Immune-mediated parasite clearance in mice infected with *Plasmodium berghei* following treatment with manzamine A

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**Abstract** Manzamine A, a sponge-derived alkaloid, was recently shown to possess in vivo antimalarial activity against the blood stages of the rodent malaria parasite *Plasmodium berghei*. A single intraperitoneal dose of 100 μmol/kg of manzamine A suppressed parasite growth but was followed by parasite recrudescence. Forty percent of mice with recrudescing parasites were able to recover and clear the fulminating parasitaemia. Examination of sera from these mice revealed that infected mice treated with manzamine A had a suppressed IFN-γ production but an increase in their IL-10 and IgG production. The prolonged survival of infected mice treated with manzamine A and the eventual clearance of recrudescing parasites in some of these mice involve a down-regulation of Th1 responses and a switch to antibody dependent-Th2 responses.

**Introduction**

Malaria continues to plague the world’s population with great impact on human health. A major reason for this devastating situation is the emergence of drug resistance to the currently available antimalarial drugs (Krogstad 1996). Hence, there is a need for the development of structurally novel antimalarials (Trigg and Kondracki 1998). We have recently demonstrated the in vivo antimalarial potential of manzamine A against the rodent malaria parasite *Plasmodium berghei*, together with its pharmacokinetics in mouse plasma and its effect on the morphology of *P. berghei* (Ang et al. 2000). Manzamine A (Fig. 1) was first isolated from a *Halichondria* sp. sponge from Okinawan waters (Sakai et al. 1986). It belongs to a structurally unique group of more than 30 β-carboline alkaloid manzamines found in various marine sponges from the Indian and Pacific Oceans. The discovery and chemistry of various manzamines have recently been reviewed (Higa and Tanaka 1996; Magnier and Langlois 1998). The considerable biological and chemical interest in manzamines has recently resulted in the complete synthesis of manzamine A (Winkler and Axten 1998). A single intraperitoneal injection of 100 μmol/kg of manzamine A suppresses parasitaemia in mice but generally does not cure it. Recrudescence occurs and the treated mice eventually develop fulminating parasitaemia. However, some manzamine A-treated mice are able to recover from such heavy infection after a single dose of treatment (Ang et al. 2000). In the present study, we examined whether there is an immune-mediated clearance of the recrudescing parasites in mice, following treatment with manzamine A. This was carried out by measuring the serum concentration of immunoglobulin G (IgG) and several key cytokines associated with the host cellular and humoral immune response, such as interferon-γ (IFN-γ), interleukin-10 (IL-10) and tumour necrosis factor-α (TNF-α).

**Materials and methods**

**Compound**

Manzamine A, provided by T. Higa from the University of the Ryukyus, was isolated and purified as a monohydrochloride salt (Sakai et al. 1986).

**Parasites and mice**

The erythrocytic stages of *Plasmodium berghei* (ANKA strain) were maintained in male Swiss albino mice via serial passaging of infected blood. Experimental animals used were male Swiss albino mice at 4 weeks of age, purchased from the Laboratory Animal Centre, Singapore. Mice were housed in cages and provided with water and food ad libitum.

**Experimental design**

The experiment involved four groups of mice: (1) control mice, (2) infected/manzamine A-treated mice, (3) infected mice and (4)
uninfected/manzamine A-treated mice. For the control group, four mice were left uninfected and untreated. For the infected/manzamine A-treated group, 22 male Swiss albino mice were intraperitoneally injected with 10⁶ P. berghei-infected mouse erythrocytes on day 0. On day 2 after infection (0.7–3.1% parasitaemia), these mice were treated with a single intraperitoneal injection of 100 μmol/kg of manzamine A. The antimalarial compound was injected as a suspension in 5% Tween-60 saline. For the infected group, eight mice were infected as above, but were intraperitoneally injected with 5% Tween-60 saline only. Parasitaemia was followed by light microscopic observation of blood films stained with 5% Giemsa solution (Merck). For the uninfected/manzamine A-treated group, 12 uninfected mice were treated with a single intraperitoneal injection of 100 μmol/kg of manzamine A. In each group, four mice were bled at different time points as indicated:

1. Control group: day 0
2. Infected/manzamine A-treated mice: days 4, 8, 12 and 60 after infection
3. Infected mice: days 2 and 4 after infection
4. Uninfected/manzamine A-treated mice: days 2, 10 and 30 after treatment

Sera were stored at −20 °C until required for the enzyme-linked immunosorbent assays (ELISA).

Preparation of malaria antigens for ELISA

Blood containing P. berghei was collected by cardiac puncture of infected male Swiss albino mice when their parasitaemia reached 50–60%. The blood was then layered on a 2x volume of Lymphosep (lymphocyte separation medium; ICN Pharmaceuticals, USA) and centrifuged (600 g, 20 min, 4 °C). The plasma and buffy coat were carefully removed and discarded. The deposited erythrocytes were resuspended in phosphate-buffered saline (PBS, pH 7.4) to the original volume of blood and then lysed with 0.1% saponin (60 min, 4 °C). The lysed suspension was centrifuged (10,000 g, 5 min, 4 °C) and washed with PBS. The soluble antigen preparation was produced by resuspension of the harvested parasites in 1 ml PBS, followed by sonication (5 min, 4 °C) and then centrifugation (10,000 g, 5 min, 4 °C). The supernatant (the soluble antigen) was stored at −80 °C until used for malaria-specific IgG ELISA.

Total IgG ELISA

The concentration of total IgG in mouse sera was determined using a commercial mouse-IgG ELISA kit, following the manufacturer’s instruction (Boehringer Mannheim, Germany). The assay captures all IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) in mouse serum. Evaluation of the concentration of total IgG was performed using a calibration curve ranging over 6.25–1,000 ng/ml. Test sera were diluted 10⁴ or 10⁶ times for assay. All experiments were carried out in duplicate.

Malaria-specific IgG ELISA

Flat-bottomed, 96-well ELISA plates (Nunc, Denmark) were coated with 50 μl of malaria antigen (1 μg/ml) per well (4 °C, overnight). The plates were then washed three times with 0.1% Tween-20 saline, saturated for 30 min with blocking reagent for the ELISA (Boehringer Mannheim, Germany) and washed repeatedly. Subsequently, 50 μl of mouse sera (as primary antibody) was added to each well at 1:1000 dilution. After incubation (room temperature, 1 h), the plates were washed as described above. The plates were then incubated (room temperature, 1 h) with 50 μl of goat anti-mouse IgG-peroxidase conjugate (Gibco BRL, USA) per well, at 1 μg/ml. Following another wash, 50 μl of ABTS substrate solution (Boehringer Mannheim, Germany) was added to each well. The optical density values were read at 405 nm, using a SpectraMax 340 microplate reader (Molecular Devices, USA). All experiments were carried out in duplicate.

Cytokine ELISA

IFN-γ, IL-10 and TNF-α concentrations in mouse sera were analysed by commercial Quantikine M ELISA kits according to the manufacturer’s protocol (R&D System, Minneapolis, USA). In each cytokine ELISA, a standard curve was generated to determine the concentration of cytokine in mouse sera. Sera were assayed undiluted in the IFN-γ and TNF-α ELISA, whereas sera were assayed at 1/2 dilution in the IL-10 ELISA. All experiments were carried out in duplicate.

Statistical analysis

Data are presented as group means and standard errors with n = 4. Data analysis was performed using Analyse-it software linked to Microsoft Excel. Differences between means were evaluated with the non-parametric Mann-Whitney U test, with P < 0.05 considered significant.

Results

Parasitaemia

Low level parasitaemia developed in the infected mice by day 2 after infection, rising to more than 60% on day 4 (Table 1). All infected mice in this group died of an unremitting parasitaemia by day 6 post-infection. On day 4 after infection (i.e. 2 days after treatment with manzamine A), parasitaemia of the infected/manzamine A-treated mice remained low (Table 1). This result confirms that a single dose of 100 μmol/kg of manzamine A suppresses the rise of parasitaemia (Ang et al. 2000). However, these mice experienced parasite recrudescence, carrying moderately high parasitaemia on day 8 and reaching a high parasitaemia level (65%) on day 12 after infection (i.e. day 10 after treatment). Among the 14 surviving mice on day 12 post-infection, four were sacrificed for ELISA assays, six eventually...