NATURE OF MALARIA COLD-REACTIVE LYMPHOCYTOTOXIC ANTIBODY

Principal Investigators : Micheal J. Gilbreath, CPT, MSC
Katchrinnee Pavanand, M.D.
Richard P. MacDermott, M.D.[1]

Associate Investigators : Robert A. Wells, LTC, MSC[2]
Barnyen Permpanich
Somchit Lulyayon
Prasit Sookto
Niphon Chuanak

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

BACKGROUND : This laboratory previously reported the presence of cold-reactive lymphocytotoxic antibody (ALA) in the sera of Thai adults infected with *P. falciparum* and *P. vivax* malaria (1). In that study 95% of the *P. falciparum* and 98% of the *P. vivax* patient's sera had lymphocytotoxic activity when assayed against normal pheripheral blood cells at 15°C. Activity in assays done at 37°C was significantly less. In addition, our earlier study showed that the relative percentage and number of T lymphocytes was reduced in Thai adults with naturally acquired malaria (2).

Cold reactive ALA present in other disease states have been shown to be predominantly IgM and directed against T lymphocytes (3, 4). However, the immunoglobulins involved and the precise effect of malaria cold-reactive ALA on lymphocyte populations has not been determined. The possibility exists that cold reactive malaria ALA may function at physiological temperatures against autologous lymphocyte subpopulations and influence the immune response during infection.

The present study was undertaken to further characterize malaria cold reactive ALA. The effect of malaria ALA on autologous lymphocytes, E-rosetting, and cytotoxicity toward lymphocytes subpopulations was investigated. In addition, the ALA activity of sera collected during the acute and convalescent periods of malaria infection was compared. Sucrose density fractionation was done on high ALA activity sera to determine if ALA activity is associated with the fractions containing IgG or IgM.

---

[1] Howard Hughes Medical Institute Laboratory and Division of Gastroenterology, Washington University School of Medicine, St. Louis, Missouri 63110.

[2] Dept of Immunology, WRAIR, WRAMC, Washington, D.C. 20012.

METHODS :

Patients : The patients participating in this study were treated by the staff of the National Malaria Eradication Center, Phrabuddabat, Thailand.

The patients screening process has previously been described (2). Half of individual patients' blood obtained was collected in heparinized tubes to obtain plasma and mononuclear cells while serum was obtained from the remainder after the non-heparinized blood was allowed to clot at room temperature. Serum and plasma were removed and used immediately or stored at -20°C.

Effect of Serum on E-rosette Formation : The rosette formation method used to assay T cells was done as previously described (2). In brief, mononuclear cell-rich populations were derived by Ficoll-Hypaque gradient sedimentation from normal peripheral blood. The cells were washed three times in cold Hank Blanced Salt Solution (HBSS) (Grand Island Biological Co., Grand Island, N.Y.) and resuspended to a concentration of 2×10^6 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 mononuclear cells. The mixture was incubated for 30 min, then HBSS was added and the percentage of cells forming E-rosettes was determined after 1 hour and 18 hours at 4°C.

Elution Experiments : Ficoll-Hypaque purified patient mononuclear cells 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 prewarmed to 37°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 HI 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 mononuclear cells at physiological temperature, thus inhibiting E-rosette formation.

Isolation of Peripheral Blood Lymphocytes and Lymphocyte Subpopulations : Peripheral blood lymphocytes were prepared from venous blood centrifuged through Ficoll-Hypaque (5) and then washed three times with Hanks Balanced Salt Solution (HBSS), then resuspended in RPMI 1640 supplemented with L-glutamine, HEPES, and 10% heat inactivated FCS. Initially each sera was screened for the presence or absence of lymphocytotoxicity against autologous, allogeneic patient, and control lymphocytes.

Aliquots of some patient sera were frozen, packed in dry ice and shipped to Washington University, St. Louis, Mo., where the sera were tested for the presence of lymphocytotoxicity against a panel of lymphocytes from 15 normal Caucasian adults of varying HLA-phenotype. Unused sera was stored at -70°C until used.

Subsequently, mononuclear cells from a small number of donors were used to determined if the sera lymphocytotoxicity was specific for lymphocyte subpopulations. Mononuclear cells were isolated from a unit of peripheral blood

from 5 of the initial healthy Caucasian volunteers by Ficoll-Hypaque centrifugation. The cells were further separated into subpopulations as previously described (6).

Briefly, the isolated cells were passed over a Sephadex G-10 column to remove adherent cells. The nonadherent macrophage deplete cells were separated into T, B, and Null cells in a two stage process. First, the cells were poured over a Sephadex G-200 anti-human $F(ab')_2$ immunoabsorbent column. Then the nonadherent T plus Null cells were further fractionated using on 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^+ cells, E-rosette$^+$ cells and esterase$^+$ cells as previously described (5). All populations were highly enriched and viability of the final preparation was greater than 95%, as assessed using the trypan blue dye exclusion procedure.

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. In a small number of assays patients' plasma was also tested for the presence or absence of lymphocytotoxicity.

Sera with high lymphocytotoxicity from dengue patients or patients with systemic lupus erythematous served as positive controls while sera from healthy Thai volunteers served as negative controls.

The microcytotoxicity assays were modified slightly when sera was tested for lymphocytotoxicity against enriched T-cell, B-cell, or Null cell targets. Sera was distributed on microtiter trays which had been covered with mineral oil and then .001 ml sera was added to each well and the trays were stored at -70°C until needed. The assays were performed by putting .001 ml of target cell suspension (2×10^6 lymphs/ml) into each well containing test sera. The target cell-serum mixture was incubated 1 hour then .050 ml rabbit complement (Pel Freeze) was added to each well and the mixture further incubated for 3 hours. .003 ml of 5% aqueous eosin was then added to each well and 2 minutes later .010 ml 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.

To study thermal characteristics of the cytotoxic antibodies, the tests were done at two temperatures. The initial cell/serum incubation was done at either 4°C or 37°C, followed by the 3 hours complement incubation at either 15°C or 37°C respectively.

Positive sera controls included sera from SLE and malarious patients while negative control sera included sera from patients with primary biliary circhosis or healthy volunteers.

Scoring of sera lymphocytotoxicity against target cells was done using the following scale :

1 - negative 0-10% cell dead (same as negative control)
2 - doubtful negative 11-20% cells dead
4 - doubtful positive 21-50% cells dead
6 - positive 51-80% cells dead
8 - strong positive 81-100% cells dead

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 4°C against mononuclear target cells in the microcytotoxicity assay.

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 (7). Each fraction was tested against normal mononuclear cells at 4°C using the microcytotoxicity assay to determine which fractions contained lymphocytotoxic activity. Preliminary experiments were performed to ensure that the sucrose concentration in the various fractions did not inhibit ALA in the microcytotoxicity assay.

RESULTS : Sera from 21 patients with *P. falciparum* and 14 patients with *P. vivax* were examined for the presence of autolymphocytotoxic antibody in assays done at 15°C and 37°C. As seen in Table 1, 9/12 *P. falciparum* sera and 7/14 *P. vivax* sera were cytotoxic against greater than 10% of the autologous mononuclear target cells in the assay done at 15°C. An equivalent level of cytotoxicity was found when allogenic patient or healthy Thai adults' mononuclear cells were used as target indicator cells. No significant lymphocytotoxic activity was found at 37°C.

In patient sera, serial diluted, cytotoxicity is removed or severely decreased at dilutions as low as 1:16 (Table 2).

Malaria patients plasma had either no ALA activity or a greatly reduced level when compared to serum from the same malaria patient (Table 3). Only 2 of 11 plasma samples tested had ALA activity above control levels. In one case the level of lymphocytotoxic activity was 46% for serum and 25 % for plasma, while the other was 23.5% for serum and 10% for plasma. More recent experiments indicate that the absence or reduction of ALA activity in plasma may be due to the use of heparin (Figure 1), however, further work is needed to determine the effect various anti-coagulants may have on ALA activity.

When a comparison was made between serum collected during the acute period of illness and serum collected 15 and 30 days later, ALA activity was highest in serum during the acute period of malaria infection (Table 4). The respective sera was stored, at -20°C, until all three of the sera samples from the individual patients could be tested in the same assay against identical target cells. Preliminary experiments indicate that repeated freeze-thawing of serum samples has no effect on ALA activity.

Table 5 summarizes the results of experiments 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. There is no significant difference in the percentage of E-rosettes formed by mononuclear cells incubated with patients 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 cells incubated in the presence of autologous serum and cells incubated without autologous serum (Table 6).

When malaria patient serum with high ALA activity is fractionated using sucrose gradient centrifugation (and the serum fractions are collected from the bottom to the top of the tube), ALA activity is only found in the early fractions as seen in Table 7. The early fraction are the ones in which IgM is found, the later fractions (7, 8) containing IgG are not cytotoxic for the mononuclear target cells. These results suggest that cold reactive ALA are IgM. However, further work is needed to better understand the role of these antibodies during malaria infection and their effect on various lymphocyte subpopulations.

It is important to consider the possibilities as to the relationship between the lymphocytotoxic antibodies and reduced numbers of T cells : 1) the reduced T cells could actually have led to lymphocytotoxic antibody production through loss of a suppressor cell regulatory effect, or 2) there may be no relationship between the two, and the T cell loss is actually due to other factors. Additional studies will be necessary in this area, as well as, in order to determine the *in vivo* relevance of lymphocytotoxic antibodies in patients with malaria.

During the past year we have performed experiments to answer the above question, by assessing the type of cell which the lymphocytotoxic antibodies react with. As seen in Table 8, the lymphocytotoxic antibodies are primarily directed against B cells, and in most instances, T cells as well as B cells. It would be therefore of interest to examine the HLA antigens against which the antibodies are directed, with emphasis on the D locus antigens.

Table 1. Lymphocytotoxic Antibody Activity of Malaria Patient Serum Against Autologous and Allogeneic Patient Cells and Normal Donor Target Cells

P. falciparum						
P.E.[+] %	Autologous Cells		Allogeneic Cells[++]		Normal Cells	
	15°C	37°C	15°C	37°C	15°C	37°C
0.5	69.0[+++]	3.5	50.0	4.0	72.5	3.5
0.1	6.5	3.5	25.5	3.0	44.0	1.5
0.1	5.0	3.0	3.5	3.5	7.0	2.0
+VC[x]	8.5	1.5	5.0	1.5	12.0	3.5
0.1	21.5	3.5	11.0	3.0	17.5	3.0
0.5	13.0	3.0	13.5	3.5	20.5	3.5
1.2	5.5	4.5	6.0	3.5	6.5	2.5
0.6	12.0	3.5	12.0	4.5	22.5	3.0
1.2	15.0	2.0	16.5	2.0	20.5	2.5
0.3	5.0	3.0	5.0	2.0	5.5	3.5
0.2	7.5	2.5	9.0	3.0	13.0	1.5
+VC	14.0	6.5	18.0	4.5	28.5	3.0
+VC	7.0	3.0	5.5	3.5	9.0	2.0
0.6	19.0	5.5	22.5	5.0	23.5	4.0
0.3	3.5	3.5	4.5	5.5	3.0	3.5
+VC	3.5	2.5	4.0	4.0	2.5	2.5
0.4	46.0	4.5	51.0	4.0	48.5	2.0
+VC	18.0	5.0	16.5	4.5	21.0	3.5
0.4	6.5	3.5	7.0	4.5	9.5	3.0
0.2	4.0	3.5	5.5	2.0	11.0	2.5
0.6	2.5	2.5	4.5	4.5	3.0	2.5

P. vivax						
P.E. %	Autologous Cells		Allogeneic Cells[++]		Normal Cells	
	15°C	37°C	15°C	37°C	15°C	37°C
+VC	15.0	1.5	16.0	1.0	37.0	4.0
0.1	5.5	5.0	14.5	5.5	14.5	3.0
+VC	51.0	5.0	67.0	5.5	56.5	2.5
0.1	5.0	3.0	6.5	2.5	9.5	2.0
0.1	42.5	3.5	46.0	5.0	52.0	4.0
0.1	4.5	3.5	6.0	2.5	3.0	2.5
0.4	22.5	3.5	22.0	4.0	31.5	2.5
0.1	76.5	5.0	91.5	5.0	84.0	4.0
0.5	4.5	3.5	3.0	2.5	4.0	2.5
0.3	17.0	3.5	18.0	2.5	22.5	2.5
+VC	9.0	3.5	11.0	4.5	22.5	1.0
0.8	5.5	2.5	8.5	3.5	14.5	2.0
0.1	7.0	4.5	5.5	5.5	17.5	3.0
0.1	63.5	5.5	61.5	5.0	75.0	4.0

[x] Parasitemia very low, confirmation based on thick smear slide.

[+] Percent of erythrocytes containing parasites.

[++] Experiments were done testing *P. vivax* serum on *P. vivax* allogeneic cells or *P. falciparum* serum on *P. falciparum* allogeneic cells.

[+++] Average percentage of cells killed per well by patient serum, determined from duplicate test wells.

Table 2. Effect of Serial Dilution of Malaria Sera on Lymphocytotoxicity+

	Neat (fresh)	Neat (freeze-thawed)	1:2++	1:4	1:8	1:16
P.v.	41.0	39.5	28.0	20.5	18.0	4.5
P.v.	56.0	60.0	51.5	47.5	38.5	21.5
P.v.	55.5	44.0	43.5	44.0	12.0	3.5
P.v.	52.5	47.5	40.0	15.0	2.5	0
P.v.	47.5	43.5	35.5	14.0	3.5	1.0
P.f.	31.5	30.0	10.5	2.5	0.5	0
P.f.	48.0	46.5	26.5	13.5	6.5	3.0
P.f.	43.5	37.5	10.0	3.0	0	0
P.f.	40.0	38.5	8.0	1.5	0	0
P.f.	41.0	38.5	7.5	7.0	0	0

\+ Average cytotoxicity determined prior to freezing serum at -20°C.

++ 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.

Table 3. Comparison of Lymphocytotoxic Activity of Individual Patient Plasma and Serum

Patient	Lymphocytotoxic Activity		
	Plasma	Serum	Control[+]
P.f.[++]	3.5[+++]	16.0	
P.f.	4.0	44.0	
P.f.	5.5	22.0	5.5
P.v.	4.5	8.0	
P.f.	4.5	7.0	
P.f.	6.0	9.5	5.5
P.f.	3.5	11.0	5.0
P.f.	3.5	12.5	5.5
P.v.	4.5	8.0	5.5
P.v.	25.0	46.0	5.0
P.v.	10.0	23.5	5.5

\+ Media with 10% Fetal Calf Serum was added to indicator target cells to determine background death of cells.

++ P.f. Patient with *Plasmodium falciparum* infection.
P.v. Patient with *Plasmodium vivax* infection.

+++ Average cytotoxicity determine from duplicate wells in assays done at 15°C

Table 4. Lymphocytotoxic Activity in Malaria Patients Convalescent Serum

Patient	Days Post-admission		
	0	15	30
P.f.+	33.5++	0.5	1.5
P.f.	24.0	1.0	1.5
P.v.	16.0	0	0
P.f.	32.5	1.5	0
P.f.	14.5	4.0	3.5
P.f.	35.5	19.5	19.0
P.f.	7.5	3.0	2.5
P.f.	3.0	12.0	27.5
P.f.	0	0.5	16.0
P.f.	0	8.0	1.0

\+ P.f. Patient with *Plasmodium falciparum* infection.
P.v. Patient with *Plasmodium vivax* infection.

++ 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.

Table 5. Effect of Malaria Patient Serum Containing Lymphocytotoxic Activity or Normal Human Serum on Percentage of E-rosette Forming Cells (% E-RFC) of Peripheral Blood Lymphocytes from Healthy Thai Adult Donor after 4 Hours Incubation in RPMI-1640 Containing 10 Percent Serum

Patient Serum	% E-RFC Before Incubation	% E-RFC After 4 Hours Incubation	
		In Malaria Patient Serum	In Healthy Thai Individual Serum
377 F P.f.	53	52.0	N.D.+
376 V P.v.	53	49.5	N.D.
375 F P.f.	50	48.0	N.D.
332 V P.v.	53	46.0	50.5
383 F P.f.	53	47.5	N.D.
376 V P.v.	46.5	40.0	45.5
380 F P.f.	46.5	44.5	N.D.
280 V P.v.	50	47.0	50.0
343 V P.v.	50	41.0	N.D.
259 F P.f.	53	52.0	49.5
360 F P.f.	53	48.0	42.0
322 V P.v.	45	43.0	N.D.
351 F P.f.	45	45.5	N.D.
352 F P.f.	44.5	51.5	54.0
366 F P.f.	50.0	47.5	55.0

+ Not done

Table 6. Influence of Washing or Overnight Incubation of Malaria Patient Mononuclear Cells on E-rosette Formation.

% E-RFC[+]			
Patient		Control	
Pre Incubation	Overnight Incubation	Pre Incubation	Overnight Incubation
41.0 (95)[++]	44.0 (91)	43.0 (98)	41.5 (92)
42.5 (94)	42.0 (87)	44.0 (98)	43.5 (94)
43.0 (98)	41.5 (92)	40.5 (98)	38.0 (91)
		32.0 (92)	31.5 (87)
		49.0 (98)	46.0 (93)
		30.0 (98)	26.0 (92)

\+ E-RFC 1 hour erythrocyte rosette forming cell.

++ Percent viable mononuclear cells at conclusion of 1 hour E-rosetting assay.

Table 7. Cold Reactive Lymphocytotoxic Activity in Sucrose Gradient Centrifugation Fractions in Sera from Patients with Malaria.

Patient	Unfractionated Sera	Percentage Lymphocytotoxicity[1]						
		Fraction Number						
		2	3	4	6	7	8	9
391 P.f.[2]	55	32.0	26.0	28.5	3.5	1.0	1.5	0.5
367 P.f.	44	7.0	7.5	4.5	1.0	0	0	0
371 P.f.	49	27.5	24.0	19.0	3.5	1.5	2.0	1.5
375 P.f.	51	41.0	41.5	40.0	0.5	0.5	0	0
366 P.v.	66	41.5	37.5	29.0	2.0	0	0.5	0.5
370 P.v.	48.5	33.0	25.5	33.5	2.0	1.0	0.5	0.5
373 P.v.	51	42.5	42.0	37.5	0	0	0	0
299 P.v.	21	-	-	-	0	0	-	-

1 Average determined from duplicate wells with serum incubated at 15°C with unfractionated peripheral blood mononuclear cells isolated from healthy Thai adults.

2 P.f. Patient with *Plasmodium falciparum* infection.
P.v. Patient with *Plasmodium vivax* infection.

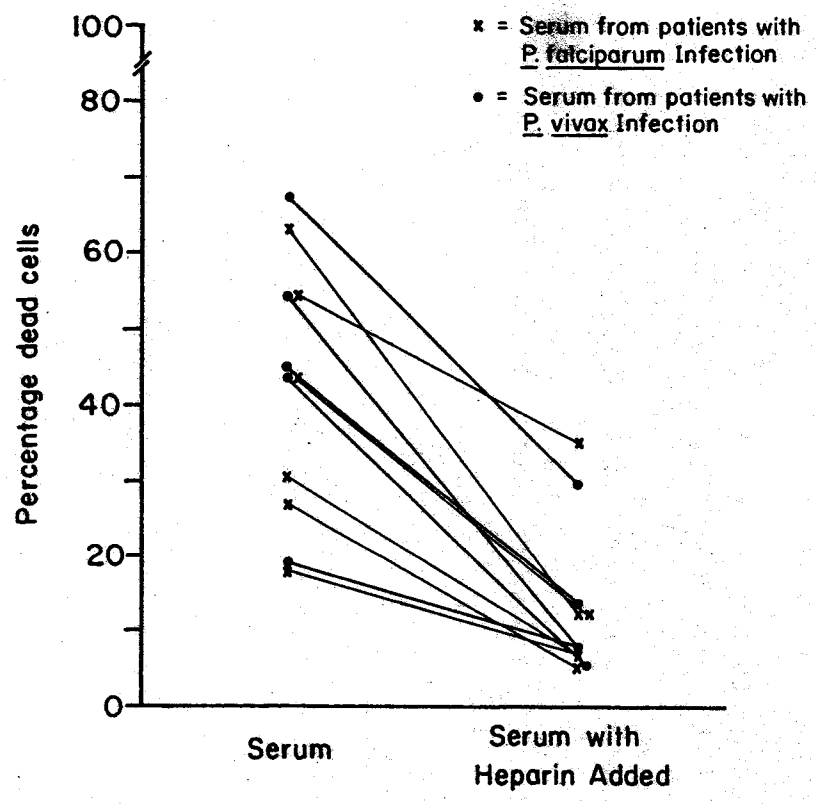
Table 8. Cell Types Against which Lymphocytotoxic Antibodies in Malaria Sera are Directed

Sera	T Cells Only	B Cells Only	Both T and B Cells	No Cells
Normal control (16)[a]	0	0	0	100%
P. falciparum (31)	3%[b]	29%	48%	19%
P. vivax (25)	12%	24%	44%	16%

[a] Number of individual sera examined in parentheses.

[b] Percent of sera reacting with T cells only, B cells only, both T and B cells, or No cells.

Figure 1.



Effect of heparin on cold-reactive anti-lymphocytotoxic antibody activity in malaria patients serum. 5 U.S.P. unit of heparin were mixed with 0.5 ml. of serum that had previously been determined to have malaria ALA. Sera with heparin and untreated sera were then assayed against identical target mononuclear cells at 15°C.

REFERENCES :

1. Wells, R.A., K. Pavanand, S. Zolyomi, B. Permpanich and R.P. MacDermott : Anti-lymphocytotoxic Antibodies in Sera of Thai Adults Infected with *Plasmodium falciparum* or *Plasmodium vivax* Malaria. (in press) 1980.

2. Wells, R.A., K. Pavanand, S. Zolyomi, B. Permpanich and R.P. MacDermott : Loss of Circulating T Lymphocytes with Normal Levels of B and "Null" Lymphocytes in Thai Adults with Malaria. Clin. Expl. Immunol. 35:202, 1979.

3. Lies, R.B., R.P. Messner and R.C. Williams, Jr. : Relative T Cell Specificity of Lymphocytotoxins from Patients with Systemic Lupus Erythematosis. Arthr. Rheumat. 16:369, 1973.

4. Winfield, J.B., R.J. Winchester, P. Wernet, S.M. Fu and H.G. Kunkel : Nature of Cold-reactive Antibodies to Lymphocyte Surface Determinants in Systemic Lupus Erythematosus. Arthr. Rheumat. 18:1, 1975.

5. Nelson, D.L. and R.P. MacDermott : Modification of Anti-Fab Immunoabsorbent Separation of Human Peripheral Blood Mononuclear Cells. In Regulator Mechanisms in Lymphocyte Activation. Edited by D.O. Lucas Academic Press, New York. pp. 587-590, 1977.

6. Boyum, A. : Isolation of Mononuclear Cells and Granulocytes from Human Blood, Scan. J. Clin. Lab. Invest. 21(Supl 97), 77, 1968.

7. Kunkel, H.G. : Macroglobulins and High Molecular Weight Antibodies, in F.W. Putnam (ed). The Plasma Proteins, Academic Press, New York, Chapter 8, 1960.