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Identification and characterization of a Ross River virus variant that grows persistently in macrophages, shows altered disease kinetics in a mouse model, and exhibits resistance to type I interferon

Brett A. Lidbury
University of Canberra

Nestor Rulli
University of Wollongong

Cristina M. Musso
University of Canberra

Susan B. Cossetto
University of Canberra

Ali Zaid
University of Wollongong

Publication Details
See next page for additional authors
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Abstract
Alphaviruses, such as chikungunya virus, o’nyong-nyong virus, and Ross River virus (RRV), cause outbreaks of human rheumatic disease worldwide. RRV is a positive-sense single-stranded RNA virus endemic to Australia and Papua New Guinea. In this study, we sought to establish an in vitro model of RRV evolution in response to cellular antiviral defense mechanisms. RRV was able to establish persistent infection in activated macrophages, and a small-plaque variant (RRVPERS) was isolated after several weeks of culture. Nucleotide sequence analysis of RRVPERS found several nucleotide differences in the nonstructural protein (nsP) region of the RRVPERS genome. A point mutation was also detected in the E2 gene. Compared to the parent virus (RRV-T48), RRVPERS showed significantly enhanced resistance to beta interferon (IFN) stimulated antiviral activity. RRVPERS infection of RAW 264.7 macrophages induced lower levels of IFN expression and production than infection with RRV-T48. RRVPERS was also able to inhibit type I IFN signaling. Mice infected with RRVPERS exhibited significantly enhanced disease severity and mortality compared to mice infected with RRV-T48. These results provide strong evidence that the cellular antiviral response can direct selective pressure for viral sequence evolution that impacts on virus fitness and sensitivity to alpha/beta IFN (IFN).

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Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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Authors

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Identification and Characterization of a Ross River Virus Variant That Grows Persistently in Macrophages, Shows Altered Disease Kinetics in a Mouse Model, and Exhibits Resistance to Type I Interferon

Brett A. Lidbury,1† Nestor E. Rulli,1,2,3,4† Cristina M. Musso,1† Susan B. Cossetto,1 Ali Zaid,1,2 Andreas Suhrbier,5 Harald S. Rothenfluh,6 Michael S. Rolph,1,3 and Suresh Mahalingam1,2,3,*

Virus and Inflammation Research Group, Faculty of Applied Science, University of Canberra, Canberra, ACT 2601, Australia;1 Institute for Glycomics, Griffith University, Gold Coast, QLD 4222, Australia2; Emerging Viruses and Inflammation Research Group, Institute for Glycomics, Griffith University, Gold Coast, QLD 4222, Australia3; LISIN, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Buenos Aires, Argentina4; Queensland Institute of Medical Research and Griffith University, Brisbane, Australia5; and Division of Immunology and Genetics, John Curtin School of Medical Research, Australian National University, Canberra, ACT 0200, Australia6

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Alphaviruses, such as chikungunya virus, o’nyong-nyong virus, and Ross River virus (RRV), cause outbreaks of human rheumatic disease worldwide. RRV is a positive-sense single-stranded RNA virus endemic to Australia and Papua New Guinea. In this study, we sought to establish an in vitro model of RRV evolution in response to cellular antiviral defense mechanisms. RRV was able to establish persistent infection in activated macrophages, and a small-plaque variant (RRVpers) was isolated after several weeks of culture. Nucleotide sequence analysis of RRVpers found several nucleotide differences in the nonstructural protein (nsP) region of the RRVpers genome. A point mutation was also detected in the E2 gene. Compared to the parent virus (RRV-T48), RRVpers showed significantly enhanced resistance to beta interferon (IFN-β)-stimulated antiviral activity. RRVpers infection of RAW 264.7 macrophages induced lower levels of IFN-β expression and production than infection with RRV-T48. RRVpers was also able to inhibit type I IFN signaling. Mice infected with RRVpers exhibited significantly enhanced disease severity and mortality compared to mice infected with RRV-T48. These results provide strong evidence that the cellular antiviral response can direct selective pressure for viral sequence evolution that impacts on virus fitness and sensitivity to alpha/beta IFN (IFN-α/β).

Arthritogenic alphaviruses are distributed globally and are maintained in nature by cycles of transmission between hematophagous arthropods (for example, mosquitoes) and enzootic vertebrate hosts (mammals, marsupials, or birds) (33, 53). Nearly all symptomatic infections with these alphaviruses manifest with joint symptoms (arthritis and arthralgia), with myalgia, rash, and lethargy also being common. Ross River virus (RRV) is an Australian alphavirus associated with chronic polyarthritis and causes up to 8,000 cases annually in Australia, with the number of cases reported in new localities increasing, (42, 55). During the late 1970s and early 1980s, a number of South Pacific island nations experienced a major outbreak of RRV disease (RRVD) affecting more than 50,000 people (17). More recently, a related virus, chikungunya virus (CHIKV), caused similar rheumatic disease in one-third of the population of Réunion Island in the Western Indian Ocean and an estimated 1.39 million cases in India (22, 23, 37, 52). This outbreak was also associated for the first time with some severe clinical manifestation and mortality (37).

In RRV-infected patients, the disease is usually severe at onset with a gradual resolution over 3 to 6 months (40). Alphavirus-induced rheumatic disease is thought to have a substantial immunopathological component. For example, in animal models tissue damage results from the induction of proinflammatory cytokines and chemokines and recruitment of macrophages in response to infection (29, 43, 55). RRV infects and replicates in human and mouse macrophages, and infection of the mouse macrophage cell line RAW 264.7 results in the establishment of a persistent productive infection (11, 31, 36, 60), which has been regarded as a model of how RRV persists in synovial tissues and produces arthritis/arthralgia (54, 55). Persistence of RRV occurs in RAW 264.7 cells despite the ability of these cells to produce beta interferon (IFN-β) (30, 36), which is known to effectively control alphavirus replication (44).

There is considerable evidence that RRV can persist and establish chronic infection. For example, Soden et al. (50) showed genetic evidence of RRV in synovial tissue of 2 patients from a cohort of 12, 5 weeks after initial symptoms of RRV infection were reported. We have also obtained evidence for chronic infection in a mouse model of RRV infection, with RRV RNA being detected in the ankle joints of mice 3 months after infection (N. Rulli and S. Mahalingam, unpublished data). A number of cell culture models of RRV persistence have also been described. Journeaux et al. (21) established long-term infection (up to 35 days) of primary human synovial cell cultures (which were found to contain “macrophage-like cells”). Small-plaque variants developed in these cultures, suggesting that RRV underwent mutation and adaptation to the
culture conditions. We have previously described persistent RRV infection in macrophages for periods up to 180 days (60). We have also recently observed persistent infection in murine and human osteoblast cultures, suggesting that these cells may also be persistently infected in vivo (N. Rulli, R. Li, P. Smith, A. Choo, C. Musso, Y. C. Su, B. Lidbury, and S. Mahalingam, unpublished data). Long-term persistence of antigen from the related alphavirus chikungunya virus has recently been found in perivascular synovial macrophages in one chronically infected patient 18 months after initial infection (19). With a macaque model, Labadie et al. observed long-term chikungunya virus infection in joints, muscles, lymphoid organs, and liver, which may explain the long-lasting disease symptoms observed in humans (26). In addition, the authors identified macrophages as the main cellular reservoirs during the late stages of chikungunya virus infection in vivo (26).

Here we describe the generation of a small-plaque mutant of RRV (RRVPERS) derived from persistently infected RAW 264.7 macrophages that had been stimulated with lipopolysaccharide (LPS) to induce an antiviral phenotype, including the production of alpha/beta interferon (IFN-α/β) (30, 34). RRVPERS displayed significantly increased resistance to IFN-β-induced antiviral activity compared to the parental RRV-T48 virus. RRVPERS also induced lower levels of type I IFN production of alpha/beta interferon (IFN-α/β/H9251) for RRV plaque assay. Fresh and warmed EMEM-FCS (500 l per well) was added to a fresh monolayer of confluent Vero cells in culture conditions. We have previously described persistent RRV infection in macrophages for periods up to 180 days (60). After the second round of purification, stocks of small- and large-plaque (parent) virus were grown in fresh Vero cell cultures, as described above; these stocks were used for all subsequent experiments and nucleotide sequence analyses. The small-plaque RRV was designated RRVPERS. These studies are covered by a license from the Australian Government’s Office of Gene Technology Regulator (license number DNIR 389/2006).

MATERIALS AND METHODS

Ross River virus and macrophage cell line. Ross River virus (RRV) derived from an infected clone of strain T48 (originally designated R664) (25) was used to initially infect the mouse macrophage cell line RAW 264.7 (ATCC TIB-71) cultured at 50 × 10^6 cells per well/ml in 24-well trays (Nunc, Roskilde, Denmark). To produce infectious virus, RPR64 was linearized by SacI digestion and transcribed in vitro from the cDNA using SP6 RNA polymerase, and the infectious RNA was transfigured into BHK-21 cells as previously described (25). Viral stocks were propagated in Vero cells (ATCC CCL-81), as previously described (26), and no virus stock exceeded two Vero cell passages prior to experimental use. Viral titers were determined by plaque assay with Vero cells (see details below).

RAW 264.7 cells were maintained in Eagle minimal essential medium (EMEM) (Thermo-Trace, Melbourne, Australia) supplemented with 5% heat-inactivated fetal calf serum (HI-FCS) (Thermo-Trace), 1% penicillin-streptomycin, 1 to 1.5% sodium bicarbonate, and 2.0 mM l-glutamine (Thermo-Trace). Following RRV infection, 5.0 ng/ml lipopolysaccharide (LPS) (Escherichia coli serotype 0111:B4; Sigma) was added to the cultures, and this LPS concentration was maintained over the entire experimental period.

Following RRV infection, 5.0 ng/ml lipopolysaccharide (LPS) to induce an antiviral phenotype, including the production of IFN-α/β (IFN-α/β/H9251) for RRV plaque assay. Fresh and warmed EMEM-FCS (500 l per well) was added to a fresh monolayer of confluent Vero cells in culture conditions. We have previously described persistent RRV infection in macrophages for periods up to 180 days (60). After the second round of purification, stocks of small- and large-plaque (parent) virus were grown in fresh Vero cell cultures, as described above; these stocks were used for all subsequent experiments and nucleotide sequence analyses. The small-plaque RRV was designated RRVPERS. These studies are covered by a license from the Australian Government’s Office of Gene Technology Regulator (license number DNIR 389/2006).

RRV neutralization by polyclonal anti-RRV sera and anti-RRV-E2 monoclonal antibodies. Marine anti-RRV polyclonal serum was added to Hanks balanced salt solution (HBSS) (pH 7.2), Thermo-Trace, Melbourne, Australia) containing 2% (wt/vol) bovine serum albumin (BSA) (Sigma, Missouri), to a final dilution of 5.0 × 10^-5. Serial 10-fold dilutions of this polyclonal serum were then prepared. An equal volume of HBSS-BSA containing 200 PFU of RRV-T48 or RRVPERS was added to the diluted antibody. The antibody-virus preparations were incubated at 37°C for 1 h, after which 100 μl was plated on confluent Vero cell monolayers and incubated at 37°C (5% CO₂, 95% humidity) for 48 h. The monolayers were stained for plaque enumeration exactly as described above.

Reaction of RRVPERS or RRV-T48 with E2-specific monoclonal antibodies (MAbs) was performed exactly as described above for polyclonal sera. MAbs designated 109C, 3C4, and E7 were generously provided by Ron Weisz (Australi- an National University) and Roy Hall (University of Queensland) from a previously described antibody panel (6).
TABLE 1. Primers used for RT-PCR isolation and nucleotide sequence analysis of the RRV E2 gene from a small-plaque variant of RRV-T4

<table>
<thead>
<tr>
<th>Primer</th>
<th>Polarity</th>
<th>Position (5')</th>
<th>Oligonucleotide sequence (5'-3')</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>+</td>
<td>8545</td>
<td>GAAACAGATACGCAGCCACCGGC</td>
<td>1,289</td>
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<td>P2</td>
<td>−</td>
<td>9834</td>
<td>CTGGCTTGCCCTCCGTCGG</td>
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<tr>
<td>P3</td>
<td>+</td>
<td>8946</td>
<td>GTCAATACAGCAGCAGCG</td>
<td>798</td>
</tr>
<tr>
<td>P4</td>
<td>−</td>
<td>9454</td>
<td>TAGGAGAAGAGCTGGTGGTGG</td>
<td>1,490 (with P1)</td>
</tr>
<tr>
<td>P5</td>
<td>−</td>
<td>10035</td>
<td>GAAATGTTGGCGTGTGGTGG</td>
<td>1,490 (with P1)</td>
</tr>
</tbody>
</table>

a Position of primer in complete RRV genome (refer to reference 10).

TABLE 2. Primers used for nucleotide sequence analysis of the RRV nsP1 to nsP4 genes from RRV-PERS

<table>
<thead>
<tr>
<th>Primer</th>
<th>Polarity</th>
<th>Position (5')</th>
<th>Oligonucleotide sequence (5'-3')</th>
<th>Product size (bp)</th>
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<td>P2</td>
<td>−</td>
<td>662</td>
<td>GAAATACCTGGTGGTATGCGG</td>
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<tr>
<td>P3</td>
<td>+</td>
<td>603</td>
<td>GAGATTCGACCCACCTCCTATT</td>
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<tr>
<td>P4</td>
<td>−</td>
<td>1253</td>
<td>TCCGTGTTGGGTGTTGACCC</td>
<td>1,050</td>
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<tr>
<td>P5</td>
<td>−</td>
<td>1147</td>
<td>TCTCGTGTTCATGTTGTC</td>
<td>1,050</td>
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<tr>
<td>P6</td>
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<td>1844</td>
<td>GCCGTCTGATCGTGTGCTT</td>
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<tr>
<td>P7</td>
<td>+</td>
<td>1744</td>
<td>TCCCTGACCGTCTGACGTCT</td>
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<tr>
<td>P8</td>
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<td>2452</td>
<td>TCGACGCGAAATGCTTCGCCTT</td>
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<tr>
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<td>4552</td>
<td>CAGATACGCCGCTGACGTTG</td>
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<td>P19</td>
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<td>4514</td>
<td>CGCGTGTCCTGACTGCTG</td>
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<td>P20</td>
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<td>6341</td>
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<td>P21</td>
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<td>6242</td>
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<td>P23</td>
<td>+</td>
<td>6840</td>
<td>TTTAGGACCGCGCTAGGAGG</td>
<td>1,050</td>
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<td>P24</td>
<td>−</td>
<td>7520</td>
<td>CAAGATGAAAGATCGTGGCTG</td>
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<td>P25</td>
<td>+</td>
<td>7311</td>
<td>CAGGATGAAAGATCGTGGCTG</td>
<td>1,050</td>
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<td>P26</td>
<td>−</td>
<td>7750</td>
<td>CTGTGTTGTTGTTGAGGCG</td>
<td>1,050</td>
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</table>
acetonemethanol, followed by overnight incubation at 4°C in PBS. The cells were then incubated at 37°C for 2 h with mouse anti-RRV hyperimmune ascitic fluid diluted (10⁻³) in PBS containing 1% FCS, followed by three washes with sterile PBS. This was followed by incubation of the cells with fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG antibody (Silenus, Melbourne, Australia) in PBS (1% FCS) for 1 h at 37°C. Cells were then washed three times with sterile PBS. Cells were counted with a Leica fluorescence microscope.

IFN-β treatment of RAW 264.7 cells. RAW 264.7 cells were cultured in EMEM-FCS containing 10, 50, or 100 IU/ml of recombinant mouse IFN-β (catalog no. PMC4024; Invitrogen, Australia) for 24 h prior to infection with 0.1 MOI of parental RRV-T48 or RRVPERS. Supernatants were collected 12 h postinfection (p.i.) and titrated by plaque assay on Vero cell monolayers for determination of virus titers.

Plasmids and DNA transfection for luciferase assay. The luciferase reporter plasmids were propagated in DH5α E. coli and purified using Qiagen (Hilden, Germany) 10 K Amicon columns and resuspended in 10 mM Tris-HCl, pH 7.4, prior to transfection. For each reaction, 100 μg of plasmid DNA was transfected using the GeneJammer transfection reagent, according to the manufacturer’s protocol (Promega). 2 × 10⁴ cells were incubated for 24 h in 96-well plates and then infected with 0.1 MOI of RRV-T48 or RRVPERS. Cells were harvested at 12 h postinfection and processed for measurement of luciferase activity (luciferase reporter assay kit; Promega) and β-galactosidase activity (β-galactosidase reporter assay kit; Promega) according to the manufacturer’s instructions. Luciferase activity was read on an Ascent Luminoskin luminometer (PerkinElmer) and β-galactosidase activity was measured on a UV-visible microplate reader at 495 nm (Bio-Rad). For Vero cell experiments, cells were transfected with 2 μg of pSH4-1 (29) Lucter and pISRE (9-27) Lucter were then incubated at 37°C for 2 h and then infected with 0.1 MOI of RRV-T48 or RRVPERS. Cells were harvested at 12 h postinfection and processed for measurement of luciferase activity (luciferase reporter assay kit; Promega) and β-galactosidase activity (β-galactosidase reporter assay kit; Promega) according to the manufacturer’s instructions. Luciferase activity was read on an Ascent Luminoskin luminometer (PerkinElmer) and β-galactosidase activity was measured on a UV-visible microplate reader at 495 nm (Bio-Rad). For Vero cell experiments, cells were transfected with 2 μg of pSH4-1 (29) Lucter and 2 μg of pCMV-βGal (Promega). Cells were then infected with RRV-T48 or RRVPERS at an MOI of 5. Twelve hours later, IFN-β (100 U/ml) was added to cell culture and luciferase expression was measured at 6 h following infection.

Quantitative real-time PCR. Total RNA was isolated from RAW 264.7 cells infected with RRV-T48 or RRVPERS at an MOI of 0.1 using TRIZol (Invitrogen Life Technologies). Real-time PCR for IFN-β was carried out on the RotorGene RG-3000 thermal cycler (Corbett Research, Australia), using Quantitect primer assay kits (Qiagen, Germany) based on quantification of the SYBR green fluorescent dye. Data were normalized to data for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time PCR was performed using the ABI Prism 7500 sequence detector (Applied Biosystems). Synthesis of cDNA from total cellular RNA was carried out with random hexamers and M-MLV reverse transcriptase (Invitrogen). The following primers and probe sequences were used for the detection of IFN-β gene expression: forward primer, 5'-CTGCTTAACTGGGACACTGACATCA-3'; reverse primer, 5'-GATCTTCCGCTGGTGGATCACTGAC-3'; and probe (FAM-TAMRA probe), 5'-CTGCTTAACTGGGACACTGACATCA-3'. The following primers and probe sequences were used for the detection of β-actin gene expression: forward primer, 5'-GGAGCGCTGATCTTCATGAGAC-3'; reverse primer, 5'-CCACCTGTCTGATCCATCA-3'; and probe (TAMRA probe), 5'-CCACCTGTCTGATCCATCA-3'. The following primers and probe sequences were used for the detection of cyclophilin gene expression: forward primer, 5'-GCTTGGAGGCTGGAGGAGT-3'; reverse primer, 5'-GACATGGGCTGGAGGAGTGT-3'; and probe (TAMRA probe), 5'-GACATGGGCTGGAGGAGTGT-3'. The following primers and probe sequences were used for the detection of IL-12 gene expression: forward primer, 5'-CCACCTGTCTGATCCATCA-3'; reverse primer, 5'-GACATGGGCTGGAGGAGTGT-3'; and probe (TAMRA probe), 5'-GACATGGGCTGGAGGAGTGT-3'. Data were normalized to data for GAPDH. Results were analyzed using the ΔCt method. The reporter gene was normalized to the housekeeping gene 18S rRNA.

RESULTS

Kinetics of small-plaque RRV growth. To study the interplay between virus and host antiviral pathways, we established persistent RRV infection in macrophages that had been treated with LPS to stimulate antiviral activity. In LPS-treated RAW 264.7 cell cultures, small plaque mutants of RRV first emerged at day 5 postinfection and coexisted with large (parent RRV) plaques. By day 14 postinfection, 90% of plaques visible on Vero cell monolayers were of the small phenotype (Table 3). In cultures treated with polymyxin B sulfate (to remove LPS) at the same time as RRV infection, the appearance of small plaques was delayed until day 14 postinfection (data not shown). RRV_PERS maintained a small-plaque phenotype after several rounds of plaque purification and growth in fresh Vero cell cultures (Fig. 1A).

To determine whether there were differences in the growth of RRV-T48 and RRV_PERS, kinetics of virus growth were

whole-cell protein extracts by immunoblotting as previously described (49). The following primary antibodies recognizing nonphosphorylated and phosphorylated (P) forms of STAT-1 and STAT-2, α-actin (loading control), and secondary antibody were purchased from the indicated manufacturers: anti-STAT-1 (sc-345), anti-STAT-2 (sc-476), anti-PI-3K (sc-21689), and anti-α-actin (from Santa Cruz Biotechnology) and peroxidase-conjugated goat anti-rabbit antibody (from Jackson Immunoresearch).

Outbred mouse mortality and morbidity studies. Mice were obtained from the Animal Resources Centre, Canning Vale, Western Australia. Fourteen-day-old Swiss outbred mice were inoculated intraperitoneally (i.p.) with 100 μl PBS–1% FCS containing various doses (from 10⁴ to 10⁶ PFU) of plaque-purified parent RRV (RRV-T48) or purified RRVPERS. Plaque assays on T48 and RRVPERS stocks used in mouse experiments were performed in parallel using the same batch of Vero cells. Both parent RRV and RRVPERS showed stock titers of >10⁶ PFU/ml. In addition, to reflect equal particle doses, specific infectivity of the RNA of each virus was determined by the overlay of agarose on transfected BHK-21 cells and the enumeration of plaques from each virus. The specific infectivities of RRV-T48 and RRVPERS were found to be similar (for RRV-T48, 1.8 × 10⁷ PFU per μg of RNA; for RRVPERS, 1.9 × 10⁷ PFU per μg of RNA). Experiments were performed to compare RRVPERS and parent RRV titers in mouse serum at 24 h p.i. with 10⁶ PFU RRV. Control mice inoculated i.p. with PBS alone showed no mortality or disease symptoms over the experimental period. All experiments were approved by the Animal Ethics Committee of the University of Canberra and Australian National University. In a separate experiment, 5-week-old Swiss outbred mice were inoculated in the right hind-leg footpad with 10⁶ PFU RRVPERS or RRV-T48. At 1 day postinfection, mice were sacrificed and popliteal lymph nodes were collected and homogenized for IFN-β ELISA analysis.

Outbred mouse RRV studies. The experimental approach used for murine RRV infection and monitoring of clinical disease was as described previously by Lidbury et al. (28, 29). Eighteen-day-old mice were inoculated subcutaneously in the pectoral area with 10⁷ PFU of RRV diluted in PBS (pH 7.2) in a 20-μl volume. Mock-inoculated animals were injected with the diluent alone. Mice were scored for disease symptoms every 24 h. Signs of disease were determined by assessing grip strength and altered gait. Mice were scored as follows: 0, no disease; 1, ruffled fur; 2, very mild hind limb weakness; 3, mild hind limb weakness; 4, moderate hind limb weakness; 5, severe hind limb weakness/dragging; 6, complete loss of hind limb function; 7, moribund; 8, dead. The experiments were approved by the Animal Ethics Committee of the University of Wollongong.

Statistical analysis. The significance of differences between experimental groups was analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni’s test. Values were reported as the means ± standard errors (SEM). For disease scores, data were analyzed by using the Mann-Whitney test. For survival studies, survival curves were analyzed by using the log rank test. Statistical analyses were performed using the GraphPad Prism software program, version 4.0b (GraphPad Software Inc.). Differences in means were considered significant at P values of <0.05.

RRVPERS maintained a small-plaque phenotype after several rounds of plaque purification and growth in fresh Vero cell cultures (Fig. 1A).

To determine whether there were differences in the growth of RRV-T48 and RRV_PERS, kinetics of virus growth were
compared in Vero, HEp-2, and RAW 264.7 cells. There were no significant differences in growth kinetics observed in Vero cells (Fig. 1B) (which do not make IFN-α/β) and in HEp-2 cells (Fig. 1C). In contrast, in RAW 264.7 cells, growth kinetics for RRV-T48 and RRVPERS differed, with RRVPERS producing >1 log10 more virus at 24 and 48 h postinfection (Fig. 1D). Importantly, growth kinetics for RRV-T48 and RRVPERS were not significantly different in RAW 264.7 cells pretreated with anti-murine IFN-α and anti-murine IFN-β antibodies (Fig. 1E). These findings suggest that the enhanced growth of RRVPERS in RAW 264.7 cells involves modulation of the IFN-α/β response.

Characterization of RRVPERS using monoclonal antibodies. Using polyclonal anti-RRV antibodies, plaque inhibition assays (PIA) showed a 50% neutralization endpoint for small-plaque RRV identical to that for the parent RRV-T48 virus (1.9 ± 10^5 versus 1.5 ± 10^5), confirming the identity of the small-plaque virus as RRV (Table 4).

**TABLE 3. Mean RRV titers in RAW 264.7 murine macrophages over 21 days p.i.**

<table>
<thead>
<tr>
<th>Day p.i.</th>
<th>Mean RRV titer (log10 PFU/ml) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.77 ± 0.02 (0 small plaques)</td>
</tr>
<tr>
<td>2</td>
<td>5.27 ± 0.16 (0 small plaques)</td>
</tr>
<tr>
<td>5</td>
<td>5.34 ± 0.11 (27, 5.8, 8.5)</td>
</tr>
<tr>
<td>14</td>
<td>5.93 ± 0.14 (89, 93, 91)</td>
</tr>
<tr>
<td>21</td>
<td>4.01 (80^4)</td>
</tr>
</tbody>
</table>

*Cells were infected at an MOI of 0.1 on day 0 (n = 3) and cultured with 5 ng/ml LPS. Virus used in this experiment was derived from the RRV-T48 genetic clone (RR64).*

*Proportion(s) (%) of small RRV plaques observed on Vero cell monolayers after RAW 264.7 macrophage infection (n = 3 except where otherwise noted) over a 21-day period. Parent virus (RRV-T48) presents as a “large” plaque on Vero cell monolayers (Fig. 1). At days 1 and 2 post-RRV-T48 infection, only large plaques were observed.*

*Counts per milliliter (pfu/ml) at various time points post-RRV infection.*

*Virus used in this experiment was derived from the RRV-T48 genetic clone (RR64).*
TABLE 4. Antibody-mediated inhibition of RRV plaque formation on Vero cell monolayers by anti-RRV polyclonal sera or RRV E2 protein-specific monoclonal antibodies

<table>
<thead>
<tr>
<th>Anti-RRV antibody</th>
<th>Highest antibody dilution to achieve 50% RRV plaque inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal anti-RRV&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5 x 10&lt;sup&gt;-5&lt;/sup&gt; / 1.9 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAb E7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.5 x 10&lt;sup&gt;-3&lt;/sup&gt; / &lt;10&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAb 10C4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.2 x 10&lt;sup&gt;-4&lt;/sup&gt; / 3.5 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAb 3C4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.2 x 10&lt;sup&gt;-6&lt;/sup&gt; / &lt;10&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> All antibodies were titrated by 1/10 serial dilution from 1/50 x 10<sup>6</sup> to 1/5 x 10<sup>10</sup> prior to mixing with 200 PFU of purified RRV.
<sup>b</sup> Polyclonal antibody to RRV raised in ascitic fluid to PEG-purified RRV-T48.
<sup>c</sup> Specific for the E2 protein of RRV.

PIA analysis using three monoclonal antibodies (MAbs) specific to the viral E2 protein revealed differences in neutralization. For two of the three MAbs tested (E7 and 3C4), 50% plaque neutralization antibody titers were significantly lower for RRVPERS than for the parent RRV-T48 virus (Table 4). The relative resistance of RRVPERS to neutralization by MAbs E7 and 3C4 suggested an alteration in the RRV-E2 sequence in the small-plaque variant.

**Nucleotide sequence of RRVPERS.** The E2 gene in RRVPERS and RRV-T48 E2 were sequenced using RRV E2-specific primers (Table 1) that covered the entire 1,266-bp sequence. E2 PCR products were also cloned into the EcoRI site of pUC-19, and forward and reverse m13 nucleotide sequencing was performed. Analysis of the E2 sequences revealed a single nucleotide substitution at position 347 (5→3) of the RRV-E2 gene sequence (RRV-T48, [GAG]; small-plaque RRV, [GUG]). This genetic alteration resulted in a nonconservative change from the acidic (negatively charged) glutamic acid residue at amino acid position 116 of the parent virus E2 protein to a nonpolar hydrophobic valine residue in RRVPERS (Table 5). Sequence analysis of the untranscribed regions (UTRs) and other genes encoding RRV structural proteins outside the E2 gene (e.g., 6K, E1, and E3) did not show any differences between RRVPERS and RRV-T48 (data not shown).

The complete nsP region of RRVPERS was sequenced and compared with sequence of the parent strain virus, RRV-T48 (NCBI accession number DQ226993). Sequence analysis was started from 21 nucleotides 5' of the polyprotein start codon (AUG) (position 59 in full-length RRV sequence) and finished at the nsP4 stop codon (UAA; position 7523). The genomic organization was the same for RRVPERS and RRV-T48. Four silent mutations and 11 mutations resulting in amino acid substitutions were identified in RRVPERS nsP1 to nsP4 (Table 5). No base deletion or nonsense mutations were found.

RRV-T48 and RRVPERS nsPs thus showed an average amino acid identity of 99%. Eighty-three percent (83%) of the amino acid substitutions are within the nsP1 and nsP2 regions. The majority of amino acid changes were nonconservative. Interestingly, a nonconservative change of arginine (basic) to proline (hydrophobic) was present near the center of the nsP1 protein (Pro-303), a region that is highly conserved among alphaviruses (10).

**RRVPERS induces less IFN-β mRNA expression and protein production.** To determine whether RRV-T48 and RRVPERS differentially affect IFN-β production, the levels of IFN-β mRNA were measured in RAW 264.7 cells infected with each virus. The increase in IFN-β mRNA expression in response to RRV-T48 infection was significantly less than that in cells infected with RRVPERS (Fig. 2A). This reduction in IFN-β mRNA expression occurred despite the fact that RRVPERS-infected cells contained about 4-fold more genomic RNA than RRV-T48-infected cells as determined by semiquantitative RT-PCR analysis of RRV-E2 gene expression (Fig. 2B and C). Using a reporter plasmid encoding luciferase under the control of the IFN-β promoter, RRVPERS-infected cells also showed a 2-fold reduction in luciferase activity compared to that of RRV-T48-infected cells (Fig. 2D). ELISA analysis confirmed these observations, with 2.5-fold more IFN-β protein in cells infected with RRVPERS than in cells infected with RRV-T48 (Fig. 2E). These observations clearly show that infection of RAW 264.7 cells with RRVPERS induced less IFN-β than infection with RRV-T48 and suggest that the increased replication of RRVPERS in RAW 264.7 cells (Fig. 1D) may due to lower levels of IFN-α/β induction.

In addition, we sought to exclude the possibility that reduced IFN-α/β production in cells infected with RRVPERS was due to differences in the number of cells infected early during infection. The percentages of cells infected with each virus at 6, 12, and 24 h postinfection were measured. At 6 h postinfection, the percentages of cells infected with each virus were found to be similar (Fig. 2F). However, at 12 and 24 h postinfection, a higher percentage of RRV-positive cells was detected in cultures infected with RRVPERS than in cultures infected with RRV-T48 (Fig. 2F). This result suggests that the differences in the induction of IFN-α/β between RRV-T48 and RRVPERS were not due to differences in the percentage of cells infected at early time points after infection.

TABLE 5. Predicted amino acid differences in the nsP1 to nsP4 and E2 proteins of persistent RRVPERS compared to sequence of the parent, RRV-T48<sup>a</sup>

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino acid position</th>
<th>Amino acid (nucleotides)</th>
<th>Conservation (+/-)</th>
<th>Nucleotide position</th>
</tr>
</thead>
<tbody>
<tr>
<td>nsP1</td>
<td>R (AGA)</td>
<td>T (ACA)</td>
<td>–</td>
<td>450</td>
</tr>
<tr>
<td>nsP2</td>
<td>L (TTG)</td>
<td>F (TTT)</td>
<td>+</td>
<td>2341</td>
</tr>
<tr>
<td>nsP3</td>
<td>S (AGC)</td>
<td>I (ATC)</td>
<td>–</td>
<td>5028</td>
</tr>
<tr>
<td>nsP4</td>
<td>K (AAG)</td>
<td>N (AAC)</td>
<td>–</td>
<td>7321</td>
</tr>
<tr>
<td>E2</td>
<td>M (GAG)</td>
<td>V (GTV)</td>
<td>–</td>
<td>8913</td>
</tr>
</tbody>
</table>

<sup>a</sup> Based on nucleotide sequence analysis of the nsP1 to -4 region and E2, 6K, and E1 (structural) regions of the RRV genome. (Silent mutations are not included). Amino acid residues are numbered from the N terminus of each nsP and E2 protein. The single-letter amino acid code is used. Conservative amino acid differences are indicated by “+.” Amino acids that could not be deduced due to nucleotide ambiguity (N) are shown as an X. Nucleotides are numbered from the 5' terminus of the RRV 5p sequence. The GenBank accession number for RRV E2 cDNA is X720462.
RRVPERS exhibits higher resistance to IFN-β/IFN-H9252-induced antiviral activity. We next investigated the effects of IFN-β treatment on the replication of RRVPERS and RRV-T48 in RAW 264.7 cells. RAW 264.7 cells were treated with 10, 50, or 100 IU IFN-β and infected 24 h later with RRVPERS or RRV-T48. The amount of virus in the cultures was determined by plaque assay, real-time PCR, and Western blotting. RRVPERS showed increased resistance to IFN-β-mediated antiviral activity, with RRVPERS-infected cells generating a substantially higher viral load than cells infected with the parent RRV-T48 virus (Fig. 3A to D). Cells were also collected for Western blotting to analyze RRV E2 glycoprotein levels. Qualitative analysis reveal that at 24 h postinfection, RRV E2 levels were higher in RRVPERS-infected cells than in those infected with RRV-T48 (Fig. 3E). These results indicate that RRVPERS has increased resistance to the antiviral activity of IFN-β.

Infection by RRVPERS shows inhibitory effects on type I IFN signaling. To determine whether the resistance of RRVPERS to IFN-β treatment is mediated by the inhibition of type I IFN signaling, a plasmid encoding the luciferase reporter gene under the control of type I IFN (ISRE)-responsive element was used. These studies were carried out with Vero cells, which are able to respond to IFN-β/IFN-H9252 but do not produce these factors themselves. Induction of luciferase can thus be attributed exclusively to exogenously added IFN-β. Vero cells were transiently transfected with the pIFN-β (-125/-72) Lucter reporter plasmid. Twenty-four hours later, cells were inoculated at an MOI of 0.1 with RRV-T48 or RRVPERS, followed by cell lysis at 12 h postinfection. Luciferase activity was normalized to β-galactosidase reporter expression. Significant differences in expression (P < 0.05) are marked with an asterisk. (E) ELISA analysis of IFN-β protein production in RAW 264.7 cells 12 h after infection with 0.1 MOI of RRV-T48 or RRVPERS. Infected cells were detected using mouse anti-RRV hyperimmune ascitic fluid and fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG antibody. Infected cells were counted using a Leica fluorescence microscope. Significant differences (P < 0.05) in cell numbers are indicated with an asterisk.
receptor signaling pathway to a greater extent than wild-type virus.

To further analyze IFN-α/β receptor signaling, we determined the levels of STAT-1 and STAT-2 phosphorylation in HEp-2 cells infected with RRV-T48 or RRVPERS at an MOI of 5 for 12 h followed by IFN-α/β (100 IU/ml) treatment for 30 min. Treatment with IFN-β resulted in high levels of phosphorylated STAT-1, but this was markedly reduced in cells infected with RRVPERS from that in cells infected with RRV-T48 (Fig. 4B and C). There were no detectable differences in phosphorylated STAT-2, total STAT-1, total STAT-2, and α-actin in cells infected with either of the viruses (Fig. 4B and C). Similar results were obtained when the same experiment was performed with Vero cells (data not shown). Uninfected cells treated with IFN-β showed levels of phosphorylated STAT-1 and STAT-2 comparable to those for cells infected with RRV-T48 and treated with IFN-β (Fig. 4C). These results suggest that RRVPERS and not RRV-T48 can interfere with the IFN-α/β receptor signaling pathway at the level of, or upstream of, STAT-1 phosphorylation. The observation that the inhibition of STAT-1 phosphorylation was not associated with detectable changes in STAT-2 phosphorylation, total STAT-1, total STAT-2, and α-actin levels suggests that shutdown of host protein synthesis was not responsible for this inhibition.

Infection of outbred mice with RRVPERS results in enhanced mortality associated with high virus titers and reduced IFN-α/β. To determine whether the altered in vitro phenotype of RRVPERS resulted in increased pathogenicity in vivo, we undertook infection studies in Swiss outbred mice, which have been used extensively in RRV pathogenesis studies (58, 59). Following infection of Swiss mice with a dose range of 10^4 to 10^6 PFU RRV (i.p.), markedly enhanced mortality was observed for mice infected with RRVPERS compared to those infected with RRV-T48 (Fig. 5A, B, and C). Serum RRV titers were 1.5 log_{10} higher for RRVPERS-infected mice than for RRV-T48-infected animals (P < 0.05) (Fig. 5D), suggesting that the increased mortality in mice infected with RRVPERS was related to enhanced viral replication.

To determine whether RRVPERS induces less IFN-β in vivo, lymph nodes from 5-week-old Swiss outbred mice at 1 day following infection with RRV-T48 or RRVPERS were homogenized and analyzed for the presence of IFN-β by ELISA. Nearly 50% less IFN-β was detected in the lymph nodes of mice infected with RRVPERS than in mice infected with the RRV-T48 (Fig. 5E).
Infection of Swiss outbred mice with RRVPERS results in enhanced severity of hind limb disease and myositis compared to results with parent virus. We recently demonstrated that Swiss outbred mice infected with RRV developed severe disease characterized by loss of hind limb gripping ability and altered gait. These disease signs correlated with inflammation of joint and skeletal muscle tissue (29, 38). Since RRVPERS has enhanced virulence in vivo (Fig. 5), we examined its ability to induce arthritis and myositis in Swiss outbred mice. Eighteen-day-old mice were infected with 10⁴ PFU RRVPERS or RRV-T48, and mice were monitored for the development of disease signs. Infection of mice with RRVPERS resulted in more-severe disease than infection with RRV-T48 (Fig. 6A). Mice infected with RRVPERS reached a level of disease requiring euthanasia by day 10 postinfection. To compare muscle tissue inflammation and pathology in mice infected with RRVPERS and RRV-T48, histological analysis of skeletal muscle was performed. In RRVPERS-infected mice, severe inflammation and tissue damage were observed in muscle tissue at 5 days postinfection (Fig. 6B, panels c and f). Inflammatory infiltrates were also observed in quadriceps skeletal muscle of RRV-T48-infected mice, but the level of inflammation was not as severe and tissue damage was not apparent at this time point (Fig. 6B, panels b and e).

**DISCUSSION**

In the present study, we characterized an RRV variant (RRVPERS) that had been selected during persistent infection of a mouse macrophage cell line under antiviral conditions. RRVPERS formed small plaques and possessed mutations in the structural and nsP regions. RRVPERS induced lower levels of type I IFN and showed enhanced resistance to antiviral stimuli associated with inhibition of IFN-α/β signaling. In association with these changes, RRVPERS was highly pathogenic, with increased disease severity and mortality following infection of mice.

Significant insights into the selection of an IFN-resistant viral phenotype have come through the study of human hepatitis C virus (HCV), which successfully persists in some patients (and cell cultures) for very long periods. The treatment of HCV patients with IFN is often stymied by the eventual development of IFN resistance by the virus. It has been suggested that exogenous IFN treatment could lead to selective pressure on the virus and the development of mutant strains able to resist IFN-mediated host defense (16, 27, 56). Sumpter et al. (56) found that persistent growth of HCV in cell culture was associated with genetic variation of viral NS5A, NS3, and NS4A, which endowed the virus with the ability to disrupt IFN regulatory factor 1 (IRF-1) and IRF-3 signaling following IFN

**FIG. 4.** Type I IFN signaling is inhibited in cells infected with RRVPERS. (A) These studies were performed with Vero cells, which do not produce IFN but are able to respond to IFN. Vero cells were transfected with the pISRE (9-27) Lucifer plasmid and then infected with RRV-T48 or RRVPERS (5 MOI). IFN-β (100 IU/ml) was added 12 h later, and luciferase expression was measured following incubation for 6 h. Luciferase activity was normalized to β-galactosidase reporter expression. Significant differences in expression (P < 0.05) are marked with an asterisk. (B and C) Western blot analysis of STAT-1 and STAT-2 expression and phosphorylation in HEp2 cells infected with RRV-T48 or RRVPERS (5 MOI) for 12 h, followed by IFN-β (100 IU/ml) treatment for 30 min or no treatment. The cell lysates were examined by Western blotting with antibodies to STAT-1, pY-STAT-1, STAT-2, or pY-STAT-2. The control included the detection of host cell protein expression by anti-α-actin antibody.
stimulation. HCV proteins have been linked to the attenuation of signaling via the IFN-α/β receptor (18), and HCV variant E2 and NS5A proteins have been demonstrated to bind PKR and interfere with IRF-1 stimulation (41, 57). The ability of HCV to modulate the activity of type 1 IFN may contribute to its ability to persist in the host. In the present study, we observed that small-plaque variants also arose in unstimulated RAW 264.7 cells but at a much lower rate than in cultures stimulated with LPS. This observation is consistent with the model that has been proposed for HCV infection (56), in which exogenous IFN can drive the evolution of strains resistant to IFN-mediated antiviral activity.

Alphaviruses efficiently induce IFN-α/β and are also generally highly sensitive to the antiviral effects of IFN-α/β (3, 46, 62). For instance, based on human and mouse studies, low levels of IFN-β can efficiently inhibit chikungunya virus (CHIKV) infection (46). Mice deficient in the IFN-α/β receptor (IFN-α/β R−/−) are highly susceptible to CHIKV infection, with infection resulting in death within 3 days (8). Similarly, RRV infection resulted in substantial mortality in IFN-α receptor-deficient mice (IFN-α R−/−), while wild-type mice were able to resist infection (J. Podger and S. Mahalingam, unpublished data). In terms of induction and sensitivity to IFN, our results show that RRVPERS induces less IFN-β and exhibits resistance to its antiviral effects compared to the parent virus. Mortality and disease severity were greatly enhanced in mice infected with RRVPERS compared to those infected with RRV-T48. The reduction in IFN-β production observed in vitro following infection with RRVPERS was also observed in vivo, with a 50% reduction in IFN-β levels in the lymph nodes of mice infected with RRVPERS compared to results for those infected with RRV-T48. It is likely that the virus-mediated
inhibition of the type I IFN response contributes to disease exacerbation following infection with RRV_pers. Our results are reminiscent of findings with Venezuelan equine encephalitis virus (VEEV), in which infection of mice with an IFN-resistant strain resulted in enhanced clinical disease (51). Similar results were obtained with an IFN-α/β-resistant eastern equine encephalitis virus (EEEV) strain; infection of mice with this strain resulted in enhanced encephalitis, an effect that mapped to both structural and nonstructural genes (1).

To counteract type I IFN responses, many viruses encode proteins that disrupt type I IFN signaling and downstream responses. These evasion strategies have been linked to viral
pathogenesis and the emergence of virus in new host populations (24, 51). Suppression of type I IFN responses has been observed for influenza virus (4, 14), dengue virus (20, 39), West Nile virus (32) and other RNA viruses (reviewed in references 15 and 35). Many viral proteins have been reported to block transcription factors that control production of type I IFN (reviewed in references 14, 15, and 35). In this study we have identified RRV as an additional member capable of developing mechanisms to suppress IFN responses. Alphavirus infection results in rapid shutdown of host cell protein synthesis in favor of viral protein synthesis, and the conventional view has been that this mechanism underlies the observed suppression of IFN-α/β production (44). For example, the nsP2 proteins of Sindbis virus and Semliki Forest virus (SFV) can inhibit type I IFN responses via host protein shutdown (5, 12), while the capsid proteins of EEEV and VEEV perform a similar IFN-targeting function (1, 2, 51). Mutating the nuclear localization sequence of nsP2 of SFV resulted in a virus that induced more IFN-α/β, suggesting that nsP2 inhibits IFN-α/β induction (5). However, those authors admit that an alternative explanation is that relocalization of nsP2 may simply result in more-effective induction of IFN-α/β (5). Alphaviruses might also exert much more specific effects on the IFN-α/β system independent of host protein shutoff. Our earlier studies with RRV demonstrated that Fc receptor bearing cells (dendritic cells [DCs] and macrophages) can be infected via a mechanism involving antibody-dependent enhancement (ADE) of infection and that this results in interleukin 10 (IL-10)-dependent inhibition of cellular IFN-α/β production (34). We have also demonstrated that the presence of high-mannose glycans on virus derived from mosquito cells interferes with type I IFN induction in myeloid DCs (47). Recently, Yin and colleagues reported that the phosphorylation of STAT-1 and STAT-2 was partially blocked by VEEV and Sindbis virus and the effect was dependent on the expression of viral nsP (61). In another study, Simmons et al. showed that VEEV can antagonize STAT-1 activation following type I IFN treatment and that the inhibition of type I IFN signaling occurred via distinct mechanisms independent of host protein shutoff (49). Cruz et al. have identified a mutation in the nsP1/nsP2 cleavage domains of Sindbis virus and RRV associated with a specific enhancement of IFN production independent of virus-induced host shutoff (9). More recently, Fros et al. showed that chikungunya virus infection blocked IFN-induced STAT-1 phosphorylation and that this inhibition was mediated by nsP2 and was independent of host shutoff (13). Several lines of evidence also suggest that shutdown of host protein synthesis is not responsible for the RRV-PERS-mediated IFN-α/β signaling inhibition. First, the reduced levels of phosphorylated STAT-1 were not associated with reduced total STAT-1 levels, which suggests that the inhibition was not due to decreased synthesis of total STAT-1. Second, phosphorylated STAT-2, total STAT-2, and housekeeping gene α-actin levels were not decreased by RRV-PERS infection, which indicates that the small-plaque variant specifically targets STAT-1. Third, the STAT-1 and STAT-2 levels in uninfected controls treated with IFN were comparable to levels in cells infected with RRV-T48 and treated with IFN, suggesting that RRV-T48 does not inhibit phosphorylation of STAT-1 or -2 and that the effects are specific to RRV-PERS.

The ability of viruses to develop resistance to host antiviral activity by genetic sequence variation has a major effect on virus virulence and persistence in the host. Mutational studies have shown that alphaviruses appear to attain these effects mainly through the action of nsPs (1, 2, 5, 9, 13, 51). We identified a number of mutations in nsP1 to nsP4 and a single mutation in E2 in RRV-PERS. Using site-directed mutagenesis, we introduced the RRV-PERS E2 mutation into the parent RRV-T48 cDNA infectious clone (adenine to uracil [GAG→GUG] at position E2-347). Interestingly, the mutation in E2 did not affect IFN induction and resistance of RRV-PERS (data not shown). Future studies will investigate whether these mutations in the nsP regions are involved in the perturbation of IFN signaling pathways that we observed. Our preliminary studies (using chimeric virus and a pcDNA plasmid expressing nonstructural genes) show that RRV-PERS nsP1 and nsP2 are able to inhibit IFN-α/β signaling (data not shown), and we are currently attempting to identify the mechanisms of action. Future studies will introduce the mutations that we identified in the RRV-PERS nsPs into the wild-type RRV infectious clone (pRR64), followed by testing of the IFN resistance phenotype and pathogenicity of the recombinant virus. If the IFN resistance phenotype of RRV-PERS is recovered, examination of the expression of downstream IFN-induced proteins will be investigated. These studies will allow us to identify the specific nsP mutation(s) that leads to the IFN suppression phenotype induced by enhanced macrophage inflammatory activity.

In conclusion, we describe in vitro and in vivo studies characterizing the genetic and phenotypic properties of a novel small-plaque, persistent strain of RRV. The enhanced suppression of IFN signaling mediated by RRV-PERS is likely to play a key role in the enhanced pathogenicity exhibited by this virus. This study demonstrates that selective pressure exerted by host antiviral activity can promote the evolution of an IFN-resistant, persistent, and highly pathogenic alphavirus strain. It is possible that a similar evolutionary mechanism may operate in vivo, and sequencing of clinical isolates from persistent alphavirus infections will provide new insights into viral pathogenesis in human disease.

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