

## CYTOKINES AND DYSREGULATION OF THE IMMUNE RESPONSE IN HUMAN MALARIA

M. FATIMA C. ALVES; LEOPOLDO L. SANTOS-NETO\*; M. IMACULADA M. JUNQUEIRA\* & C. EDUARDO COSTA\*

Departamento de Imunologia e Patologia, Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, 74001-970 Goiânia, GO, Brasil \*Laboratório de Imunologia Celular, Departamento de Patologia, Faculdade de Ciências da Saúde, Universidade de Brasília, 70910-900 Brasília, DF, Brasil

*The dysregulation of the immune response by malaria parasite has been considered as a possible constraint to the effectiveness of malaria vaccination. In spite of the important role interleukin-1 (IL-1) plays on the immunoregulation, and its ability to mimic various features of clinical malaria, reports on IL-1 in malaria are lacking. We found that only 2 out of 35 subjects with acute malaria showed increased levels of serum IL-1 alpha by enzyme immunoassay. To assess whether IL-1 could interfere with T-lymphocyte responses, blood mononuclear cells from patients infected with Plasmodium falciparum, P. vivax, or healthy subjects were cultured with phytohemagglutinin, and lymphocyte proliferation measured 72h later by <sup>3</sup>H-thymidine incorporation. Our data showed that T-lymphocyte responses are depressed both in P. falciparum (10,500 ± 2,900) and P. vivax malaria (13,000 ± 3,300), as compared to that of healthy individuals (27,000 ± 3,000). Addition of IL-1 partially reversed depression of malaria lymphocytes, but had no effect on normal cells. On the other hand, T-lymphocytes from malaria infected-subjects presented a minimal decrease in proliferation, when cultured in the presence of exogenous PGE2. These data indicate the occurrence of two defects of immunoregulation in malaria: a deficiency of IL-1 production by monocytes/macrophages, and an increased resistance of lymphocytes to the antiproliferative effect of PGE2.*

Key words: Cytokines – immune response – *Plasmodium falciparum* – *Plasmodium vivax* – T-lymphocyte – responses – IL-1

Malaria vaccines have been considered as the most important potential weapon for malaria control. A considerable progress towards this aim has been achieved through the characterization of immunogenic sequences, and by cloning and production of peptides, both by genetic recombination and chemical synthesis. However, although some malaria vaccines have already been submitted to human trials (Herrington et al., 1987; Ballou et al., 1987; Patarroyo et al., 1988; Sherwood et al., 1991), it is possible to foresee that some crucial questions have to be answered before vaccination become a real asset for malaria control. It is important to know how to overcome the problem played by the high polymorphism of some relevant epitopes, to cope with the difficulties imposed by genetic restriction on the immune response towards vaccine peptides, and to better understand the mechanisms of the dysregulation of the immune response caused by malaria parasites and, potentially, by malaria vaccines.

Several manifestations of dysregulation of the immune reactivity are demonstrated during the acute phase of malaria, namely, lymphocyte polyclonal activation, autoantibody production, and depression of the immune response (Weidanz, 1982). The mechanisms responsible for these alterations are not known. It is likely that the parasite or its products may play a part by acting on the cells of the immune system. In fact, the macrophage, a cell in charge of presenting antigens to lymphocytes and modulating their response, may show different functional alterations during acute malaria, including a deficient ability to process (Loose et al., 1972) and present antigens to lymphocytes (Warrewn & Weidanz, 1976), and a delayed capacity of mobilization and activation (Tosta et al., 1983). It is possible that some of these dysfunctions are due to alterations in the capacity of macrophages to produce cytokines. In support to this possibility, it has been shown that adherent cells from the spleen of

infected mice synthesize subnormal amounts of interleukin-1 (IL-1), starting from the 4th day of infection, when they also produce some effectors capable to suppress the immune response (Wyler et al., 1979). We now present evidence suggesting that the decreased proliferation of lymphocytes from humans infected with either *Plasmodium falciparum* or *Plasmodium vivax* is associated with a deficient production of IL-1 by monocytes, and also that lymphocytes from infected individuals show a diminished sensitivity to the anti-proliferative effect of prostaglandin E2 (PGE2).

#### MATERIALS AND METHODS

This basic protocol was followed: fractionated or unfractionated mononuclear cells, from infected or normal subjects, were cultured with phytohemagglutinin (PHA) in the presence of added IL-1 or PGE2, and proliferation of lymphocytes assessed by isotope incorporation, while blood IL-1 was measured by an enzyme immunoassay.

*Study groups* – Ten patients infected with *P. vivax* and 13 with *P. falciparum* malaria were studied and compared with 23 normal controls.

Informed consent was obtained from the patients. Blood was collected within the first week of infection, most frequently on the 4th day after beginning of the symptoms.

*Quantitation of IL-1 alpha* – This cytokine was measured in plasma by an enzyme immunoassay using a kit donated by Oncomembrane. Recombinant IL-1 alpha was used as a reference standard. The protocol followed was essentially that suggested by the manufacturer.

*Mononuclear cell separation* – The volume of heparinized blood was duplicated by addition of Hanks' solution and 5mL samples applied onto 3mL cushions of a solution of colloidal silica covered by polyvinylpyrrolidone (Percoll), density 1.077. After centrifugation at 1200 xG for 15min at 4°C, the mononuclear cell layer was collected, washed twice with Hanks' solution and quantified by nigrosin exclusion.

*Mononuclear cell fractionation* – To evaluate whether individual variations in the proportions of blood monocytes and lymphocytes

could interfere with the degree of proliferation of the latter cells, assay were performed with both unfractionated and fractionated cells. To get a constant proportion of 1 monocyte per 9 lymphocytes, this protocol was followed: 2 x 10E5 mononuclear cells were suspended in serum-free RPMI medium incubated in flatbottomed 96-well microculture plates (Costar), in a wet chamber, at 37°C for 1h, to obtain about 10,000 adherent monocytes/well, after washing the wells with RPMI medium to discard non-adherent cells. A suspension of lymphocytes with minimal contamination of monocytes (< 4%) was prepared by passing mononuclear cells through a column of nylon wool, according to the method of Greaves et al. (1976), and 90,000 effluent lymphocytes were applied to each well containing adherent monocytes.

*Lymphocytes proliferation assay* – Samples of 100,000 fractionated or unfractionated mononuclear cells in RPMI 640 medium, supplemented with 10% fetal calf serum (Cultilab), 20M HEPES, 2mM glutamine, 2mM NaHCO3 and 4mg/l gentamycin were dispensed into triplicate wells of a microculture plate, and cultured with optimal (5 µg/ml) or suboptimal (2.5 µg/ml) concentrations of PHA (Sigma), in a wet chamber, with an atmosphere of 5% CO2 in air, at 37°C, for 72h. Sixteen hours before finishing the cultures, 0.5 µCi of 3H-thymidine (Amersham, specific activity of 15 Ci/mmol) was added per well. Lymphocyte proliferation was assessed by measuring radioactivity by beta scintigraphy. To evaluate the effects of different mediators on lymphocyte proliferation, recombinant human IL-1 alpha (Hoffmann-La Roche) or PGE2 (Sigma) were added to cultures in the presence of a sub-optimal concentration of PHA, and 1 µg/ml indomethacin, as an inhibitor of PGE2 synthesis.

*Statistical analyses* – Results were analyzed either by the Student's test or by the Wilcoxon test.

#### RESULTS

The proliferative response of lymphocytes from both *P. falciparum* and *P. vivax*-infected individuals was significantly lower than that of normal controls ( $P < 0.001$ ), when cultured with PHA, as assessed by 3H-thymidine (Table I). A similar pattern was obtained when a pre-established proportion of 9 lymphocytes per monocyte was kept constant by using fractionated

TABLE I

Proliferation of lymphocytes from *Plasmodium falciparum*, *P. vivax*-infected individuals, and from normal controls, after 72h incubation with phytohemagglutinin (5 µg/ml), and measure by the incorporation of 3H-thymidine (cpm)

Groups	n	Mean	SEM	Range
<i>P. falciparum</i>	13	10,500 <sup>a</sup>	2,900	1,860 - 31,970
<i>P. vivax</i>	10	13,000 <sup>a</sup>	3,300	1,800 - 33,970
Control	23	27,000	3,000	9,500 - 50,000

a: P < 0.001 (Student's t test) as compared to Control group.

TABLE II

Effect of the inhibition of endogenous PGE<sub>2</sub> by indomethacin (1 µg/ml) on the proliferation of lymphocytes from *Plasmodium falciparum* or *P. vivax*-infected individuals, and from normal controls after 72h incubation with PHA (2.5 µg/mL), measured by the incorporation of 3H-thymidine (cpm)

Groups	n	Without indomethacin (SEM)	With indomethacin (SEM)	P
<i>P. falciparum</i>	13	14,100 (3,800)	16,900 (4,400)	0.035
<i>P. vivax</i>	10	16,900 (5,200)	19,600 (5,400)	0.028
Control	23	30,900 (3,300)	34,700 (3,500)	0.004

TABLE III

Effect of exogenous PGE<sub>2</sub> (3x10E-7M) on the proliferation of lymphocytes from *Plasmodium falciparum* or *P. vivax*-infected individuals, and normal controls after 72h incubation with PHA (2.5 µg/ml) and indomethacin (1 µg/ml), measured by the incorporation of 3H-thymidine (cpm)

Groups	n	Without PGE <sub>2</sub> (SEM)	With PGE <sub>2</sub> (SEM)	P
<i>P. falciparum</i>	13	16,900 (4,400)	11,000 (3,600)	0.659
<i>P. vivax</i>	10	19,600 (5,400)	14,300 (3,500)	0.133
Control	23	34,700 (3,500)	25,000 (3,500)	0.0005

populations of mononuclear cells (data not shown).

Addition of 0.9 ng/ml recombinant human IL-1 alpha to the cultures of lymphocytes from infected subjects partially corrected their depressed proliferation but had no effect whatsoever on normal lymphocytes. Fig. 1 shows that this cytokine caused a significant increment of proliferation in both *P. falciparum* (P = 0.005) and *P. vivax* malaria (P = 0.018), but

not of normal lymphocytes (P = 0.3). When a tenfold increase in the concentration of IL-1 was used, a similar pattern of stimulation of malaria lymphocytes was observed.

Since IL-1 stimulates the synthesis of PGE<sub>2</sub> by monocytes/macrophages, the effect of this mediator on T-lymphocytes was investigated. As shown in Table II, inhibition of endogenous PGE<sub>2</sub> by indomethacin caused a significant increase of lymphocyte proliferation in all 3

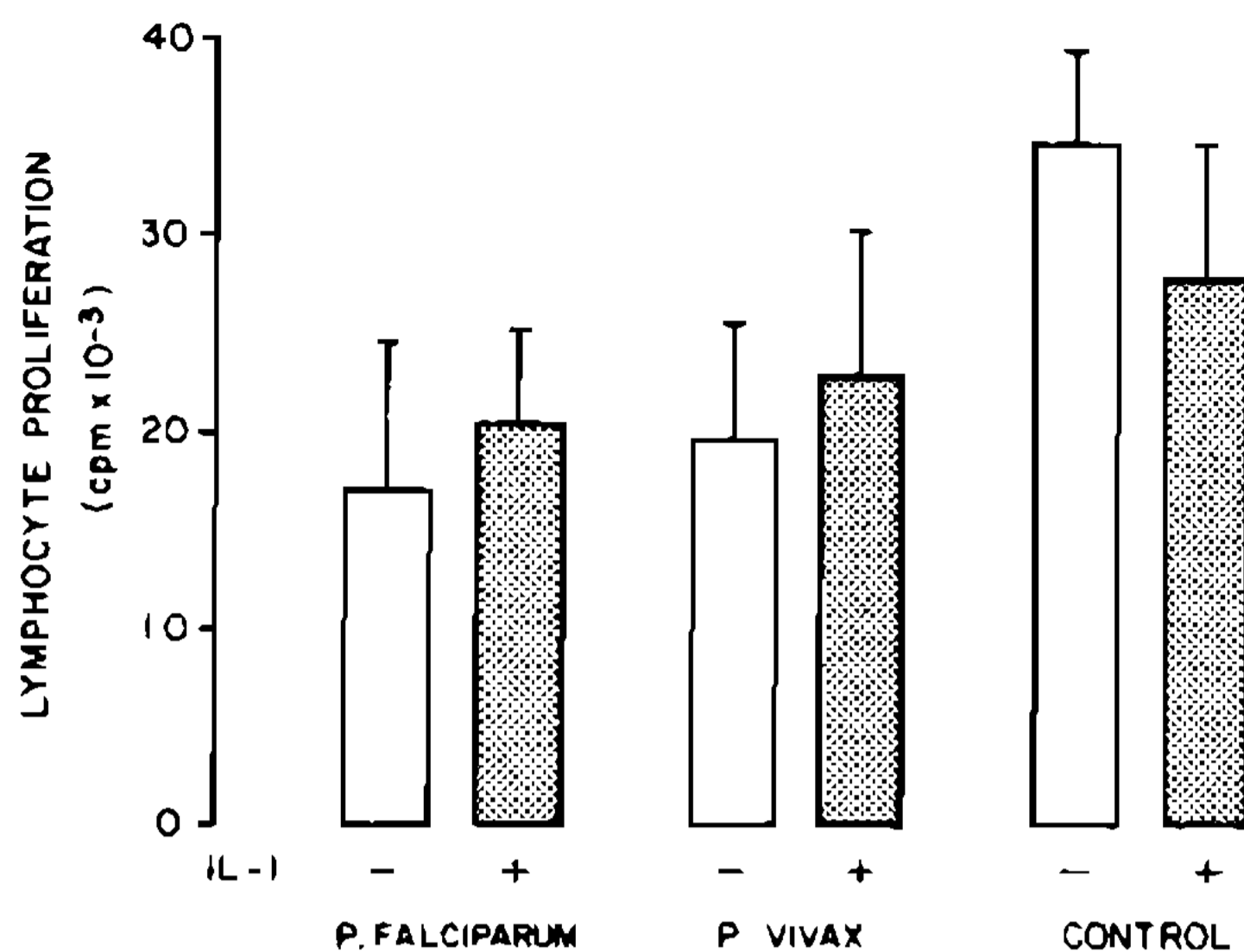


Fig. 1: effect of the addition of 0.9 ng/ml recombinant human IL-1 alpha on the proliferation of lymphocytes from malaria-infected and non-infected subjects, after 72h culture with PHA (2.5  $\mu$ g/ml) and indomethacin (1  $\mu$ g/ml), and measured by the incorporation of <sup>3</sup>H-thymidine. Lymphocytes from *Plasmodium falciparum* ( $P = 0.005$ ) and *P. vivax*-infected individuals ( $P = 0.018$ ), but not from non-infected subjects ( $P = 0.289$ ) show increased proliferation.

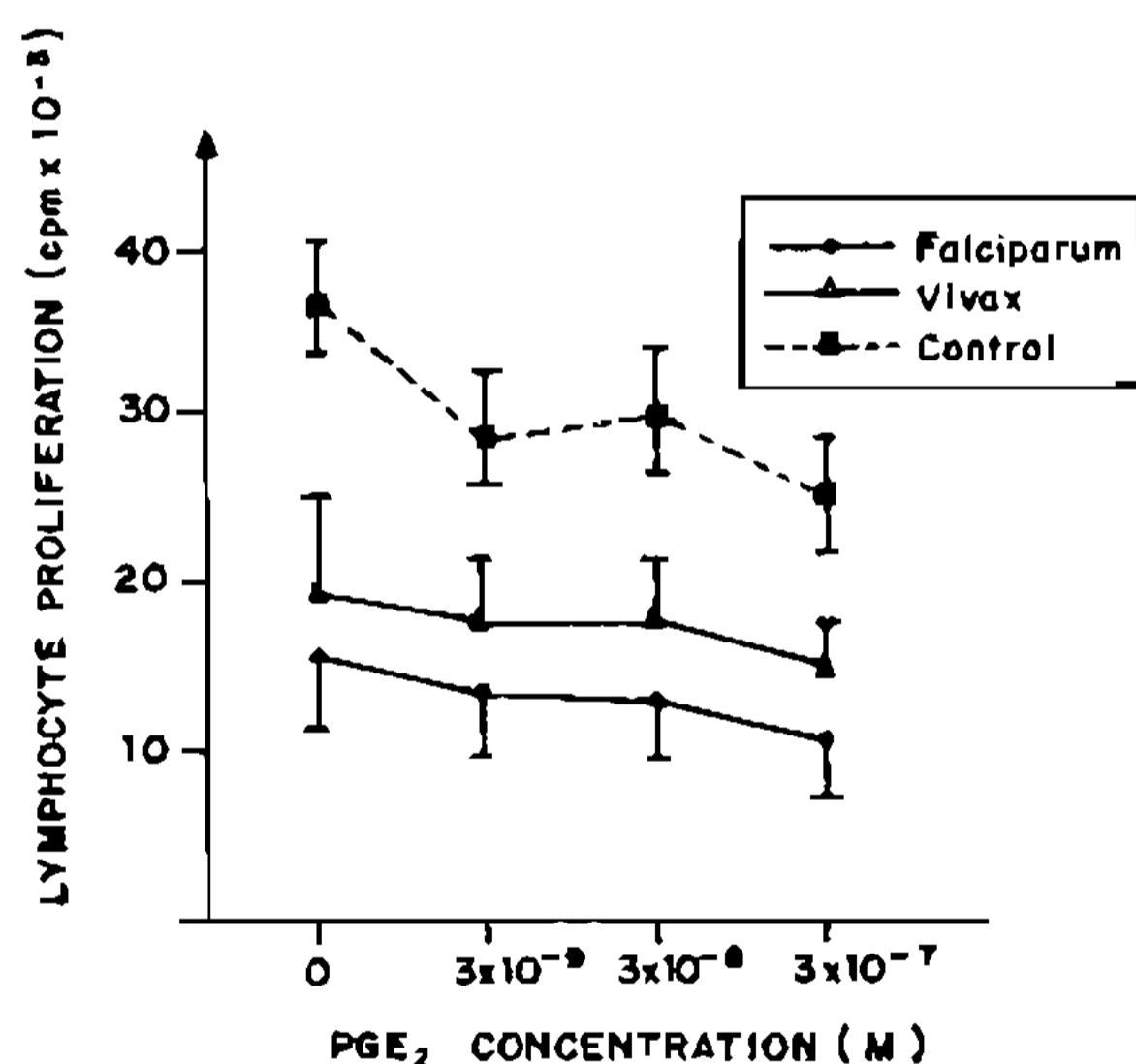


Fig. 2: dose-response effect of exogenous prostaglandin E<sub>2</sub> on the proliferation of lymphocytes cultured for 72h with PHA (2.5  $\mu$ g/ml) and indomethacin (1  $\mu$ g/ml), and measured by the incorporation of <sup>3</sup>H-thymidine. Results presented as the mean  $\pm$  SEM.

groups. If exogenous PGE<sub>2</sub> is added to cultures, lymphocytes from both *P. falciparum* and *P. vivax*-infected individuals showed a decreased inhibition of their antiproliferative response to PHA as compared to that of normal cells (Fig. 2).

However, the degree of inhibition by PGE<sub>2</sub> was not significant with either *P. falciparum* ( $P = 0.659$ ) or *P. vivax* ( $P = 0.133$ ), differently with which occurred with normal lymphocytes ( $P = 0.0005$ ) (Table III).

## DISCUSSION

Mitogen-induced T-lymphocytes proliferation has been widely used for studying the factors associated to lymphocyte differentiation and activation, including the interactions of these cells with monocytes/macrophages. Using this *in vitro* system, we presented evidence that proliferation of lymphocytes from both *P. falciparum* and *P. vivax*-infected individuals was depressed, when compared to that of healthy individuals.

Different possibilities could be considered for explaining this finding. Firstly, depression could be due to a decreased number of lymphocytes in the total population of blood mononuclear cells used in cultures. This possibility was ruled out by setting experiments using preestablished proportions of lymphocytes and monocytes, after fractionation of these two cell populations. No difference was found in lymphocyte proliferation between fractionated and unfractionated cultures.

Another possibility is that the dysfunction of malaria lymphocytes to an insufficient production of some stimulatory cytokine by monocytes/macrophages. Three of such cytokines are IL-1, IL-6 and tumor necrosis factor alpha (TNF). The latter two are reported to be increased in serum during the acute phase of malaria (Grau et al., 1989; Kern et al., 1989), probably as a consequence of the activation of macrophages, their main source of production (Ferrante et al., 1990). Surprisingly, no reference was found on the alterations of IL-1 in malaria, in spite of its well recognized immunoregulatory effect on T and B lymphocyte and macrophage differentiation/activation, and its ability to mimic most of the pathological findings of malaria infection, characteristics common to IL-6 and TNF (reviewed by Titus et al., 1991). Interestingly, it has been shown that IL-1 inhibits the hepatic development of *P. falciparum* sporozoites (Maheshwari, 1990) and, when given in low concentrations to mice protect them against cerebral malaria (Curfs et al., 1990). In the present work, it was shown that only rarely serum levels of IL-1 alpha are increased during acute malaria. In fact, in 33 out of 35 infected individuals the levels of circulating IL-1 was found below the detection level of 5 ng/ml. Addition of recombinant human IL-1 alpha to the cultures of mononuclear cells significantly increased the proliferation of lym-

phocytes from malaria-infected subjects but not of that from healthy people. This ability of IL-1 to stimulate T-cell proliferation is well recognized (reviewed by Dinarello, 1989), and has been considered as a hallmark of its immunoregulatory activity. Thus, a deficient IL-1 production in malaria is expected to lead to a dysregulation of the immune response. A possible explanation for our findings is that monocytes/macrophages from malaria-infected individuals produce sub-normal concentrations of IL-1. Therefore, addition of exogenous cytokine would restore T lymphocyte activation and proliferation. The fact that a total reversion of lymphocyte function was not observed suggests that some other factor is also involved in the activation of these cells. This is not surprising since it is recognized that the mechanisms involved both in lymphocyte activation, and in IL-1 production and action are very complex and depend on different and interacting factors. For instance, IL-6 which acts synergistically with IL-1 in T-cell activation (Houssiau et al., 1988), suppress IL-1 production (Schindler et al., 1990), while IL-1 is capable by itself to induce IL-6 synthesis by monocytes (Tosato & Jones 1990). The reasons for a deficient production of IL-1 by monocytes/macrophages in malaria are not established. No relationship was found with the species of the parasite, parasitemia, or duration of malaria. However, our preliminary data suggest that this deficiency recovers after the infection is cured, and appears to be under an adaptive control, since lymphocyte proliferation is less affected in individuals who have suffered several episodes of malaria.

The relationship of IL-1 with other products of macrophages are also very complex. PGE<sub>2</sub>, whose synthesis is stimulated by IL-1, may both inhibit (Knudsen et al., 1986) or stimulate IL-1 production (Kassis et al., 1989). A possible excess of PGE<sub>2</sub> production by macrophages in malaria could lead to a deficient lymphocyte proliferation both by its direct antiproliferative effect on lymphocytes, and also indirectly through the inhibition of IL-1 production. However, our data do not support this possibility since the increment of lymphocyte proliferation, which occurred after blocking endogenous PGE<sub>2</sub> with indomethacin, was not different in infected and healthy individuals. Our finding that lymphocytes from malaria-infected individuals are somewhat resistant to the antiproliferative effect of PGE<sub>2</sub> may add a new dimension to the understanding of

the mechanisms involved in the genesis of polyclonal activation of lymphocytes, which occurs in malaria, and has the hypergammaglobulinemia as a prominent feature (Weidanz, 1982), and anemia as a possible consequence (Daniel-Ribeiro et al., 1986). It is possible to speculate that if lymphocytes, during malaria infection, show some degree of resistance to the antiproliferative control exerted by PGE<sub>2</sub>, they will more easily proliferate under the influence of mitogens and antigens of plasmodial origin (Greenwood & Vick 1975).

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