibodies used were biotinylated goat anti-mouse or goat anti-rabbit immunoglobulin G. The avidin-biotin-peroxidase complex was then used to localize the biotinylated antibody. Finally, diaminobenzidine was used as a substrate for color development. For Western blotting, brain extract as well as cell extract were subjected to electrophoresis on a 10% polyacrylamide-sodium dodecyl sulfate (SDS) gel. After separation on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were electroblotted to polyvinylidene difluoride membranes. Blots were then blocked in PBS containing 5% nonfat milk and 0.05% Tween 20 for 1 h at room temperature with shaking. Then, blots were incubated with the respective antibodies overnight at 4°C or for 2 h at room temperature. After three washes with PBS containing 0.05% Tween 20, blots were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody, followed by extensive washes in PBS-0.05% Tween 20. Proteins were detected by enhanced chemiluminescence (Amersham Biosciences). Band signals corresponding to immunoreactive proteins were measured and scanned by image densitometry using Adobe Photoshop 6.0 software.

Immunofluorescence and confocal microscopy. Primary neurons grown on coverslips were infected with each of the viruses and then fixed with 4% paraformaldehyde at day 5 after infection. Viral antigens were detected by using fluorescein isothiocyanate conjugated with anti-RV N monoclonal antibodies (Centocor, Pennsylvania). The expression of STAT1, STAT2, and STAT3 was detected by using rabbit anti-STAT polyclonal antibodies (Chemicon). Anti-rabbit secondary antibody conjugated with Alexa 488 (Molecular Probes) was used for 1 h at room temperature. Propidium iodide was used for counterstaining (15 min, room temperature). After washing, the coverslips were mounted with aqueous antifade mounting medium and examined under a Leica TCS NT confocal microscope. The percentage of cells with nuclear translocation of STAT proteins was evaluated by counting six areas, and the average number of translocated cells was calculated.

RESULTS

SHBRV is more pathogenic but induces fewer inflammatory changes than B2C. To compare the pathogenicity of the two viruses, SHBRV and B2C, the i.c. and i.m. pathogenic indices were determined (32, 35, 36) by subtracting the log virus titer/ml in BHK cells from the log i.c. LD_{50}/ml or the log i.m. LD_{50}/ml , and the results are shown in Fig. 1A. Almost 1,000 times more viral particles were required for attenuated B2C than for SHBRV to kill infected mice by either the i.c. or the i.m. route, suggesting that SHBRV is more pathogenic than B2C in the mouse model.

Although it was previously reported that very little inflammation and neuronal loss is observed in rabies patients (40), laboratory-adapted viruses induce extensive inflammation and necrosis (34, 54). To examine the pathological changes in mice infected with each virus, mice were transcardially perfused and brains were removed for histopathology and immunohistochemistry. It was found that attenuated B2C induced extensive pathological changes, particularly inflammation, including perivascular cuffing, gliosis, and infiltration of macrophages and lymphocytes. Necrosis and apoptosis were also observed frequently in brain tissues infected with B2C. On the other hand, only a few histological changes were observed in mice infected with SHBRV by the i.c. or i.m. routes (Fig. 1B). To quantify the inflammatory reactions, CD3-positive cells were measured using anti-CD3 antibodies in the cortex in mice infected by the i.c. route as described previously (32). Three serial sections were selected from each animal for measurement, and the average number of CD3-positive cells was obtained and analyzed for statistical significance by a one-way analysis of variance. As shown in Fig. 1C, significantly ($P < 0.01$) more CD3-positive cells were detected in B2C- than in

A: Pathogenic Index

	B2C	SHBRV
IC	1.1×10^{-4}	0.15
IM	6.2×10^{-7}	3.3×10^{-4}

B:

C:

FIG. 1. SHBRV is more pathogenic and induces less inflammation than B2C. The pathogenic indices for B2C and SHBRV were determined by subtracting the log virus titer/ml in BHK cells from the log i.c. LD₅₀/ml or the log i.m. LD₅₀/ml (A). Pathological changes in the cortex (i.c.) or the thalamus (i.m.) were observed in paraffin sections infected with either B2C or SHBRV (B). Hippocampal sections were made for immunohistochemistry to quantify CD3-positive cells using anti-CD3 antibodies (C). Con, sham-infected control mouse brain; B2C, B2C-infected mouse brain; SHB, SHBRV-infected mouse brain. *, *P* < 0.05 for SHB versus control; **, *P* < 0.01 for B2C versus SHB and *P* < 0.001 for B2C versus control.

SHBRV-infected mice, indicating that more inflammatory cells infiltrated B2C-infected than SHBRV-infected mouse brain.

Pathogenic SHBRV induces fewer changes in host gene expression than B2C. To investigate the different host responses to infection with the attenuated and the wt RV, mice were infected i.c. with 10 i.c. LD₅₀ of the pathogenic SHBRV or the laboratory-adapted B2C. Alternatively, mice were infected i.m. with 10 i.m. LD₅₀ of each virus. Sham-infected mice were used as controls. Mice were sacrificed when they developed severe paralysis, and flash-frozen brains were used for total RNA extraction and cRNA synthesis. The cRNA was then used to hybridize to the mouse whole genomic microarray with mouse

expression set 430A. The data were analyzed by a combination of the GeneChip operating software and dChip. The normalized data for 22,626 mouse genes were collected. Changes over twofold are considered for either up- or down-regulation. When compared with controls, there are 792 genes up-regulated and 301 genes down-regulated in animals infected i.c. with B2C, while there are 525 genes up-regulated and 107 genes down-regulated in animals infected i.c. with SHBRV. In comparison, there are 890 genes up-regulated and 694 genes down-regulated in animals infected i.m. with B2C, while there are 259 genes up-regulated and 198 genes down-regulated in animals infected i.m. with SHBRV. Overall, pathogenic SHBRV induced fewer changes in host gene expression than

TABLE 1. Host gene expression profiling in mice infected i.c. or i.m. with B2C or SHBRV^a

No. of genes	Regulation after i.c. infection with:		No. of genes	Regulation after i.m. infection with:	
	B2C	SHBRV		B2C	SHBRV
419	↑	↑	215	↑	↑
62	↓	↓	63	↓	↓
373	↑	—	671	↑	—
237	↓	—	631	↓	—
0	↑	↓	4	↑	↓
2	↓	↑	0	↓	↑
104	—	↑	44	—	↑
45	—	↓	131	—	↓
All other genes	—	—	All other genes	—	—

^a ↑, up-regulation; ↓, down-regulation; —, no up- or down-regulation.

B2C in either i.m.- or i.c.-infected mice. Although there is a number of genes whose expression is altered by one virus infection but not by the other, there are very few genes whose expression is up-regulated by one virus and down-regulated by another. Table 1 compares the numbers of genes up- and/or down-regulated in animals infected with each virus and by each route.

Attenuated B2C, but not wt SHBRV, activates gene expression of innate immune responses. Analysis of the microarray

data by gene ontology revealed that pathogenic and attenuated RVs differentially induce host gene expression in many of the gene clusters (Fig. 2). For immunity and antiviral genes as well as genes involved in apoptosis, there are more genes up- than down-regulated in mice infected with either virus. In addition, there are more genes up-regulated in mice infected with B2C than with SHBRV. For genes involved in neuronal functions, there are more genes down- than up-regulated, particularly in mice infected with B2C. For transcription factors, the numbers

FIG. 2. Expression patterns of host genes. Hierarchical cluster analysis of host gene expression was performed by gene ontology. The results were filtered to retain only those genes involved in the immunity and antiviral, apoptosis, neuron-specific, and transcriptional factors. Open bars, up-regulation; shaded bars, down-regulation.

TABLE 2. Expression profile of genes involved in the IFN- α/β signaling pathway in mouse brain infected with B2C or SHBRV i.c. or i.m.

Gene category and identification	Fold change over control			
	Infected i.c. with:		Infected i.m. with:	
	B2C	SHBRV	B2C	SHBRV
Interferon genes				
IFN- α 2	2	N ^b	3	N
IFN- α 4	8	6	9	N
IFN- α 5	9	N	8	N
IFN- β	3	N	3	2
Interferon signaling genes				
Cbp/p300-transactivator	2	2	2	N
Stat1	32	18	14	9
Stat2	13	4	10	4
Stat3	4	5	5	N
IRF-1	6	3	9	2
IRF-2	3	N	5	N
IRF-7	11	6	30	7
Jak-2	2	N	2	N
PKR	3	N	6	4
Interferon effector genes				
2'-5'-OAS-1B	15	10	13	6
2'-5'-OAS-1G	13	5	13	5
2'-5'-OAS-2	4	N	6	3
2'-5'-OAS-3	2	N	5	N
2'-5'-OAS-like 1	7	3	11	3
2'-5'-OAS-like 2	42	18	45	26
ADAR, RNA specific	3	2	-2	N
MHC I antigen	3	N	4	N
Mx 1	388	91	119	18
Mx 2	56	15	147	24
Interferon-activated genes				
202B	315	147	478	49
203	5	2	23	N
204	49	12	478	16
205	45	11	147	12
IFN-induced protein 1 ^a	37	21	24	N
IFN-induced protein 2 ^a	256	128	208	74
IFN-induced protein 3 ^a	45	18	60	30

^a IFN-induced transmembrane protein with tetratricopeptide repeats 1, 2, and 3.

^b N, no change.

of genes up-regulated and down-regulated are similar for B2C by each route of infection. In this paper, only the genes involved in the innate immune and antiviral responses are analyzed in detail, particularly those up-regulated. The modification of other host genes will be described in more detail elsewhere.

Analysis of the gene profiles involved in the innate immune and antiviral responses revealed that the attenuated B2C (by either i.c. or i.m. inoculation) induced the expression of genes important in the innate immune responses, particularly the IFN- α/β induction and IFN- α/β signaling pathway. Genes encoding inflammatory cytokines and chemokines are also up-regulated by infection with B2C. On the other hand, wt SHBRV is a poor inducer of the innate immune responses (Fig. 2). Many of the genes important for the immune and antiviral responses are not up-regulated in SHBRV-infected animals. For those genes up-regulated by both virus infections,

TABLE 3. Expression profile of inflammatory genes in mouse brain infected with B2C or SHBRV i.c. or i.m.

Gene category and identification	Fold change over control			
	Infected i.c. with:		Infected i.m. with:	
	B2C	SHBRV	B2C	SHBRV
Toll-like receptors				
TLR1	3	N ^a	2	2
TLR2	6	4	5	N
TLR3	10	6	11	4
Chemokines				
(C-C) MCP-5	60	60	39	37
(C-C) MCP-1	28	8	12	3
(C-C) MIP-1 α	104	34	111	N
(C-C) MIP-1 β	5	N	4	N
(C-C) RANTES	56	12	91	5
(C-C) MCP-3	37	11	7	3
(C-X-C) MIP-2 α	6	7	3	3
(C-X-C) IP-10	84	37	158	26
(C-X-C) L11	16	3	25	3
(C-X-C) BLC	3	N	3	N
(C-X-C) MIP-2 β	4	5	3	3
(C-X-C) MIG	34	5	42	3
Cytokines^b				
IL-6	4	3	15	3
IL-12 β	3	2	N	N
IL-12 receptor β 1	3	N	20	11
IL-13 receptor α 1	5	N	N	N
TGF- β -regulated gene 1	3	N	2	N
TGF- β induced, 68 kDa	7	2	28	N
TNFR-12 α	15	17	7	N
TNFR-5	2	N	N	N
TNF- α -induced protein 2	7	8	3	N
Complement genes				
c1q- α	2	2	N	N
c1q- β	3	3	2	2
c1q- γ	3	2	3	N
c1r	56	20	18	2
c1s	3	N	158	5
c2	4	N	4	2
c3	30	8	119	N
c4	5	4	8	4

^a N, no change.

^b TGF, transforming growth factor; TNF, tumor necrosis factor; TNFR, TNF receptor.

usually the increase is 2- to 30-fold higher in animals infected with B2C than with SHBRV (Tables 2 and 3).

In mice infected with B2C by the i.c. or i.m. route, most of the genes involved in the IFN- α/β pathway are up-regulated. These include IFN- α/β genes, genes involved in the IFN- α/β -mediated signaling and transcription activation, and genes encoding proteins implicated in antiviral activities (Table 2). Up-regulated IFN genes include IFN- α 2, IFN- α 4, and IFN- α 5 as well as IFN- β . Interferon signaling genes (Cbp/p300-interacting transactivator, Stat1, Stat2, Stat3, and Jak-2) and interferon regulatory factors (IRF-1, -2, and -7) are up-regulated. IFN- α/β -induced proteins implicated in the antiviral activities, including double-stranded RNA-dependent protein kinase (PKR), the 2',5'-oligoadenylate synthetases (OAS), RNA-specific adenosine deaminase (ADAR), myxovirus resistance (Mx), and major histocompatibility (MHC) class I, are also

up-regulated in B2C-infected animals. The up-regulated genes for 2',5'-OAS include OAS-1B, -1G, -2, and -3 as well as OAS-like 1 and 2. Along the IFN signaling pathway, many of the IFN-activated and inducible genes are highly up-regulated (IFN-activated genes 202B, 203, 204, and 205 and IFN-induced transmembrane protein with tetratricopeptide repeats 1, 2, and 3). The most up-regulated gene is the antiviral Mx1, which is increased 388-fold in animals infected with B2C by the i.c. route.

On the other hand, many of genes important in the IFN- α/β pathway are not up-regulated in SHBRV-infected mice. The IFN genes are not up-regulated except for IFN- $\alpha 4$ (sixfold) by i.c. and IFN- β (twofold) by i.m. For the IFN signaling and effector genes, Cbp/p300-interacting transactivator, Stat3, Jak-2, IRF-2, 2',5'-OAS-2, 2',5'-OAS-3, ADAR, MHC I, PKR, IFN-activated gene 203, and IFN-induced transmembrane protein with tetratricopeptide repeat 3 are not up-regulated in mice infected with SHBRV by the i.c. or the i.m. route. Some of the genes in the IFN- α/β pathway were up-regulated in mice infected with SHBRV, but the increase was 2- to 30-fold lower than that in mice infected with B2C (Table 2).

Components in the inflammatory pathway, including toll-like receptors (TLR), chemokines, cytokines, and complement components, are also up-regulated in B2C-infected animals (Table 3). The expression of TLR1, TLR2, and TLR3 is up-regulated. Proinflammatory chemokines in both the C-C and C-X-C families, including RANTES (CCL5), MCP-1 (CCL2), MCP-3 (CCL7), MCP-5 (CCL12), MIP-1 α (CCL3), MIP-1 β (CCL4), MIP-2 α (CXCL-1), and MIP-2 β (CXCL-2), and IP-10 (CXCL-10), are all up-regulated, with some increased more than 100-fold. Many of the cytokines and cytokine receptors are up-regulated, for example, the proinflammatory cytokine interleukin-6 (IL-6). Complement components, such as C1q, C1r, C1s, C2, C3, and C4, are up-regulated. In mice infected with SHBRV, TLR1 and TLR2 are not up-regulated in animals infected by the i.c. or i.m. routes. For chemokines, only MCP-5 is up-regulated in SHBRV-infected mice to a similar level as in B2C-infected animals. MIP-1 α , MIP-1 β , and CXC chemokine BLC are not up-regulated in mice infected with SHBRV by the i.c. or i.m. route. The up-regulation of other chemokines in mice infected with SHBRV is 2- to 20-fold lower than that in mice infected with B2C (Table 3). Likewise, expression of many cytokine, cytokine receptors, and complement components is not up-regulated in mice infected with SHBRV.

Confirmation of microarray data by real-time PCR. To validate the microarray data, real-time PCR was performed on selected genes from each of the categories, including IFN (IFN- $\alpha 2$ and IFN- $\alpha 5$), IFN regulatory genes (Stat1, Stat2, Stat3, IRF2, and IRF7), IFN effector genes (OAS-1G and Mx1), and chemokine genes (MCP-1, IP-10, and RANTES). GAPDH was used as a reference gene. Primers for amplification of these genes are listed in Table 4. The results from the real-time PCR were compared with the data obtained by microarray hybridization and are summarized in Table 5. The increases in mice infected with either SHBRV or B2C over the controls are similar for some genes in both the microarray data and real-time PCR results. For other genes, such as Stat1, Stat2, OAS-1G, Mx1, IP-10, and RANTES, real-time PCR was more sensitive and detected greater increases than the mi-

TABLE 4. Primers used for real-time PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	GGAGAAGCTGCCAATGGATA	TTACGCTTGCACTTCTGGTG
IFN α -2	TCTGTGCTTTCCTCGTGATG	TTGAGCCTTCTGGATCTGCT
IFN α -5	CTGCCTGAAGGACAGAAAGG	TCATTGAGCTGCTGATGGAC
Stat1	CACATTCACATGGGTGGAAC	TCTGGTGCTTCCTTTGGTCT
Stat2	ACCAGTGGGACCACTACAGC	ATCTCAAGCTGCTGGCTCTC
Stat3	TCATGGGTTCATCAGCAAG	TGGTCGCATCCATGATCTTA
IRF2	CTTATCCGAACGACCTTCCA	CTTGCTGTCCAGATGGGACT
IRF7	CCTCTTGCTTCAGGTTCTGC	GGCCCTTGATACATGATGGTC
OAS-1G	GGCTGTGTACCCATGTGTTT	CAGAAGCACGGAATCTGATG
Mx1	CAAAGCCCTGGAAGAGTCTG	CGGATCAGGTTTTCAGCTTC
MCP-1	AGGTCCCTGTCTGCTTCTG	TCTGGACCCATCTCTTCTTG
IP-10	GGTCTGAGTGGGACTCAAGG	TCTTTTTCATCGTGGCAATG
Rantes	CCCTCACCATCATCTCTACT	CCTTCGAGTGACAAACACGA

croarray hybridization. Nevertheless, the ratios between B2C and SHBRV increases were similar in both the microarray and the real-time PCR.

The increased expression of Stat genes resulted in increased synthesis of STAT proteins. To determine if increased Stat gene expression results in increased protein synthesis, the levels of STAT1, STAT2, and STAT3 (protein yield) in the i.c.- or i.m.-infected mice were measured by Western blotting using anti-STAT antibodies, and the band intensity was measured by densitometry. As shown in Fig. 3, the expression of STAT1 and STAT2 increased more than sevenfold in mice infected with B2C by either the i.c. or the i.m. route, whereas STAT1 and STAT2 increased only two- to fourfold in mice infected with SHBRV. On the other hand, STAT3 expression was up-regulated similarly in animals infected with each of the viruses, and its level increased about twofold over the controls. The levels of increased STAT proteins were proportional to that of increased Stat transcripts as detected by microarray and real-time PCR (Tables 2 and 5). The level of β -tubulin expression was almost the same in infected or uninfected animals. To further determine the expression pattern of the STATs, primary neurons were infected with each virus at a multiplicity of infection of 0.1, and the cells were harvested at day 5 for Western blotting. The levels of STAT1 and STAT2 increased four- to sevenfold in cells infected with B2C, but only about twofold in cells infected with SHBRV. Likewise, the level of STAT3 increased about twofold in cells infected with either B2C or SHBRV. These data indicate that the increased expression of Stat genes resulted in increased STAT protein synthesis.

STAT1 and STAT2, but not STAT3, are activated by RV infections. STATs, particularly STAT1 and STAT2, play an important role in the IFN- α/β signaling pathway. IFN- α/β binds to IFN- α/β receptors, which activates STATs by phosphorylation (51). Phosphorylated STATs form specific multimeric complexes that then translocate to the nucleus and initiate transcription (13). To determine if STATs are activated by RV infection, neurons infected with B2C or SHBRV were fixed with 4% paraformaldehyde and subjected to immunocytochemistry with anti-STAT antibodies and confocal microscopy. The percentage of cells with nuclear translocation was quantified in neurons at 1, 3, and 5 days postinfection (p.i.). There were only a few translocated cells for any of the STAT proteins at 1 or 3 days p.i. (data not shown) in cells infected

TABLE 5. Comparison of real-time PCR and microarray data for mice infected with SHBRV or B2C

Gene	Fold change ^a					
	Microarray			Real-time PCR		
	B2C vs control	SHBRV vs control	B2C vs SHBRV	B2C vs control	SHBRV vs control	B2C vs SHBRV
IFN- α 2	2	NC	NA	2	1.3	1.6
IFN- α 5	9	NC	NA	3.1	1.9	1.6
Stat1	32	18	1.8	55	22	2.5
Stat2	13	4	3.3	19	4.8	4.4
Stat3	4	5	0.8	6	3.3	1.8
IRF2	3	NC	NA	2.3	1.1	2
IRF7	11	6	1.9	25	12	2
OAS-1G	13	5	2.6	78.6	37	2.1
Mx1	388	91	4.3	$+\infty^*$	$+\infty^*$	6
MCP-1	28	8	3.5	11	4.6	2.4
IP-10	84	37	2.3	2394	819	2.9
RANTES	56	12	4.8	413	267	1.5

^a NC, no change compared with control; NA, not applicable; *, no signal for control.

with either virus. As shown in Fig. 4, significantly more cells with nuclear translocation were observed for STAT1 and STAT2 in cells infected with B2C at day 5 p.i. Only a few cells with nuclear translocation were detected for STAT3 in cells infected with B2C. In addition, significantly more cells with nuclear translocation for STAT1 and STAT2 proteins were observed in cells infected with B2C than with SHBRV. Together, these data suggest that STAT1 and STAT2, but not STAT3, are involved in the IFN activation and effector pathway in RV infections.

Evasion of the innate immune responses by pathogenic SHBRV correlates with the restriction of RV G expression. Previously, it has been reported for pathogenic RV that restriction of the expression of G contributes to its pathogenicity (37, 54). To determine if restriction of G expression also occurs in pathogenic SHBRV infections and might correlate with the evasion of the innate immune responses by pathogenic SHBRV, the expression levels of RV G and N were evaluated on brain sections by using immunohistochemistry. As shown in Fig. 5A, the levels of N expression were similar in mice infected i.c. with each virus, whereas the level of G expression was almost undetectable in SHBRV-infected mice. In contrast, the level of G expression was high in B2C-infected mice. To

confirm this finding, brain extracts were made from mice infected with SHBRV or B2C by either the i.c. or i.m. routes. Furthermore, cellular extracts were prepared from primary neurons infected with each virus. All these extracts were used for Western blotting to detect the level of G and N expression. As shown in Fig. 5B, the level of G expression was consistently threefold lower in both animals and cells infected with SHBRV than with B2C, while the levels of N expression were similar in animals and cells infected with either virus. These data suggest that G expression is consistently inhibited in pathogenic RV infections in vivo as well as in vitro and, as a result, the restriction of G expression may be one of the mechanisms by which pathogenic SHBRV evades the activation of the innate immune responses.

DISCUSSION

Host innate immune responses are the first line of defense against infections. During the pathogen-host coevolution, many viruses have developed ways to evade the host innate immune responses, particularly the IFN pathways (48). Previously, various groups have reported that RV infection can

FIG. 3. Expression of STAT proteins in mouse brain or in primary neuronal cell culture after RV infection. STAT (1, 2, and 3) proteins were detected by Western blotting in brain tissues from mice infected by the i.c. or i.m. routes as well as in primary neurons. As a loading control, β -tubulin was detected in the same sample preparation using anti- β -tubulin (anti-T) antibody in the Western blot assays. The protein band intensity was determined by densitometry, using that from the control as 100%.

A.

B.

FIG. 4. Nuclear translocation of STAT proteins in primary neurons after RV infection. Primary neurons were infected with each of the viruses at 0.1 focus-forming units/cell and then fixed at day 5 after infection. Viral antigen (N) was detected by using fluorescein isothiocyanate-conjugated anti-RV N monoclonal antibodies. The expression of STAT1, STAT2, and STAT3 was detected by using rabbit anti-STAT polyclonal antibodies and anti-rabbit secondary antibody conjugated with Alexa 488 (green). Propidium iodide (PI) was used for counterstaining (red). The cells were examined under a Leica TCS NT confocal microscope (A). The percentage of cells with nuclear translocation was quantified for each of the STAT proteins (B). Significantly more nuclear-translocated cells were observed for STAT1 ($P < 0.001$) and STAT2 ($P < 0.001$) in cells infected with B2C and for STAT2 ($P < 0.01$) in cells infected with SHBRV (as indicated by **).

FIG. 5. Expression of RV proteins in mouse brain or in primary neuronal culture after RV infection. RV proteins (N and G) were detected by immunohistochemistry in mouse brain sections (A) or by Western blotting in brain tissues from mice infected by the i.c. or i.m. routes as well as in primary neurons (B). As a loading control, β -tubulin was detected in the same sample preparation using anti- β -tubulin (anti-T) antibody in the Western blot assays. The ratio between G and N was determined after measurement of the band density.

activate the innate immune responses. For example, RV infection triggers the expression of inflammatory cytokines (4, 5) and chemokines (22, 41) in vitro or in vivo. In all these studies, only laboratory-adapted RV was used. In the present paper, we compared a laboratory-adapted and attenuated RV with a wt pathogenic RV and found for the first time that the pathogenic RV evades, while the attenuated RV activates, the host innate immune responses. As detected by the microarray technology and real-time PCR, almost all the genes involved in the activation of the IFN- α/β pathway and many of the inflammatory chemokines and cytokines are up-regulated in animals infected with attenuated RV B2C by either the i.c. or the i.m. routes. However, many of these genes are not up-regulated in animals infected with pathogenic SHBRV. For those genes involved in the IFN- α/β pathway that are up-regulated in SHBRV-infected animals, the magnitude of increase is at least 2- to 30-fold lower than that in B2C-infected mice. Furthermore, attenuated RV induces extensive CNS inflammation while pathogenic RV does not.

The attenuated B2C activates the innate immune responses, particularly the IFN- α/β signaling pathway. Recently, Nakamichi et al. (41) also reported the up-regulation of IFN- α/β in RAW macrophages after stimulation with laboratory-adapted, attenuated RV. In a companion paper (44) as well as a paper published very recently (12), RV infection induces the expres-

sion of IFN- β . In our present study, not only IFN- β but also IFN- α 2, -4, and -5 are found to be up-regulated by infection with attenuated RV. Furthermore, those genes that are involved in IFN-mediated signaling and transcription activation of cellular gene expression are up-regulated. These include interferon signaling genes (Stat1, -2, and -3 and Jak-2) and interferon regulatory factors (IRF-1, -2, and -7). As summarized by Samuel (48), IFN- α/β -induced proteins implicated in the antiviral activities include PKR, the 2',5'-OAS, ADAR, Mx, and MHC class I. Genes encoding these proteins are all up-regulated in B2C-infected animals. These molecules are involved in mRNA translation inhibition, RNA degradation, RNA editing, and cytotoxic T-lymphocyte responses. In the IFN signaling pathway, many of the IFN-activated and inducible genes are highly up-regulated (IFN-activated genes 202B, 203, 204, and 205 and IFN-induced transmembrane protein with tetratricopeptide repeats 1, 2, and 3). Thus, attenuated RV activates the IFN- α/β pathway. The role of IFN- α/β in resisting RV infection has previously been investigated. Direct administration of IFN- α/β or IFN-inducing poly(I · C) resulted in various degrees of protection against RV infection in mice, hamsters, rabbits, or monkeys (25, 27). Hooper et al. (28) reported that higher virus titers were detected in IFN- α/β receptor knockout (IFNAR^{-/-}) mice than immunologically intact mice when infected with an attenuated CVS-F3. It also

took a longer time for the IFNAR^{-/-} mice (21 days) than normal counterparts (8 days) to clear the virus from the CNS. In addition, fully immunocompetent mice developed higher levels of virus neutralization antibodies than IFNAR^{-/-} mice. All these data indicate that IFN- α/β plays a role in RV resistance through both innate and adaptive immune responses.

In addition to the IFN- α/β pathway, attenuated RV also stimulates the expression of many genes encoding inflammatory molecules such as chemokines, cytokines, TLRs, and complement components. Inflammatory cytokine IL-6 (30) is highly up-regulated in B2C-infected animals. Many of the inflammatory chemokines (both C-C and C-X-C families) are also highly up-regulated, particularly MCP-1, -3, and -5, MIP-1 α , RANTES, IP-10, and MIG. Chemokine CCL-5 has been previously detected in migratory T cells in the CNS of mice infected with RV (22). Recently, Nakamichi et al. (41) reported that CXCL-10 was highly up-regulated and other chemokines were not up-regulated in RV-infected macrophages. The disparities between that study and ours reported here may be due to the different types of cells involved. In the study by Nakamichi et al. (41), only macrophages were used, whereas in the present study the expression of chemokines was detected in the brain, where there are other cell types beside neurons, such as astrocytes, microglia, and infiltrating CD3-positive T cells. It has been reported that chemokine (MCP-1) expression can also be affected by monocyte-astrocyte interactions (3). Thus, it is possible that interactions among neurons, astrocytes, microglia, and infiltrating CD3-positive T cells are responsible for the up-regulation of so many chemokines as observed in our present study. Activation of TLRs also induces inflammation (8). TLR1, TLR2, and TLR3 are all found to be up-regulated in B2C-infected mice. The increased expression of the chemokines, cytokines, and TLRs corresponds to the severe inflammatory reaction and significant increase in CD3-positive cell infiltration observed in mice infected with B2C. In addition, complement C1, particularly C1r, is highly up-regulated in B2C-infected mice. Although the classic or alternative complementary cascades may not be involved in RV resistance in the CNS (28), increased expression of C1 may be a consequence of activated microglia during RV-induced CNS inflammation (16). Inflammatory reaction and infiltration of T cells have been reported to play a major role in blocking RV spread in the CNS (5, 10) as well as RV clearance from the CNS (28).

It is thus clear that attenuated RV activates the innate immune responses, including the IFN- α/β pathway and inflammatory reactions. Up-regulation of these genes is detected in both i.c.- and i.m.-infected mice by both microarray and real-time PCR. In addition, infection of mice with other laboratory-adapted and attenuated RVs also resulted in up-regulation of genes involved in the innate immune responses (data not shown). Furthermore, our data are supported by other recent work that has demonstrated that many of these genes involved in IFN signaling and inflammation are also up-regulated in human postmitotic N2T cells after infection with laboratory-adapted RV (44). On the other hand, pathogenic SHBRV induces very little or no inflammation and little or no up-regulation of gene expression in the IFN- α/β and inflammatory pathways. The activation of the innate immune responses by attenuated RV may play a protective role in the host against RV infection, which may explain why a few viral particles of the

pathogenic RV can kill infected animals, whereas about 1,000 times more viral particles are required for the attenuated RV to kill infected animals in the mouse model. The evasion of the innate immune responses observed in SHBRV-infected mice may contribute to the highly neuroinvasive characteristic of the virus (18, 39).

IFN- α/β exerts its antiviral activities by binding to IFN- α/β receptors, which activates STATs by phosphorylation (51). Phosphorylated STATs form specific multimeric complexes that translocate to the nucleus and initiate transcription (13). Stat1, Stat2, and Stat3 genes are all up-regulated in RV-infected mice as detected by the microarray hybridization and real-time PCR (Tables 2 and 5). Furthermore, up-regulation of Stat expression resulted proportionally in increased protein synthesis. The level of STAT1 and STAT2 is higher in animals or cells infected with B2C than with SHBRV. On the other hand, STAT3 expression increases similarly in animals or cells infected with either virus. Most importantly, significantly more STAT1- and STAT2-translocated cells were found after infection with RV, particularly with B2C. Only a few STAT3-translocated cells were observed in RV infection. These data may indicate that RV infection, particularly with attenuated virus, not only results in the increased transcription and synthesis of STAT1 and STAT2, but also in the activation of the STAT1 and STAT2, presumably by phosphorylation, leading to nuclear translocation. These data also suggest that STAT1 and STAT2, but not STAT3, are involved in the IFN- α/β activation and effector pathway in RV infections. This is in agreement with results from other studies that STAT1 and STAT2 promote the synthesis of effector proteins that inhibit viral replication (1, 13). Increased expression of Stats has been reported in RV-infected mice (44) and neuronal cells (45).

To counter the host's antiviral activities, viruses developed ways to impair the induction of innate immunity, particularly the IFN- α/β pathways (48). Poxviruses encode soluble IFN receptor homologues that prevent IFNs from acting through their natural receptors to elicit an antiviral response (50). Adenovirus VAI RNA antagonizes the antiviral state of IFN by preventing PKR activation (33). Poliovirus infection leads to the degradation of PKR (7). In a recent review, Conzelmann (12) summarized the mechanisms by which nonsegmented negative-stranded RNA viruses interfere with the transcriptional activation of IFN- α/β . For example, the V or the C protein from paramyxoviruses (simian virus 5, Sendai virus, and mumps virus) mediates the degradation of STAT1 via the ubiquitination pathway (14, 42, 43, 49). The VP35 protein of Ebola virus inhibits the induction of the IFN- β promoter and double-stranded RNA/virus-mediated activation of IFN-stimulated response element-derived gene expression (6). The NS1 protein of influenza virus is an IFN- α/β antagonist (23). The P protein of RV has recently been reported to interfere with the phosphorylation of IRF-3, thus exerting an antagonistic function for IFN- α/β (9). In our study, we found that the activation of the innate immune responses, particularly the up-regulation of IFN- α/β , correlates with the level of RV G expression. Not only in the CNS, but also in primary neurons, attenuated RV expresses G abundantly while pathogenic RV expresses three-fold less G, despite the fact that both viruses express a similar amount of N. The restriction of G expression also results in virus yield 3 logs lower in the brain of mice infected with wt

SHBRV than those infected with B2C (data not shown), similar to the findings of Faber et al. (18). Thus, we propose that one way by which pathogenic RV evades the innate immune responses is by restriction of G expression. It has been reported that RV inhibits G expression in order to be pathogenic (17, 18, 38). Thus, it is possible that restriction of G expression helps pathogenic RV to evade the innate immune responses. Although double-stranded RNA has been reported as the major factor for the induction of IFN- α/β in attenuated RV-infected cells (44), it is also possible that RV G can activate the TLRs, particularly TLR-3, thus stimulating the expression of the IFN- α/β pathway in RV-infected cells. It has been reported that viral surface glycoproteins can activate TLRs (8). TLR-3 can sense RV infection in human postmitotic neurons to produce IFN- β (44), and TLR-3 is also up-regulated in mice infected with RV in our studies.

In addition to genes involved in the innate immune and antiviral responses, many other host genes, such as those involved in apoptosis, are also up-regulated in B2C- but not in SHBRV-infected mice. Up-regulation of more genes involved in apoptosis in B2C than SHBRV may explain the observation that B2C induced apoptosis (36) while SHBRV did not (54). On the other hand, RV infection resulted in down-regulation of many of the neuron-specific genes. Actually, there are more neuronal genes down-regulated than up-regulated, particularly in B2C-infected mice. This is not surprising, since we have previously reported the down-regulation of neuron-specific genes, such as the preproenkephlin gene, by *in situ* hybridization in rats infected with CVS-24 (21). Furthermore, Prosnjac et al. (45) have reported that by using subtraction hybridization most of the host genes were down-regulated in RV-infected mice. It was also found in the present study that there were as many transcriptional factors up-regulated as down-regulated in RV-infected mice. Previously we have reported that transcriptional factors such as *egr-1* and *c-jun* are up-regulated in RV-infected rats (21). The importance of the modification in the expression pattern for neuron-specific genes and the transcription factors in RV pathogenesis is not entirely clear and warrants further investigation.

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