RNase L Antiviral Activity Is Not a Critical Component of the Oas1b-Mediated Flavivirus Resistance Phenotype

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ABSTRACT In mice, resistance to central nervous system (CNS) disease induced by members of the genus *Flavivirus* is conferred by an allele of the 2'-5' oligoadenylate synthetase 1b gene that encodes the inactive full-length protein (Oas1b-FL). The susceptibility allele encodes a C-terminally truncated protein (Oas1b-tr). We show that the efficiency of neuron infection in the brains of resistant and susceptible mice is similar after an intracranial inoculation of two flaviviruses, but amplification of viral proteins and double-stranded RNA (dsRNA) is inhibited in infected neurons in resistant mouse brains at later times. Active OAS proteins detect cytoplasmic dsRNA and synthesize short 2'-5'-linked oligoadenylates (2'-5'A) that interact with the latent endonuclease RNase L, causing it to dimerize and cleave single-stranded RNAs. To evaluate the contribution of RNase L to the resistance phenotype *in vivo*, we created a line of resistant RNase L^{-/-} mice. Evidence of RNase L activation in infected RNase L^{+/+} mice was indicated by higher levels of viral RNA in the brains of infected RNase L^{-/-} mice. Activation of type I interferon (IFN) signaling was detected in both resistant and susceptible brains, but *Oas1a* and *Oas1b* mRNA levels were lower in RNase L^{+/+} mice of both types, suggesting that activated RNase L also has a proflaviviral effect. Inhibition of virus replication was robust in resistant RNase L^{-/-} mice, indicating that activated RNase L is not a critical factor in mediating this phenotype.

IMPORTANCE The mouse genome encodes a family of Oas proteins that synthesize 2'-5'A in response to dsRNA. 2'-5'A activates the endonuclease RNase L to cleave single-stranded viral and cellular RNAs. The inactive, full-length Oas1b protein confers flavivirus-specific disease resistance. Although similar numbers of neurons were infected in resistant and susceptible brains after an intracranial virus infection, viral components amplified only in susceptible brains at later times. A line of resistant RNase L^{-/-} mice was used to evaluate the contribution of RNase L to the resistance phenotype *in vivo*. Activation of RNase L antiviral activity by flavivirus infection was indicated by increased viral RNA levels in the brains of RNase L^{-/-} mice. *Oas1a* and *Oas1b* mRNA levels were higher in infected RNase L^{-/-} mice, indicating that activated RNase L also have a proflaviviral affect. However, the resistance phenotype was equally robust in RNase L^{-/-} and RNase L^{+/+} mice.

KEYWORDS flavivirus, RNase L, West Nile virus, yellow fever virus, flavivirus resistance gene, Oas1b, Oas1a, Mx1, yellow fever virus, flavivirus

The genus *Flavivirus* in the family *Flaviviridae* includes West Nile virus (WNV), dengue virus, yellow fever virus (YFV), and Zika virus. Human infections with mosquito-borne viruses can be asymptomatic or induce disease of various degrees of severity (1). A genetically controlled resistance phenotype that is specific for members of the genus *Flavivirus* was detected segregating in wild *Mus musculus* populations in studies conducted in the 1920s, 1970s, and 1990s (2–5). Resistance is conferred by a single autosomal dominant allele (6–8). Resistant mice are permissive to infection by flaviviruses but produce lower virus titers and do not develop disease symptoms after

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intracranial injection of virus doses that are lethal in animals homozygous for the susceptibility allele. A positional cloning approach was used to identify the *Oas1b* gene as the locus controlling this resistance (9). The mRNA transcribed from the *Oas1b* susceptibility allele contains a premature stop codon and produces a truncated protein (*Oas1b-tr*) that lacks the C-terminal transmembrane domain. Susceptible C57BL/6 *Oas1b-tr* mice were converted to the resistant phenotype by knocking in a part of the coding region of the resistance allele by homologous recombination and then establishing a line of C57BL/6 *Oas1b-FL* mice expressing the full-length *Oas1b* protein. These mice are fully resistant to flavivirus-induced disease, confirming that expression of the full-length *Oas1b* protein is essential for the resistance phenotype (10).

Oas1b is a member of the OAS gene family (11). The human genome encodes single copies of the *OAS1*, *OAS2*, *OAS3*, and *OASL* genes. In contrast, the mouse genome encodes single copies of *Oas2* and *Oas3*, two copies of *OasL*, and eight copies of *Oas1* (*Oas1a-Oas1h*). Only the mouse *Oas1a* and *Oas1g* proteins have 2',5'-oligoadenylate synthetase activity (12, 13). OAS genes are interferon (IFN)-stimulated genes (ISGs). OAS proteins interact with double-stranded RNA (dsRNA) and function as cellular RNA sensors of virus infections (14). Upon activation, OAS proteins polymerize ATP and generate short 2'-5'-linked oligoadenylates (2'-5'A) that activate the cytosolic endoribonuclease RNase L to dimerize and cleave single-stranded cellular and viral RNAs (15). *OAS3* has been shown to be the major source of 2'-5'A in cells infected with different types of viruses (16). We previously showed that WNV genomic RNA is susceptible to cleavage by RNase L and that a susceptible C57BL/6 RNase L^{-/-} mouse embryo fibroblast (MEF) cell line produced 5 to 10 times higher WNV yields and higher viral RNA levels than a C57BL/6 RNase L^{+/+} MEF cell line (17). A previous *in vivo* study showed that 70% of susceptible C57BL/6 RNase L^{+/+} mice but only 50% of C57BL/6 RNase L^{-/-} mice survived a peripheral WNV infection, while no difference in survival was observed between these mice after an intracranial WNV infection (18).

In the present study, the efficiency and progression of two different flavivirus infections were compared in the brains of resistant and susceptible mice that differed only in their *Oas1b* alleles. At early times after infection, the relative number and distribution of neurons infected by either WNV NY99 or YFV 17D as well as the intensity of the fluorescence signals of viral components in these cells were similar in susceptible and resistant mouse brains, but the number of infected neurons and the intensity of the viral fluorescence signals increased with time after infection only in the susceptible mouse brains. The contribution of RNase L activity to the resistance phenotype *in vivo* was studied using a line of resistant RNase L^{-/-} mice. Evidence of an antiviral effect of RNase L was indicated by higher viral RNA levels in the brains of RNase L^{-/-} mice. The higher levels of *Oas1a* and *Oas1b* mRNA observed in the RNase L^{-/-} mice suggested that activated RNase L contributes to the turnover of these mRNAs, which would be expected to have a proflaviviral effect. However, the *Oas1b* resistance phenotype was equally robust in C57BL/6 *Oas1b-FL* mice with and without RNase L, indicating that RNase L activity does not play an essential role in the resistance phenotype.

RESULTS

Comparison of flavivirus replication in resistant and susceptible mouse brains.

The efficiency and distribution of neuron infection by WNV or YFV in the brains of resistant C57BL/6 (*Oas1b-FL/RNase L^{+/+}*) mice and susceptible C57BL/6 (*Oas1b-tr/RNase L^{+/+}*) mice was compared. On different days following intracranial inoculation with 100 PFU of WNV NY99 or 500 PFU of YFV-17D, 6- to 8-week-old resistant and susceptible mice were perfused with 4% paraformaldehyde and the brains were processed for immunofluorescence assay (IFA). Viral RNA replication in infected cells was detected with anti-dsRNA antibody, and viral translation was detected with anti-NS3 antibody. Neurons were detected with anti-NeuN antibody. At 2 days postinfection (dpi) with WNV NY99, the percentage of NeuN-positive cells that were infected was quantified in different areas of the brain. In the cortex ~50%, in the thalamus ~35%, and in the hippocampus ~85% of the NeuN-positive cells in the brains of both

Oas1b-FL/RNase L^{+/+} and Oas1b-tr/RNase L^{+/+} mice contained similar low-intensity viral dsRNA and NS3 signals in the perinuclear region (Fig. 1A, B, and C). At 4 dpi, both the intensity and intracellular area of the viral dsRNA and NS3 signals had markedly increased in the infected neurons of the susceptible Oas1b-tr/RNase L^{+/+} brains but had not amplified in the infected neurons of the resistant Oas1b-FL/RNase L^{+/+} brains. The percentage of NeuN-positive cells that were infected had increased to ~85% in the cortex and to ~90% in the thalamus and remained at ~85% in the hippocampus of the susceptible mouse brains. In contrast, the percentage of infected NeuN-positive cells did not increase appreciably in the cortex or thalamus and decreased slightly in the hippocampus of the resistant mice.

Due to the slower time course of a YFV 17D infection, the percentage of NeuN-positive cells that were infected was determined using brains harvested at 4 and 9 dpi. At 4 dpi, the percentage of NeuN-positive cells that were infected in the thalamus of resistant Oas1b-FL/RNase L^{+/+} mice was ~45%, while that in the same brain region of susceptible Oas1b-tr/RNase L^{+/+} mice was ~55%. In both strains of mice, the infected neurons contained low-intensity perinuclear dsRNA signals (Fig. 1D). By 9 dpi, the intensity and intracellular area of the dsRNA signal had greatly increased in the infected neurons of the susceptible mice, whereas no change from what was observed at 4 dpi had occurred in the infected neurons in the resistant mouse brains. At 9 dpi, the percentage of NeuN-positive cells that were infected had increased to ~75% in the thalamus of the susceptible mice, but the percentage of infected NeuN-positive cells had not increased in the thalamus of the resistant mice. The thalamus data obtained with a YFV infection were similar to the data obtained with a WNV infection. Additional data obtained from the cortex and hippocampus of the YFV-infected mice were also similar to the WNV data. The data confirm that the Oas1b-mediated resistance is very effective at preventing the amplification of flavivirus replication and at promoting survival even after intracranial inoculation of a highly virulent flavivirus that results in neuron infection in multiple regions of the brain.

Resistant RNase L^{-/-} mice are fully resistant to lethal doses of WNV and YFV.

RNase L is activated by 2-5A produced by Oas proteins. RNase L was previously shown to have anti-WNV activity (17). Although Oas1b is an inactive 2-5A synthetase, Oas1a and other Oas proteins, such as Oas3, are active synthetases, and the expression of these ISGs is upregulated in most tissues in response to IFN signaling (19). An initial study of the contribution of RNase L to the resistance phenotype utilized a simian virus 40 (SV40)-transformed C3H/RV MEF cell line stably expressing a dominant-negative mutant of RNase L (17). To obtain a more biologically relevant experimental model for testing the contribution of RNase L to the resistance phenotype *in vivo*, we created a line of resistant RNase L^{-/-} mice. F1 progeny from a cross between susceptible C57BL/6 Oas1b-tr/RNase L^{-/-} mice (20) and resistant C57BL/6 Oas1b-FL/RNase L^{+/+} mice (10) were mated, and the F2 mice were genotyped to identify double homozygous mice that were then used to establish a line of Oas1b-FL/RNase L^{-/-} mice.

We chose to analyze the contribution of RNase L activity *in vivo* using a brain infection because the Oas1b-mediated resistance phenotype is very robust in brains, and an intracranial route of infection rather than a peripheral one increases the consistency and extent of brain neuron infection (Fig. 1) (17, 18, 21). Groups of 6- to 8-week-old mice of the two flavivirus-susceptible C57BL/6 lines, Oas1b-tr/RNase L^{+/+} and Oas1b-tr/RNase L^{-/-}, and the two resistant C57BL/6 lines, Oas1b-FL/RNase L^{+/+} and Oas1b-FL/RNase L^{-/-}, were inoculated by the intracranial route with either 500 PFU of YFV-17D or 100 PFU of WNV NY99 and monitored for 25 days for signs of disease. After inoculation with YFV-17D, both Oas1b-tr/RNase L^{+/+} and Oas1b-tr/RNase L^{-/-} mice became moribund and were euthanized between 10 and 20 dpi (Fig. 2A). Oas1b-tr/RNase L^{+/+} and Oas1b-tr/RNase L^{-/-} mice inoculated with WNV NY99 became moribund and were euthanized between 5 and 11 days after infection (Fig. 2B). In contrast, none of the Oas1b-FL/RNase L^{+/+} or Oas1b-FL/RNase L^{-/-} mice infected with either virus displayed any detectable disease signs during the observation period.

C. Hippocampus **D. Thalamus**

FIG 1 Analysis of flavivirus infections in the brains of resistant and susceptible mice. Six- to 8-week-old *Oas1b*-tr/RNase L^{+/+} or *Oas1b*-FL/RNase L^{-/-} mice were injected intracranially with 100 PFU of WNV NY99 (A to C) or 500 PFU of YFV 17D (D) diluted in sterile PBS in a volume of 10 μ l. At different days postinfection, mice were perfused with 4% paraformaldehyde and the brains were sectioned and processed for IFA using anti-NeuN antibody (green) to detect neurons. Virus infection was detected using anti-WNV NS3 antibody (red) and anti-dsRNA antibody (white) (A to C) or anti-dsRNA antibody (red) alone (D). Hoechst 33258 (blue) was used to stain nuclei. (A to D) The region of the brain analyzed by IFA is indicated in the diagram at the upper left. The percentage of infected NeuN-positive cells in each brain region was calculated as described in Materials and Methods. Scale bars, 13 μ m. Enlarged panel scale bars, 8 μ m. (D) Scale bars, 18 μ m. Enlarged panel scale bars, 9 μ m.

FIG 2 Comparison of the effect of RNase L knockout on survival of resistant and susceptible mice infected intracranially with a flavivirus. (A) Survival of 6- to 8-week-old Oas1b-tr/RNase L^{+/+} ($n = 14$), Oas1b-tr/RNase L^{-/-} ($n = 10$), Oas1b-FL/RNase L^{+/+} ($n = 15$), and Oas1b-FL/RNase L^{-/-} ($n = 15$) mice infected with 500 PFU of YFV-17D. (B) Survival of 6- to 8-week-old Oas1b-tr/RNase L^{+/+} ($n = 11$), Oas1b-tr/RNase L^{-/-} ($n = 13$), Oas1b-FL/RNase L^{+/+} ($n = 11$), and Oas1b-FL/RNase L^{-/-} ($n = 14$) mice infected with 100 PFU of WNV NY99. Virus diluted in sterile PBS was injected intracranially in a volume of 10 μ l. Mice were monitored daily for symptoms and euthanized when moribund.

The data indicate that the lack of RNase L does not alter the survival phenotype of either the resistant or susceptible mice after an intracranial flavivirus inoculation.

Analysis of WNV titers in the brains of RNase L^{-/-} and RNase L^{+/+} resistant and susceptible mice. Flavivirus titers in the brains of flavivirus-infected resistant mice were previously shown to be lower than those in the brains of susceptible mice (21–23). Although neither the percent survival nor time of death was altered in the infected susceptible RNase L^{-/-} mice and all of the resistant RNase L^{-/-} mice survived, it was possible that the RNase L^{-/-} mice produced higher brain virus titers than the RNase L^{+/+} mice. To investigate this possibility, groups of 6- to 8-week-old susceptible Oas1b-tr/RNase L^{+/+} and Oas1b-tr/RNase L^{-/-} and resistant Oas1b-FL/RNase L^{+/+} and Oas1b-FL/RNase L^{-/-} mice were inoculated intracranially with 100 PFU of WNV, and virus titers in brain homogenates were determined by plaque assay (Fig. 3A). At 2 dpi, the levels of infectious virus were low (10^2 to $10^{2.5}$ PFU/g) in the brains of all four strains of mice. At 3 dpi, the virus titers in the brains of the Oas1b-tr/RNase L^{-/-} mice had increased to $10^{4.4}$ PFU/g. In contrast, in the brains of Oas1b-tr/RNase L^{+/+} mice, the virus titers had not increased above the level at day 2. At 4 dpi, the Oas1b-tr/RNase L^{+/+} and Oas1b-tr/RNase L^{-/-} virus titers had increased to $10^{5.4}$ PFU/g and $10^{5.6}$ PFU/g, respectively. The virus titers in the brains of the resistant Oas1b-FL/RNase L^{+/+} and Oas1b-FL/RNase L^{-/-} mice remained low from 3 through 6 dpi. Average peak virus yields were reached in all four of the mouse strains at 5 dpi and were $10^{6.5}$ PFU/g in the Oas1b-tr/RNase L^{-/-} brains, 10^6 PFU/g in the Oas1b-tr/RNase L^{+/+} brains, 10^3 PFU/g in the Oas1b-FL/RNase L^{+/+} brains, and $10^{2.2}$ PFU/g in the Oas1b-FL/RNase L^{-/-} brains. Both types of susceptible mice became moribund by 7 dpi, while both types of resistant mice displayed no disease signs. Brain virus titers in both types of resistant mice decreased to undetectable levels by 8 dpi. The susceptible Oas1b-tr/RNase L^{-/-} mice produced significantly increased virus yields compared to those of the Oas1b-tr/RNase L^{+/+} mice only at 3 dpi, while resistant Oas1b-FL/RNase L^{+/+} and Oas1b-FL/RNase L^{-/-} mice produced similarly low virus yields at all times tested.

WNV genome RNA levels in the brains of RNase L^{-/-} and RNase L^{+/+} resistant and susceptible mice. RNase L was previously shown to cleave WNV genome RNA (17). To determine whether the presence of RNase L decreases viral genomic RNA levels in the brains of infected mice, groups of 6- to 8-week-old susceptible Oas1b-tr/RNase L^{+/+} and Oas1b-tr/RNase L^{-/-} and resistant Oas1b-FL/RNase L^{+/+} and Oas1b-FL/RNase L^{-/-} mice were infected intracranially with 100 PFU of WNV. Total RNA was extracted from brains harvested on various days postinfection, and the level of viral genomic RNA

FIG 3 Analysis of the effect of RNase L on virus yields and RNA in the brains of resistant and susceptible mice infected with WNV NY99. Groups of 6- to 8-week old *Oas1b*-tr/*RNase L*^{+/+}, *Oas1b*-tr/*RNase L*^{-/-}, *Oas1b*-FL/*RNase L*^{+/+}, and *Oas1b*-FL/*RNase L*^{-/-} mice were injected intracranially with 100 PFU of WNV NY99 diluted in sterile PBS in a volume of 10 μ l. (A) Brains were removed aseptically, and half of each brain was used to determine virus titers in tissue homogenates by plaque assay on BHK cells. Titers of each sample were determined in duplicate. Horizontal bars represent the means. ***, $P < 0.005$; ****, $P < 0.001$; determined by 2-way analysis of variance (ANOVA) compared to the *Oas1b*-tr/*RNase L*^{+/+} values. (B) Total RNA was extracted from the other half of each brain. Viral RNA was quantified by qRT-PCR, and RNA copy number was determined. The level of viral RNA in each sample was normalized to that of *Gapdh* mRNA in the same sample. Horizontal bars represent the means. *, $P < 0.05$; ****, $P < 0.001$; determined by 2-way ANOVA compared to *Oas1b*-tr/*RNase L*^{+/+} values. Each data point represents the value determined for a single mouse brain.

was determined by real-time quantitative reverse transcription-PCR (qRT-PCR) (Fig. 3B). Equivalent low levels of viral RNA were detected in the brains of the susceptible *Oas1b*-tr/*RNase L*^{+/+} and of both resistant *Oas1b*-FL/*RNase L*^{+/+} and *Oas1b*-FL/*RNase L*^{-/-} mice at 2 dpi. However, the viral RNA levels were significantly higher at 2 dpi in the brains of the *Oas1b*-tr/*RNase L*^{-/-} mice. By 4 dpi, the viral RNA levels had markedly increased in both the *Oas1b*-tr/*RNase L*^{-/-} and *Oas1b*-tr/*RNase L*^{+/+} brains and continued to increase on day 5. In contrast, in the resistant *Oas1b*-FL/*RNase L*^{+/+} and *Oas1b*-FL/*RNase L*^{-/-} brains, the viral RNA levels had increased only slightly above the day 2 levels at 4 and 5 dpi and decreased on day 6. Viral RNA levels were significantly higher in the brains of susceptible *Oas1b*-tr/*RNase L*^{-/-} mice than those of the other strains at 2 dpi, and a slight increase in the viral RNA levels was detected in the *Oas1b*-FL/*RNase L*^{-/-} brains at 4 and 6 dpi. These results are consistent with the activation of RNase L antiviral activity by virus infection in the *RNase L*^{+/+} brains.

Comparison of the type 1 interferon response to WNV infection in the brains of *RNase L*^{-/-} and *RNase L*^{+/+} resistant and susceptible mice. The induction of gene expression regulated by the type 1 IFN pathway following an intracranial WNV infection (100 PFU) was analyzed in the brains of 6- to 8-week-old *Oas1b*-tr/*RNase L*^{+/+}, *Oas1b*-tr/*RNase L*^{-/-}, *Oas1b*-FL/*RNase L*^{+/+}, and *Oas1b*-FL/*RNase L*^{-/-} mice. Total cellular RNA was extracted from the brains of infected and mock-infected animals, and *Ifn β* , *Mx1*, *Oas1a*, and *Oas1b* mRNA expression was analyzed by qRT-PCR. At 2 dpi, the relative induction of *Ifn β* mRNA in the brains of each of the four mouse strains was 10-fold or less (Fig. 4A). At 4 dpi, *Ifn β* mRNA was induced by an average of 250-fold in

FIG 4 Comparison of *Ifnβ*, *Mx1*, *Oas1a*, and *Oas1b* mRNA expression in the brains of WNV-infected susceptible and resistant mice. Six- to 8-week-old Oas1b-tr/RNase L^{+/+}, Oas1b-tr/RNase L^{-/-}, Oas1b-FL/RNase L^{+/+}, and Oas1b-FL/RNase L^{-/-} mice were injected intracranially with 100 PFU of WNV NY99. Brains were aseptically removed and total RNA was extracted. (A to D) The levels of *Ifnβ* (A), *Mx1* (B), *Oas1a* (C), and *Oas1b* (D) mRNA were determined by qRT-PCR. C_t values were normalized to the levels of *Gapdh* mRNA in each sample, and the fold change relative to the average level in the brains of control mock-infected animals was calculated. Each data point represents the RNA value determined for a single mouse brain. Horizontal bars represent the means. **, *P* < 0.01; ***, *P* < 0.005; ****, *P* < 0.001; determined by 2-way ANOVA.

susceptible Oas1b-tr/RNase L^{+/+} brains and by 500-fold in Oas1b-tr/RNase L^{-/-} mouse brains. At 6 dpi, *Ifnβ* mRNA expression was induced by approximately 1,000-fold in the brains of both susceptible mouse strains. In contrast, *Ifnβ* mRNA levels had increased only slightly from the 2-dpi levels in the resistant Oas1b-FL/RNase L^{+/+} and Oas1b-FL/RNase L^{-/-} brains. The lower induction in *Ifnβ* mRNA expression detected in the brains of the resistant mice compared to that of the susceptible brains was consistent with the lower virus titers produced in the resistant brains at 4 and 6 dpi (Fig. 3A).

At 2 dpi, the relative induction of *Mx1* mRNA expression in WNV-infected, susceptible Oas1b-tr/RNase L^{+/+} brains was about 200-fold, while that in the Oas1b-tr/RNase L^{-/-} brains was about 750-fold. There was a 70-fold induction of *Mx1* mRNA expression in the resistant Oas1b-FL/RNase L^{+/+} brains and a 30-fold induction in the Oas1b-FL/RNase L^{-/-} brains (Fig. 4B). At 4 and 6 dpi, the fold induction of *Mx1* mRNA expression was 2,500 in the Oas1b-tr/RNase L^{+/+} brains and 2,000 in the Oas1b-tr/RNase L^{-/-} brains. At 4 dpi, the fold induction of *Mx1* mRNA in the Oas1b-FL/RNase L^{+/+} brains was

160, while that in the *Oas1b*-FL/*RNase L*^{-/-} brains was about 470. At 6 dpi, the levels of *Mx1* mRNA had decreased slightly in the brains of both strains of resistant mice. The induction of *Mx1* mRNA expression was lower in the resistant brains than in the susceptible brains, consistent with the lower levels of IFN- β mRNA expression. The *Mx1* expression levels in the brains of mice with and without *RNase L* were not significantly different.

For *Oas1a* mRNA, a 3-fold induction in susceptible *Oas1b*-tr/*RNase L*^{+/+} brains, a 2-fold induction in resistant *Oas1b*-FL/*RNase L*^{+/+} brains, an 11-fold induction in *Oas1b*-tr/*RNase L*^{-/-} brains, and a 20-fold induction in *Oas1b*-FL/*RNase L*^{-/-} brains was observed at 2 dpi (Fig. 4C). At 4 dpi, the fold induction of *Oas1a* mRNA was 12 in susceptible *Oas1b*-tr/*RNase L*^{+/+} brains, 2 in resistant *Oas1b*-FL/*RNase L*^{+/+} brains, 65 in *Oas1b*-tr/*RNase L*^{-/-} brains, and 360 in *Oas1b*-FL/*RNase L*^{+/+} brains. At 6 dpi, the relative levels of *Oas1a* mRNA in *Oas1b*-tr/*RNase L*^{-/-}, *Oas1b*-FL/*RNase L*^{+/+}, and *Oas1b*-FL/*RNase L*^{-/-} brains had not increased from the 4-dpi levels, whereas the expression levels in the *Oas1b*-FL/*RNase L*^{+/+} mouse brains had increased slightly. By 4 dpi, the relative expression levels of *Oas1a* mRNA in both the resistant and susceptible *RNase L*^{-/-} brains were higher than the levels in the corresponding *RNase L*^{+/+} brains, with a higher increase observed in the resistant *RNase L*^{-/-} brains.

The expression of *Oas1b* mRNA in WNV-infected susceptible *Oas1b*-tr/*RNase L*^{+/+} mouse brains was induced by 5-fold on day 2, by 6-fold on day 4, and by 12-fold on day 6 (Fig. 4D). In the brains of *Oas1b*-tr/*RNase L*^{-/-} mice, *Oas1b* mRNA expression levels were induced by 12-fold at 2 dpi, by 30-fold at 4 dpi, and by 22-fold at 6 dpi. The premature stop codon present in the *Oas1b*-tr mRNA is expected to increase the turnover rate of this mRNA. Expression of *Oas1b* mRNA in the brains of the *Oas1b*-FL/*RNase L*^{+/+} mice was induced by 25- to 40-fold at all times tested. In contrast, in the brains of the *Oas1b*-FL/*RNase L*^{-/-} mice, *Oas1b* mRNA was induced by 56-fold at 2 dpi, 410-fold at 4 dpi, and 220-fold at 6 dpi. The results indicate that *RNase L* is activated in the infected brains. The lower levels of *Oas1b* mRNA in the *Oas1b*-FL/*RNase L*^{+/+} brains compared to those in the *Oas1b*-FL/*RNase L*^{-/-} brains at 4 and 6 dpi and the higher expression of *Oas1a* mRNA in the brains of the resistant and susceptible *RNase L*^{-/-} mice suggest a role for *RNase L* in the turnover of these mRNAs consistent with previous observations (24).

DISCUSSION

Our previous studies of the characteristics of the *Oas1b*-mediated, flavivirus genetic resistance phenotype utilized congenic C3H.PRI-Flv^r and C3H/He mice that were generated by a backcross breeding protocol and differed by an ~240 centiMorgan region in the resistant mice inherited from the PRI parent that contained the resistance allele (25). A subsequent study utilized congenic BALB/c strains that differed by a region containing 27 genes (26). As one means of addressing whether additional genes exerted effects on the resistance phenotype, three lines of susceptible BALB/c transgenic mice were generated that expressed the endogenous *Oas1b*-tr protein and also a recombinant *Oas1b*-FL protein under the control of a CAG promoter after random insertion of a cDNA plasmid into the cell genome (26). Even though very high basal levels of the recombinant *Oas1b* were expressed in the brains of these mice, they were only partially resistant to a peripheral WNV infection, and the expression of the transgene was shown to be reduced by a WNV infection (26). The resistant mouse strain utilized in the present study was made by replacing the *Oas1b*-tr allele C-terminal coding sequence in the susceptible C57BL/6 *Oas1b*-tr/*RNase L*^{+/+} mouse genome with the corresponding *Oas1b*-FL allele sequence from the resistant C3H.PRI-Flv^r genome by homologous recombination (10). The generation of this mouse strain provided an improved congenic pair that differ by only a single gene insertion.

The efficiency and progression of intracranial WNV and YFV infections in the brains of susceptible C57BL/6 *Oas1b*-tr/*RNase L*^{+/+} and resistant C57BL/6 *Oas1b*-FL/*RNase L*^{+/+} mice were compared. Unique aspects of this study are the use of congenic resistant and susceptible mouse strains that differ by only a single *Oas1b* gene

insertion, the comparison of brain infections with the neurovirulent WNV NY99 and the YFV 17D vaccine strain, and the comprehensive IFA analysis of infected neurons in different regions of the brain at different times after infection. At early days after an intracranial flavivirus infection, the number and distribution of infected neurons in the brains of resistant and susceptible mice were equivalent and the intensity and area of the perinuclear dsRNA and NS3 IFA signals in the infected neurons were similar, indicating that the infection efficiency did not differ in resistant and susceptible brains. At later days postinfection with either YFV or WNV, the intensity and area of the viral signals increased markedly in the infected susceptible neurons but were not amplified in the infected resistant neurons. Consistent with these observations, the WNV titers and genome RNA levels were lower in the brains of the resistant mice than in those of the susceptible mice. These results were consistent with the data from previous studies showing that intracellular WNV genome RNA levels in resistant C3H/RV MEFs were lower than those in susceptible C3H/HeJ MEFs (17), and that Murray Valley encephalitis virus replicative form RNA (dsRNA) and genome RNA levels were markedly lower in brain extracts from resistant C3H/RV than from susceptible C3H/He mice. The overall conclusion from the data is that the Oas1b-mediated inhibition of a flavivirus infection does not affect initial infection efficiency but reduces viral replication and virus spread at later times.

Data from our previous studies indicated that RNase L has anti-flavivirus activity. Activated RNase L cleaves WNV genomic RNA, and an C57BL/6 Oas1b-tr/RNase L^{-/-} MEF cell line produced 5- to 10-fold higher WNV yields than a C57BL/6 Oas1b-tr/RNase L^{+/+} MEF cell line (17). At early times after infection with a flavivirus, viral RNA replication is low, with similar levels of minus and plus RNAs produced, and the nascent plus strands are preferentially used for protein translation (27). It was previously shown that during the early stage of the virus replication cycle, flavivirus RNA is membrane associated but is partially exposed and able to activate the OAS pathway, leading to degradation of some of the early viral RNA (28, 29). As the infection progresses, replicating viral RNA is sequestered in endoplasmic reticulum invaginations and viral factory organelles and protected from cleavage by RNase L (30). The activation of RNase L and the susceptibility of viral RNA to RNase L degradation was indicated by the 10-fold higher WNV genome RNA levels detected at 2 dpi in the brains of Oas1b-tr/RNase L^{-/-} mice compared to those in the brains of Oas1b-tr/RNase L^{+/+} mice. The large increase in WNV RNA levels in susceptible Oas1b-tr/RNase L^{+/+} and Oas1b-tr/RNase L^{-/-} brains at 4 dpi coincided with the amplification of the dsRNA signal in infected neurons in susceptible brains observed by IFA and indicated that RNA replication was occurring. A detectable effect of RNase L was not observed at later times, consistent with the expected sequestering of the viral RNA.

A previous *in vivo* study found that 70% of C57BL/6 Oas1b-tr/RNase L^{+/+} but only 50% of C57BL/6 Oas1b-tr/RNase L^{-/-} mice survived a peripheral infection with WNV (18). However, after an intracranial injection of 10 PFU of WNV NY99, none of the mice of either strain survived and no decrease in the survival time in C57BL/6 Oas1b-tr/RNase L^{-/-} compared to that of C57BL/6 Oas1b-tr/RNase L^{+/+} mice was observed (18). In the current study, as expected, none of the susceptible C57BL/6 Oas1b-tr/RNase L^{+/+} or C57BL/6 Oas1b-tr/RNase L^{-/-} mice survived an intracranial injection of 100 PFU of WNV NY99, and no difference was observed in the median survival time. However, evidence of an antiviral effect of activated RNase L was indicated by the increased virus yields and viral RNA levels observed at early times after infection in the brains of the Oas1b-tr/RNase L^{-/-} mice. The results indicate that although RNase L is activated in the brains of WNV-infected C57BL/6 Oas1b-tr/RNase L^{+/+} mice, the RNase L antiviral activity in the brains of susceptible mice is not sufficient to alter the infection outcome or survival time even when a 10-fold lower dose of WNV NY99 is used.

We previously utilized stable expression of a dominant-negative RNase L mutant to decrease RNase L activity in a resistant SV40-transformed C3H/RV MEF line and observed a 5- to 10-fold increase in WNV yield, depending on the multiplicity of infection (MOI) (17). However, the virus yields produced by these cells were still lower than those

produced by a susceptible C3H/HeJ MEF line expressing the empty vector. For the present study, to obtain a more biologically relevant experimental system, a line of C57BL/6 Oas1b-FL/RNase L^{-/-} mice was generated by breeding and used to test the contribution of RNase L to the Oas1b-mediated resistance phenotype *in vivo*. We used a brain infection because the Oas1b-mediated resistance phenotype is very robust in brains and an intracranial route of infection rather than a peripheral one to increase the consistency and extent of brain neuron infection (17, 18, 21). None of the resistant C57BL/6 Oas1b-FL/RNase L^{+/+} or C57BL/6 Oas1b-FL/RNase L^{-/-} mice displayed any signs of disease, and all survived an intracranial infection with either WNV or YFV. No difference in the brain virus titers and only a slight increase in viral RNA levels was detected in the brains of the Oas1b-FL/RNase L^{-/-} mice compared to those of the Oas1b-FL/RNase L^{+/+} mice. Both the viral RNA levels and viral yields remained low in these mice through 6 dpi and declined thereafter. The data suggest that the strong effect of Oas1b on reducing viral RNA levels in infected neurons masks the antiviral effect of activated RNase L through cleavage of viral RNA.

Although Oas1b is an ISG, data from a previous study showed that the expression of the resistance phenotype in mice and in MEFs did not require either type 1 IFN pretreatment or production, and the resistance phenotype in MEFs was not inhibited by treatment with anti-IFN antibody (22). However, pretreatment of resistant MEFs with type 1 IFN further reduced virus yields (17). Although some of the flavivirus nonstructural proteins inhibit the type 1 IFN signaling pathway (31), which would be expected to reduce upregulation of ISGs, we previously observed that a subset of ISGs, including Oas1a and Oas1b, was upregulated by a WNV infection in MEFs in a type 1 IFN-independent manner (17, 32). A comparison of the type 1 IFN- β response in the brains of WNV-infected resistant Oas1b-FL/RNase L^{+/+} and susceptible Oas1b-tr/RNase L^{+/+} mice in the present study indicated that although the kinetics of upregulation of *Ifn β* mRNA were similar in the two mouse strains, the level of expression was higher in the brains of the susceptible than the resistant mice at all times, which is consistent with the lower virus yields produced by the resistant mice. The induction of *Ifn β* mRNA was similar in RNase L^{+/+} and RNase L^{-/-} mice of the same type. Even though the induction of *Ifn β* mRNA was lower in the brains of resistant mice, *Mx1*, *Oas1a*, and *Oas1b* mRNAs were upregulated in the brains of both types of mice by 2 dpi. However, the levels of *Oas1b* mRNA in the two resistant strains were moderately higher than those in the two susceptible strains. Simon-Chazottes et al. (26) suggested that promoter sequence variations between the *Oas1b* genes of resistant and susceptible mice contribute to lower *Oas1b* mRNA induction by a WNV infection in susceptible mice, but this would not be the case for the pair of resistant and susceptible mice used in the present study because the promoters for the two alleles were identical. Another possible explanation is that the premature stop codon in the *Oas1b-tr* mRNA increases its turnover rate by the nonsense decay pathway (33–35).

The *Oas1a* and *Oas1b* mRNAs were upregulated in the Oas1b-FL/RNase L^{+/+} mice at 2 dpi but did not increase much thereafter, whereas the *Oas1a* and *Oas1b* mRNA levels in the Oas1b-FL/RNase L^{-/-} brains increased by 4 dpi. Also, *Oas1a* mRNA levels in the Oas1b-tr/RNase L^{-/-} brains were higher than those in the Oas1b-tr/RNase L^{+/+} brains at 4 dpi. A previous study showed that RNase L cleavage activity is involved in regulating the levels of some ISG mRNAs (24). The decreased levels of *Oas1a* and *Oas1b* mRNA observed in the Oas1b-FL/RNase L^{+/+} brains compared to those of the Oas1b-FL/RNase L^{-/-} brains indicate that RNase L activity was activated in response to a WNV infection in resistant and susceptible RNase L^{+/+} mice. The decreased levels of *Oas1a* mRNA would be expected to have a proviral effect in both resistant and susceptible RNase L^{+/+} mice, while the decreased levels of *Oas1b* mRNA would have a proflavivirus effect only in the resistant mice. However, the resistance phenotype was very robust in the resistant RNase L^{+/+} mice, indicating that RNase L activity is not a required component of the Oas1b-mediated flavivirus resistance phenotype.

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney 21/W12 (BHK) cells were maintained in minimal essential medium (MEM) containing 2.5% fetal bovine serum (FBS) and 10 $\mu\text{g/ml}$ gentamicin at 37°C in a 5% CO₂ atmosphere (36).

YFV-17D lyophilized vaccine (Wyeth Laboratories) was reconstituted in sterile phosphate-buffered saline (PBS) according to the manufacturer's instructions. The virus titer was 4×10^6 PFU/ml. An aliquot of WNV strain NY99 was obtained from Robert Tesh (World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch). A WNV NY99 virus stock was prepared by infecting confluent BHK monolayers at an MOI of 0.1. Culture fluid was harvested at 42 h postinfection (hpi), clarified by low-speed centrifugation, aliquoted, and stored at -80°C . The titer of this stock was 6.5×10^6 PFU/ml. The infectivity of the YFV and WNV samples was determined by plaque assay on BHK cells as previously described (37). Briefly, virus was serially diluted in MEM supplemented with 2.5% FBS and adsorbed onto confluent BHK monolayers for 1 h at 37°C. After the incubation period, the virus inoculum was aspirated and the cells were washed and then overlaid with 2 ml/well of 1% SeaChem agarose (Bio-Whittaker Molecular Applications) mixed 1:1 with 2 $\times$ MEM containing 2.5% fetal calf serum. The cells were incubated at 37°C for 72 hpi and then stained using 0.05% crystal violet in 10% ethanol. All samples were assayed in duplicate.

Generation of an *Oas1b*^{+/+}/*RNase L*^{-/-} mouse strain. All animal experiments were approved by the Georgia State University Institutional Animal Care and Use Committee and were performed in compliance with the Public Health Service policy on humane care and use of laboratory animals. C57BL/6 breeding pairs were obtained from Jackson Laboratory and used to initiate a colony at Georgia State University. C57BL/6 mice are homozygous for the susceptibility allele of the *Oas1b* gene, which expresses *Oas1b*-tr. A strain of flavivirus-resistant mice, C57BL/6 *Oas1b*-FL/*RNase L*^{+/+}, was previously generated by partially replacing the *Oas1b*-tr allele sequence in the susceptible C57BL/6 *Oas1b*-tr/*RNase L*^{+/+} mouse genome with the *Oas1b* allele sequence from the resistant C3H.PRI-Flv^r genome using a homologous recombination strategy (10). C57BL/6 mice homozygous for *RNase L* knockout (*Oas1b*-tr/*RNase L*^{-/-}) were obtained from Robert Silverman (Cleveland Clinic). C57BL/6 *Oas1b*-FL/*RNase L*^{+/+} mice were bred to the *Oas1b*-tr/*RNase L*^{-/-} mice. The F1 progeny were then mated and the F2 progeny were genotyped by Transnetyx for each allele of interest (10, 20). Animals homozygous for both the *Oas1b*-FL and *RNase L*^{-/-} alleles were bred to establish a colony of *Oas1b*-FL/*RNase L*^{-/-} mice.

Mouse survival experiments. Groups of 6- to 8-week-old mice were anesthetized using 2% isoflurane until a surgical plane was reached. The mice were then injected with 500 PFU of YFV-17D or 100 PFU of WNV NY99 in 0.01 ml of sterile PBS by the intracranial route using a 26.5-gauge needle fitted with a plastic guard to control the depth of insertion. Infected mice were initially observed once daily and then twice daily when symptoms of encephalitis appeared. Posture, grooming behavior, condition of the fur, activity, and presence of limb paralysis were assessed to determine disease severity. Animals assessed to be moribund by the approved endpoint criteria were euthanized and counted as having died the next day.

Analysis of infectious virus, viral RNA, and ISG mRNA levels in the brains of infected mice. On different days following intracranial inoculation of 100 PFU of WNV, groups of mice were sacrificed every day starting on day two. Mice were deeply anesthetized with isoflurane. Once at a surgical plane, the mice were decapitated and the brains aseptically removed. The brains were cut in half on the sagittal plane using a sterile razor blade. Each half was weighed and stored at -80°C . One-half of each brain was placed into a Dounce homogenizer on ice, and the correct amount of ice-cold Dulbecco's MEM containing 2.5% fetal calf serum to give a 10% (wt/vol) homogenate was added. After being Dounce homogenized 20 times, the homogenate was centrifuged at $2,000 \times g$ at 4°C. The supernatant was collected and centrifuged at $10,000 \times g$ at 4°C. The clarified supernatant was aliquoted, stored at -80°C , and used to determine virus infectivity by plaque assay on BHK cells.

The other half of each brain was stored in RNA_{later} stabilization solution (ThermoFisher Scientific) at -80°C . These brain halves were Dounce homogenized 20 times in homogenizers containing the correct amount of TRI Reagent (Molecular Research Center) needed to give a 10% (wt/vol) homogenate. Total RNA was extracted according to the manufacturer's protocol. The concentration and quality of the RNA were determined using a NanoDrop UV-visible (UV-VIS) spectrophotometer (Implen). The viral RNA was quantified by real-time quantitative reverse transcription-PCR (qRT-PCR) using a TaqMan RNA-to-CT 1-step kit (Applied Biosystems) according to the manufacturer's protocol. Reaction mixtures contained 300 μg of RNA. The primer and probe set targeted the WNV NY99 NS1 region (Sigma-Aldrich). The primer sequences were the following: forward primer, 5'-GGCAGTCTCTGGGTGAAGT-3'; reverse primer 5'-GCTTCCGCTCTCTGTGGTGG-3'; TaqMan probe, 5'-6-carboxyfluorescein-TGTACGTGGCCTGAGACGCATACCTTG T-6-carboxytetramethylrhodamine-3'. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA was detected with TaqMan mouse *Gapdh* primers and probe (number 4308313; Applied Biosystems). WNV genomic RNA was normalized to cellular *Gapdh* mRNA in the same sample and was quantified using a standard curve generated from serially diluted WNV NY99 genomic RNA of a known concentration. Each sample was assayed in triplicate for each target. The cycling parameters were reverse transcription at 45°C for 15 min, TaqMan enzyme activation at 95°C for 10 min, denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min (cycle repeated 40 times).

Mouse *Ifn β* , *Mx1*, *Oas1a*, and *Oas1b* mRNAs in mouse brain homogenates were quantified by qRT-PCR performed in a 10- μl reaction mixture containing 500 ng of total RNA (determined with a NanoDrop UV-VIS spectrophotometer), 1 μM the target gene primer pair, and 0.2 μM the target gene probe. Applied Biosystems Assays-on-Demand 20 $\times$ primer and fluorogenic TaqMan 6-carboxyfluorescein/minor groove binder probe mixes Mm00439552 for IFN- β , Mm00487796 for *Mx1*, Mm00836412 for *Oas1a*, and

Mm0049297 for *Oas1b* were used. The *Oas1b* probe used spans the first and second exons of this gene and detects *Oas1b-FL* mRNA in resistant and *Oas1b-tr* mRNA in susceptible mouse brains. *Gapdh* mRNA was utilized as the endogenous control for each sample. The reaction conditions described above for viral RNA were also used for the ISG mRNAs. Values for target genes obtained in infected brains were normalized to *Gapdh* threshold cycle (C_t) values in the same sample and presented as fold change compared to values obtained from mock-infected brains.

Immunofluorescence assay of brain tissue. On different days following intracranial inoculation with 500 PFU of YFV-17D or 100 PFU of WNV NY99, mice were deeply anesthetized with isoflurane and then transcardially perfused first with ice-cold PBS and then with 4% paraformaldehyde. The brains were then removed and cut into 1-mm sections using a tissue slicer (Stoelting). The sections were stored at 4°C in 4% paraformaldehyde overnight and then transferred to 30% sucrose and moved to PBS when they sank. Prior to additional sectioning, the tissue sections were washed three times with PBS to remove any residual sucrose, embedded in O.C.T. compound (Sigma-Aldrich), and then cut into 10- to 20- μ m sections. The sections were permeabilized with 0.2% Triton X-100 for 20 min, blocked with 5% normal horse serum (NHS) and 0.1% Triton X-100 for 1 h, and then incubated with primary antibodies diluted in 5% NHS–0.1% Triton X-100 solution overnight at 4°C. The antibodies used were mouse anti-dsRNA (1:1,000; English & Scientific Consulting, Hungary), goat anti-WNV NS3 (1:500; R&D Systems), and chicken anti-NeuN (1:250; Millipore). Following incubation with the primary antibodies, the sections were washed three times with PBS and then incubated with donkey anti-mouse Alexa Fluor 594 and donkey anti-chicken Alexa Fluor 488-conjugated secondary antibodies (ThermoFisher Scientific) diluted in 5% NHS–0.1% Triton X-100 supplemented with Hoechst 33258 (Invitrogen) for 1 h. The sections were then washed in PBS and mounted on microscope slides with Prolong Gold antifade reagent (Invitrogen). Images were obtained with a Zeiss LSM 700 scanning microscope and then were processed using Velocity software (PerkinElmer). Quantification of the percent infected NeuN-positive cells was determined by analyzing 20 random fields from each brain region from three brains of each strain for overlapping signals from dsRNA and from NeuN. Images were chosen for analysis using a blinded method by scanning only on the Hoechst channel.

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