

## NATURE OF MALARIA COLD-REACTIVE LYMPHOCYTOTOXIC ANTIBODY

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**OBJECTIVE :** To determine the target cell population and the chemical nature of lymphocytotoxic antibodies in sera from patients infected with malaria.

**BACKGROUND :** We have previously reported the presence of cold-reactive anti-lymphocyte antibodies (ALA) in the sera of Thai adults naturally infected with *P. falciparum* and *P. vivax* malaria (21). ALA was found in 95% of the *P. falciparum* and 98% of the *P. vivax* patients' sera when assayed at 15°C against peripheral blood target cells from uninfected individuals. Activity in assays done at 37°C was significantly less.

Cold reactive ALA present in other disease states have been shown to be predominately IgM and directed against T lymphocytes (7,24). However, cold-reactive IgM lymphocytotoxic antibodies directed against B cells also occur in approximately 20% of normal individuals (15). Thus ALA in malarious patients may be directed against more than the B cell subpopulations since greater than 12% of the target cells were killed by the majority of sera in our previous study.

Additionally we have previously found that Thai adults naturally infected with either *P. falciparum* or *P. vivax* have a decrease in both the percentage and absolute number of lymphocytes in general and T lymphocytes in particular (20). Although the mechanisms leading to this loss of lymphocytes are unclear our recent interest has focused on the relationship of ALA and the decrease T cell numbers found in the peripheral blood of actively infected malarious patients.

We have also observed in previous studies a loss of functional Con A inducible T suppressor cells and defective lectin-induced and spontaneous cell-mediated cytotoxicity (Gilbreath, et al., submitting for publication), and serum suppressor factors (8), in peripheral blood of malarious Thais.

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While the T cell depression and defective cytotoxic capability observed in infected patients could represent a transient relocation of lymphocyte pools, it would be reasonable to hypothesize that the loss of T cells could be due to the destruction or modulation of a specific T cell subclass by serum factors including anti-lymphocyte antibodies.

Since it is possible that ALA play a role in modulating the immune response of patients toward malaria we have further characterized ALA in the sera of malaria patients, with regard to the type of antibody, their relationship to the disease, the antibody titers, and the cell types against which they are directed.

## MATERIALS AND METHODS :

Patients : The patient screening process and the collection of sample specimens has previously been described (20). Basically, the patients were all out-patients who were mildly ill, recently infected, and not anemic from the region surrounding Phrabuddhabat, Thailand, which is endemic for malaria. The patients participating in this study were then treated by the staff of the National Malaria Project. Individual patients' blood was collected in heparinized tubes to obtain plasma and mononuclear cells. Serum was obtained by allowing a second aliquot of blood to clot in non-heparinized tubes at room temperature.

Isolation of Peripheral Blood Lymphocytes and Lymphocyte Subpopulations : Peripheral blood lymphocytes were prepared from venous blood centrifuged through Ficoll Hypaque (1) and then washed three times with Hanks Balanced salt Solution (HBSS), then resuspended in RPMI 1640 supplemented with 2 mM glutamine, 50 µg of penicillin, 50 µg of streptomycin per ml, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, and 10% heat inactivated FCS. Initially each serum was screened for the presence or absence of lymphocytotoxicity against autologous, allogeneic patient, and control lymphocytes from healthy donors, then aliquots of the unused sera were stored at -20°C for use in subsequent assays. Preliminary studies indicated that lymphocytotoxicity was unaffected by repeated freeze-thawing for 2 years or longer. Low volumes of some sera precluded testing positive ALA sera in all the experiments.

Mononuclear cells were isolated from a unit of peripheral blood from 5 healthy Caucasian donors by Ficoll-Hypaque centrifugation and used to determine if the sera lymphocytotoxicity was specific for lymphocyte subpopulations. The cells were further separated into subpopulations as previously described (12).

Briefly, the isolated cells were passed over a Sephadex G-10 column to remove adherent cells. The nonadherent cells were then separated into T, B, and Null cells in a two stage process. First, the cells were poured over a Sephadex G-200 anti-human F(ab')<sub>2</sub> immunoabsorbent column. Then the nonadherent T plus Null cells were further fractionated using an overnight incubation with SRBCs followed by Ficoll-Hypaque density centrifugation. The adherent cells (B cells) were eluted from the column with medium containing 1% human immunoglobulin, incubated overnight with SRBC, and centrifuged over Ficoll-Hypaque.

Surface characteristics determinations of the enriched cell populations were performed to quantitate the percentage of SIg<sup>+</sup> cells, E-rosette<sup>+</sup> cells and esterase<sup>+</sup> cells as previously described (12). All populations were highly enriched and viability of the final preparation was greater than 95%, as assessed using the eosin dye inclusion procedure.

Preparation of Ox RBC Antibody Indicator Cells : 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 immunization and the 3rd immunization 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,  $\alpha$  chain specific goat anti-rabbit IgG as well as sheep and rabbit gammaglobulin and goat anti-rabbit albumin.

Isolation of T<sub>m</sub> and T<sub>s</sub> Cells : Suppressor T cells (T<sub>s</sub>) and helper T-cells (T<sub>m</sub>) were identified by rosette formation as described by Moretta et al. (9,10). Briefly, to isolate T lymphocytes which have receptors for IgG, purified T cells were mixed with ox erythrocytes (OE) coated with rabbit anti-OE IgG antibodies (IgG-OE), pelleted, and incubated at 4°C for 1 hour (9,10). Rosetting cells (T<sub>m</sub>) were isolated from nonrosetting cells and purified by pelleting through a Ficoll-Hypaque density gradient. This yielded a population of > 98% rosetting cells. Erythrocytes were then lysed with ammonium chloride buffer.

T<sub>m</sub> enriched and T<sub>s</sub> depleted cells were incubated overnight in RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.), supplemented with 20% fetal bovine serum (FBS). Cell bearing receptors for IgM (T<sub>m</sub>) present in the T<sub>s</sub> depleted fraction were purified on density gradient after rosetting for 1<sup>1/2</sup> hour on ice with OE coated with rabbit anti-OE IgM antibodies (IgM-OE). The rosette were dissociated by vortex agitation. T<sub>m</sub> cells were then separated from OE on a density gradient.

Lymphocytotoxic Antibody Assays : The methodology for the lymphocytotoxic antibody assays has been described previously (21). Briefly, at either 4°C, 15°C or 37°C, 0.1 ml of each sera or a fraction of the sera obtained by sucrose gradient fractionation was added in duplicate to appropriate wells for 30 min followed by the addition of 0.1 ml of fresh rabbit serum (as a source of complement).

To determine complement dependency, test sera were run in parallel, adding complement to one group and deleting the complement from the other. To maintain the volume, 0.1 ml of media was added to the plate not receiving complement (when complement was not added, all test sera were negative for lymphocytotoxicity). Then after an additional 3 hours incubation at either temperature the percentage of dead cells was determined by eosin dye inclusion. In preliminary experiments we determined that unabsorbed rabbit complement was interchangeable with rabbit complement previously absorbed with pooled unfractionated sera.

MNC in the assays. Thus, unabsorbed rabbit complement was used in the majority of the assays.

Sera with high lymphocytotoxicity from dengue patients or patients with systemic lupus erythematosus served as positive controls while sera from healthy Thai volunteers or patients with primary biliary cirrhosis served as negative controls. Some sera with lymphocytotoxic activity were subsequently tested for the presence of lymphocytotoxicity against a panel of lymphocytes from 15 normal Caucasian adults of varying HLA-phenotype. Reproducibility of the assay checked by repeating the same sera directed against the same targets at different times, demonstrated essentially identical results for the sera tested. In a small number of assays patients' plasma was also tested for the presence or absence of lymphocytotoxicity, however little or no activity was present in the plasma samples tested. Preliminary study indicates that the addition of small amounts of heparin to sera containing positive ALA can either remove or substantially reduce ALA activity.

The microcytotoxicity assays were modified slightly when some sera were tested for lymphocytotoxicity against enriched T-cell, B-cell, or Null cell targets. Sera was distributed on Terasaki microtiter trays which had been covered with mineral oil and then 1 lambda serum was added to each well and the trays were stored at  $-70^{\circ}\text{C}$  until needed. The assays were performed by putting 1 lambda of target cell suspension ( $2 \times 10^6$  lymphs/ml) into each well and the mixture further incubated for 3 hours. Three lambda of 5% aqueous eosin was then added to each well and 2 min. later 10 lambda of formalin was added to the wells and the tray covered with 50 x 75 mm microscope slide. Target cell death was determined using a phase contrast microscope.

Serum Dilution Studies : Some of the sera with high levels of ALA activity during the initial screening were diluted serially using commercial pooled human AB sera. The serum dilutions were then assayed at  $15^{\circ}\text{C}$  against mononuclear target cells in the microcytotoxicity assay. Some of these sera were tested against Chang liver target cells, however, no ALA was detected at  $15^{\circ}\text{C}$  or  $37^{\circ}\text{C}$  when these cells were used as target cells.

Effect of Serum on E-rosette Formation : The rosette formation method used to assay T cells was done as previously described (20). In brief, MNC populations were derived by Ficoll-Hypaque gradient sedimentation from normal peripheral blood. The cells were washed three times in cold Hanks balanced Salt Solution (HBSS) (Grand Island Biological Co., Grand Island, N.Y.) and resuspended to a concentration of  $2 \times 10^6$  cells/ml. Then 10 microliters of either pre-SRBC absorbed malaria patients' serum containing ALA activity or control serum was mixed with 0.1 ml of the MNC. The mixture was incubated for 30 min then HBSS was added and the percentage of cells forming E-rosettes was determined after 1 hour or 18 hours at  $4^{\circ}\text{C}$ .

Elution Experiments : Ficoll-Hypaque purified patient MNC were divided into three groups. One group was resuspended in cold RPMI 1640 medium containing either 10% heat inactivated (HI) FCS or medium containing 10% autologous serum. The second group was washed 3 times with medium pre-warmed to  $37^{\circ}\text{C}$ . One hour E-rosette assays were immediately performed on both groups of cells. A third group of cells was incubated in medium containing either

autologous serum or HI FCS and incubated overnight at 37°C before 1 hour E-rosette assays were done. The number of E-rosettes formed by the various cells was compared to see if the reduced number of E-rosetting cells in the peripheral blood of acutely ill malaria patients might be due to ALA coating E-rosette forming MNC at physiological temperature, thus inhibiting E-rosette formation.

Sucrose Density Fractionation of Serum : Sucrose density centrifugation of serum was performed by layering the serum over a 10% to 40% linear sucrose gradient from which 12 equal fractions were subsequently collected after centrifugation (6). Each fraction was tested against normal MNC at 4°C using the microcytotoxicity assay to determine which fractions contained lymphocytotoxic activity. Preliminary experiment were performed to ensure that the sucrose concentration in the various fractions was not cytotoxic to the target indicator cells and did not inhibit ALA in the microcytotoxic assay. All fractions were concentrated to their original volume for use in lymphocytotoxic assays. Quantitative estimation of human IgG, IgM, and IgA in the fractions was performed using commercially available regular and low level radial immunodiffusion plates (Hyland diagnostics, Deerfield Illinois).

## RESULTS :

Incidence of Autologous Lymphocytotoxicity : The ALA of thirty-five sera obtained from *P. falciparum* and *P. vivax* malarious patients during the acute stage of illness and tested in a microcytotoxicity assay at 15°C against unfractionated mononuclear cells (MNC) from autologous, allogeneic patients and healthy control donors is shown in Fig. 1. The mean percentage killing by these sera against autologous MNC was  $18.3 \pm 3.5$  (mean  $\pm$  S.E.M.). In contrast, the killing of MNC from allogeneic patients or control donors was  $19.7 \pm 3.7$  and  $24.6 \pm 3.8$ , respectively. The difference in ALA activity found when autologous target cells were tested compared to the activity found when allogeneic target cells from patients' or healthy donors was significantly different when a 2 tailed, paired student t-test was used to analyze the data ( $p < 0.025$  and  $P < 0.005$ , respectively). However, it is evident from Fig. 1 that most malarious patients who have antibodies against MNC of others also have antibodies, although they may be of a lower titer, against their own MNC.

Dilution Studies : When patients' positive ALA sera is serially diluted and tested against allogeneic MNC target cells from healthy Thai donors ALA is absent or substantially decreased at dilutions as low 1:16 (Table 1).

Distribution of ALA of Sera Against Panel of Target MNC from Ten Healthy Donors : Sixteen of thirty-one *P. falciparum* (52%) and 19 of 25 *P. vivax* (76%) sera demonstrated high (> 40%) cold-reactive lymphocytotoxicity against unfractionated mononuclear target cells from ten healthy Caucasian donors. By contrast, none of sixteen sera from healthy controls showed ALA. Many of the ALA positive sera were highly cytotoxic (Table 2).

Acute and Convalescent Periods : When a comparison was made on a limited number of paired antisera collected during the acute period of illness and antisera collected 15 and 30 days later during the convalescent period the antibodies more often had higher titers during the acute period of malaria

infection than antibodies during the convalescence period (Table 3). If a difference of 10 percent in reactivity is considered to be significant, 10 sera were stronger in the acute period, 2 sera were stronger in the convalescent period (of which once can be considered a treatment failure), and 1 was of about equal strength in 13 groups of malarious patients' sera tested. Furthermore none of six paired patient sera which did not contain any antibodies during the acute period developed antibodies during the convalescent period of illness.

Immunoglobulin Activity : Serum lymphocytotoxic activity is mediated by a protein with the characteristics of an IgM antibody. Treatment with 2-mercaptoethanol followed by iodoacetamide removes all or substantially reduced the lymphocytotoxicity of the malarious patients sera. Seven individual sera were examined by sucrose density gradient analysis and the lymphocytotoxicity was consistently found in the IgM containing fraction near the bottom of the gradient (tubes 2, 3, and 4 in a 12 tube gradient) (Table 4). The cytotoxicity in the fraction was inhibited by rabbit antiserum specific for human IgM (U chain).

More importantly, in four of six individual serum (No. 1, 3, 4, 5) examined lymphocytotoxicity greater than 10% was found in the IgM containing fraction when assayed at 37°C. Lymphocytotoxic activity was always less than 6% in the patients neat sera at 37°C. Thus, there is ALA reactivity against unfractionated mononuclear target cells at 37°C in the patients' sera which becomes apparent after fractionation and which is easily detectable.

In order to investigate the possibility that IgG or IgA present in fractions 7, 8 and 9 could be blocking IgM lymphocytotoxicity in the neat sera at either 15°C or 37°C we did "add-back" experiments. In these experiments we pooled the patients IgM fractions (3 and 4) and the patients' IgG - IgA fractions (7 and 8) respectively. We then added to the IgM fraction equal volumes of either media, autologous IgG/IgA, or IgG/IgA fractions from a healthy donor. The samples were then assayed against unfractionated peripheral blood MNC obtained from healthy Thai donors and the percentage cytotoxicity of the respective test samples was determined. As shown in Table 5 the IgM fraction of the various patients was equally cytotoxic irrespective of whether it was combined with media, autologous IgG/IgA or IgG/IgA from an uninfected donor. In 4 of the experiments (No. 1, 3, 4 and 6) the addition of IgG-IgA appeared to slightly enhance IgM lymphocytotoxicity at 37°C, while one slightly reduced cytotoxicity (No. 5) and one showed no change in lymphocytotoxicity (No. 2). However in 2 of the 4 experiments in which enhancement of lymphocytotoxicity was noted when IgG/IgA was added to the autologous IgM fraction the level of lymphocytotoxicity seen at 37°C for the IgG/IgA and media was too high to permit us to make any assessment of the ability of these patients' IgG/IgA to block or enhance IgM induced lymphocytotoxicity.

Specificity of ALA for Peripheral Blood MNC Subpopulations : Thirty-one sera from patients with acute *P. falciparum* (P.f.) and 25 sera from patients with acute *P. vivax* (P.v.) malaria were tested against T enriched, B enriched and null cell enriched target subpopulations. In a small number of experiments patient serum with strong ALA against unfractionated MNC was tested against

monocyte/macrophage enriched (> 85% esterase positive) target subpopulations.

As seen in Table 6, the lymphocytotoxic antibodies are primarily directed against B cells, and in most instances, T cells as well as B cells. One *P.f.* sera and 3 *P.v.* sera reacted with only T cells at 4°C, 5 *P.f.* and 4 *P.v.* reacted with B cells at 4°C, 4 *P.f.* and 2 *P.v.* reacted with B-cells at both 4°C and 37°C, while 10 *P.f.* and 9 *P.v.* reacted with T cells and B cells at 4°C and 5 *P.f.* and 2 *P.v.* reacted with T cells at 4°C and B-Cells at both 4°C and 37°C. No reactivity was seen against Null cells. Two of six sera showed cold ALA against the monocyte/macrophage enriched population, however no activity was found at 37°C.

Effect of ALA on E-rosette Formation : Experiments were performed to determine if malaria patients' sera with high ALA activity can influence the ability of normal donor peripheral blood mononuclear cells to form E-rosettes. No significant difference was found in the percentage of E-rosettes formed by mononuclear cells incubated with patient's serum or normal serum, even when the mononuclear cells are incubated with the serum overnight.

When malaria patient cells were washed and incubated at 37°C overnight to remove any factors that might be absorbed to the surface of the patient cells that could interfere with E-rosetting, no change was seen between the cell incubated in the presence of autologous serum and cells incubated without autologous serum.

Effect of 19S and 7S Sucrose Density Gradient Sera Fractions on Different Cell Populations : Sera from 3 patients with *P. falciparum* malaria showing strong ALA activity against unfractionated MNC were fractionated into fractions containing 19S (IgM) and 7S (IgG/IgM) antibodies using sucrose gradient centrifugation. The fractions (3 and 4 of a 12 fraction gradient) containing IgM (19S) and fraction (8 and 9) containing IgG/IgA (7S) were reacted with T cell enriched, B-cell enriched (non-rosetting non-phagocytic cell (B - Null) and monocyte/macrophage (> 85 esterase) enriched cell populations. As seen in Table 7 all of the patient IgM fractions were more reactive against T cells at 15°C than at 37°C. One IgM fraction reacted strongly against the B cell-subpopulation at 15°C while another one reacted weakly. Patients IgG/IgA fractions did not react against any of the target cells, nor did IgM fractions obtained from 3 healthy Thai donors. No reactivity against the monocyte/macrophage population was found.

Specificity of ALA for Peripheral Blood T Cell Subpopulations : Three patient sera containing high ALA activity and 3 control sera from healthy Thai donors were fractionated using sucrose gradient centrifugation. The individuals' respective 7S or 19S fractions were then combined with one another or with an equal volume of medium before being assayed for cytotoxicity against the various target cells. Although the cytotoxicity of the 3 patients' sera varied in respect to activity and target cell specificity the 19S fractions generally were more cytotoxic at 15°C than the patients 7S or the control donors 19S fractions. Sera was reactive against both the T<sub>H</sub> and T<sub>H</sub> target at 15°C, but no activity was found in assays done at 37°C when either the 7S or the 19S fractions were assayed using enriched B + Null cell targets. However, weak HLA reactivity at 37°C was not ruled out in these experiments.

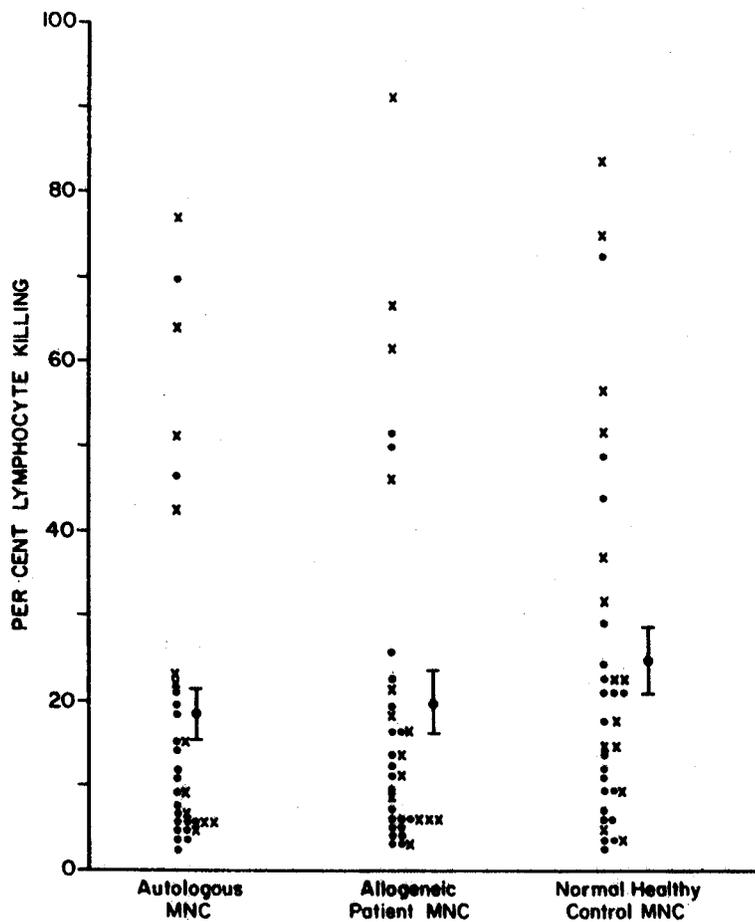


Fig. 1. The ALA of thirty-five sera from mononuclear cell donor male patients with *P. falciparum* (•) or *P. vivax* (x) malaria tested against autologous and allogeneic target mononuclear cells (MNC) in assays done at 15°C. No ALA (>6.5%) was detected when the sera was tested in assays done at 37°C. Sera from healthy male Thai donors was always assayed in parallel with the patients' sera but never demonstrated any significant ALA at 15°C. Percentage lymphocytotoxicity is based on value after control background for the assay was subtracted.

Table 1. Effect of Serial Dilution of Malaria Sera on Lymphocytotoxicity

	% parasitemia <sup>a</sup>	Neat (fresh)	Neat (freeze-thawed)	1:2 <sup>b</sup>	1:4	1:8	1:16
P.v. <sup>c</sup>	0.1	41.0 <sup>d</sup>	39.5	28.0	20.5	18.0	4.5
P.v.	0.3	56.0	60.0	51.5	47.5	38.5	21.5
P.v.	0.4	55.5	44.0	43.5	44.0	12.0	3.5
P.v.	0.1	52.5	47.5	40.0	15.0	2.5	0
P.v.	+ve	47.5	43.5	35.5	14.0	3.5	1.0
P.f.	8.5	31.5	30.0	10.5	2.5	0.5	0
P.f.	0.1	48.0	46.5	26.5	13.5	6.5	3.0
P.f.	+ve	43.5	37.5	10.0	3.0	0	0
P.f.	0.6	40.0	38.5	8.0	1.5	0	0
P.f.	1.5	41.0	38.5	7.5	7.0	0	0

<sup>a</sup> Percentage parasitemia determined by examination of thick smear slide.

<sup>b</sup> Test sera diluted with pooled commercial human AB sera (microbiological Associates, batch C893018). Assays done at 15°C. No activity detected in assays done at 37°C.

<sup>c</sup> P.v. indicates patients with *Plasmodium vivax* infections.  
P.v. indicates patients with *Plasmodium falciparum* infections.

<sup>d</sup> Average cytotoxicity determined prior to freezing serum at -20°C.

Table 2. Distribution of ALA of Sera Against Panel of MNC from Health Donors

Sera Source	No. of Sera	Positive <sup>a</sup> Sera	Percent Donor Panel MNC Killed					
			0-19	20-39	40-49	50-59	60-79	80-100
<i>P. falciparum</i>	31	52	8	6	1	2	4	9
<i>P. vivax</i>	25	76	3	3	1	3	5	10
Healthy Donors	16	0	16	0	0	0	0	0
SLE <sup>b</sup>	3	67	1	0	0	0	0	2
P.B. <sup>c</sup>	4	25	3	0	0	0	0	1
Dengue <sup>d</sup>	13	62	3	2	1	1	3	3

<sup>a</sup> Percentage of donor MNC panel killed by sera. Positive sera = > 40% of donor panel killed. No positive killing of panel MNC by the sera was observed in assays performed at 37°C.

<sup>b</sup> Sera from patients with systemic lupus erythematosus.

<sup>c</sup> Sera from patients with primary biliary cirrhosis.

<sup>d</sup> Sera from patients with dengue fever.

Table 3. Lymphocytotoxic Activity in Malaria Patient's Sera During Acute and Convalescent Period of Illness

Patient	% Parasitemia	Days Post - Admission		
		Acute 0	7-15	24-30
P.f.	1.2 <sup>a</sup>	33.5 <sup>b</sup>	0.5	1.5
P.f.	4.2	24.0	1.0	1.5
P.v.	.05	16.0	0	0
P.f.	0.7	32.5	1.5	0
P.f.	+ve <sup>c</sup>	14.5	4.0	3.5
P.f.	+ve	35.5	19.5	19.0
P.f.	4.5	7.5	3.0	2.5
P.f.	0.5	3.0	12.0	27.5 <sup>d</sup>
P.f.	+ve	0	0.5	16.0
P.f.	+ve	0	8.0	1.0
P.v.	+ve	1.0	3.0	0
P.v.	+ve	48.5	33.0	29.5
P.v.	.04	0	0	5.0
P.f.	+ve	0	0.5	0
P.f.	0.3	0	3.5	1.5
P.f.	+ve	0	0	0
P.f.	1.2	21.0	9.0	1.0
P.f.	+ve	24.0	12.5	13.5
P.f.	+ve	21.5	22.0	17.5
P.f.	+ve	22.5	20.0	13.5

<sup>a</sup> Percentage of donor MNC panel killed by sera. Positive sera = > 40% of donor panel killed. No positive killing of panel MNC by the sera was observed in assays performed at 37°C.

<sup>b</sup> Average percent cytotoxic activity in duplicate wells of assays done at 15°C. Assays of individual patients' sera were done in a single experiment using identical target cells.

<sup>c</sup> Parasitemia verified using Giemsa stained thick and thin slide smear.

<sup>d</sup> Indicates that patients experienced relapse or re-infection.

Table 4. Lymphocytotoxic Activity in Sucrose Gradient Centrifugation Fractions in Serum from Patients with Malaria

Patient	% Parasitemia <sup>b</sup>	Temp	Neat	Percentage Lymphocytotoxicity <sup>a</sup>						
				Fraction Number						
				F <sub>2</sub>	F <sub>3</sub>	F <sub>4</sub>	F <sub>6</sub>	F <sub>7</sub>	F <sub>8</sub>	F <sub>9</sub>
P.v.	0.3	15°	67.5	32	31	24.5	0	<1	1	0
		37°	5.5	18	17.5	13	8	9.5	0	0
P.f.	0.1	15°	40.5	4.5	3.0	3.0	1	<1	0	0
		37°	0	<1	0	3.0	1	<1	0	0
P.v.	0.1	15°	50	26.5	36.5	32.5	<1	0	<1	0
		37°	3	8.5	13.0	4.5	0	<1	<1	0
P.f.	0.1	15°	47.0	35.0	26.0	16.5	1	2	1.5	1
		37°	2.5	12.5	12.0	9.5	3.5	1.5	0	2
P.v.	+ve	15°	47	40	38.5	35	0	0	0	0
		37°	<1	12.5	12.5	9.5	2.5	<1	0	0
P.f.	0.1	15°	55.5	38.5	38.0	35.5	0	<1	0	0
		37°	1.0	7.5	4.5	3.5	0	1.5	1	1
P.f.	0.7	15°	54	31	25	27.5	2.5	0	<1	0
		37°	ND	ND	ND	ND	ND	ND	ND	ND

<sup>a</sup> Average cytotoxicity determined from duplicate wells with serum incubated with unfractionated peripheral blood mononuclear cells from healthy Thai adults as described in Materials and Methods. IgM was only detected in fractions 3 and 4 and IgG/IgA was only detected in fractions 6,7,8 of the patients serum fractions using radial immunodiffusion plates.

<sup>b</sup> Percentage parasitemia determined as described in Table 1.

Table 5. Effect of IgG/IgA on IgM Lymphocytotoxicity

Exp. No.	IgM + media		IgG + media		IgM + IgG auto		IgM + IgG control	
	15°	37°	15°	37°	15°	37°	15°	37°
1	28.5	1	0	0	35	7.5	33.5	4.5
	<1	0	1	0	N.D.	N.D.	0	0
2	11.5	11.5	2.5	4.5	2.5	11.0	1.5	3
	1.5	3	<1	0	N.D.	N.D.	0	0
3	17.5	5	<1	1	17	12.5	11.5	9.5
	2	<1	1	5.5	N.D.	N.D.		
4	49	7.5	0	15.5	47.5	14	35	7.0
	10	4	1.5	2.5	N.D.	N.D.		
5	37.5	8.5	1	3.5	30	3.5	30.5	8.5
	8	2.5	0	2.0	N.D.	N.D.		
6	39	1	0	12.0	37.5	5.5	30.5	4
	7.5	<1	2.5	3.5	N.D.	N.D.		

Sera from four healthy donors were fractionated in a similar fashion using sucrose gradient centrifugation and assayed in parallel with the patients' serum fractions. None of the control sera demonstrated any ALA activity at either 15°C or 37°C above the background value of 5%.

Table 6. Cell Types Against which Lymphocytotoxic Antibodies in Malaria Sera are Directed

Sera	T Cells Only	B Cells Only	Both T and B Cells	No Cells
Normal control (16) <sup>a</sup>	0	0	0	100%
<i>P. falciparum</i> (31)	3% <sup>b</sup>	29%	48%	19%
<i>P. vivax</i>	12%	24%	44%	16%

<sup>a</sup> Number of individual sera examined in parentheses

<sup>b</sup> Percent of sera reacting with T cells only, B cells only, both T and B cells, or No cells.

Table 7. Lymphocytotoxic Activity of Malaria Patients' Sera Fractions Against Enriched Cell Populations

Exp. No.	Fraction	T Cells		B Cells		Macrophage	
		15°C	37°C	15°C	37°C	15°C	37°C
1 (0.4/51.5) <sup>b</sup>	IgG	<2.0 <sup>a</sup>	<2.0	<2.0	2.5	<3.0	<2.0
	IgM	2.5	3.5	14.0	<2.0	<3.0	<2.0
	IgMc <sup>c</sup>	<4.0	<2.0	4.5	<2.0	<3.0	<2.0
2 (0.8/31.5)	IgG	2.0	4.0	0	<2.0	0	0
	IgM	9.5	4.5	6.0	2.5	<2.0	0
	IgMc	2.5	3.5	<2.0	<2.0	0	0
3 (0.1/32.5)	IgG	3.0	4.5	0	0	0	0
	IgM	8.0	5.5	2.5	3.0	<2.0	<2.0
	IgMc	2.5	3.5	<2.0	<2.0	0	0

<sup>a</sup> Percentage lymphocytotoxicity. Average background (5%) has already been subtracted from these values.

<sup>b</sup> Percentage parasitemia and average lymphocytotoxicity of the patients unfractionate serum against unfractionated MNC at 15°C.

<sup>c</sup> IgM serum fractions from 3 healthy donor served as controls and were tested in parallel with patients IgM and IgG associated fractions.

Table 8. Cytotoxicity of 19S and 7S Serum Sucrose Gradient Centrifugation Fraction to T Cell Subpopulations

Exp. No.	Fraction	B + Null		T - Helper		T-Suppressor	
		15°C	37°C	15°C	37°C	15°C	37°C
1 (39) <sup>a</sup>	IgG + IgM	40 <sup>b</sup>	21	33.5	1.0	1.0	4.5
	IgG	2.5	17.5	0	0	0	7.5
	IgM	21	12.5	16.0	0	17	0
	IgMc <sup>c</sup>	0	18.0	0	0	8.5	7.0
2 (3.15)	IgG	7.0	6.5	3.5	1.5	0	1.5
	IgM	7.0	8.0	6.5	2.0	2.0	0.5
	IgMc	6.5	6.5	4.5	2.0	0.5	4.0
3 (32.5)	IgG	3.5	5.5	1.5	0	0	0.5
	IgM	5.5	7.5	4.0	0	0	0
	IgMc	5.0	5.0	2.5	0	0	3.0

<sup>a</sup> Cytotoxic activity of unfractionated serum against mononuclear cells for healthy donors.

<sup>b</sup> Average cytotoxicity against fractionated peripheral blood lymphocytes subpopulation obtained from healthy Thai donors was determined as described in Materials and Methods. Values represent percentage of dead target cells after subtracting percentage of spontaneous lysed target cells. Spontaneous target cell death after overnight incubation ranged from 7% to 13%.

<sup>c</sup> Purified 19S serum fraction obtained from a healthy Thai donor.

DISCUSSION : This laboratory has previously reported on the observation that a high percentage of sera from adult Thais with naturally acquired *P. falciparum* and *P. vivax* malaria contain cold-reactive lymphocytotoxic activity optimally detected at 15°C (21). Because of the association of cold-reactive lymphocytotoxic activity (LCA), including lymphocytotoxic antibodies (ALA), with dysfunction in immunoregulation in other diseases; and because dysfunctions in the immune response such as decreased number of peripheral T-cells (20), hypergammaglobulinemia (19), decreased Con A inducible suppressor cell capacity and deficiencies in lectin-induced and mitogen induced cellular cytotoxicity (Gilbreath et al. submitting for publication), have been found to exist in malarious individuals we were interested in characterizing cold-reactive lymphocytotoxicity and exploring the possible significance of LCA during malarial infection in man.

Our present data suggests that lymphocytotoxicity is not due to HLA antibody activity. Malarious serum lymphocytotoxicity is not associated with any history of transfusions or allogeneic stimulation. LCA is characterized by being autocytotoxic, and LCA is comparable for autologous and allogeneic mononuclear cells (MNC) targets thus making specificity with HLA seem unlikely.

It is apparent that the cold-reactive malaria LCA is similar to cold-reactive LCA reported for other disease states (24) in that the activity is reduced or eliminated when serially diluted between 1:4 to 1:16. Although no correlation has been shown between percentage of parasitemia and the degree of cytotoxicity it is important to note that cold-reactive LCA is maintained in the serum for several weeks following acute malarial infection suggesting that continued exposure to the infecting agent is necessary for the maintenance of detectable LCA. Although it is acknowledged that many intercurrent infections, apart from malaria, can lead to production of LCA and possibly obscure the relationship between malarial infection and LCA the data suggest a relationship since LCA wanes with treatment and subsequent recovery from malaria infection.

The autocytotoxic antibodies observed in several diseases have been shown to be specific for subpopulations of peripheral mononuclear cells (3). This laboratory recently reported the loss of circulating T lymphocytes with normal levels of B and "Null" lymphocytes in Thai adults with malaria and suggested anti-lymphocytic antibodies as one possible mechanism to account for the loss of T lymphocytes (20).

However, in view of the occurrence of LCA in a wide variety of human disease states (14) it seemed likely that their specificity for various MNC subpopulations may be quite heterogenous. As expected the LCA in unfractionated malarious serum for various MNC subpopulations was quite variable. Most reactivity was against B cells and to a lesser degree T cells while no anti-Null cell activity was found. A small number of sera contained weak anti-macrophage activity. We have previously observed that malaria patients' lymphocytes have a normal capacity to respond in the mixed leukocyte reaction but a decrease stimulation capability, while a normal response to PHA and Con A is seen (8). Since stimulatory capacity in the MLR is based on "LD" or "Ia" antigens and is different from responding capacity it is possible that IgM anti-B cell antibodies could cover Ia antigen leading to suppressed MLC

stimulatory capacity. The failure of elution technique to enhance, or malarious sera to block the formation of sheep Rosettes with T lymphocytes strengthens the contention that ALA are directed against lymphocyte antigens and have no functional relationship to T cell rosette formation. This hypothesis is supported by the fact that malaria serum with ALA activity fails to inhibit formation of E-rosettes by mononuclear blood cells from healthy donors and by the fact that repeated washing or incubation of malaria patient's mononuclear cells in pre-warmed media (37°C) containing 10% FCS did not increase the number of the patients' E-rosette forming cells.

The absence of cytotoxicity in unfractionated serum at 37°C raises the possibility that lymphocytotoxic antibodies in malarious sera may not cause *in vivo* complement-mediated lymphocytolysis as has been suggested (23). It is possible that optimal cytotoxicity at 15°C represents a compromise between lower and higher temperature for binding and complement fixation, respectively. *In vivo* and *in vitro* observations on lymphocytotoxins in other human diseases suggest that at 37°C lymphocytes may be able to process lymphocytotoxic antibodies effectively. This has been proposed to result from pinocytosis (18) or shedding of the cytotoxic factor and its receptor (22). At temperatures less than 37°C, altered lymphocyte metabolism might impair this process with resultant cell lysis.

The sedimentation characteristics, the association with light chain determinants, and reduction of cytotoxic activity by 2-mercaptoethanol lead us to believe that the cold-reactive lymphocytotoxic factor is 19S IgM antibody. The unexpected cytotoxicity seen when the 19S gradient fractions were tested at 37°C suggest that lymphocytotoxic IgM antibodies present in malarious serum may have *in vivo* fractions. One explanation for the lack of cytotoxicity of unfractionated serum toward unfractionated MNC is that blocking antibodies present in the IgG/IgA associated sucrose gradient fractions may interfere with IgM cytotoxic antibodies. However, our data fails to show any decrease in IgM associated lymphocytotoxicity when either malarious patient or healthy control IgG/IgA sucrose gradient eluates are mixed with IgM associated eluates containing cytotoxic activity. A more likely explanation for the enhanced lymphocytotoxicity at 37°C is that the degree of avidity of the cytotoxic antibodies in the malarious serum vary and IgM eluates are more reactive at 37°C in their concentration. A similar phenomenon was noted by Cicciarelli et al. (2) in their studies on the identification of surface IgM as the target antigen of cold lymphocytotoxins.

Interestingly, purified IgM eluates demonstrates a higher level of ALA at 15°C than 37°C against highly enriched T cell and B/Null cell populations, yet no significant ALA is seen when adherent cells (macrophage-monocytes) are used as target indicators. This data and the fact that highly cytotoxic sera previously absorbed with enriched T-cells or B-cells continued to demonstrate ALA when tested at 15°C against the non-absorbing target cell type indicates the presence in sera of ALA with separate B and T cell specificities. The ALA detected at 37°C in these experiments, albeit weaker, suggest that serum ALA are present in low titer and/or avidity in malarious individuals which are capable of interacting in a cytotoxicity manner with peripheral blood cells at physiological temperatures *in vivo*.

We have recently shown, using an autologous Con A-induced suppressor cell assay, that malarious patients Con A-induced suppressor cell activity in peripheral blood MNC is significantly reduced in comparison to Con A-induced suppressor cell activity of MNC from healthy donors (Gilbreath et al. submitted for publication). One explanation for this reduced activity is that a quantitative reduction in the suppressor T cell subpopulation may exist in malarious patients. Indeed, in preliminary experiments we have found a quantitative reduction in the peripheral blood suppressor T cells in malarious Thai during the acute state of malarial infection that returns to normal within several weeks after treatment. Although it is unlikely that ALA account for direct lysis of lymphocyte subpopulations at physiological temperatures, the possibility that non-HLA warm reactive lymphocytotoxic antibodies directed against one or both these T cell subpopulations cannot be exclude. Warm-reactive ALA may be present in too low a concentration to be detected in our assay system or may function in a non-cytotoxic manner at physiologic temperatures. The low level of ALA directed against both enriched B/Null and T-cell targets, at 37°C by some IgM eluates obtained from healthy donors may indicate that IgM ALA are routinely found in serum in low amounts. Furthermore, since lymphocytotoxins have not been found in 100% of sera from patient with diseases in which a high incidence of lymphocytotoxic antibodies have been reported, they may not play any role in disease pathogenesis. On the other hand ALA may be ubiquitous in human sera and serve a normal immunoregulatory role, but only be detectable after strigent purification of sera from healthy individuals and subsequent testing in assays with greater sensitivity.

ALA in malarious patients' serum may function as auto-regulatory feedback antibodies. Since they react with B lymphocytes they could function in modulating antibody production by the B lymphocytes. Hypothetically, auto-reactive B-cells may develop during malarial infection with the potential to synthesize antibodies against autologous cell subpopulations. Therefore it would be important to determine whether polyclonal B-cell activation, which has been demonstrated in murine malaria (16) also exists in human as a result of malarial infections. Whether or not lymphocytotoxic activity in malarious serum results from activation of auto-reactive B-cells during the course of malaria infection can be of major theoretical and practical significance for future anti-malarial vaccine programs especially if an autoimmunosuppression role is established for these lymphocytotoxic antibodies.

A rather nonspecific immunoregulatory role of lymphocytotoxic antibody would be consistent with the observation that serum cold-reactive lymphocytotoxins have been reported in a variety of human disease states (14) as well as during pregnancy (11) and following vaccination (5). Thus, the cold reactive lymphocytotoxic antibodies may occur as a by-product of exposure to many different unrelated antigens.

In fact, an elevated level of red blood cell membrane fragments containing a variety of parasite antigens in the circulation of a malarious individual may upon reaching a threshold level be able to activate autoreactive B cells in a polyclonal fashion. Although there is little evidence at present to support this hypothesis one might speculate that the autolymphocytotoxins observed in pernicious anemia patients (4) are due to the polyclonal activation of auto-reactive B-cells in response to altered RBC antigens.

For example maturation and rupturing of parasitized erythrocytes may serve as an efficient method for presenting to the B-cell either solubilized antigens from the RBC or altered RBC membranes which can then effectively stimulate production of ALA by auto-reactive B-cells. These ALA may cross react with antigenic structures on lymphocyte sub-populations, i.e. the "Ii" RBC antigen system. Similarly parasite derived antigens may induce the production of ALA which cross-react with receptors on subpopulations of MNC. In addition, anti-idiotypic antibody, reactive with determinants within or near a specific antigen binding site could function in this manner since ALA have been shown to have anti-lymphocyte receptor activity (13). Finally non-antiidiotypic antibodies could attack to other than idiotypic portions of the immunoglobulin molecule, and could interfere sterically with the proper attachment of antigens to the active site giving rise to aberrant antibodies with lymphocytotoxic activity.

Presently the role, if any, of cold reactive lymphocytotoxic factors in malaria is unknown, however, continued research on the subject is warranted in view of the immunoregulatory role of similar factors in other diseases.

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