Modulation of IFN-γ, TNF-α, IL-10 and IL-12 Production by Artesunate in Mitogen Treated Splenocytes

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Abstract: This study compared the effects of artesunate and chloroquine on mitogen-induced splenocyte release of several important cytokines involved in the pathogenesis of malaria. Concanavalin A (Con A) or Lipopolysaccharide (LPS) was used to stimulate splenocytes from healthy C57BL/6 mice in an *in vitro* system. Splenocytes were stimulated for 24 or 48 h at 37°C and in 5% CO_2 in the presence or absence, respectively, of different concentrations of chloroquine and artesunate. Culture supernatants were tested for TNF-α (after 24 h stimulation), IFN-γ (after 48 h stimulation), IL-10 (after 24 h stimulation) and IL-12p40 (after 48 h stimulation), using ELISA. Chloroquine, but not artesunate, inhibited ConA-induced IFN-γ release over the drug concentration range of 3-30 µM. Artesunate inhibited ConA IFN-γ release at the concentration of 90 µM, compared with controls (p = 0.0138). Over the dose range 3-30µM, artesunate stimulated production of IL-12p40, while chloroquine caused dose-dependent inhibition (p = 0.03). Both chloroquine and artesunate produced dose-dependent inhibition of TNF-α, chloroquine being the stronger inhibitor over the same dose range. Artesunate had no effect on IL-10 production at the concentration of 3 µM but at the highest test concentration of 90 µM, artesunate almost eliminated the response. The differences in immunomodulatory activity observed between chloroquine and artesunate might explain the differences in therapeutic response in malaria and possibly the recrudescence associated with artesunate but not with chloroquine monotherapy. Further studies are necessary to understand better the role of the different immunological effects of antimalarial drugs on malaria treatment outcomes.

Key words: Artesunate, cytokines, chloroquine, malaria, immunomodulation, LPS

INTRODUCTION

Artemisinin-based malaria treatment is replacing chloroquine as first-line treatment of malaria in most malaria endemic areas (Breman *et al.*, 2004; Davis *et al.*, 2005). The use of artemisinin derivatives has been associated with recrudescence of the disease, particularly following monotherapy and this appears to be happening more often than with treatment with the aminoquinolines (Giao *et al.*, 2001; Nguyen *et al.*, 1993). The association between the immunomodulatory properties of an antimalarial drug and treatment outcomes in malaria has been reported for chloroquine and for several other antimalarials (Kwiatkowski *et al.*, 1993; Kwiatkowski and Bate, 1995). Treatment outcome had been good with chloroquine monotherapy until chloroquine-resistance developed; this has been attributed in part to the drug's immunomodulatory activity (Picot *et al.*, 1997). The fact that sulphadoxine-pyrimethamine may exert complete anti-parasite efficacy while being less effective than chloroquine in relieving clinical symptoms (Bojang *et al.*, 1998) further suggests a therapeutic benefit of enhanced immunological activity in malaria treatment.

In addition to influencing malaria treatment outcomes, the immunomodulatory properties of the respective drugs might be important in understanding their clinical activity as antimalarials. Chloroquine is used to treat rheumatoid arthritis on the basis of its immunomodulatory properties and there have been a number of clinical trials of chloroquine in the treatment of other immunological diseases (Paton and Aboulhab, 2005; Reeves *et al.*, 2004; Dean *et al.*, 2003). Artemisinin derivatives have been reported to stimulate lymphocyte proliferation and they have been associated with delayed-

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type hypersensitivity reactions (Lin *et al.*, 1984; Sun *et al.*, 1991; Tawfik *et al.*, 1990). Artesunate enhances anti-SRBC antibody formation in mice (Kwiatkowski and Bate, 1995). Kwiatkowski and Bate (1995) found that at standard doses the TNF-suppressive properties of chloroquine are greater than of quinine and far greater than of either artemether or artesunate. These results suggest that cytokines play a major role in the pathogenesis of malaria and modulation of their release might play an important role in the therapeutic response. There are, however, no reports known to us on the effects of artemisinin derivatives on release of pro and anti-inflammatory cytokines.

In this study, we have investigated the effects of artesunate on mitogen-induced release of TNF-α, IFN-γ, IL-10 and IL-12 in an *in vitro* system, compared with chloroquine, previously one of the most effective antimalarial agents.

MATERIALS AND METHODS

Experimental animals: Wild strains of C57/BL6 mice from clones at the animal unit, Faculty of Health Sciences, University of Cape Town were housed in cages in groups of 5 at an ambient temperature of 22°C and relative humidity 80%. The diet was a standard pellet with continuous availability of clean drinking water.

Isolation of splenocytes: Healthy uninfected C57BL/6 mice 7-10 weeks of age were euthanased by intra-peritoneal injection of a lethal dose of sodium thiopentone. The spleens were removed under sterile conditions and placed in iced 1 mL RPMI 1640 medium (Sigma, Cat. No. R-0883). Spleen cells were isolated by perfusing each spleen with 2-3 mL of RPMI 1640 supplemented with Gentamicin (25 μg mL^{-1}) (Sigma, UK), 2 mM L-glutamine (Gibco) and 10% heat-inactivated Fetal Calf Serum (FCS). Cell aggregates were left to sediment for a few seconds and the cell suspension was then transferred into a fresh sterile tube. The cell suspensions were centrifuged for 10 min at 1500 rpm at 4°C and resuspended in 5 mL of lysis buffer and kept on ice for 60 sec. The cell suspension was centrifuged for 10 min at 1500 rpm at 4°C and resuspended into 20 mL culture medium. The cells were counted and plated in a concentration of 0.5×10^6 cells well^{-1} in 96-well plates (Falcon, Becton Dickinson) (5×10^6 cells mL^{-1}) with a volume of 100 μL well^{-1}. Viability was determined by exclusion of trypan blue dye. Splenocytes were pre-treated with chloroquine and artesunate (Sigma-Aldrich) in the following concentrations: 3, 10, 30 and 90 μM for 3 h at 37°C and 5% CO_2. The splenocytes

were then stimulated *in vitro* with 10 mg mL^{-1} Concanavalin A (Con A) (Sigma) or Lipopolysaccharide (LPS) diluted in RPMI for 24 or 48 h at 37°C and 5% CO_2. Splenocyte culture supernatants were tested for TNF-α (after 24 h stimulation), IFN-γ (after 48 h stimulation), IL-10 (after 24 h stimulation) and IL-12p40 (after 48 h stimulation), using ELISA.

Enzyme linked immunosorbent assay: Ninty six-well Maxisorb microtiter plates (Nalge Nunc International, Naperville, Il, USA) were incubated overnight with capturing antibody (10 μg mL^{-1}) at 4°C. Plates were washed 4 times with washing buffer and incubated overnight with 200 mL blocking buffer at 4°C. The blocking buffer was removed and recombinant mouse cytokine standards were added in 3-fold dilutions at concentrations ranging 100 μg mL^{-1} -0.6 pg mL^{-1}. Samples were added undiluted and the plates incubated overnight at 4°C. Plates were washed 4 times and incubated overnight with a biotinylated-detecting antibody (2 μg mL^{-1}) at 4°C. Plates were washed 4 times and incubated with streptavidin-alkaline phosphatase (BD Pharmingen, USA) (1:1000 dilution) for 2 h at room temperature. The plates were subsequently washed, incubated with p-nitrophenyl phosphate (1 mg mL^{-1}) (Boehringer Mannheim, Germany) and the enzymatic reaction read at 405 nm using a microplate spectrophotometer (Molecular Devices, spectra MAXGemini). Interleukin 12 (IL-12p40), Interleukin 10 (IL-10), Tumour Necrosis Factor-α (TNF-α) and Interferon-γ (IFN-γ) were measured by specific ELISA, after stimulation with Lipopolysaccharide (LPS) and Concanavalin A (ConA) in the presence or absence of different concentrations of artesunate and chloroquine.

Use of clinically relevant concentrations and exposure duration: The decision as to what drug concentrations to use in this study was based on the results of 2 studies (Walker *et al.*, 1983; Teja-Isavadharm *et al.*, 2001). In these studies maximum plasma concentrations of 200-800 and 186-406 nmol L^{-1} of chloroquine and artesunate, respectively, were reported. Higher drug concentrations were used since it has been reported that both drugs are highly tissue bound (White, 1985). The duration of exposure was based on the observation that complete parasite clearance from the blood stream occurs between 24 and 48 h with both drugs (Barennes *et al.*, 2004).

Data analysis: Statistical analysis was performed using the student's t-test. For all tests, a p<0.05 was considered significant. Graphical presentations were made using Microsoft Excel 2000.

Ethical issues: The study was approved by the Animal Research and Ethics Committee of the University of Cape Town. The study animals were handled in conformity with international ethics norms.

RESULTS

The 4 study cytokines were not detectable in culture without addition of a mitogen. Trypan blue staining showed no increase in cell death upon culturing in the presence of either chloroquine or artesunate at any of the doses tested.

Effect of artesunate on TNF-α production: Maximum production of TNF-α was reached after 24 h stimulation with Lipopolysacharide (LPS) at 10 μg mL^{-1}. Artesunate had no effect on TNF-α production by mitogen-treated splenocytes. Chloroquine inhibited TNF-α production in a dose-dependent manner at a dose of 10 μM (p = 0.03) (Fig. 1).

Interferon gamma production: Maximum production of IFN-γ by splenocytes stimulated with ConA was attained after 48 h. Chloroquine inhibited ConA IFN-γ release more than artesunate over the study concentration range (Fig. 2). ConA IFN-γ production is almost eliminated at the concentration of 90 μM.

Effect on IL-12p40 production: Maximum production of IL-12p40 by splenocytes stimulated with Lipopolysaccharide (LPS) was reached at 48 h. Artesunate over the concentration range 10-30 μM had no inhibitory effect on IL-12p40, while chloroquine produced dose-dependent inhibition of IL-12p40 production over the entire concentration range tested (Fig. 3). A stimulatory effect on IL-12p40 production was observed with artesunate at a concentration of 3 μM (p = 0.01).
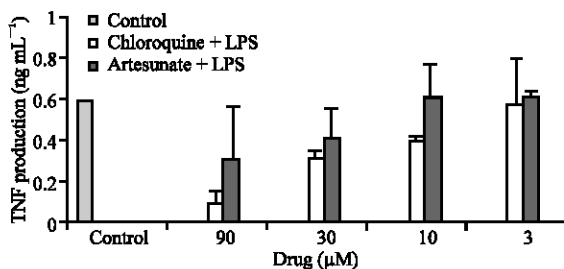

Fig. 1: The effects of a concentration range of chloroquine and artesunate on LPS- induced TNF-α production for 24 h. Results are expressed as means±Standard Error of the Mean (SEM) of duplicates from 3 independent experiments

Effect on IL-10 production: Maximum production of IL-10 by splenocytes stimulated with ConA was reached after 24 h. Artesunate inhibited production of IL-10 at concentrations >30 μM. At the highest concentration
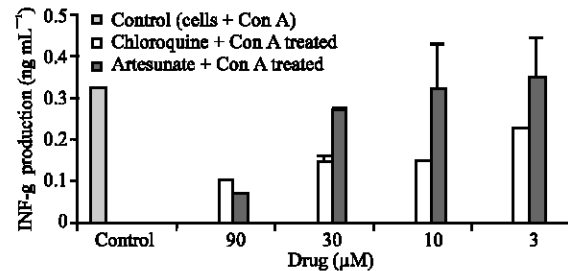

Fig. 2: The effects of a concentration range of chloroquine and artesunate on LPS- induced IFN-γ production for 24 h. Results are expressed as means±Standard Error of the Mean (SEM) of duplicates from 3 independent experiments
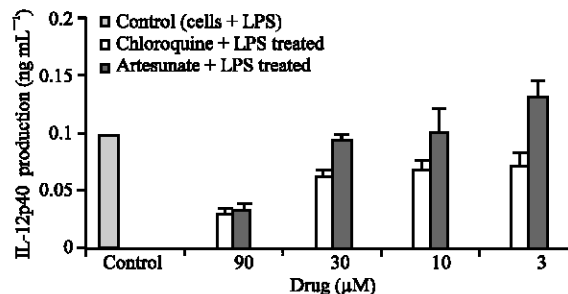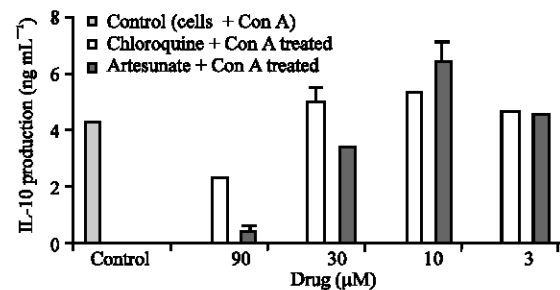

Fig. 3: The effects of a concentration range of chloroquine and artesunate on LPS-induced IL-12p40 production for 24 h. Results are expressed as Means±Standard Error of the Mean (SEM) of duplicates from three independent experiments

Fig. 4: The effects of a concentration range of chloroquine and artesunate on LPS- induced IL-10 production for 24 h. Results are expressed as means±Standard Error of the Mean (SEM) of duplicates from 3 independent experiments

90 μM, artesunate virtually eliminated the IL-10 response (Fig. 4). Chloroquine also inhibited the production of IL-10 at the concentration of 90 μM, although to a less extent than artesunate.

DISCUSSION

This study reports differences between the effects of artesunate and chloroquine in the production of cytokines stimulated in splenocytes by mitogens. Artesunate only showed similarities to chloroquine in its effect on release of IL-10, being more potent than chloroquine.

Few studies have explored the effect of artesunate on cytokine production (Ittarat *et al.*, 1999; Yang *et al.*, 1993). They indicate that the drug has immunomodulatory properties. In this study, artesunate did not inhibit the pro-inflammatory cytokines but it did enhance IL-10 production suggesting anti-inflammatory activity. IL-10 is a critical component in the down-regulation of pro-inflammatory immune reactions and in the up-regulation of anti-inflammatory responses. Artemisinin has been reported to increase phagocytic capacity of peritoneal macrophages, interferon production and delayed-type hypersensitivity responses (Qian *et al.*, 1981, 1987; Ye *et al.*, 1982). Taken in conjunction with our findings that artesunate showed no increase in interferon production by either Con A-or LPS-stimulated splenocytes, it appears that there may be different immunological effects of artesunate and the parent compound artemisinin. Sodium artesunate, a water-soluble derivative of artemisinin, markedly enhances anti-SRBC antibody formation when given to mice intraperitoneally in doses of 50 mg kg^{-1} daily for 5 days (Chen *et al.*, 1988). Artemisinin and its derivatives dihydroartemisinin and arteether suppress humoral response in doses ranging from 400-600 mg kg^{-1} (Tawfik *et al.*, 1990). Our results are consistent with the previously observed dose-related response with artemisinin and its derivatives.

The effects of chloroquine on IFN-γ and TFN-α production that we have found are in agreement with the results in 2 other studies (Landewe *et al.*, 1995; Picot *et al.*, 1993). The inhibitory effect of chloroquine on LPS-induced TNF-α production in cultured splenocytes is concentration-dependent and is likely to be caused by the disruption of iron homeostasis rather than by a lysosomotropic mechanism.

These findings have implications for the treatment of malaria and they provide a basis for other possible clinical uses of artesunate. Chloroquine, which is falling into disuse in the treatment of malaria because of increasing resistance to the drug, is a better suppressant of pro-inflammatory cytokines than artesunate. This property could maintain chloroquine in the picture with the beginning of considerations for triple drug therapy in malaria.

CONLCUSION

However, extrapolation of such *in vitro* results and conclusions to the clinic requires caution. An *in vivo* test system is far more complex than the closely controlled *in vitro* system. Nevertheless, testing the effects of chloroquine and artesunate *in vitro* provides an opportunity to predict the immune modulatory action of the drugs *in vivo*, provided the concentrations used *in vitro* are relevant to the *in vivo* situation and not toxic to the cells. The other limitation is that we used splenocytes from non-infected mice; the use of mice infected with malaria could have provided results with higher predictive values in malaria infection. The splenocytes were used to model for the immunological cells in malaria. Better still use of leucocytes from parasiteamic individuals would provide information, which is more relevant to clinical malaria. The differences in immunomodulatory properties observed between chloroquine and artesunate might explain the recrudescence associated with monotherapy with the latter.

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