IMMUNOPRECIPITATING ANTIBODIES AGAINST BLOOD STAGE ANTIGENS OF *PLASMODIUM FALCIPARUM* IN CEREBRAL AND NON-CEREBRAL MALARIA PATIENTS

NITAYA THAMMAPALERD, SAVANAT THARAVANIJ,
JINTANA PATARAPOTIKUL AND SORNCHAI LOOAREESUWAN*

Department of Microbiology and Immunology, *Department of Clinical Tropical Medicine and Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand.

INTRODUCTION

Little is known about factors contributing to the development of cerebral malaria which occurs only in a small percentage of patients with falciparum malaria (Daroff *et al.*, 1967; Harinasuta *et al.*, 1982). In comparison with acute uncomplicated malaria (AM), patients with cerebral malaria (CM) had less frequencies of previous malaria infection (Tharavanij *et al.*, 1984; Tapchaisri *et al.*, 1985), lower anti-RESA (ring-infected erythrocyte surface antigen) seropositive rate and lower geometric mean anti-RESA antibody (Tharavanij *et al.*, 1987 unpublished data), and higher frequencies of endotoxaemia (Usawattanakul *et al.*, 1985). CM patients had significantly lower mean IgG anti-sporozoite antibody titer against *Plasmodium falciparum* than AM patients (Tapchaisri *et al.* 1985). With respect to humoral immune responses to the blood stage antigens, reduced responses were restricted only to patients with complicated cerebral malaria (CCM) and not in patients with uncomplicated cerebral malaria (UCCM) and only when the indirect haemagglutination (IHA) and RESA tests were used (Tharavanij *et al.*, 1984; 1987 unpublished data). Except for the RESA test, serological tests employed in our previous studies measure only the past malaria experience but not protective immunity. Therefore investigations were carried out to repeat the study using putative tests for 'protective' immunity, one of which is immunoprecipitation. This technique has been used to identify the protective antigens interacting with sera from immune persons (Perrin and Dayal, 1982; Brown *et al.* 1982) or with monoclonal antibodies (MAB) possessing parasite growth inhibitory or merozoite invasion inhibitory activities (Perrin and Dayal 1982; Saul *et al.*, 1984; Campbell *et al.*, 1984; Banyal and Inselburg 1985). The objective of this study was to determine whether sera from AM, CCM and UCCM patients reacted to the same or different antigenic molecules and if the latter is the case, would such differences be in any way related to the development of cerebral malaria.

This investigation received financial support from UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and in cooperation with the Wellcome-Mahidol University-Oxford Tropical Medicine Research Programme.

MATERIALS AND METHODS

Serum samples: Sera used in this study were randomly selected from a serum bank of CM and AM patients admitted to Phra Pokklao Hospital, Chantaburi, 240 km east of Bangkok from July to December 1980. All but one patient selected were those hospitalised within three days after the onset of clinical symptoms. Included in the study were single sera from 15 AM patients taken on the day of admission, paired specimens from 14 CCM and 17 UCCM patients taken 8.4 ± 2.1 and

6.4 ± 2.2 days apart respectively. The criteria for CCM categorisation included clinical pictures of multi-organ involvement especially liver and kidney (total bilirubin of ≥ 2 mg/dl, BUN ≥ 45 mg/dl), anemia (haematocrit of < 21%), or high parasitaemia (≥ 20%) (Tharavanij *et al.*, 1984). Sera from eight healthy controls who lived in Bangkok, a non-malaria endemic area and did not have histories of malaria in the past were also used. All sera were kept frozen in small aliquots at –70°C until used.

Parasite and metabolic labelling: Otherwise stated the SO strain of *P. falciparum* was used (Tharavanij *et al.*, 1981). Two other isolates (H11 and QD3) were also used to compare antigenic profiles between isolates. The H11 was isolated in July 1983 from an AM patient with an initial parasitaemia of 1,288,200 parasites per µl and the QD3 in June 1983 from a CM patient, all of whom were admitted to Phra Pokklao Hospital, Chantaburi. Continuous cultures of the parasites were maintained in RPMI-1640 (Gibco, Grand Island, N.Y.) in the presence of 10% heat inactivated AB serum and gentamicin (30 µg/ml) in a candle jar according to the technique of Trager and Jensen (1976). The parasite growth was synchronised by treatment with 5% aqueous solution of sorbitol as described by Lambros and Vanderberg (1979). The synchronously grown parasites mostly at the trophozoite stages were concentrated by Percoll gradient centrifugation according to the technique of Saul *et al.* (1982), and the concentrated parasites were grown further in 1.5 ml of methionine-free RPMI-1640 medium in the presence of 10% AB serum, gentamicin (30 µg/ml) and 75 µCi of ^{35}S–methionine (New England Nuclear, U.S.A.) for a period of 5–6 hours. The parasitised cells were harvested by centrifugation at 9,980 × g for 5 min at room temperature using an Eppendorf 5412 centrifuge, washed once in physiological saline and then extracted twice at 4°C each with 50 µl of TEN buffer (10mM Tris–HCl, 10mM EDTA and 0.4M NaCl) containing 1mM phenylmethylsulphonyl fluoride (PMSF) and 1% Triton X–100, followed by centrifugation at 9,980 × g for five min. The supernatants from each extract were pooled and kept at –70°C until used.

Immunoprecipitation: The technique used by Dr. P. Dubois, Institut Pasteur, Paris (pers. comm.) was adopted. Briefly, an aliquot of parasite extract with 2×10^5 CPM was diluted in a detergent buffer (TEN buffer with 1% Triton X-100) to give a final volume of 100 µl to which 1 µl of 0.1M PMSF and 0.1M (N-∝-p-tosyl-L-lisine chloromethyl ketone) were added. The mixture was then mixed with 10 µl of serum to be tested by using a vortex mixer and let stand at 4°C overnight. The immune complexes formed were removed by adding 100 µl of 20% washed formalin-fixed protein A bearing *Staphylococcus aureus* (Immunoprecipitin, Bethesda Research Laboratories, Maryland, U.S.A.) in the detergent buffer containing 0.1% ovalbumin, mixed thoroughly in a Vortex mixer and kept in an ice-bath with intermittent mixing for one hour. Thereafter, 800 µl of detergent buffer containing 0.1% ovalbumin (Sigma Chemical, Missouri, U.S.A.) was added to stop the reaction and the Staph A cells were washed further five times by centrifugation at 9,980 × g, the first two washings in the detergent buffer with ovalbumin, the subsequent two washings in the detergent buffer alone (without ovalbumin) and the last washing in the TEN buffer only. Fifty µl of a denaturation buffer (0.125M Tris-HCl, 6% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol and 0.1% Coomassie blue) was added to the cell pellet and boiled for five min. Following centrifugation, the supernatant was removed and subjected to electrophoresis in a vertical slab gel apparatus (Bio-Rad, U.S.A.) in 7.5% polyacrylamide for 2-3 hours according to the technique of Laemmli (1970).

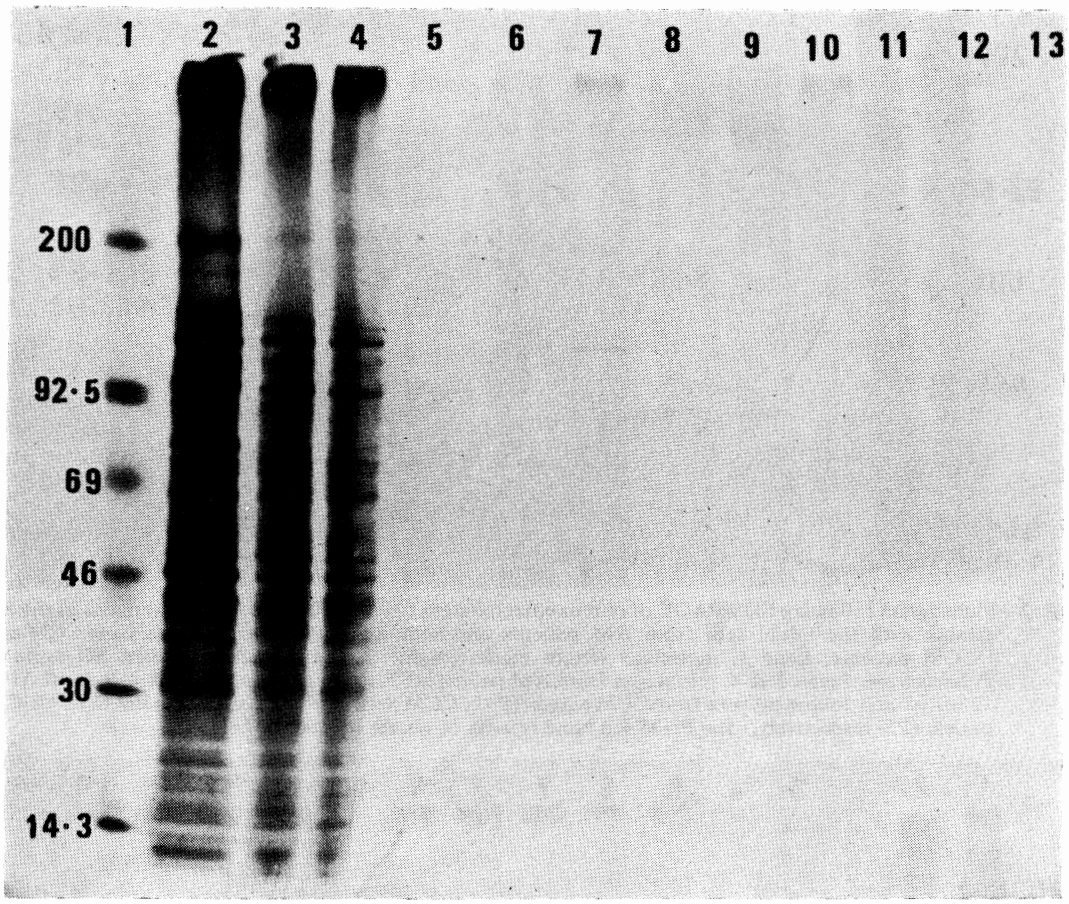

Fig. 1—SDS-PAGE analysis of metabolically labelled extracts of the SO, Hll and QD3 isolates of *Plasmodium falciparum* (lanes 2-4) and immunoprecipitates of metabolically labelled parasite antigens with sera from three heathy controls (lanes 5-13). Lane 1, molecular weight marker; Lane 2, SO isolate; Lane 3 Hll isolate; Lane 4, QD3 isolate. No qualitative differences of bands are seen from the three isolates but quantitiatively, bands from the SO are a little more intense than the other two isolates. Lane 5, 6, 7, control serum (Nl) reacting with antigens from the SO, Hll and QD3 isolates respectively. Lanes 8, 9, 10 serum (N2) reacting with antigens from the SO, Hll and QD3 respectively. Lanes 11,12, 13, serum(N3) reacting with antigens from SO, Hll and QD3.

Standard ^{14}C–methylated molecular weight markers (Amersham, Buckinghamshire, England) were simultaneously electrophoresed. The gel slabs were treated with En^{3}Hance (New England Nuclear, U.S.A.), dried and fluorographed on Kodak X–Omat S films.

Statistical analysis: Chi square analysis with Yate's correction was used.

RESULTS

Comparison of the extracts of different isolates of *P. falciparum:* The extracts of three metabolically labelled *P. falciparum* isolates comprising the SO, H11 and QD3 were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Qualitatively, the

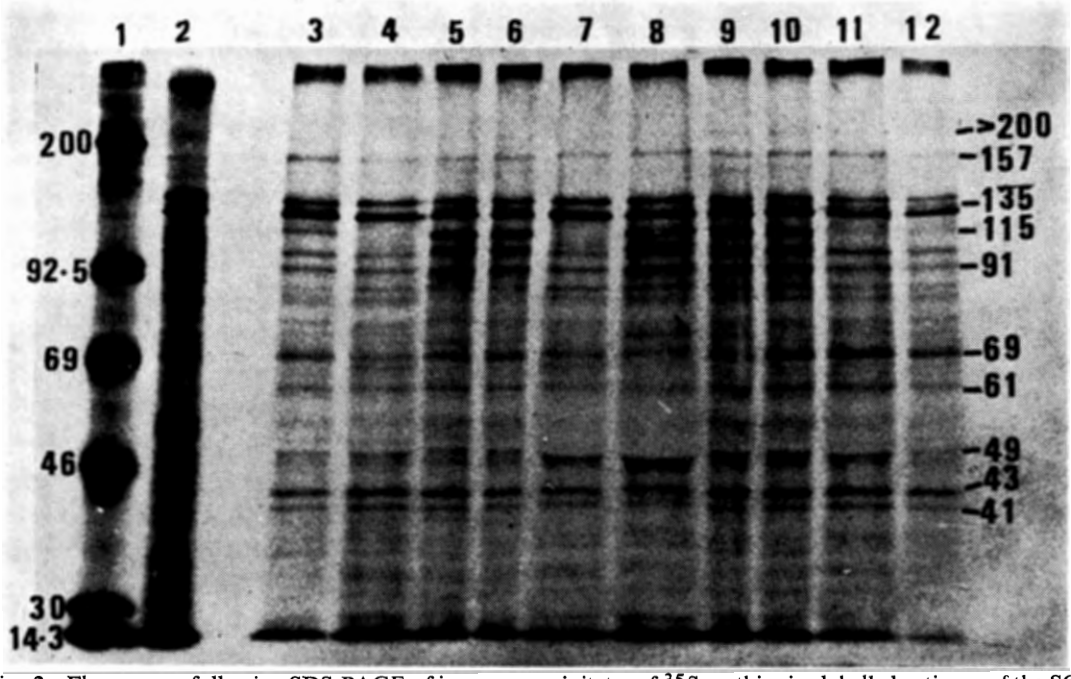

Fig. 2—Fluorogram following SDS-PAGE of immunoprecipitates of ^{35}S-methionine labelled antigens of the SO strains with the initial sera from AM patients, and both initial and follow-up sera from CCM and UCCM patients. Lane 1, molecular weight marker; Lane 2, the extract of labelled SO strain of *P. falciparum;* Lanes 3 & 4, initial sera from AM patients (F7, F11); Lanes 5 & 6, 7 & 8, 9 & 10, 11 & 12 initial and follow-up sera from CCM patient (P57), CCM patient (P60), UCCM patient (P1), UCCM patient (P2) respectively. The Pf135 Kd band is weak or absent in lanes 4, 7, 11, 12.

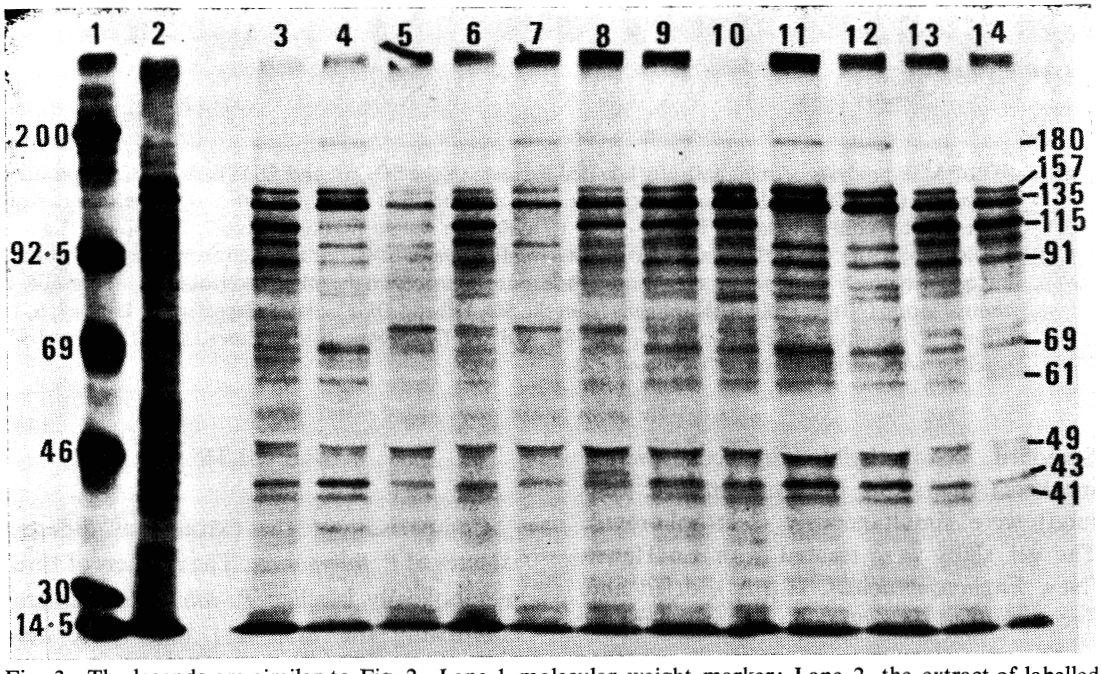

Fig. 3—The legends are similar to Fig. 2. Lane 1, molecular weight marker; Lane 2, the extract of labelled SO strains of *P. falciparum;* Lanes 3 & 4, initial sera from two AM patients (F12, M1); Lanes 5 & 6, 7 & 8, 9 & 10, 11 & 12, 13 & 14 initial and follow-up sera from CCM patient (P63), CCM patient (P64), UCCM patient (P5), UCCM patient (P6), UCCM patient (P12) respectively. The Pf135 Kd band is weak or absent in lanes 4,5,7, 11 and 12.

Table 1

Number of sera with immunoprecipitating antibodies against major components of metabolically labelled parasite protein antigens.

Mr (KD)	AM (15)*	CCM (14)		UCCM (17)		NHS (8)
	initial	initial	follow-up	initial	follow-up	
> 200	15**	14	14	14	14	0
180	15	14	14	17	17	0
157	15	14	14	17	17	8
135	3	2	6	14	15	0
130	15	14	14	17	17	8
115	11	12	14	13	13	0
103	7	8	13	12	13	0
96	11	12	13	15	17	0
91	13	12	13	17	17	0
73	11	11	12	16	16	0
71	8	7	7	10	10	0
69	15	14	14	17	17	0
61	14	14	14	17	16	0
49	15	14	14	17	17	8
46	15	14	14	17	17	0
45	6	5	7	7	9	0
43	15	14	14	17	17	0
41	14	13	13	17	17	8
14.3	15	14	14	17	17	8

* Number of patients tested.
** Number of patients positive for a given immunoprecipitating band.
AM = Acute uncomplicated malaria.
CCM = Complicated cerebral malaria.
UCCM = Uncomplicated cerebral malaria.
NHS = Normal human serum.

three isolates gave similar numbers of bands but quantitatively the SO strain gave slightly more intense image than the other two isolates (Fig. 1). The SO isolate was therefore chosen in the subsequent study.

Immunoprecipitation of sera from cerebral and non-cerebral malaria patients: Prominent immunoprecipitating bands observed were > 200, 180, 157, 135, 130, 115, 103, 96, 91, 73, 71, 69, 61, 49, 46, 45, 43, 41 and 14.3 Kd molecules (Fig. 2, 3). Among these bands, some (157, 130, 49 and 14.3 Kd molecules) were also apparent when normal human sera were used but the intensities were much weaker (Fig. 1). The frequencies of positive immunoprecipitating antibodies against major antigenic components are shown in Table 1. On the day of admission, the positive frequencies to individual antigenic molecules in AM, UCCM and CCM patients were not statistically significant except for the Mr 135 Kd molecule to which sera from UCCM pa-

tients showed significantly higher positive frequencies (82.4%) than those from AM (20%)and CCM (14.3%) patients ($p < 0.002$). In follow-up samples, four CCM and one UCCM patients who were initially non-reactive to the Mr 135 Kd molecules became reactive. Nevertheless, the positive frequency to the Mr 135 Kd molecule in UCCM patients (88.2%) was still significantly higher than that in CCM patients (42.9%, $p = 0.02$).

DISCUSSION

In the study on immunoprecipitation, it is important to select the antigens from the parasites which gave a large number of bands in the SDS-PAGE. When the extracts of three isolates of *P. falciparum* were compared, no qualitative differences in the number of bands were observed. The SO strain was selected for further study for two reasons: First, the bands in the SDS-PAGE was slightly more intense than the other two isolates (Fig. 1). Second, this strain has been well adapted to *in vitro* culture condition, has been producing good yield of parasites and has been used in several studies from our laboratory (Tharavanij *et al.*, 1981; 1982; 1984; 1986).

With the immunoprecipitation technique, candidate antigens of asexual blood stages of *P. falciparum* could be identified. Recently, these molecules have been categorised into five groups by Mitchell and Tam (1986) comprising 1) merozoite/schizont membrane-associated antigens, 2) high molecular mass rhoptry proteins, 3) low molecular mass rhoptry proteins, 4) membrane-associated antigens of infected RBC, and 5) antigens in the parasitophorous vacuoles. If CM patients are more immunologically naive than AM patients, sera from CM patients taken within a confined period of time after the onset of clinical illnesses should be less frequently reactive to certain proteins than those from AM patients. This turns out not to be the case, since sera from CM and AM patients taken on the day of admission within three days after the onset of clinical symptoms reacted similarly to all except the Pf135 Kd molecules. Positive reactions to the Pf135 Kd protein in AM and CCM patients (20% and 14.3% respectively) were significantly lower than those of UCCM patients (82.4%). The reason for this finding is unclear. Neither do we know the source from which this molecule is derived. With this molecular mass, we can only suspect that the Pf135 Kd is merozoite derived (Campbell *et al.*, 1984). In the follow-up studies in 14 CCM and 17 UCCM patients, four CCM and one UCCM patients were serologically converted. Nevertheless, the positive rate (42.9%) in CCM patients was still significantly lower than that of UCCM patients (88.2%). Our result suggests that if immunological naiveness in term of protective immunity is the feature in cerebral malaria, the immunoprecipitation test used is inadequate to demonstrate the fundamental differences in immune responses between CM and AM patients.

SUMMARY

Immunoprecipitating antibodies were determined in paired sera of 31 patients with cerebral malaria (CM), of whom 14 had complicated cerebral malaria (CCM) and 17 had uncomplicated cerebral malaria (UCCM), 15 single specimens of patients with acute uncomplicated (AM) malaria taken on the day of admission and 8 healthy controls. All but one patient were admitted within the first three days of the onset of fever. More than 20 precipitating bands were observed, of which the predominating molecules were the Mr > 200, 180, 157, 135, 130, 115, 103, 96, 91, 73, 71, 61, 49, 45, 43, 41 and 14.3 Kd. In general, there were no significant differences in the positive rates among the AM, CCM and UCCM patients except for the pf135 Kd molecule which was more frequently reactive in UCCM patients than the AM and CCM

patients. If immunological naiveness in term of protective immunity is the feature in CM patients, the immunoprecipitation test used is inadequate to demonstrate the fundamental differences in immune responses between CM and AM patients.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. P. Dubois, Institut Pasteur, Paris for instructions on the immunoprecipitation test. Grateful thanks to Dr. Chaisit Dharakul, Director and the staff of Phra Pokklao Hospital, Chantaburi, and Dr. M.J. Warrell, Wellcome Research Unit, Faculty of Tropical Medicine, Mahidol University for their help in the collection of sera. Thanks also to Dr. Chutatip Premyotin, Department of Medicine, Rajvithi General Hospital, Bangkok for procurement of human sera for *in vitro* cultivation of malaria parasites.

REFERENCES

DAROFF, R.B., DELLER, J.J., KASTL, A.J. and BLOCKER, W., (1967). Cerebral malaria. *JAMA, 202* : 679.

BANYAL, H.S. and INSELBURG, J. (1985). Isolation and characterization of parasite-inhibitory *Plasmodium falciparum* monoclonal antibodies. *Amer. J. Trop. Med. Hyg., 34* : 1055.

BROWN, G.V., ANDER, R.F., MITCHELL, G.F. and HEYWOOD, P.F., (1982). Target antigens of purified human immunoglobulins which inhibit growth of *Plasmodium falciparum in vitro. Nature, 297* : 591.

CAMPBELL, G.H., MILLER, L.H., HUDSON, D., FRANCO, E.L. and ANDRYSIAK, P.M. (1984). Monoclonal antibody characterization of *Plasmodium falciparum* antigens. *Amer. J. Trop. Med. Hyg., 33* : 1051.

HARINASUTA, T., DIXON, K.E., WARRELL, D.A. and DOBERSTYN, E.B., (1982). Recent advances in malaria with special reference to Southeast Asia. *Southeast Asian J. Trop. Med. Pub. Hlth., 13* : 1.

LAMBROS, C. and VANDERBERG, J.P., (1979). Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J. Parasitol., 65* : 418.

LAEMMLI, U.K., (1970). Cleavage of structural proteins during the assembly of the head of bacteriphage T4, *Nature, 227* : 680.

MITCHELL, G. and TAM, L., (1986). *In :* Proceedings of the Asia and Pacific Conference on Malaria: Practical considerations on malaria vaccines and clinical trials. W.A. Siddiqui, (ed.), Publication of the Department of Tropical Medicine and Medical Microbiology, John A. Burns School of Medicine, University of Hawaii, pp. 228-230.

PERRIN, L.H. and DAYAL, R., (1982). Immunity to asexual erythrocytic stages of *Plasmodium falciparum:* Role of defined antigens in the humoral response. *Immunol. Rev., 61* : 245.

SAUL, A., MYLER, P., ELLIOTT, T. and KIDSON, C. (1982). Purification of mature schizonts of *Plasmodium falciparum* on colloidal silica gradients *Bull. W.H.O., 60* : 755.

SAUL, A., MYLER, P., SCHOFIELD, L. and KIDSON, C., (1984). A high molecular weight antigen in *Plasmodium falciparum* recognized by inhibitory monoclonal antibodies. *Parasite Immunol., 6* : 39.

TAPCHAISRI, P., ASAVANICH, A., LIMSUWAN, S., THARAVANIJ, S. and HARINASUTA, K.T., (1985). Antibodies against malaria sporozoites in patients with acute uncomplicated malaria and patients with cerebral malaria. *Amer. J. Trop. Med. Hyg., 34* : 831.

THARAVANIJ, S. and PRASERTSIRIROJ, V., (1981). Simultaneous preparation of merozoites and concentrated schizonts of *Plasmodium falciparum*. *Southeast Asian J. Trop. Med. Pub. Hlth., 12* : 518.

THARAVANIJ, S., TANTIVANICH, S., CHONGSA-NGUAN, M. and PRASERTSIRIROJ,V., (1982). Comparison of various serological test results using antigens from different strains of *Plasmodium falciparum*, *Southeast Asian J. Trop. Med. Pub. Hlth.. 13* : 174.

THARAVANIJ, S., WARRELL, M.J., TANTIVANICH, S., TAPCHAISRI, P., CHONGSA-NGUAN, M., PRASERTSIRIROJ, V. and PATARAPOTIKUL, J., (1984). Factors contributing to the development of cerebral malaria. I. Humoral immune responses. *Amer. J. Trop. Med. Hyg., 33* : 1.

THARAVANIJ, S., CHONGSA-NGUAN, M., KETRANGSI, S., PATARAPOTIKUL, J., TANTIVANICH, S. and TAPCHAISRI, P., (1986). Cross-sectional seroepidemiological survey of malaria in endemic areas with different activities of malaria control. *Southeast Asian J. Trop. Med. Pub. Hlth., 17* : 524.

TRAGER, W. and JENSEN, J.B., (1976). Human malaria parasites in continuous culture. *Science, 193* : 673.

USAWATTANAKUL, W., THARAVANIJ, S., WARRELL, D.A., LOOAREESUWAN, S., WHITE, N.J., SUPAVEJ, S. and SOIKRATOKE, S., (1985). Factors contributing to the development of cerebral malaria. II. Endotoxin. *Clin. Exp. Immunol., 61* : 562.