

SUBPOPULATIONS OF T CELLS (T_g and T_m) IN
PATIENTS WITH MALARIA

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OBJECTIVE : To quantitate subpopulations of T cells (T_g suppressor and T_m helpers) in the peripheral blood of patients with malaria.

SUMMARY : In the present study we utilized rosetting techniques to enumerate the putative suppressor (T_g) and helper (T_m) T-cell subpopulations in the peripheral blood of adult Thais with malaria. A lower percentage of both T_g and T_m subpopulations and a lower number and percentage of total T cells was found in these patients during the acute period of infection than in the peripheral blood of healthy donors. However, the percentages of total T, T_g and T_m cells were higher during the convalescent period and were comparable to the values found in the peripheral blood of healthy donors. The significance of these findings are discussed.

No correlations were found between the percentage of these T-cell subpopulations and the level of parasitemia or the hematocrit.

INTRODUCTION : A number of alterations have been found in malarious individuals humoral and cellular immune responses. These include hypergammaglobulinaemia (Tobie et al., 1966), nephrotic syndrome due to immune complex deposition (Houba et al., 1970), and impaired antibody response to certain antigens (Greenwood et al., 1971). Evidence from this laboratory indicates that malarious Thai adults have serum antilymphocyte antibodies (Wells et al., 1980); a true decrease in circulating T cells, but no real change in Null or B cell numbers (Wells et al., 1970); serum blastogenesis inhibitory factors (MacDermott et al., 1980); and defective cellular cytotoxic capabilities (Gilbreath et al., submitted for publication).

While the precise mechanisms for these abnormalities are unknown they may be primary phenomena in malarial infections or may be related to alterations in immunoregulatory mononuclear cells such as thymus derived (T) cell subpopulations (Talal, 1979). The previous findings of antilymphocyte antibodies, defective T cell capabilities and deficiency of suppressor cell activity prompted us to examine regulatory T-cell subpopulations, T_g and T_m cells, in the circulation of malarious Thais.

T_g lymphocytes express Fc receptors for IgG and have suppressor functions while T_m lymphocytes, which perform helper functions, can be identified by the

presence of Fc receptors for IgM (Moretta, 1977). Changes in the proportion of T_g and T_m cells have been reported in various disease states (Gupta & Good, 1977; Mendes et al., 1974; Moretta et al., 1977), but there are no reports on numerical changes in T cell subsets in the peripheral blood of *P. falciparum* or *P. vivax* patients. We therefore conducted this investigation using the rosetting technique of Moretta et al., (1978) for detecting Fc_g and Fc_m receptors, since it was adaptable to our field situation.

MATERIALS AND METHODS :

Patients: Peripheral blood mononuclear cells (MNC) were obtained from male patients with naturally acquired *P. falciparum* (12 patients) or *P. vivax* (8 patients) malaria who were diagnosed as having malaria by the medical staff of the Malaria Eradication Center (MEC), Phrabuddabat, Thailand. All the patients had low levels of parasitemia (< 0.09%) and denied being on any medication at the time of testing. All patients were subsequently treated as "out patients" by the MEC medical staff. An age- and sex-matched healthy Thai volunteer who had not previously had malaria served as a control donor for each patient and was bled at the same time of the day so that the cells could be tested simultaneously with the patients' cells. Different, normal, fresh controls were used concurrently in all assays.

Isolation and fractionation of peripheral blood (PB) mononuclear cell suspensions: Mononuclear cells were obtained from 15 ml heparinized PB (10 units heparin/ml) and isolated using Ficoll-Hypaque density gradient centrifugation (Boyum, 1968) as previously described (Wells et al., 1979). Briefly, MNC were washed three times and resuspended in final medium, RPMI 1640 (GIBCO) containing 2 mM glutamine, 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer, 50 U of penicillin, 50 ug of streptomycin per ml, and 20% heat-inactivated fetal bovine serum (FBS).

T cell enriched suspensions were obtained by E-rosetting of lymphocytes with sheep erythrocytes (SRBC) followed by centrifugation of rosetted cells over Hypaque-Ficoll gradients as previously described in detail (Wells et al., 1979). The E-rosetting cells (T cell-enriched) separated into the pellet fraction leaving non-rosette forming cells (T-cell depleted) at the interface.

Surface characteristic determinations of the enriched cell populations were routinely performed to quantitate the percentage of SIg^+ cells, E-rosette⁺ cells and esterase⁺ cells as previously described (MacDermott & Stacey, 1981). All T-cell populations were highly enriched (< 95%) and viability of the final preparation was greater than 98% as assessed by using the eosin dye inclusion procedure. Contamination by monocytes was routinely less than 3%.

Anti-Ox red blood cells (RBC) antibodies: IgG and IgM fractions of anti-Ox RBC antibody were raised in locally obtained rabbits by injecting washed Ox RBC intraperitoneally each week for a total of 3 weeks. The rabbits were bled one week following the first and 3rd immunizations for the IgM and IgG isolation, respectively. The euglobulin fraction was precipitated with 2% boric acid. The IgM and IgG fractions were prepared by chromatography of the resuspended precipitate on Sepharose 6B, followed by concentration of IgM containing fractions with an Amicon XM-300 membrane filter and the IgG containing fraction with a PM-30 membrane filter. These fractions were checked for

cross contamination by immunodiffusion with mu chain specific goat anti-rabbit IgM, gamma chain specific goat anti-rabbit IgG, as well as sheep and rabbit gammaglobulin and goat anti-rabbit albumin (Cappel Laboratories, Inc. Cochranville, Pa.). Because of the report by Gleeson-White et al., (1950) of differences between cattle erythrocytes from individual animals we used erythrocytes from a single Ox in this study.

Identification of T-cell subpopulations: T-cell subpopulations were determined as previously described in detail (Moretta et al., 1975). Briefly, to identify T cells bearing receptors for the Fc portion of IgG (T_g), 0.1 ml of purified T cells at a concentration of 2×10^6 cells/ml was mixed with 0.1 ml of a 1% suspension of Ox E (OE) coated with highly purified rabbit anti-OE IgG antibody (7S EOA) (5.2 mg/ml). This mixture was spun into a pellet and incubated at 4°C for 60 min. To identify T cells bearing receptors for the Fc portion of IgM (T_m), purified T cells were incubated at 37°C overnight in RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.) and 20% fetal calf serum. After overnight incubation, the T cells were suspended in RPMI 1640 at 2×10^6 cells/ml, and 0.1 ml was added to 0.1 ml of a 0.1% suspension of OE coated with purified rabbit anti-OE IgM (19S OEA) (0.33 ug/ml). The T-cell 19S OEA mixture was spun into a pellet and incubated at 4°C for 60 min. The optimal concentrations of the respective anti-Ox immunoglobulin fractions used in this study were determined in preliminary studies.

After incubation of T cells with either IgG or IgM OEA cell suspensions, the buttons were gently resuspended, placed on a glass slide, and the percentage of rosette-positive cells was determined by using a phase contrast microscope. Lymphocytes binding three or more Ox RBC were classified as rosettes. The percentage of T-cells having neither receptor (T non-g, non-m, or T null) can be determined by subtracting the sum of the percentage of T_g cells and T_m cells from 100.

Enzymatic treatment of T cells: In certain experiments purified T cells from normal subjects and patients were examined for the proportion of T_g cells and T_m cells both before and after treatment with trypsin to determine if a serum factor might be influencing the expression of the Fc receptor for IgG or IgM in the patients. The trypsin treatment protocol consisted of determining the proportion of T_g and T_m in separate aliquots of purified T cells which had been incubated with either RPMI 1640 media or trypsin 10 mg/ml (Sigma Chemical Co., St. Louis, MO.) for 20 min at 37°C followed by three washes in RPMI 1640, and then processed as described above. When tested by eosin dye inclusion viability of the T cells after incubation was greater than 98% in all cases.

Statistical calculations: Statistical significance of the results was assayed by the 2-tailed, student's t-test for paired samples.

RESULTS :

Lymphocyte counts in patients and controls: The percentage and number of T cells, and the percentage of T_g and T_m cells in the peripheral blood of malarious and healthy Thais are tabulated in Table I. The percentage and number of total T cells, and the percentage of T_g and T_m cells was

significantly lower in the malarious group than in the Thai control group. No correlation was found between the level of parasitemia or the hematocrit and the percentage of T_g or T_m cells in the malaria patients.

Effect of enzymatic treatment of T cells upon expression of IgG Fc or IgM Fc receptors: The effect of trypsin treatment on T cells from malarious patients who manifested low proportions of T_g cells did not result in an increase in detectable IgG Fc receptor-bearing T cells suggesting that IgG Fc receptors were not being blocked by any patient serum factors (trypsin sensitive). Enzymatic treatment of T cells from normal individuals, likewise, did not affect the proportion of cells with detectable IgG Fc receptors. Furthermore, we failed to observe an increase in T_g cells after a 24 hr incubation of patients' T lymphocytes at 37°C indicating that the decreased number of receptors available for rosetting was not due to the blocking of the T_g receptors on patients' T lymphocytes by cytophilically attached IgG.

The percentage of T_m cells in the T cell populations obtained from both the patients and healthy donors was higher (5-7%) in the aliquots of cells treated with trypsin (prior to overnight incubation in IgM free media) than in the non-treated aliquots. However, it is conceivable that both patient and control values were higher after trypsin treatment due to the enzyme affecting membrane equilibrium and possibly increasing the turnover rate of the surface structure.

Level of T_g and T_m cells in individual patients during the clinical course of the disease: We next looked to see if changes in the % HCT, total T cells, and percentage T_g and T_m cells were associated with remission. Values are listed in Table II for five patients who agreed to return after 14 days for follow-up testing.

Low numbers of parasites were detected in thick blood smears from 2 of the 5 individuals when tested 14 days after the initial testing, however quantitation of the T cell subpopulation for the two parasitemic individuals was also done in order to see if these T subpopulations values differed from the value obtained for the 3 non-parasitemic individuals.

When patients T cells, T_g cell and T_m cell percentages were quantitated during the acute period of illness and two weeks later, patients that were not parasitemic during the convalescent period had higher percentages of T_g and T_m cells in their peripheral blood. The percentages were comparable to the percentages found in the peripheral blood of their concurrent control donors. Although the percentage of total T cells was also higher during the convalescent period than the acute period of infection the percentage of total T cells remained lower than the percentage of total T cells found in the concurrent control's peripheral blood. Little or no change was noted in patients' hematocrit level during the convalescent period.

DISCUSSION : The T-lymphocytes are functionally heterogeneous in that they are subdivided into two major functional categories; effector T cells that mediate functions of cellular immunity, and the immunoregulatory T-helper and T-suppressor cells. T-lymphocytes with IgM Fc receptor (T_m) contain cells having helper activity for differentiation of B cells into immunoglobulin synthesizing

and secreting plasma cells in pokeweed mitogen and antigen driven systems, while T-lymphocytes with IgG Fc receptors (T_g) contain cells with suppressor activity in these systems. Recently it has been shown that the immunoregulatory cell subpopulations can be identified using an Ox red blood cell rosetting technique developed by Moretta et al., (1977) which can be adapted and utilized with minimal difficulty in field situations that otherwise prohibit the use of more sophisticated technologies requiring either a fluorescent microscope or a fluorescence-activated cell sorter.

By using this rosetting technique we have found that alterations exist in the general T cell subpopulations of malarious Thais with specific decreases in both the T_g and T_m subpopulations. Although the percentages of T_g cells that we observed in the peripheral blood of the control subjects were low, the patients' T_g values were always less than the values found for their respective controls. However, it should also be noted that the Ox and rabbits used in this study were not from an established breeding colony, thus our rabbits may have been generally low responders in respect to the development of IgG antibodies and the resultant ratio of specific IgG anti-OEA to total IgG antibodies may have been low.

It has also been suggested that without enzymatic treatment of sheep red blood cells, T cells with low affinity receptors for sheep red blood cells may not form rosettes thus leading to a lower percentage of T cells being identified. Since T_g cells have been reported to have low affinity receptors to sheep red blood cells, it is possible that the lower percentages of T_g cells obtained in this study stem from the fact that we did not enzymatically treat our SRBC prior to isolating SRBC rosetting cells. However, in preliminary experiments we did treat our SRBC with neuraminidase and found that the percentage of rosetting cells was not increased by this process, therefore the procedure was not performed in subsequent assays. Since the patient and control cells were processed in the same way we feel that the differences in the patient and control T_g values are real and are consistent with our previously report of deficient Con A suppressor cell activity in malarious Thais (Gilbreath et al., submitted for publication). At the very least the data indicate that a difference exists in the relative percentage of patient and control T_g cell with high or strong SRBC rosette forming affinities.

It is interesting that a higher percentage of T_m cells was found when T cells from healthy Caucasians were compared to T-cells obtained at the same time from healthy Thais. The limited information would suggest that the relative percentage of the T cell subpopulations may vary, in part on an individual's ethnic background.

Although the mechanism for this decrease in helper and suppressor T cells in malaria is unknown the data suggest that the decrease is due more to a general T-cell lymphocytopenia than to a specific depletion of either one or both of the specific subpopulations. Wells et al., (1980) suggested that lymphocytotoxic antibodies present in sera of some malarious individuals may be responsible for this T-cell lymphopenia. However, in our recent characterization of lymphocytotoxic activity in malarious Thais' sera we found that the cytotoxicity is associated with the 19S immunoglobulin fractions, shows optimal cytotoxicity at 15°C and is cytotoxic to enriched B-cell, T-cell, T_m cell and

T_g cell subpopulations (Gilbreath et al., submitted for publication). Based on this information it is difficult to support the hypothesis that anti-lymphocyte antibodies are functioning in vivo in a cytotoxic fashion during malarial infections to remove specific lymphocyte subpopulations. Also, the fact that the decrease in T cell, T_g cell or T_m cell percentages could not be normalized by trypsinization in this study argues against the expression of receptors of a given T-cell subpopulation being blocked by malarious serum factors indicating that a true depletion of total T cells in general and the specific T_m and T_g regulatory cells exist in malarious Thais.

It is also of interest that in 3 of the 5 follow-up patients who initially had reduced percentages of total T cells, T_m cells and T_g cells, and who did not have any detectable parasites in their blood 14 days after treatment showed an increase in the percentage of all three cell populations. This suggests that an association exists between the abnormalities in total T cells, T_m cells and T_g cell and the patients' clinical state. In respect to the T_g cells this data supports our earlier finding (Gilbreath et al., submitted for publication) in which we demonstrated that reduced Con A induced suppressor cell activity was present early in malaria infection but returned to normal soon after treatment.

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Table 1. T_g and T_m cells in patients with malaria and in age-matched controls.

	Z HCT (mean S.D.)	WBC/mm ³ (mean S.D.)	Total T lymphocytes (x 10 ⁶ /ml) (mean S.D.)	Z total T cell (mean S.D.)	Z T cell ^g (mean S.D.)	Z T Cells (mean S.D.)	Ratio T _g :T _m (mean S.D.)
Caucasian control (n = 16)	48.0 ± 2.0	7400 ± 672	3.3 ± 0.8	45.5 ± 1.6	6.8 ± 1.2	29.8 ± 4.5	4.4 ± 0.6
Thai controls (n = 17)	46.5 ± 2.5	7455 ± 1262	5.5 ± 2.1	44.0 ± 2.7	6.4 ± 1.0	22.6 ± 4.0	3.5 ± 0.8
Thai patients (n = 17)	39.1 ± 3.9**	6746 ± 1502*	3.9 ± 1.2***	32.7 ± 5.3***	4.2 ± 1.0**	10.4 ± 4.3**	2.7 ± 1.3**

* Difference between the Thai controls and patients values are statistically significant at p < 0.05.

** Difference between the Thai controls and patients values are statistically significant at p < 0.005.

*** Difference between the Thai controls and patients values are statistically significant at p < 0.01.

