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Examination of Peripheral Blood Mononuclear Cells and Sera from Thai Adults Naturally Infected with Malaria in Assays of Blastogenic Responsiveness to Mitogenic Lectins and Allogeneic Cell Surface Antigens†

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We have previously observed that Thai adults who are infected with malaria have a loss of peripheral blood T cells, and that patient sera contain lymphocytotoxic antibodies. In the present study, we examined peripheral blood mononuclear cells from Thai adults naturally infected with *Plasmodium falciparum* and *Plasmodium vivax* for the capacity to undergo blastogenesis in response to phytohemagglutinin, concanavalin A, pokeweed mitogen, and allogeneic cell surface antigens in a one-way mixed leukocyte reaction. In addition, sera from actively infected patients were examined with regard to suppressive capabilities toward normal lymphocyte blastogenesis by using the same assays. We found that patient mononuclear cells exhibited normal reactivity to phytohemagglutinin, concanavalin A, and pokeweed mitogen when compared with controls. However, peripheral blood mononuclear cells from patients had a decreased stimulatory capacity in the allogeneic mixed leukocyte reaction, and *P. vivax*, but not *P. falciparum*, lymphocytes exhibited decreased responsiveness in the mixed leukocyte reaction. Furthermore, sera from patients with active malaria induced decreased responsiveness by normal mononuclear cells to phytohemagglutinin and concanavalin A, but not pokeweed mitogen; pooled *P. falciparum* sera caused decreased responsiveness to allogeneic cell surface antigens in the mixed leukocyte reaction. These studies indicate that despite the lost of circulating T cells during the course of infection with malaria, blastogenic responsiveness remains intact, and that sera from patients with malaria are capable of exerting negative immunoregulatory effects.

Delineation of the host immune response to infection with malaria should include examination of infected humans. Studies in this area have only recently begun to be carried out utilizing peripheral blood mononuclear cells (MNC) from patients. We have previously observed that Thai adults naturally infected with either *Plasmodium falciparum* or *Plasmodium vivax* have a decrease in the percentage and concentration of T lymphocytes, an increase in the percentages but no change in the concentrations of B and "null" lymphocytes, and no change in either the percentage or concentration of Fc receptor-bearing lymphocytes (7). Thus, peripheral blood MNC from patients who have malaria exhibit a true loss of T cells without any real change in B cells, Fc receptor-bearing cells, or null cells. In addition, we have recently demonstrated that sera of Thai adults naturally infected with both *P. falciparum* and *P. vivax* contain cold-reactive lymphocytotoxic antibodies with marked reactivity at 15°C (6). A number of individuals also had lymphocytotoxic antibodies which were effective at 37°C. Although the subpopulations against which these antibodies are directed have not yet been elucidated, the ability of serum components to interact with peripheral blood MNC in functional assays clearly needs to be ascertained. Therefore, to examine the functional capabilities of peripheral blood cells from patients with malaria and to also examine the effects of their sera on cellular immune function, we have begun to examine the peripheral blood MNC and sera of Thai adults

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naturally infected with *P. falciparum* and *P. vivax* in a number of in vitro cellular immune assays. The present work describes our results using mitogen-induced lymphocyte transformation and mixed leukocyte culture systems.

MATERIALS AND METHODS

Patients. Peripheral blood was obtained from normal volunteers or Thai adults (ages 16 to 50) naturally infected with *P. falciparum* or *P. vivax*. The patients were mildly ill, recently infected outpatients from the region surrounding Pruddhabaht, which is endemic for malaria. Individual serum samples were also obtained from infected Thai adults or from uninfected individuals (controls) living in the same regions as the patients. No infected or control individuals donating cells or sera had a history of receiving blood transfusions, and none was on medication. The degree of parasitemia in all infected individuals was assessed by examination of Giemsa-stained peripheral blood smears. The percent parasitemia values for *P. vivax* patients ranged between "positive" and 1.0%, whereas those for *P. falciparum* patients ranged between positive and 1.1%. Patients were then treated by the staff of the National Malaria Project.

MNC isolation. Peripheral blood MNC were isolated from heparinized blood by diluting the blood 1:2 in Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) followed by Ficoll-Hypaque centrifugation (1).

After three washes in Hanks balanced salt solution the isolated peripheral blood MNC were adjusted to a concentration of 5×10^6 cells per ml in assay medium RPMI 1640 (GIBCO) containing 2 mM glutamine, 50 U of penicillin, 50 μg of streptomycin per ml, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, and 20% human serum. Cell viability was determined by trypan blue exclusion; viability was greater than 95% in all specimens.

Lectin-induced mitogenesis. The method used for analysis of lectin-induced blast transformation has been reported previously (4). In brief, cells were adjusted to 1.5×10^6 leukocytes per ml in assay medium, and 1.5×10^5 leukocytes (0.1 ml) were added to each well of flat-bottomed microtiter plates (Costar, Cambridge, Mass.). The optimal stimulatory concentration for each lectin was determined in separate experiments and used throughout the study: mitogens were added to a final concentration of 5 μg of phytohemagglutinin (PHA; Calbiochem, La Jolla, Calif.) per ml, 10 μg of concanavalin A (ConA; Calbiochem) per ml, or 25 μg of pokeweed mitogen (PWM; GIBCO) per ml in a 0.2-ml volume. Control wells received 0.1 ml of medium in addition to 0.1 ml of cells (total, 0.2 ml). Tests were performed in triplicate. Cells were incubated at 37°C in a 5% CO_2–95% air, humid environment. Cultures were pulsed by addition of 0.4 μCi of tritiated thymidine (New England Nuclear) at 72 h (for PHA and ConA) or 120 h (for PWM) and harvested 24 h later. [^{3}H]thymidine incorporation by cells in each well was determined by impregnating filter disks with leukocyte cultures by using a multiple automated sample harvester (Microbiological Associates, Bethesda, Md.) and counting the radioactivity of each filter in a Hewlett-Packard liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.). Hydromix (Yorktown Research, Hackensack, N.J.) was used as scintillation fluid.

Recognition of allogeneic cell surface antigens. The methods used for the allogeneic mixed leukocyte reaction (MLR) have also been described previously (3). In brief, stimulator cells were prepared by treating cells from one individual at a concentration of 1.5×10^6/ml with 50 μg of mitomycin C (Sigma Chemical Co., St. Louis, Mo.) per ml for 45 min at 37°C. The mitomycin C-treated cells were then carefully washed three times in Hanks balanced salt solution with 5% pooled human serum and were then suspended in RPMI 1649 medium with 20% sera and adjusted to a final concentration of 2.5×10^6 cells per ml. Responder cells were diluted in assay medium to a final concentration of 2.5×10^6 cells per ml. In flat-bottomed microtiter test plates, 0.1 ml (2.5×10^5 cells) of responder cells and/or 0.1 ml (2.5×10^5 cells) of stimulator cells were added to wells; each cell type was cultured alone (nonspecific blastogenesis control) and with PHA (mitomycin C control). Patients' lymphocytes were tested as both stimulators and responders with the cells of individual allogeneic normal volunteers; each of the individual normal volunteer's cells were tested against another normal control. All tests were done in triplicate. Cells were cultured at 37°C in a 5% CO_2–95% air, humid environment for 120 h and pulsed with [^{3}H]thymidine (0.4 μCi) for 24 h. Samples were processed as described above for estimation of [^{3}H]thymidine incorporation. The means of triplicate cultures in both lectin-induced mitogenesis and MLR were used to determine stimulation index (counts per minute [cpm] from stimulated cultures divided by cpm from nonstimulated cultures) and Δcpm (cpm from stimulated cultures minus cpm from nonstimulated cultures). The effect of patient sera was studied on responsiveness of individual normal peripheral blood leukocytes to lectins and in the allogeneic MLR. In these assays 20% serum from a pool of 10 *P. vivax* or 10 *P. falciparum* patients or controls was used in the cultures in place of the 20% pooled human sera normally used.

Statistics. The Student *t* test was used to determine statistical significance, and a *P* value of less than 0.05 was considered significant.

RESULTS

Examination of patient peripheral blood MNC. We first examined the ability of peripheral blood MNC from patients infected with *P. falciparum* or *P. vivax* to respond to selected mitogens in culture. The responsiveness of patient peripheral blood MNC to ConA, PHA, and PWM is summarized in Table 1. The responsiveness of the patient cells was equal to that of normal controls. Thus, with regard to stimulation by mitogenic lectins, peripheral blood MNC from mildly ill patients naturally infected with malaria did not show a decreased responsiveness.

We next turned our attention to the allogeneic

TABLE 1. *Responsiveness of peripheral blood MNC from normal controls and malaria patients to mitogens*

Source of MNC[a]	Responsiveness to mitogen[b]					
	ConA		PHA		PWM	
	Δcpm	SI	Δcpm	SI	Δcpm	SI
P. vivax (9)	27,068 ± 2,511	41 ± 9	65,620 ± 3,758	91 ± 15	31,853 ± 3,228	49 ± 12
Control (9)	28,993 ± 2,603	39 ± 4	61,471 ± 4,457	89 ± 16	29,349 ± 4,954	33 ± 5
P. falciparum (6)	23,562 ± 2,001	34 ± 5	53,957 ± 3,279	74 ± 6	26,092 ± 2,333	37 ± 4
Control (6)	28,711 ± 1,962	49 ± 7	57,537 ± 3,127	100 ± 17	27,508 ± 2,528	49 ± 10

[a] Peripheral blood MNC were taken from normal (control) volunteers or patients infected with *P. vivax* or *P. falciparum*. Numbers within parentheses indicate the number of experiments.

[b] Mean stimulation index (SI) ± standard error of the mean.

MLR to examine the capacity of infected patients' MNC to respond to or stimulate allogeneic cells from individual normal volunteers. When cells from patients with *P. falciparum* were used as responders in MLR, a normal response to cell surface antigens on allogeneic normal cells was observed (Table 2). In contrast, MNC from *P. vivax* patients had a statistically significant decreased responsiveness ($P < 0.05$) in allogeneic MLR. When cells from patients with malaria were used as stimulators in the MLR, individual normal responding cells exhibited significantly ($P < 0.05$) decreased blast transformation. Thus, although MNC from malaria patients functioned normally in response to mitogens, *P. vivax* MNC were abnormal as responders in MLR, and both *P. vivax* and *P. falciparum* MNC were suboptimal in their capabilities to function as stimulators in the one-way allogeneic MLR.

Modulation of normal MNC function by patient sera. We next examined the effects of sera from patients with malaria on mitogenic responsiveness and on the MLR by using normal human MNC from single individuals as the indicator cells in both systems. In experiments using 20% pooled sera from patients with *P. falciparum* or *P. vivax*, the mitogenic responsiveness of normal peripheral blood MNC to both PHA and ConA was markedly reduced (Table 3). There was no statistically significant decrease in the mitogenic responsiveness to PWM. We also investigated the effect of individual sera from patients with malaria on responsiveness to the mitogens, and the results were similar to that seen with the pooled sera.

Finally, we also studied the effect of 20% pooled sera from *P. falciparum* patients on normal allogeneic cells in MLR. The pooled patient sera decreased the stimulation index from 9.9 ± 1.4 to 5.1 ± 0.6. Thus, pooled sera from patients with *P. falciparum* appeared to have an inhibitory effect on the normal blastogenic response to allogeneic cell surface antigens in vitro ($P < 0.01$).

TABLE 2. *Responsiveness of peripheral blood MNC from normal individuals and malaria patients in one-way mixed lymphocyte cultures*[a]

Infecting organism	Stimulator cells	Responsiveness to MLR	
		Normal responder cells	Patient responder cells
P. vivax (22)	Normal	8.5 ± 1.6[b]	5.2 ± 0.9
	Patient	5.9 ± 0.9	ND
P. falciparum (22)	Normal	11.3 ± 2.2[c]	12.2 ± 3.2[d]
	Patient	5.1 ± 1.0	ND

[a] Individual responder and individual stimulator cells were incubated for 120 h at 37°C and pulsed an additional 24 h with [^{3}H]thymidine; stimulation index was determined. Results are expressed as mean stimulation index ± standard error of the mean for the number of experiments (each a separate patient) performed (in parentheses). ND, Not done.

[b] Value obtained with normal responders and stimulators was significantly different from values obtained with normal responders and patient stimulators ($P < 0.5$) or patient responders and normal stimulators ($P < 0.05$).

[c] Value obtained with normal responders and stimulators was significantly different from value obtained with normal responders and patient stimulators ($P < 0.02$).

[d] Value obtained when patient's cells were used as responders was significantly different from value obtained when patient's cells were used as stimulators ($P < 0.05$).

DISCUSSION

In the present experiments, peripheral blood MNC from Thai adults infected with either *P. falciparum* or *P. vivax* malaria exhibited normal responsiveness to the mitogenic lectins ConA, PHA, and PWM. It thus appears that, despite the loss of T cells in the peripheral blood of patients with malaria (7), the functional capabilities of the remaining MNC as assessed by mitogen-induced blastogenic responsiveness is essentially intact. The major cell which responds to ConA and PHA is the T cell (4); however, both B cells and null cells are also capable of responding to PHA and ConA as long as 5 to 20% T cells are also present. There are several possibilities to explain the normal MNC responsiveness to ConA and PHA in malaria patients with an apparent loss of circulating T cells: (i) B

TABLE 3. *Effect of pooled sera from patients (10 P. vivax and 10 P. falciparum patients) on responsiveness of normal peripheral blood MNC to mitogens*

Pooled sera	Responsiveness to mitogen[a]		
	PHA	ConA	PWM
P. vivax patients (15)	81 ± 9	53 ± 10	74 ± 13
Normal controls (15)	160 ± 15	127 ± 20	105 ± 23
Statistical significance	$P < 0.001$[b]	$P < 0.01$[b]	$P < 0.3$
P. falciparum patients (16)	81 ± 9	69 ± 9	86 ± 12
Normal controls (16)	157 ± 9	112 ± 11	81 ± 14
Statistical significance	$P < 0.001$[b]	$P < 0.01$[b]	$P < 0.9$

[a] Mean stimulation index ± standard error of the mean for the number of individual experiments (in parenthesis).
[b] Statistically significant difference.

and null cells, as well as the remaining T cells, may be the cells responding to these lectins, since at least 30% of the peripheral blood MNC in patients with malaria are T cells; (ii) T cells which were lost during the acute infection may not be the cells which would normally respond to mitogenic lectins, and thus the population of cells remaining could respond normally; and (iii) even though the responsiveness of MNC from patients with malaria to mitogens is intact, their functional capabilities in other assays (cell-mediated cytotoxicity, antibody synthesis) may be deficient.

In the allogeneic MLR the principal responding cell is the T cell, whereas macrophages, null cells and B cells function as stimulators (3). The normal degree of stimulation in the MLR is much less than that observed in lectin-induced mitogenesis, because a smaller percentage of cells proliferate in response to allogeneic cell surface antigens than proliferate in response to nonspecific mitogenic lectin stimulation; 60 to 70% of the cells become blasts when cultured with the mitogenic lectins, whereas only 2 to 5% of the cells become blasts in MLR. Thus, mixed leukocyte culture reactivity is potentially a more subtle test of the capacity of a discrete number of cells to specifically recognize and be stimulated by allogeneic cell surface antigens. Furthermore, in the MLR null cells, B cells, and macrophages do not function as responders (3). In our present experiments, no loss of MLR cellular responsiveness was observed with *P. falciparum* cells, whereas *P. vivax* cells functioned poorly. It is conceivable that T cells which are lost in *P. falciparum* patients are non-mixed leukocyte culture responsive T cells, whereas those lost with *P. vivax* infection may be T cells which normally respond in the MLR. Alternatively, it is possible that even though the reactivity in MLR is normal with *P. falciparum* cells, other specific T-cell functions might be impaired.

Despite normal responsiveness in the MLR, peripheral blood MNC from patients with *P. falciparum* did not exhibit normal stimulatory capabilities. Cells from *P. vivax* patients also had decreased stimulating capabilities. Because the percentage and concentration of null cells, B cells, and macrophages are not decreased in patients with malaria (7), it must be assumed that there is a functional stimulatory impairment despite the cells being present.

Thus, with regard to the allogeneic, one-way MLR by patient MNC, we have observed unexpected dichotomies: (i) normal responsiveness with *P. falciparum* MNC despite the apparent loss of the responding cell type (as suggested in our work cited above) and (ii) decreased stimulatory capabilities despite the presence of the appropriate cells in both *P. falciparum* and *P. vivax*. One possible explanation for these results is that serum factors in patients may influence cell function. Indeed, we found in the course of these experiments that pooled sera from both *P. falciparum* and *P. vivax* patients induced marked inhibition of normal peripheral blood MNC responsiveness to PHA and ConA. In contrast, inhibition of responsiveness to PWM was not observed. Pooled sera from *P. falciparum* patients also inhibited responsiveness of normal cells in the MLR. We have previously observed (6) that sera from malaria patients contain cold-reactive (15°C) and warm-reactive (37°C) lymphocytotoxic antibodies. Because malaria sera contain antibodies capable of reacting with normal peripheral blood MNC, it is possible that these sera modulate cell function through lymphocytotoxic activity and produce the inhibition of allogeneic MLR and mitogen-induced proliferation. The decrease in allogeneic MLR could be due to either a responding cell or stimulating cell defect. We have not yet determined, however, against what cell types the lymphocytotoxic antibodies are directed. Furthermore, although we have shown that sera from patients with malaria will inhibit responsiveness to PHA, ConA, and allogeneic cell surface antigens, we have not established that lymphocytotoxic antibodies are involved in mediating the suppression.

It should be noted that nonspecific assays such as mitogenic responsiveness or the MLR may not represent functions directly involved in the host response to the malaria parasites. Therefore, effector functions such as cell-mediated cytotoxicity, phagocytosis, and antibody synthesis may be more relevant and, thus, will

be of great interest for future studies. If the capability of malaria sera to modulate the host immune response is of importance, defense mechanisms which are more likely to be involved in host protection should be examined in future studies.

Previous studies by Spira and co-workers noted that the blastogenic reactivity of spleen cells from rats infected with malaria was markedly depressed in response to mitogens (5). However, in their study the decreased mitogenic responsiveness was primarily seen when the parasitemia was very high, in the 25 to 35% range. In contrast, the patients in our present study had parasitemias primarily in the 0.1 to 1% range with normal response to lectins. Greenwood and Vick described the effect of sera from children with acute malaria on MNC from a normal donor (2). They found that the majority of sera from the children with malaria were toxic to the lymphocytes. It is possible that the toxicity and suppression of stimulation that they observed was similar to the lymphocytotoxic reactivity which we observed in our previous work and the suppression of mitogenesis by pooled sera which we have reported in this paper. We should point out that we do not feel that the inhibition by malaria sera is a nonspecific toxic effect, since in our studies there was no effect in the PWM assays. Therefore, the effect of malaria sera is greatest on cells responsive to PHA and ConA and not all cells in general.

In a study examining malaria antigen as a specific stimulant, Wyler and Brown found no significant difference between MNC from infected patients as compared with normal controls (8). These authors also examined plasma from infected patients for the ability to suppress antigen-induced proliferation, and found no suppression. It is noteworthy that several differences between their study and ours exist which may have contributed to the difference in results. These include: (i) their use of 10% plasma in contrast to 20% sera in our work, (ii) their use of malaria antigen to induce specific blast transformation in contrast to our use of mitogens and cell surface antigens in nonspecific stimulation, and (iii) differences in the source of the material studied—West African children in contrast to Thai adults. The results of our study indicate that despite an apparent loss of circulating T cells during malaria, the blastogenic responsiveness of peripheral blood MNC after stimulation with mitogens or cell surface antigen remains intact. On the other hand, the stimulatory capabilities of these cells are reduced with regard to allogeneic MLR, and sera from malaria patients exhibit marked suppressive capabilities. Whether these abnormalities in immune function result in alteration of immunoregulation or of immune effector function remains to be determined.

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LITERATURE CITED

1. **Böyum, A.** 1968. Separation of leukocytes from blood and bone marrow. Scan. J. Clin. Lab. Invest. **21**(Suppl. 97): 77.
2. **Greenwood, B. M., and R. M. Vick.** 1975. Evidence for a malaria mitogen in human malaria. Nature (London) **257**:592-594.
3. **MacDermott, R. P., L. Chess, and S. F. Schlossman.** 1975. Immunologic functions of isolated human lymphocyte subpopulations. V. Isolation and functional analysis of a surface Ig negative, E rosette negative subset. Clin. Immunol. Immunopathol. **4**:415-424.
4. **MacDermott, R. P., G. S. Nash, M. J. Bertovich, N. S. Merkell, and I. J. Weinrieb.** 1978. Human B-cell mitogenic responsiveness to lectins: the requirements for T cells. Cell Immunol. **38**:198-202.
5. **Spira, D. T., J. Golenser, and I. Gery.** 1976. The reactivity of spleen cells from malarious rats to nonspecific mitogens. Clin. Exp. Immunol. **24**:139-145.
6. **Wells, R. A., K. Pavanand, B. Permpanich, S. Zolyomi, and R. P. MacDermott.** 1980. Anti-lymphocytotoxic antibodies in sera of Thai adults infected with *Plasmodium falciparum* or *Plasmodium vivax* malaria. Clin. Exp. Immunol. **39**:663-667.
7. **Wells, R. A., K. Pavanand, S. Zolyomi, B. Permpanich, and R. P. MacDermott.** 1979. Loss of circulating T lymphocytes with normal levels of B and "Null" lymphocytes in Thai adults with malaria. Clin. Exp. Immunol. **35**:202-209.
8. **Wyler, D. J., and J. Brown.** 1977. Malaria antigen-specific T-cell responsiveness during infection with *Plasmodium falciparum*. Clin. Exp. Immunol. **29**:401-407.