ARTESUNATE AND A MAJOR METABOLITE, DIHYDROARTEMISININ, DIMINISH MITOGEN-INDUCED LYMPHOCYTE PROLIFERATION AND ACTIVATION

P Veerasubramanian, P Gosi, C Limsomwong and DS Walsh

Department of Immunology and Medicine, US Army Medical Component, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand

Abstract. Artemisinin derivatives are potent antimalarial compounds that may have immunomodulatory properties. Artesunate (range 0.01-2 μg/ml) or dihydroartemisinin (range 0.01-8 μg/ml; DHART) were added to peripheral blood mononuclear cells (PBMC) or whole blood (WB) cultures before or simultaneously upon stimulation with phytohemagglutinin (PHA), a T cell mitogen. Lymphoproliferation was then measured by ^{3}[H]-thymidine incorporation, and CD4+ and CD8+ T cell activation was assessed by expression of CD69 or CD25 using flow cytometry. Reverse transcriptase polymerase chain reaction depicted PBMC mRNA production for interleukins 2, 4, 12, and 15, interferon-γ, and tumor necrosis factor-α. Artesunate concentrations between 0.1-1.5 μg/ml reduced lymphoproliferation in PHA-stimulated PBMC and WB cultures in a generally dose-dependent manner; inhibition by DHART was similar. Removing artesunate from PBMC before PHA was added abolished the reduction. PBMCs cultured with artesunate or DHART simultaneously with PHA showed modestly reduced proportions of CD4+ and CD8+ T cells expressing CD69 and CD25. Artesunate had little effect on qualitative cytokine mRNA levels in PHA-stimulated PBMC cultures. Artesunate and DHART may diminish some PBMC responses to immunologic stimuli. Further work is warranted to define the mechanisms involved, and whether this affects malaria treatment.

INTRODUCTION

Artemisinin ("qinghaosu"), isolated in 1972 by Chinese scientists, is a potent antimalarial substance extracted from the plant *Artemisia annua* (Hien and White, 1993). Two active artemisinin derivatives, artemether and artesunate, were subsequently developed for the treatment of severe falciparum malaria. Oral, parenteral, and rectal preparations of the artemisinins are widely available, particularly in Southeast Asia, where multiple drug resistant *P. falciparum* malaria is responsive to artemisinins (Hien and White, 1993; Wilairatana *et al*, 2000).

Artesunate, arteether, and their major metabolite dihydroartemisinin (DHART) may have immunomodulatory properties. For example, mice injected with artesunate show enhanced humoral and non-specific immune functions (Guojun and Yi, 1983). Artemisinin, artesunate, and DHART may also enhance T cell responses and accelerate immuno-reconstitution in mice after bone marrow transplantation (Yang *et al*, 1993). In contrast, artemisinins may inhibit antibody and cellular immune responses (Tawfik *et al*, 1990; Chen

Correspondence: Dr Douglas Walsh, Director, Kondele Research Unit (Walter Reed Project), Kisumu, Kenya.
Tel: (254) 0733-616-602
E-mail : douglas.s.walsh@us.army.mil

et al, 1994; Noori *et al*, 2004). In endothelial cells, artesunate reduces activation after lipopolysaccharide exposure, possibly by inhibiting TNF-α production (He and Liu, 2004). In patients with severe malaria, treatment with artesunate, in comparison with quinine, was associated with less plasma TNF-α, a mediator of red cell vascular cytoadherence (Ittarat *et al*, 1999).

We conducted a series of *in vitro* experiments to assess dose ranging effects of artesunate, the most widely used artemisinin, and DHART, the major metabolite of artesunate, on mitogen-driven peripheral blood mononuclear cell proliferation, activation, and cytokine production.

MATERIALS AND METHODS

Lymphoproliferation assays (LPAs)

Conventional LPAs were conducted with cultures of peripheral blood mononuclear cells (PBMC) isolated by Ficoll density gradient centrifugation or heparinized whole blood obtained from 2 healthy adult blood donors (Pichyangkul *et al*, 2001). For each sample, mononuclear cell counts were made by hemocytometer to calculate the number of cells/ml of blood. Each experiment was conducted at least twice in triplicate sets of wells.

PBMC were washed twice in RPMI-1640 medium (Gibco BRL, Grand Island, NY) and re-suspended in RPMI complete medium (RPMI-1640 supplemented with 10% fetal bovine serum, and 0.04 IU/ml of gentamicin) in 96-well, round-bottom plates at a concentration of 2.5×10^5 cells per well. Heparinized whole blood was diluted 1:5 in RPMI complete media and adjusted to a final concentration of 2.5×10^5 mononuclear cells per well.

For the LPAs, powder formulations of artesunate and DHART (Walter Reed Army Institute of Research; lot numbers BM17450 and BN46116, respectively) were solubilized in RPMI-1640 media. Phytohemaglutinin (PHA), at a final concentration of 10 μg/ml per well, was used as the mitogen in all experiments. All plates were incubated at 37°C and 5% CO_2 for 3 days after adding PHA. Then, the wells were pulsed with 20 mCi/ml of ^{3}H-thymidine for 18 hours and harvested onto glass fiber filter using an automated plate harvester (Tomtec, Orange, CT). Proliferative responses were measured by the uptake of ^{3}H-thymidine in a liquid scintillation counter (Wallac 1205 Beta plate, Turku, Finland), expressed as counts per minute.

LPAs assessing artesunate were conducted in 2 ways: artesunate pre-treatment, followed by PHA, or artesunate and PHA added simultaneously. For artesunate pre-treatment experiments: 1) in PBMC, artesunate (final concentrations of 0.01, 0.1, 0.5, 1.0, 1.5, and 2.0 μg/ml) was added; 24-48 hours later, PHA was added without washing cells, with harvest 3 days later; or, 2) in PBMC, artesunate (final concentrations of 0.01, 0.1, 0.5, 1.0, 1.5, and 2.0 μg/ml) was added. Twenty-four hours later, cells were washed with RPMI to remove artesunate; fresh media containing PHA only was added, with harvest 3 days later. For experiments in which artesunate and PHA were added simultaneously: 1) in PBMC or whole blood, artesunate (final concentrations of 0.01, 0.1, 0.5, 1.0, 1.5, and 2.0 μg/ml) and PHA were added simultaneously, with harvest 3 days later. An artesunate alone control was also conducted: in PBMC, artesunate (final concentrations of 0.01, 0.1, 0.5, 1.0, 1.5, and 2.0 μg/ml) was added, followed by harvest 48 hours later to assess for cell viability and intrinsic mitogenicity of artesunate.

LPAs assessing DHART were conducted in 2 ways: DHART pre-treatment, then PHA, or DHART and PHA added simultaneously. For DHART pre-treatment experiments, in whole blood, DHART (final concentrations of 0.01, 0.1, 0.5, 1.0, 1.5, 2.0, 4.0, and 8.0 μg/ml) was added. Three days later, PHA was added without washing, with harvest 3 days later. For

experiments in which DHART and PHA were added simultaneously, in PBMC, DHART (0.01, 0.1, 0.5, 1.0, 1.5, 2.0, 4.0, and 8.0 μg/ml, final concentrations) and PHA were added, with harvest 3 days later.

Flow cytometry analysis of T cell activation

We used fluorescein (FITC) or phycoerythrin (PE) conjugated monoclonal antibodies to study lymphocytes that included anti-CD4 (helper T cell), anti-CD8 (cytotoxic T cell), anti-CD69 (early activation), and anti-CD25 (IL-2 receptor; late activation) (Becton Dickinson, San Jose, California), in a double staining technique (De Paolo *et al*, 1984).

PBMC were cultured with artesunate and PHA for 5 days or DHART and PHA for 3 days and harvested daily in most experiments. Triplicate wells were pooled to obtain pellets of 0.75-0.9 x 10^6 cells that were re-suspended in 100 μl of RPMI 1640 media. Ten microliters of fluorescein or phycoerthrin-conjugated monoclonal antibody was added to the cell suspension and incubated at 4ºC for 30 minutes in the dark. The cells were washed in RPMI 1640 once and centrifuged. The pellet was re-suspended in 0.5 ml of PBS containing 1% paraformaldehyde and stored at 2 to 8ºC in the dark until analysis.

Analysis of cell surface immunofluorescence was performed on a FACScan Flow Cytometer (Becton Dickinson, Mountain View, California). For each stained sample, 10,000 cells were analyzed for staining positivity after linear light scatter gating on lymphocytes. The fluorescent distribution was analyzed using Consort C30 software. Throughout the study, unstained and mixed FITC and PE Calibrite™ beads were used to calibrate the cytometer. Results were expressed as a proportion of positively stained cells obtained from histogram and two-parameter analysis.

Cytokine mRNA analysis

In PBMCs co-cultured with artesunate (0.5, 1 and 1.5 μg/ml) and PHA, compared with PBMC cultured with PHA alone, cells were harvested on days 0, 1, and 2 for qualitative cytokine mRNA analysis by established reverse transcriptase polymerase chain (RT PCR) methods (Caceres-Dittmar *et al*, 1993; Pirmez *et al*, 1993). Total RNA was isolated from 1-3 x 10^6 cells and a cDNA template for each RNA sample was synthesized using octapeptide primers. Primer sequences used to amplify cDNA templates for interleukins 2, 4, 12 (p40 and p35 subunits), 15, IFN-γ and TNF-α and the PCR conditions were described previously (Caceres-Dittmar *et al*, 1993; Pirmez *et al*, 1993). PCR products were then resolved by gel electrophoresis. The intensity of bands on the gel for each cytokine were scored as: + (present), - (not present), or ± (faint). To better insure adequate amplification conditions, β-actin mRNA from all cell preparations was amplified as an internal control.

RESULTS

Lymphoproliferation assays (LPAs)

Artesunate, added to PBMC or whole blood cultures either before or simultaneously with PHA, inhibited lymphoproliferation in most experiments, generally in a dose dependent manner (Fig 1a, 1d, 1e). In one experiment, when artesunate was co-cultured with PBMC for 24 hours, washed out, and then PHA stimulated, there was no LPA inhibition (Fig 1 b). Six concentrations of artesunate alone had no stimulatory activity on PBMC (Fig 1c).

DHART, added to whole blood before PHA stimulation, or to PBMC simultaneously with PHA, showed modest inhibition of LPA responses, especially at the higher doses, some in dose dependent fashion (Fig 2, a-b). Throughout LPA experiments, cells from randomly selected wells containing artesunate or DHA stained with trypan blue confirmed cell viability was > 95%.

Flow cytometry analysis T cell activation

For flow cytometry analyses, the simul-

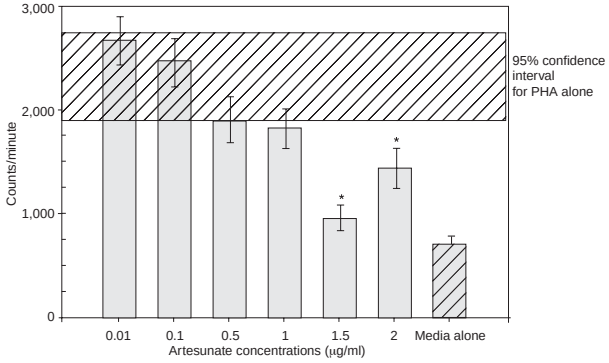

Fig 1 (a)–Mean (+/- SEM) LPA responses in PBMC co-cultured with artesunate for 24 to 48 hours, then reacted with PHA.
*p<0.05 *vs* PHA alone, ANOVA with Dunnett's multiple comparison test

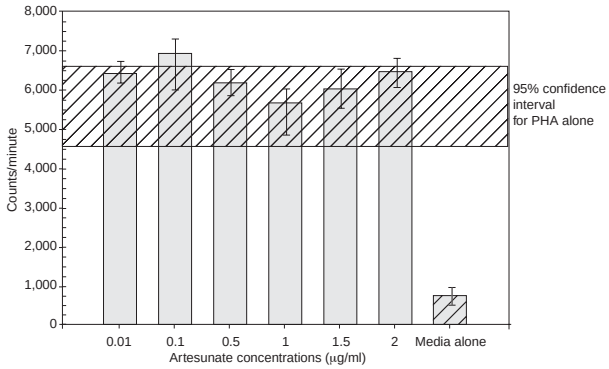

Fig 1 (b)–Mean (+/- SEM) LPA responses in PBMC co-cultured with artesunate for 24 hours. The cells were then washed to remove artesunate, then reacted with PHA.

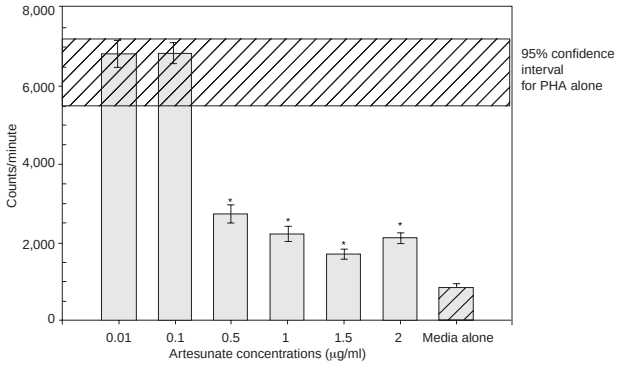

Fig 1 (c)–Mean (+/- SEM) LPA responses in PBMC co-cultured with artesunate and PHA simultaneously for 3 days.
*p<0.05 *vs* PHA alone, ANOVA with Dunnett's multiple comparison test

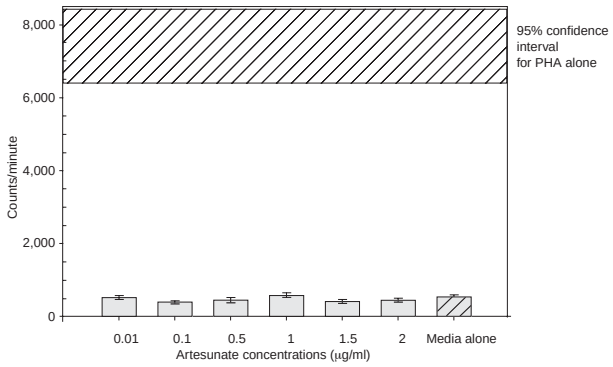

Fig 1 (d)–Mean (+/- SEM) LPA responses in PBMC co-cultured with artesunate alone for 48 hours, followed by addition of ^{3}[H] thymidine.

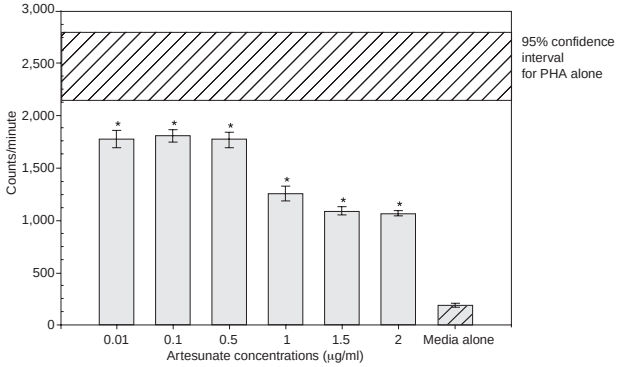

Fig 1 (e)–Mean (+/- SEM) LPA responses for whole blood co-cultured with artesunate for 3 days, then reacted with PHA.
*p<0.05 *vs* PHA alone, ANOVA with Dunnett's multiple comparison test

taneous addition of DHART and PHA to PBMC showed modest, consistent reductions in CD4+ and CD8+ cells expressing CD25 and CD69, in comparison with PBMC cultured with PHA alone (Fig 3, a-b). When artesunate was added to PBMC for 3 days and then stimulated by PHA, there were minimal changes in CD4+ and CD8+ T cell expression of CD25 and CD69, regardless of artesunate concentration, in comparison with PBMC cultured with PHA alone (data not shown). When artesunate and PHA were cultured simulta-

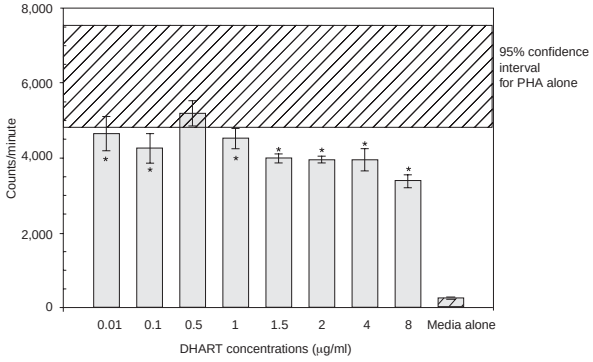

Fig 2 (a)–Mean (+/- SEM) LPA responses for whole blood co-cultured with DHART for 3 days, then reacted with PHA.
*p<0.05 *vs* PHA alone, ANOVA with Dunnett's multiple comparison test

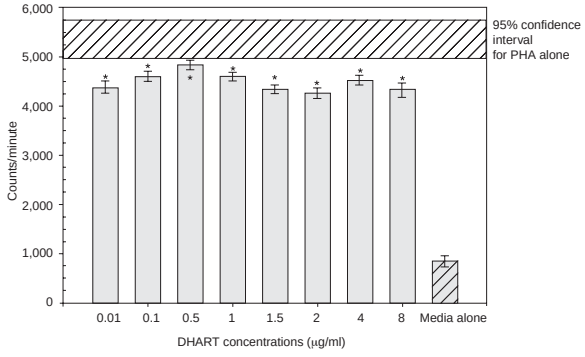

Fig 2 (b)–Mean (+/- SEM) LPA responses for PBMC co-cultured simultaneously with DHART and PHA for 3 days.
*p<0.05 *vs* PHA alone, ANOVA with Dunnett's multiple comparison test

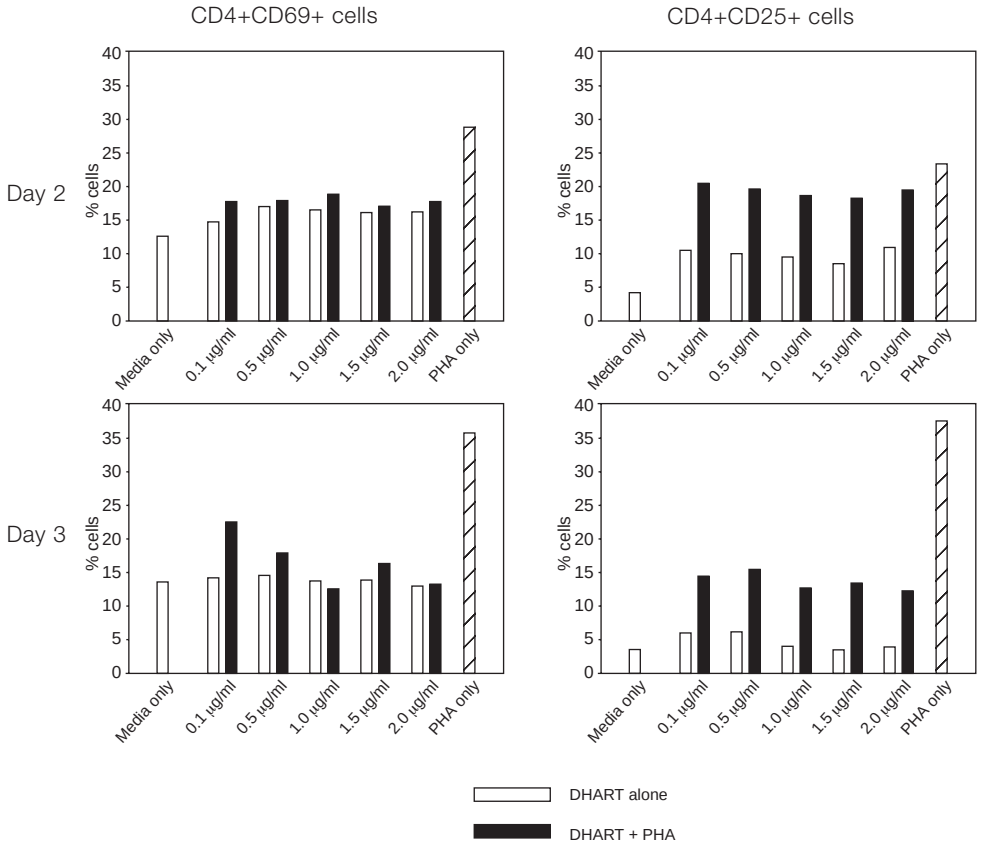

Fig 3 (a)–PBMCs co-cultured with DHART + PHA simultaneously (black bars) and harvested after 2 or 3 days had lower proportions of CD4+CD69+ and CD4+CD25+ cells, in comparison with PBMCs co-cultured with PHA only.

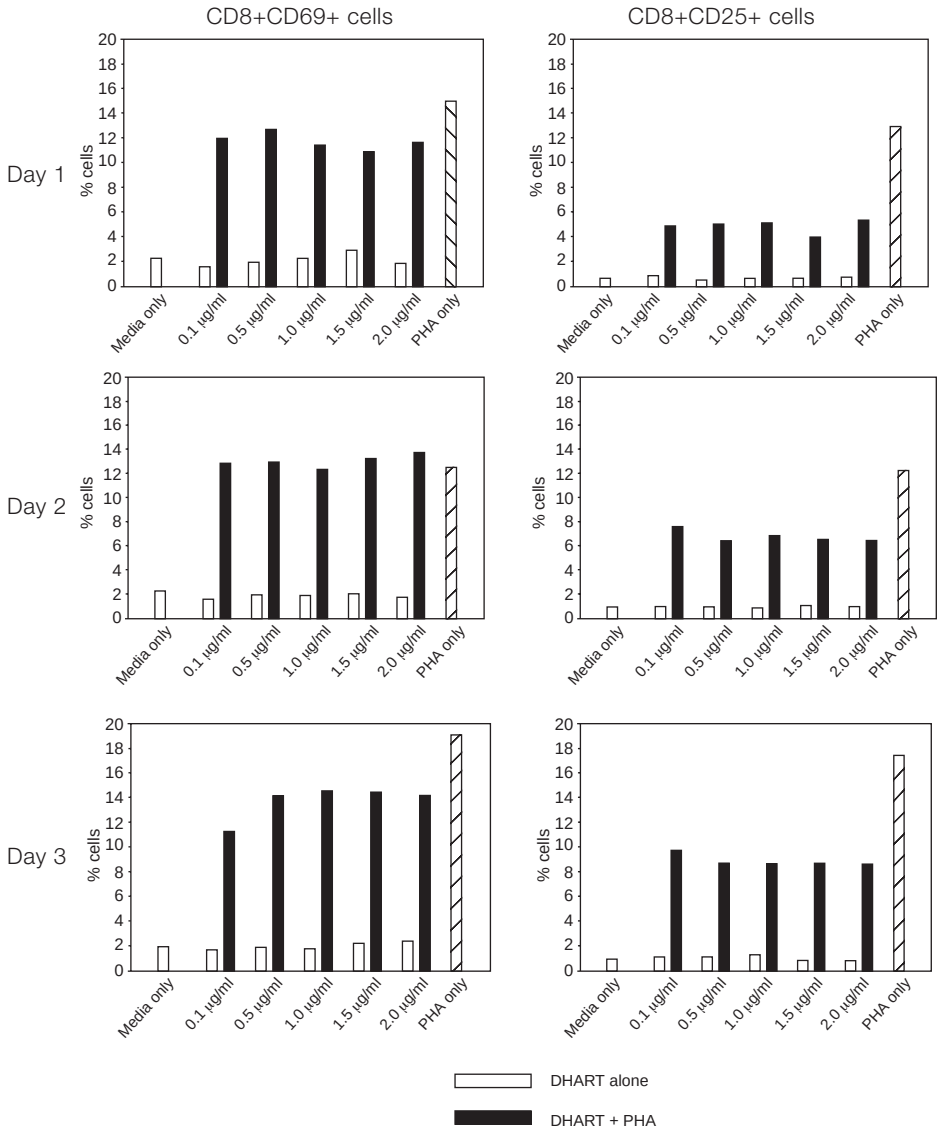

Fig 3 (b)–PBMCs co-cultured with DHART + PHA simultaneously (black bars) and harvested after 1, 2 or 3 days had lower proportions of CD8+CD69+ and CD8+CD25+ cells on most days, in comparison with PBMCs co-cultured with PHA only.

neously with PBMC, there were modest, sporadic reductions in CD4+ and CD8+ cells expressing CD25 and CD69 (data not shown).

Cytokine mRNA analysis

Qualitative cytokine mRNA analyses scores for PBMC co-cultured with artesunate and PHA simultaneously, in comparison with PHA alone, were largely unaffected by artesunate on harvest days 0, 1, or 2 for nearly all cytokines (Table 1). For TNF-α, on harvest days 0 and 2, PBMC cultures with PHA only were negative, but nearly all wells with artesunate added, regardless of concentration, were positive. All cell harvests analyzed for β-actin mRNA were positive.

Table 1
Qualitative cytokine RT-PCR for PBMC co-cultured with 3 concentrations of artesunate and PHA, or artesunate alone.

			Qualitative cytokine mRNA scores[a]							
		Dose	β-actin	IL-2	IFN-γ (p40)	IL-12	IL-12 (p35)	IL-4	TNF-α	IL-15
		Media only (- control)	+	–	–	–	–	–	–	–
Set A Harvest: day 0	Artesunate concentration	0.5 μg/ml	+	+	+	–	–	+	+	±
		1.0 μg/ml	+	+	+	–	–	+	+	+
		1.5 μg/ml	+	+	+	–	–	+	+	±
		PHA only (+ control)	+	+	±	–	–	+	–	+
Set B (artesunate only) Harvest: day 1	Artesunate concentration	0.5 μg/ml	+	–	–	–	–	–	–	–
		1.0 μg/ml	+	–	–	–	–	–	–	–
		1.5 μg/ml	+	–	–	–	–	–	–	–
Set C Harvest: day 1	Artesunate concentration	0.5 μg/ml	+	+	+	–	–	+	+	±
		1.0 μg/ml	+	+	+	–	–	+	+	±
		1.5 μg/ml	+	+	+	–	–	+	+	±
		PHA only (+ control)	+	+	+	–	–	+	+	+
Set D Harvest: day 2	Artesunate concentration	0.5 μg/ml	+	+	+	–	–	+	+	±
		1.0 μg/ml	+	+	±	–	–	+	+	±
		1.5 μg/ml	+	+	±	–	–	+	+	–
		PHA only (+ control)	+	+	±	–	–	+	–	–

[a]Scores: (+) = present, (-) = not present, (±) = faint. Light gray shading highlights cytokine mRNA expression in PBMC cultures with artesunate alone; dark gray shading highlights differences in TNF-α mRNA scores between PBMC cultured with artesunate and PHA versus PHA alone

DISCUSSION

Artesunate inhibited lymphoproliferation in PBMC or whole blood at most of the higher doses, and generally followed a dose response pattern between concentrations of 0.01 and 1.5 μg/ml. In one exception, artesunate removal from PBMC cultures before PHA stimulation abolished the reduction in lymphoproliferation, suggesting that artesunate-mediated inhibition requires drug presence and is reversible. DHART inhibition of lymphoproliferation was more modest, even at doses as high as 4 and 8 μg/ml, and the pattern was less dose-dependent. Observations that artesunate or DHART diminished PHA-driven lymphoproliferation in both PBMC and whole blood culture systems, in a generally dose dependent but reversible fashion, supports biological validity and the contention that artemisinins have immunomodulatory properties (Chen and Maibach, 1994; Chen *et al*, 1994; Wenisch *et al*, 1997; He and Liu, 2004).

The inhibition in the LPAs was generally larger and more consistent when artesunate or DHART was added to PBMC or whole blood simultaneously with PHA, rather than when added before PHA stimulation. The reason for this is unclear, but may be associated with diminished effects of artesunate or increased variability in cells cultured over a period of 6 days for step-wise additions, versus only 3 days for simultaneous additions. We are unaware of any mechanism by which artesunate or DHART could bind or inactivate PHA when added simultaneously.

For the first time, we report that artesunate or DHART added to PBMC before or simultaneous to stimulation with PHA confer modest reductions in proportions of CD4+ and CD8+ T cells expressing CD69 and CD25, early and late T cell activation markers, respectively. Similar to LPA trends, larger and more consistent reductions in T cell activation were observed when PBMCs were co-cultured with artesunate or DHA simultaneously with PHA. The inhibitory effects of DHART were also relatively larger and more consistent for CD8+ T cells, in comparison with CD4+ cells.

RT-PCR to assess mRNA production for 6 malaria-relevant cytokines in PBMC cultures containing 3 concentrations of artesunate and PHA depicted little effect of artesunate. This finding failed to support the notion that artemisinins inhibit TNF-α production (He and Liu, 2004; Noori *et al*, 2004), but is consistent with others showing little effect on TNF-α (Kwiatkowski and Bate, 1995). In addition to parasite killing, artemisinins may reduce pathogenesis by inhibiting cyotoadherence (Udomsangpetch *et al* 1996). These observations underscore contradictory immunological effects, suggesting areas for further investigation.

Defining whether artemisinins inhibit pro-inflammatory cytokine production is relevant because several, especially TNF-α, mediate clinical manifestations of severe malaria (Othoro *et al*, 1999). Notably, many effective anti-malarial compounds also exert immunodepressive effects, including reduced cytokine production, a desirable feature for malaria (Koranda, 1981; Isaacson *et al*, 1982). In one falciparum study, plasma TNF-α levels were markedly reduced in patients treated with artesunate, compared with quinine, but it was unclear if low TNF-α was related to fast parasite clearance or the artesunate treatment itself (Ittarat *et al*, 1999).

The mechanism of the inhibitory effects of artesunate or DHART on PHA-driven lymphoproliferation is unknown. The artemisinin molecule is composed of a sesquiterpene lactone with a bridged peroxide linkage (Hien and White, 1993), the component that likely confers potent antimalarial activity and, in this regard, may be sufficient to thwart large ATP-requiring processes such as lymphoproliferation (Fishwick *et al*, 1998a,b). Arteeether, a related

artemisinin derivative, and DHART induce a selective pattern of damage to the brain stem in rhesus monkeys that may relate to oxidative metabolism (Brewer *et al*, 1994). However, millions of patients treated with artemisinin compounds have shown no toxicity and, like others, we did not see any evidence for artesunate or DHART toxicity on cultured cells (Chen *et al*, 1994; Wenisch *et al*, 1997).

Artesunate, a water soluble formulation, is the most rapidly acting of the artemisinins probably because it is immediately bioavailable as DHART after intravenous injection (Hien and White, 1993). It is also rapidly absorbed after oral or intramuscular administration, and rapidly eliminated (Teja-Isavadharm *et al*, 1996; Benakis *et al*, 1997; Bethell *et al*, 1997). In PBMC and whole blood cultures, artesunate would be rapidly metabolized to DHART. Thus, we expected that artesunate and DHART would show similar trends in their affects on lymphoproliferation and activation in PBMC stimulated with PHA.

Falciparum malaria causes immune activation, often considered unfavorable. Thus it would be useful to determine whether artemesinins can down-regulate immune activation, especially the production of pro-inflammatory cytokines such as IL-2 and TNF-α that mediate pathogenesis. This would further enhance their therapeutic value.

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