Influence of the CCR-5/MIP-1 α Axis in the Pathogenesis of Rocio Virus Encephalitis in a Mouse Model

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Abstract. Rocio virus (ROCV) caused an outbreak of human encephalitis during the 1970s in Brazil and its immunopathogenesis remains poorly understood. CC-chemokine receptor 5 (CCR5) is a chemokine receptor that binds to macrophage inflammatory protein (MIP-1 α). Both molecules are associated with inflammatory cells migration during infections. In this study, we demonstrated the importance of the CCR5 and MIP-1 α , in the outcome of viral encephalitis of ROCV-infected mice. CCR5 and MIP-1 α knockout mice survived longer than wild-type (WT) ROCV-infected animals. In addition, knockout mice had reduced inflammation in the brain. Assessment of brain viral load showed mice virus detection five days post-infection in wild-type and CCR5 $^{-/-}$ mice, while MIP-1 $\alpha^{-/-}$ mice had lower viral loads seven days post-infection. Knockout mice required a higher lethal dose than wild-type mice as well. The CCR5/MIP-1 α axis may contribute to migration of infected cells to the brain and consequently affect the pathogenesis during ROCV infection.

INTRODUCTION

Rocio virus (ROCV) belongs to the family *Flaviviridae* and the genus *Flavivirus*, which includes more than 70 members, 40 of them with medical importance, such as yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV), St. Louis encephalitis virus (SLEV), and Japanese encephalitis virus (JEV). Those viruses are related to encephalitis, hemorrhagic, and hepatic diseases, as well as febrile illness in humans and other vertebrates.¹ Flaviviruses are spherical enveloped virus approximately 50 nm in diameter with a single-stranded positive-sense RNA genome of approximately 11 kb. The genome encodes three structural (capsid, membrane, and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The entire genome of ROCV has 10,794 nucleotides and a unique open reading frame of 10,275 nucleotides that is flanked by 5'- and 3'-non coding regions of 92 and 427 nucleotides, respectively.²

Rocio virus was first isolated from a human who died of encephalitis in the Ribeira Valley along the southern coast of São Paulo State, Brazil, in 1975.³ This region was devastated by a human epidemic of severe encephalitis caused by ROCV that started in the early 1970s and lasted until the 1980s.⁴ Since that time, the virus has not been isolated or related to cases of viral encephalitis. However, some studies reported neutralizing antibodies against ROCV in persons from rural areas of southeastern and northeastern Brazil, indicating that this virus still circulates⁵ and that new outbreaks should not be neglected.

In humans, ROCV infection is potentially fatal after a 7–14-day incubation period, mainly in young and elderly persons. These patients have acute fever, headache, anorexia, nausea, vomiting, myalgia, and malaise. Encephalitis signs appeared later, including confusion, reflex disturbances, motor impair-

ment, meningeal irritation, and cerebellar syndrome. The disease also produces serious sequelae such as visual, olfactory, and auditory disturbances, lack of motor coordination, equilibrium disturbance, swallowing difficulties, incontinence, and defective memory.⁶

Rocio virus is apparently transmitted by mosquitoes of the genera *Aedes*, *Psorophora*, and *Culex*. The virus is probably maintained in nature by a cycle involving wild birds as reservoirs because it was isolated from a wild bird (*Zenothrichia capensis*). In 2004, two birds captured in southern Brazil had antibodies against ROCV. Thus, ROCV may represent a threat to public health.^{7,8}

Chemokines are an important group of small secreted proteins (8–14 kD) of the immune response that are involved in numerous aspects of trafficking, tissue localization, and activation of leukocytes.^{9,10} Although some chemokines are considered usually homeostatic by their implication in trafficking of specific cells under physiologic conditions, other chemokines have pro-inflammatory properties and their production is induced in response to immunologic, inflammatory, infectious signals.¹¹ The effective immune response against pathogens, including virus, is extensively described to be dependent of the recruitment of inflammatory cells by chemokines.^{12–15} Among these chemokines, CCL3 (MIP-1 α), and its receptor CC-chemokine receptor 5 (CCR5)¹⁰ appear to have a critical role in regulating cell recruitment to different classes of virus, including those of the genus *Flavivirus*,^{16,17} and exhibits a variety of pro-inflammatory activities.^{18,19} The CCR5 chemokine receptor is expressed on several cells of the immune system, including CD8 and CD4 (Th1) lymphocytes, granulocytes, macrophages, microglia, and dendritic cells.²⁰

The immunopathogenesis of infection with ROCV is not completely understood. A previous study by our group showed that the inflammatory process in the central nervous system (CNS) of infected BALB/c mice was sustained by a mixed Th1- and Th2-type immune response. In addition, in the inflammatory process in the CNS, a severe tissue lesion was detected and associated with neuronal death. Neuronal damage most likely occurred as a consequence of ROCV infection and

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apoptosis induced by cytokines produced by glial and macrophage activated cells.²¹

One study reported that flavivirus (e.g., WNV) encephalitis is characterized by entry of macrophages, CD4⁺ and CD8⁺ T cells, natural killer cells, and dendritic cells in the CNS, and recruitment of these cells is dependent on CCR5 receptor.¹⁶ However, no information is available regarding specific chemokines responsible for cell recruitment and their influence on disease outcome during ROCV infection. Therefore, we studied the role of the chemokine MIP-1 α and its receptor CCR5 in ROCV infection in a mouse model.

MATERIALS AND METHODS

Animals. Female 4–6-week-old wild-type (WT) C57BL/6 mice and mice with targeted disruption of the CCR5 receptor or MIP-1 α chemokine (CCR5 and MIP-1 α KO) were obtained from the Animal Facility and maintained in isolated cages at the Virology Research Center at the School of Medicine of the University of São Paulo, Ribeirão Preto, Brazil. Experiments were approved by the Ethical Committee on Vertebrate Animal Experiments of the University of São Paulo (no. 080/2004). All animals were housed with food and water available *ad libitum*.

Virus. The ROCV strain SPH34675 (passage 3) used for mouse infection was kindly supplied by Dr. Terezinha Lisieux Coimbra (Adolfo Lutz Institute, São Paulo, Brazil). Virus stocks were obtained from brains of intracerebrally infected suckling mice. In brief, newborn mice were inoculated with approximately 10 μ L of viral stocks. Animals were then killed after onset of symptoms approximately two days after inoculation. Brains were aspirated and macerated in a 7.5% bovine serum albumin solution in phosphate buffered-saline (PBS). Centrifugation at 2,000 $\times$ g for 10 minutes at 4°C was conducted and supernatants were stored at –70°C as virus stock until use.

Wild-type (WT) and KO mice 50% lethal dose (LD₅₀). To identify genetic factors involved in the host response to ROCV infection, the LD₅₀ of the virus was evaluated in WT and KO mice. Viral titration was performed by LD₅₀ method in WT, MIP-1 α –/–, and CCR5–/– mice as follows. Five mice of each group were inoculated intraperitoneally with 200 μ L of each serial dilution of ROCV (10^{–1} to 10^{–7}, diluted in sterile PBS). Mice were monitored twice a day for 21 days, and the LD₅₀ was calculated as described by Reed and Muench.²²

Virulence of ROCV in WT and KO mice. Mice were inoculated intraperitoneally with 4.3 LD₅₀ (log₁₀)/0.02 mL and killed on days 1, 3, 5, 7, 15 and 21 post-infection. Three animals were killed in each indicated period. A brain fraction was excised and fixed in PBS/10% formalin for 24 hours for histopathologic analysis. Another fraction (approximately 100 mg) was frozen in 1 mL of Trizol reagent (Invitrogen–Life Sciences, Rockville, MD) and used for real-time polymerase chain reaction (PCR) to determine viral load in the brain. Survival experiments consisted in groups of 5–7 mice inoculated as described above and monitored daily for survival. Animals that had disease symptoms (severe leg paralysis) were euthanized.

Histopathologic analysis. Brain samples from infected and negative control groups were embedded in paraffin and used to prepare histologic slides. Slides were stained with hematoxylin and eosin and examined under a light microscope to detect inflammatory cell infiltrates distributed along the organ, as well as histologic changes.

Immunocytochemical analysis for antigen detection in brain.

Paraffin-embedded sections of brains from infected and control animals were deparaffinized with three xylol washes and serial washes in 100%, 90%, and 70% ethanol, followed by a final wash in PBS. For ROCV antigen detection, samples were incubated for 2 hours at 37°C with a monoclonal antibody (dengue virus 2 4G2), which reacts with all flaviviruses and binds to envelope protein.²³ Sections were washed and incubated with goat anti-mouse IgG-horseradish peroxidase conjugate (Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C. Sections were treated with 3,3'-diaminobenzidine (Sigma-Aldrich) and counterstained with Mayer's hematoxylin.

Real-time PCR for viral load in brain. RNA was extracted by using Trizol (Invitrogen–Life Sciences) according to the manufacturer's instructions. Reverse transcription was performed with approximately 1 μ g of extracted RNA, SuperScript II[®] enzyme (Invitrogen–Life Sciences) and random primers (PdN₆) according to the manufacturer's recommendations. Power SYBR[®] Green Master Mix kit (Applied Biosystems, Warrington, United Kingdom) was used for real-time PCR, and reactions were processed in ABI5700 apparatus (Applied Biosystems). Real-time PCRs were conducted in a final volume of 25 μ L containing 12.5 μ L of SYBR[®] Green PCR Master Mix, 0.5 μ M of each primer, and 5 μ L of cDNA. Primer sequences ROCV forward (5'-GGATCCATGCCGAATGAAACTGGACAAG-3') and ROCV reverse (5'-AAGCTTATGTAGGTTCTCGGTCATGGTG-3') were used to amplify part of the E protein gene of ROCV (GenBank accession no. AF372409.1). Standard PCR conditions were 94°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, and at 60°C for 1 minute. A dissociation curve was constructed by using increasing temperatures from 60°C to 90°C.

RESULTS

Wild-type and KO mice LD₅₀. As shown in Table 1, viral titers were different in each mice group. Wild-type mice required a 6.5 (log₁₀) LD₅₀ to induce lethality of 50% of the animals. Conversely, MIP-1 α –/– or CCR5–/– knock-out mice required a dose of 4 and 5 LD₅₀, respectively.

Mouse survival. Assessment of survival rate was performed in C57/B6 wild type, CCR5–/–, and MIP-1 α –/– mice infected with 4.3 LD₅₀ (log₁₀) of ROCV. Wild-type mice showed a markedly decreased survival rate compared with CCR5–/– and MIP-1 α –/– mice at 21 days post-infection. Forty percent of CCR5–/– and MIP-1 α –/– mice survived > 21 days, and WT mice survived $\leq$ 8 days post-infection (Figure 1). In this group, all animals started to show signs of encephalitis, such as hind limb paralysis, tremors, muscle weakness and ruffled pile, at approximately 4–5 days post-infection, resulting in animal death within 8 days. Both CCR5–/– and MIP-1 α –/– mice showed

TABLE 1

50% lethal dose (LD₅₀) of Rocio virus obtained for wild-type, MIP-1 α –/–, and CCR5–/– mice*

Group	LD ₅₀ (log ₁₀)
Wild type	6.5
MIP-1 α –/–	4
CCR5–/–	5

*Groups of five animals were infected with different amounts of Rocio virus and monitored daily for survival until 21 days post-infection. Viral titers of each group was obtained by LD₅₀, which was calculated according to the method of Reed and Muench.²²

FIGURE 1. Survival rate of mice infected with 4.3 (LD_{50}) of Rocio virus (ROCV). Groups of five female 6-week-old C57/B6 wild-type (WT), $CCR5^{-/-}$, and $MIP-1\alpha^{-/-}$ mice were infected with 4.3 $LD_{50}/0.02$ mL of ROCV and monitored daily for survival up to 21 days post-infection. Results are representative of five animals per group, and the survival rates of KO and WT mice were significantly different ($P = 0.0038$, by Mantel-Cox log-rank test). Statistical analysis was performed by using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA).

delayed signs of encephalitis after infection, resulting in a considerably high rate of survival after 21 days post-infection.

Brain viral load. To evaluate an association between animal death and high viral load in the CNS, a brain fraction of infected mice was assessed by real-time PCR for different

periods post-infection (Figure 2). Rocio virus was detected 5 days post-infection in WT and $CCR5^{-/-}$ mice and 7 days post-infection in $MIP-1\alpha^{-/-}$ mice. Viral load in brains of $CCR5^{-/-}$ mice was lower (but not statistically significant) than that in brains of WT mice. However, ROCV was detected in

FIGURE 2. Rocio virus (ROCV) load in brains of wild-type (WT), $CCR5^{-/-}$ (A) and $MIP-1\alpha^{-/-}$ (B) mice evaluated by real-time polymerase chain reaction. Groups of four female 6-week-old C57/B6 WT, $MIP-1\alpha^{-/-}$, and $CCR5^{-/-}$ mice were infected with 4.3 50% lethal doses (LD_{50}) of ROCV and killed on indicated days. Results are representative of four animals per time point. * $P < 0.05$ when compared with WT mice at 7 days post-infection, by two-way analysis of variance, followed by Bonferroni post-test). ND = not detected. ♦ Data not available because WT mice did not survive 8–10 days post-infection. Statistical analysis was performed by using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA).

FIGURE 3. Histopathologic analysis of brain tissues of Rocio virus (ROCV)-infected mice. Brain tissues of wild-type (WT), MIP-1 α , and CCR5 knockout mice were stained with hematoxylin and eosin and observed by light microscopy. **A**, WT infected mouse killed 0 hours (h) post-infection (negative control). **B**, WT infected mouse killed 1 day post-infection. **C**, WT infected mouse killed 5 days post-infection. **D**, WT infected mouse killed 7 days post-infection, showing a large amount of inflammatory cells in the meninge/cortex. **E**, MIP-1 $\alpha^{-/-}$ infected mouse killed 0 hours post-infection. **F**, MIP-1 $\alpha^{-/-}$ infected mouse killed 1 day post-infection. **G**, MIP-1 $\alpha^{-/-}$ infected mouse killed 5 days post-infection, showing no inflammatory foci in brain tissue. **H**, MIP-1 $\alpha^{-/-}$ infected mouse killed 7 days post-infection. No inflammatory cells in the cortex/meninge region of the central nervous system (CNS) were observed in such mice. **I**, MIP-1 $\alpha^{-/-}$ infected mouse killed 15 days post-infection, showing inflammatory foci in the CNS. **J**, CCR5 $^{-/-}$ infected mouse killed 0 hours post-infection. **K**, CCR5 $^{-/-}$ infected mouse killed 1 day post-infection. **L**, CCR5 $^{-/-}$ infected mouse killed 5 days post-infection. **M**, CCR5 $^{-/-}$ infected mouse killed 7 days post-infection. **N**, CCR5 $^{-/-}$ infected mouse killed 15 days post-infection, showing inflammatory foci in CNS. **Arrows** indicate inflammatory cells or foci.

CCR5 $^{-/-}$ mice until 15 days post-infection. In MIP-1 $\alpha^{-/-}$ mice, viral load was significantly lower than that in WT mice at 7 days post-infection ($P < 0.05$).

Histopathologic analysis and ROCV antigen detection in brains of infected mice. Brains of ROCV-infected mice in different groups showed distinct pathologic changes at different days post-infection. A higher amount of inflammatory cells were observed in nervous tissues of ROCV-infected WT mice at 7 days post-infection compared with the amount in knock-out mice at the same time point (Figure 3). Only a discrete inflammatory infiltrate was detected in CCR5 $^{-/-}$ and MIP-1 $\alpha^{-/-}$ mice brain tissues at 7 days post-infection, and it was intense at 15 days post-infection (Figure 3). In an attempt to analyze the presence of the ROCV in brains of infected animals, ROCV antigens were evaluated by immunohistochemical analysis. Our results demonstrated that virus was detected in all mice groups, and there were no significant differences.

DISCUSSION

A better understanding of immunopathogenic mechanisms of severe encephalitis caused by flavivirus, such as ROCV, is an important aspect for disease control. However, data on experimental encephalitis caused by ROCV infection are scarce. It was shown that ROCV is able to infect house sparrows experimentally, and this finding suggests that birds may be involved in the biological cycle of this virus.²⁴ A chronic persistence infection was also observed after intraperitoneal infection of ROCV into golden hamsters in which virus replicated and was excreted continuously.²⁵ Recently, our group showed that severe encephalitis developed in BALB/C mice

infected with ROCV and these mice had inflammatory cell infiltrates in the CNS,²¹ which suggested that infection of the CNS recruits leukocytes, and that this recruitment generates tissue damage in an attempt to control infection.

The attraction of leukocytes to the brain is controlled by chemokines that interact with their receptors expressed on these migrating leukocytes, which depending on the type and the number of the migrated cells to these tissues, may have an important role in the pathologic process of the disease. In our experimental model of ROCV infection, the influence of the chemokine MIP-1 α (CCL3), the most potent ligand of CCR5 (a C-C motif chemokine) in the pathogenesis of ROCV encephalitis was shown. In this study, we demonstrated that the CCR5/MIP-1 α axis contributes to intense migration of inflammatory cells to the brain, and that this migration is associated with the pathogenesis of ROCV.

To investigate the role of CCR5 and its ligand MIP-1 α in ROCV infection, we infected a group of mice deficient for CCR5 (CCR5 $^{-/-}$) and another group that was deficient for MIP-1 α (MIP-1 $\alpha^{-/-}$) in a mouse model of acute disease. Early events in the inflammatory process are critical to disease progression, which showed the role of the CCR5/MIP-1 α axis. We observed that CCR5- and MIP-1 α -deficient mice had an increased survival rate when compared with that of WT mice. This increased survival rate was related to less cellular infiltrates observed in brain by histopathologic analysis.

A previously study by our group demonstrated that ROCV-infected monocytes and macrophages induced high levels of nitric oxide production.²⁶ In addition to the antiviral effect of nitric oxide, this free radical can induce septic shock, mainly by extensive smooth muscle relaxation, which results in low

blood pressure.²⁷ Our group also reported the ability of ROCV to induce apoptosis in neurons, which caused neuronal death.²¹ However, it was not possible to state if neuronal death was caused directly by viral replication or was a consequence of the inflammatory process.

In a study with WNV, it was observed that CCR5 expression in brain cells was crucial for an antiviral effect and a survival factor. CCR5 affected WNV viral load because CCR5-deficient mice showed a rapidly fatal disease progression.¹⁶ Conversely, in the present study, CCR5^{-/-} mice had a lower viral load than WT mice. This finding could be a characteristic of ROCV infection. We also observed that CCR5 deficiency resulted in an augmented survival rate.

Deficiency of CCR5 has been extensively studied for infection with human immunodeficiency virus (HIV) because it represents a crucial host factor used by HIV for cell entry. Therapy based on CCR5 antagonism is available for HIV treatment and reinforces the role of this chemokine receptor in the outcome of the infection.²⁸ According to our results, the absence of CCR5 is beneficial for ROCV-infected mice because it prolongs survival and decreases brain viral load.

Similar to HIV infection, CCR5 may represent a co-receptor for viral infection, although additional studies are required to confirm this hypothesis. Lymphocytic choriomeningitis virus infection in a mouse model caused an equivalent viral load and no difference in mortality when CCR5^{-/-} and CCR5^{+/+} mice were investigated.²⁹ Thus, viruses can show different interactions with CCR5, as we observed in our results. Considering that CCR5 is the only receptor for the chemokines MIP-1 α , MIP-1 β , and RANTES (CCL5), which are normally expressed on T cells, macrophages, dendritic cells, and microglia^{14,20} it was somewhat surprising to observe prolonged survival during ROCV infection in CCR5-deficient mice because WNV infection causes rapid death in mice with the same deficiency.¹⁶ Although the mechanism of ROCV infection during disease progression is still unknown, it may require an association of chemokines and its receptors to co-ordinate the complex leukocyte-trafficking patterns in response to infection.

In conclusion, we demonstrated that CCR5 has a significant role in ROCV meningoencephalomyelitis caused by ROCV infection, probably by mediating lymphocyte recruitment in brain, which, in turn, contributes to disease severity. In addition, we provide new insights into immunopathogenesis caused by ROCV infection by demonstrating the participation of chemokines in disease progression. These findings may contribute to understand the biology of ROCV, which is important because this virus is still circulating in rural areas of Brazil.

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