ACCELERATION OF VIRAL REPLICATION AND UP-REGULATION OF CYTOKINE LEVELS BY ANTIMALARIALS: IMPLICATIONS IN MALARIA-ENDEMIC AREAS

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Abstract. Antimalarial drugs are widely used in malaria endemic areas, both for chemoprophylaxis and also empirically to treat patients with fever. Previously, we have reported that chloroquine enhances the severity of Semliki forest virus (SFV) and encephalomyocarditis virus infection. The studies presented herein show that a broad spectrum of antimalarial drugs augmented the replication of SFV in mice, concomitant with greater tissue damage and up-regulation of mRNA levels of various inflammatory cytokine genes, including interleukin-1 receptor antagonist (IL-1Ra), IL-1α, IL-1β, IL-6, IL-12p40, and interferon-γ inducing factor. Furthermore, chloroquine enhances IL-1Ra production in RAW cells in vitro. Since IL-1Ra is known to be up-regulated in a number of viral infections, we propose that a further enhancement of its expression by antimalarials may be responsible for the increased severity of viral infection in our studies. Thus, the widespread use of antimalarials in malaria-endemic areas may predispose the population to viral infections. Further studies are in progress to delineate mechanism(s) involved in cytokine up-regulation and acceleration of viral replication.

Cytokines are important mediators in inflammatory pathology of the central nervous system (CNS), including encephalitides and demyelinating diseases such as multiple sclerosis.1 Interleukin-1 (IL-1) is an important cytokine implicated in the host response to injury and infection. The production, secretion, and biologic response of IL-1 is tightly regulated, and a naturally occurring inhibitor, IL-1 receptor antagonist (IL-1Ra), has been demonstrated to prevent binding of IL-1 to its receptor, thereby impairing its further biologic effects. Although IL-1Ra is a competitive inhibitor of IL-1, binding to the receptors with high affinity, high quantities (10–100 times more IL-1Ra than IL-1) are required to abrogate the effects of IL-1. The balance between IL-1 and IL-1Ra probably influences the regulation of the host response, and onset and severity of the inflammatory reaction. In this regard, selective over-production of IL-1Ra could be detrimental to the host when mounting an immune response. Increased levels of IL-1Ra have been noted in a variety of viral infections.4–9 However, the role of IL-1Ra in viral-host interactions has not been clearly elucidated.

We have previously shown that chloroquine enhances the severity of symptoms and mortality following Semliki forest virus (SFV) and encephalomyocarditis virus (EMCV) infection, along with an increase in viral titers in various organs.10 Although antimalarial chemoprophylaxis is widely practiced in areas where malaria is endemic, the possible effects of such agents on viral infection have largely not been explored. Studies presented in this paper show that antimalarials as a group enhance the replication of viruses, with greater tissue pathology being noted on histology. Infection with SFV led to an up-regulation in mRNA levels of various cytokines including IL-12p40, interferon-γ (IFN-γ) inducing factor (IGIF), IL-1Ra, IL-1α, and IL-1β. Simultaneous treatment of infected animals with antimalarials resulted in a further up-regulation of IL-1Ra and IL-12p40 in the brain; IGIF, IL-1α, IL-1β, and IL-12 (p35) in the spleen; and IL-1Ra in the liver. Also, in vitro treatment of RAW cells with chloroquine led to an induction of IL-1Ra production. Since IL-1Ra has been suggested to play a role in the pathogenesis of several viral infections, it may be possible that antimalarials accelerate viral replication and severity of illness through its up-regulation.

MATERIALS AND METHODS

Infection of mice and treatment schedules. BALB/c mice of either sex (Charles River Laboratories, Kingston, NY), 10–15 g, were used in these studies. The animals were used in compliance with the United States Public Health Service policy on humane care and use of laboratory animals. Semliki forest virus was obtained from the American Type Culture Collection (Rockville, MD) and EMCV was originally obtained from C. Buckler (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). We chose to study SFV, a neurotropic virus, not only because it is an established model in our laboratory, but also because it is easy to follow a disease course based on animal symptomology, i.e., progressive paralysis. Furthermore, CNS pathology can be objectively quantified, since the normal CNS does not show inflammatory infiltrate or the presence of inflammatory cytokines. Encephalomyocarditis virus was additionally selected because it is also neurotropic, but unlike SFV, belongs to a different group, and is non-enveloped.

Chloroquine, primaquine, quinine sulfate, and pyrimethamine were obtained from Sigma Chemicals (St. Louis, MO). Sulfadoxine was a gift from the Central Drug Research Institute (Lucknow, India). Genticin and cefotaxime were obtained from Gibco-BRL Life Technologies Inc. (Gaithersburg, MD).

Estimation of 50% lethal dose (LD50) of the viruses was carried out as detailed earlier.10 Infections were initiated with 10 LD50 of the virus via the subcutaneous route. In all experiments, mice were injected intraperitoneally with a single dose of one of the drugs in the following doses: chloroquine, quinine sulfate or primaquine, 200 μg/mouse; sulfadoxine, 300 μg/mouse; pyrimethamine, 50 μg/mouse. The doses of antimalarials that we used are representative of equivalent
ENHANCEMENT OF VIRAL REPLICATION BY ANTIMALARIALS

TABLE 1

Mean survival times (MSTs) and viral titers of semliki forest virus–infected mice treated with different antimalarials*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Cumulative no. of mice died postinfection (days)</th>
<th>Percent survival MST (days)</th>
<th>Viral titer (Log_{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus + saline (control)</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus + quinine sulfate</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Virus + pyrimethamine</td>
<td>5</td>
<td>2</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Virus + sulfadoxine</td>
<td>5</td>
<td>3</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Virus + primaquine</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mice treated with various antimalarials died earlier, thus a shorter MST. Furthermore, on day 4 postinfection, higher viral titers were seen in animals treated with antimalarials, as calculated by cytopathic effect assay (50% tissue culture infectious dose).

RESULTS

Shortening of survival and enhancement of viral titers by treatment with antimalarials. Treatment with antimalarials resulted in an earlier appearance of disease symptoms (such as roughening of hairs and paralysis of limbs) and accelerated mortality in infected mice. The MST of mice treated with antimalarials was consistently shorter, when compared with that of untreated infected mice. Estimation of viral titers in the brains of SFV-infected mice showed that mice treated with antimalarials had 50–200-fold higher titers than untreated mice (Table 1). Replication of EMCV was also significantly increased by antimalarials (20–500-fold), with titers being maximal in the brain and spleen, followed by liver, lung, and kidney (Figure 1). Treatment with genticin doses used clinically for malaria and adjusted for the different pharmacokinetics in rodents. Three animals in each group were left uninfected while five per group were simultaneously infected and treated with one of the antimalarials. Control groups included uninfected mice, saline controls (infected mice injected with saline intraperitoneally), and drug controls (uninfected mice treated with antimalarials). All experiments were repeated at least three times. As additional controls, we also studied the effects of two antibiotics on the mean survival time and virus titers. The antibiotics used were genticin (400 μg/mouse), which has been shown not to possess antimalarial activity, and cefotaxime (1,200 μg/mouse).

Viral titration and mean survival time. Viral titers (50% tissue culture infectious dose) in various organs were determined by a cytopathic effect assay. Titters for SFV were assayed in baby hamster kidney (BHK) cell lines, while EMCV was assayed in L_{4}c cells. The BHK and L_{4}c cells were grown in Eagle’s minimum essential medium with 10% fetal bovine serum. Viral kinetics performed have shown that SFV titers are maximal at the fourth day postinfection, and EMCV titers are maximal on the third day. Therefore, SFV-infected mice were killed on the fourth day postinfection, and brains, spleens, lungs, livers, and kidneys. Viral titers in the solid organs were determined after homogenizing the organs in Eagle’s minimum essential medium (10% w/v). Mean survival time (MST) was calculated as reported earlier.

Histopathology. To evaluate tissue damage, formalin-fixed, paraffin-embedded sections of brains and spinal cords of mice infected with SFV, and treated with chloroquine, quinine sulfate, primaquine, or sulfadoxine were examined at fourth day postinfection. Tissue sections (5 μ) were stained with hematoxylin and eosin and examined microscopically. Leukocyte margination in the blood vessels, perivascular inflammatory infiltrate, and neuronal destruction in the form of neuronophagia, perikaryolysis, and karyorrhexis were looked for as features of encephalomyelitis. Demyelination was confirmed by staining with Luxol fast blue.

Ribonuclease protection assay. We analyzed the expression of cytokine mRNA levels in brains, spleens, and livers of mice treated with chloroquine, quinine sulfate, primaquine, or sulfadoxine, and infected with SFV, using the RiboQuant multiprobe ribonuclease protection assay (RPA) kit (Pharmingen, San Diego, CA). The RPA was also performed on samples from untreated infected mice. Animals from each group were killed on the fourth day postinfection and brains, spleens, and livers were collected, snap-frozen, and stored at −70°C. The RNA was isolated from the frozen tissue samples using TRIZol (Life Technologies Inc., Gaithersburg, MD) and quantitated; equal concentrations of RNA were confirmed by electrophoresis on formaldehyde-agarose gels. The protocols used for the RPA were according to the manufacturer’s instructions. Briefly, 20 μg of each RNA sample was hybridized at 56°C for 12–14 hr with a 32P-UTP-labeled probe. The probe was prepared by transcribing the mouse cytokine template set using T7 RNA polymerase. After hybridization, samples were subjected to RNase digestion for 45 min at 30°C. The ribonuclease-protected bands were then resolved on denaturing urea–polyacrylamide gels, followed by autoradiography. The templates used allowed us to study the differential regulation of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p35), IL-12 (p40), IL-15, IL-1Ra, IFN-γ, IGIF, and macrophage-inducing factor (MIF). L-32 and glyceraldehyde 3-phosphate dehydrogenase mRNAs served as housekeeping gene controls in the assay to ensure equal loading of RNAs.

In vitro studies. RAW 264.7 cells (American Type Culture Collection) a mouse macrophage cell line, were grown in Dulbecco’s modified Eagle’s medium (Gibco-BRL Life Technologies Inc.) with 5% serum, 1% HEPES, 1% gluta- mine, and 1% antibiotic solution. When 70% confluent, they were treated with chloroquine (50 μM or 100 μM) for 3 hr at 37°C. The RNA was extracted from treated and untreated cells by the TRIZol method, and the RPA was performed as earlier described. Lipopolysaccharide (LPS; 100 ng/ml)-stimulated cells were used as positive controls for the experiment.
and cefotaxime did not affect survival or viral titers (Table 2).

**Brain and spinal pathology in SFV-infected mice.** On histopathologic evaluation, all the infected animals showed features of encephalomyelitis. However, the intensity of inflammation, neuronal destruction, and demyelination was higher in the tissues of animals treated with antimalarials when compared with untreated infected mice (Figure 2). The brainstem and spinal cord were affected more than the cerebrum and cerebellum. Tissues from drug controls (antimalarial treated but uninfected animals) showed no pathology, and were comparable to saline-treated controls.

**Differential up-regulation of cytokine mRNAs.** Infection with SFV led to an up-regulation in mRNA levels of various cytokines including IL-12p40, IGIF, IL-1Ra, and IL-1α and IL-1β. Following treatment with antimalarials, a further significant increase was seen in the transcripts of IL-1Ra and IL-12(p40) in the brains of SFV-infected mice, and a moderate increase was seen for IL-6, IL-α, and IL-1β (Figure 3). We also found an up-regulation of IGIF, IL-1α, IL-1β, and IL-12(p35) in the spleens and IL-1Ra in the livers of these mice. These patterns were highly reproducible on repeating the experiments. In vitro treatment of RAW cells with chloroquine led to an induction of IL-1Ra production (Figure 4).

**DISCUSSION**

The earlier appearance of symptoms, shortened survival, higher viral titers, greater tissue pathology, and a concomitant up-regulation of cytokine mRNAs clearly demonstrates that viral replication was accelerated following treatment with various antimalarials. Interestingly, this effect was not restricted to any one antimalarial, and was seen to occur in infections with different virus types (SFV and EMCV). We have obtained similar results when mice are infected with herpes simplex virus-1 and treated with chloroquine (Mareshwari RK, unpublished data). Genticin and cefotaxime, two antibiotics, did not yield any such effect, thereby suggesting that enhancement of disease severity, mortality, and greater viral replication, are specific and restricted to the antimalarials tested. Currently, studies are in progress to set up a model of dual infection of mice with virus and malarial parasite to further evaluate the effect(s) of antimalarials in such a setting.

Using in vitro models, the effect of chloroquine on the regulation of cytokines has been studied. Chloroquine has been shown to reduce IL-2 production and thus inhibit T cell proliferation, inhibit production of tumour necrosis factor-α, IL-6, and IFN-γ by peripheral blood mononuclear cells, and induce IL-1 production by endothelial cells. These results are obtained based on the immunosuppressive effect of chloroquine using high doses. We have, however, used only a single dose of antimalarials at doses comparable with those clinically used for malaria.

Cytokines are known to play an important role in SFV encephalitis, and SFV has been widely used to study mechanisms in neuroimmunology. Various interleukins including IL-1α, IL-2, IL-4, and IL-6 have been shown to be up-regulated in the CNS of SFV-infected mice. We noted an increase in mRNAs of mainly IL-1Ra, IL-12p40, and IL-1 in brains following infection with SFV. These cytokines showed an additional significant increase in mice treated with different antimalarials. Analysis of RNAs from the spleens and livers showed a differential up-regulation of cytokine mRNAs with IGIF, IL-1α, IL-1β, and IL-12 (p35) being increased in the spleen, and mainly IL-1Ra in the liver. No appreciable changes in cytokine mRNAs were seen in uninfected mice treated with antimalarials.

Levels of IL-1Ra are increased by a number of viruses, as seen by up-regulation in monocytes/macrophages by cytomegalovirus and the Tat gene of human immunodeficiency virus (HIV), induction in neutrophils by Epstein-Barr virus (EBV), and elevated IL-1Ra/IL-1β ratios in serum of patients with hepatitis. More recently, a significant level of IL-1Ra production by human monocytes over that of IL-1α and IL-1β, was seen in HIV infection, and higher IL-1Ra concentrations were reported in the cerebrospinal fluid of HIV-infected patients. We propose that the up-regulation of IL-1Ra in mice treated with various antimalarials may have been responsible for disrupting immune defense responses in the early stages of SFV infection. This could have led to earlier establishment of the virus in the tissues, and thereby accelerated replication, and greater severity of encephalitis. Such an up-regulation by antimalarials may explain observations about chloroquine reported by others, such as enhancement of EBV expression in Burkitt’s lymphoma, increased incidence of herpes zoster in children, and transactivation of HIV Tat protein.

We are also studying other possible mechanism(s) in-
involved in the enhancement of viral replication by antimalarials. Since the effects of the single dose of drugs appear quite early, it is possible that antimalarials allow greater viral entry across the blood-brain barrier by abrogating initial immune responses by natural killer cells, or causing endothelial cell damage in the CNS microvasculature. Semliki forest virus has been shown to infect CNS endothelial cells, furthering the leakage across the blood-brain barrier, followed by enhanced expression of intercellular adhesion molecule on the endothelial cells, allowing exudation of more inflammatory cells, with the effects possibly mediated by cytokines. Although chloroquine has been shown to inhibit T cells, the presence of demyelination in infected mice following treatment suggests that T cell responses remained...
Figure 3. Cytokine mRNA levels in mice brain. Mice were infected with Semliki forest virus (SFV) (10^5 50% lethal doses given subcutaneously) and simultaneously treated with chloroquine (Chl) (200 μg/mouse), quinine sulfate (Quin) (200 μg/mouse), primaquine (primaquin) (200 μg/mouse), or sulfadoxine (sulfadoxin) 300 μg/mouse. SFV infected (+ SFV) and uninfected (- SFV) mice injected with saline or antimalarials served as controls. Animals from each group were killed on fourth day postinfection and brains were collected. The ribonuclease protection assay (RPA) was carried out using the RiboQuant Multiprobe RPA kit (Pharmingen, San Diego, CA) and RNA isolated from the pooled brain samples. The figure represents one of three different experiments. Marked differences can be seen in levels of IL-12p40, IL-1Ra, IGIF, and IL-1 mRNA. IL-12 = interleukin-12; IL-1Ra = IL-1 receptor antagonist; IGIF = interferon-γ-inducing factor; IFN-γ = interferon-γ; MIF = macrophage-inducing factor; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.
active. Virus activation of inflammatory cells is known to induce reactive oxygen species and pro-oxidant cytokines such as IL-1. Chloroquine has been suggested to render the organism more susceptible to oxidative stress, and this may be an additional pathway leading to greater inflammatory damage in chloroquine-treated animals.

Notwithstanding the molecular mechanisms, the demonstration of enhancement of viral replication by antimalarials is of immense clinical relevance. Given the fact that the majority of the world population resides in areas endemic for malaria, and the added exposure to viral infections in the environment therein, the widespread practice of empirical use of antimalarials in the treatment of fevers could increase morbidity, if the fever were of viral etiology. The added use of antimalarials in the treatment of fevers could increase morbidity, if the fever were of viral etiology. The added exposure to viral infections in the environment therein, the widespread practice of empirical use of antimalarials in the treatment of fevers could increase morbidity, if the fever were of viral etiology.

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