ANTIBODIES AGAINST CIRCUMSPOROZOITE PROTEINS OF *PLASMODIUM FALCIPARUM* INDUCED BY NATURAL INFECTION

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INTRODUCTION

Previous studies by the indirect immunofluorescent (IFA) test and/or circumsporozoite precipitation (CSP) reaction showed that people living in malaria endemic areas and malaria patients had specific antibodies against *Plasmodium falciparum* and *P. vivax* sporozoites as the result of natural infections by the parasites (Nardin *et al.*, 1979; Tapchaisri *et al.*, 1983, 1985). These antibodies are usually IgG and are also species and stage specific. However, the sporozoite antigens responsible for the induction of the immune responses in these individuals are unknown. The present study was aimed at determining the antigenic components of *P. falciparum* sporozoites reacting with sera from individuals living in a malaria endemic area and also patients with either acute uncomplicated malaria (AM) or cerebral malaria (CM).

MATERIALS AND METHODS

Sera and monoclonal antibodies (MABs): Sera were obtained from 10 healthy individuals living in Khao Kaeng Riang Village, Kanchanaburi Province, West Thailand, 10 AM patients and 10 CM patients admitted to Phra Pokklao Hospital in Chantaburi Province, East Thailahd. Details on the criteria of the clinical diagnosis have been described (Tharavanij *et al.*, 1984). These individuals have been previously shown by IFA and/or CSP reactions to possess serum antibodies against *P. falciparum* and/or *P. vivax* sporozoites (Tapchaisri *et al.*, 1983, 1985). The age range, serum IFA and CSP reactivities against *P. falciparum* and/or *P. vivax* of these individuals are summarized in Table 1.

Negative control sera were obtained from 10 healthy blood donors living in Bangkok, a non-malarious area. These individuals had no history of malaria and were IFA and CSP negative against *P. falciparum* and *P. vivax* sporozoites (Tapchaisri *et al.*, 1983).

Positive control sera were derived from a pool of hyperimmune mice immunized against either *P. falciparum* or *P. vivax* sporozoites by repeated intravenous injections of approximately 10^5 sporozoites per mouse at weekly intervals for 4-6 weeks These sera were IFA and CSP positive only against the homologous species of the parasites.

MABs against CS proteins of *P. falciparum* (2A10) and *P. vivax* (2F2) were kindly provided by Dr. Ruth S. Nussenzweig, Department of Medical and Molecular Parasitology, New York University School of Medicine, New York, U.S.A. These MABs have been shown to reduce infectivity of homologous sporozoites in chimpanzees and inhibit penetration of the parasites into cultured human hepatoma cells (Nardin *et al.*, 1982; Hollindale *et al.*, 1984).

This investigation was supported by the U.S. Agency for International Development Contract No. DPE-0453-C-00-2002-00 through New York University School of Medicine, the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, and in co-operation with the Wellcome-Mahidol University, Oxford Tropical Medicine Research Programme.

Table 1

The age range, serum IFA and CSP reactivities against *P. falciparum* and/or *P. vivax* sporozoites of healthy individuals, patients with acute uncomplicated falciparum malaria and cerebral malaria.

Individuals (Total Number)	Age (Years)*	IFA Positive**				CSP Positive***
		P. falciparum		*P. vivax*		*P. falciparum*
		IgG	IgM	IgG	IgM	
Healthy (10)	21-76 (49)	10 (1:32-1:128)	1 (1:8)	7 (1:8-1:128)	3 (1:8-1:16)	0
Acute Malaria (10)	16-54 (32)	10 (1:64-1:2,048)	0	2 (1:8-1:16)	3 (1:16-1:32)	8
Cerebral Malaria (10)	11-34 (20)	9 (1:32-1:2,048)	1 (1:2,048)	1 (1:16)	4 (1:8-1:128)	3

*Range and (mean)
**Number positive at 1 : 8 dilution (titer range)
***Number positive at undiluted serum.

Malarial antigens: Details on the preparation of *P. falciparum* and *P. vivax* sporozoites from infected salivary glands of *Anopheles balabacensis* have been previously described (Tapchaisri *et al.*, 1983; Nardin *et al.*, 1979).

Concentrated schizonts from *in vitro* cultures of SO strain of *P. falciparum* were prepared by percoll gradient centrifugation as previously described (Tharavanij *et al.*, 1984). The preparation contained approximately 50% schizonts, 30% trophozoites, 10% rings and 10% non-infected red cells.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis: The electrophoresis was carried out in a vertical slab gel apparatus using the method of Laemmli and Farve (1973). A 4% acrylamide stacking gel and a 10% acrylamide separating gel were used. Samples containing various amounts of *P. falciparum* or *P. vivax* sporozoites in RPMI 1640 medium were heated at 100°C for 3 minutes in the presence of sodium dodecyl sulphate (SDS) and 2-mercaptoethanol before loading onto the gel. After electrophoresis the gel was electroblotted onto a 0.45 μm nitrocellulose membrane (Bio-Rad Laboratories, Calif., U.S.A.) according to the method of Towbin *et al.*, (1979). After blotting the unreacted sites on the membrane were blocked by soaking the strips in phosphate buffered saline (PBS) containing 5% bovine serum albumin (BSA) and 0.02% sodium azide (PBS-BSA) at 4°C for 18 hours. The blots were treated with 10 ml of test serum (1 : 50 dilution in PBS-BSA) or MAB (20 μg/ml in PBS-BSA) at 26°C for 2 hours with gentle rocking. The strips were then washed 5 times with PBS containing 0.05% Tween 20 and then treated with ^{125}I-labeled protein A ($1-5\times 10^5$ cpm/ml in PBS-BSA) at 26°C for 30 minutes with rocking. The Protein A (Phamacia Fine Chemicals, Uppsala, Sweden) was labeled with ^{125}I (sodium salt, New England Nuclear, Mass., U.S.A.) by the iodogen method (Markwell

and Fox, 1978). In a CM patient (P45) who had high IgM titer and CSP-positive against the parasites, the serum IgM antibody reactivity against the antigens of *P. falciparum* was shown by allowing the paper strip to react with goat anti-human immunoglobulins and washed prior to incubation with the radioactive protein A. The nitrocellulose strips were washed as above, dried, and autoradiographed on Kodak X-Omat RP films (Eastman Kodak Co., N.Y., U.S.A.) with a Dupont Lightning Plus enhancing screen at −70°C.

Radioimmunoprecipitation (RIP) of *P. falciparum* sporozoite antigens: Approximately 1-2 × 10^6 viable *P. falciparum* sporozoites in a volume of 0.5 ml were labeled at 26°C for 3 to 4 hours in a methionine-deficient medium containing 400 μCi/ml of ^{35}S-methionine (New England Nuclear) according to the method of Yoshida *et al.*, (1980). The labeled parasites were washed three times by centrifugation with the complete medium containing protease inhibitors (antipain and leupeptin each at 25 μg/ml and aprotinin at 2 trypsin inhibiting units/ml) then extracted with 1% Triton X-100 at 26°C for 1 hour. The supernatant containing the extracted antigens was obtained by centrifugation. The extracted antigens (20 μl) were incubated with an equal volume of a 1 : 4 dilution of the test serum in 150 mM NaCl-5 mM EDTA-50 mM Tris buffer pH 7.4 (NET) or 20 μg in 20 μl of MAB in NET buffer at 26°C for 30 minutes then at 4°C for another 18 hours. The antigen/antibody complex formed was allowed to react with 100 μl of 10% formalin-fixed staphylococcus protein A cells (Bethesda Research Laboratories, Md., U.S.A.) at 26°C for 30 minutes. The suspension was centrifuged and the pellet was washed three times with NET buffer containing 0.05% Triton X-100. Dissociation of the antigen-antibody complex was made by heating the samples in SDS and 2-mercaptoethanol at 100°C for 5 minutes and the supernatants collected were subjected to SDS-PAGE. After electrophoresis the gel was treated with EN^{3}HANCE (New England Nuclear), dried and autoradiographed as above.

RESULTS

Identification of *P. falciparum* sporozoite antigens reacting with specific antibodies : Various numbers of *P. falciparum* sporozoites (ranging from 1,250 to 10,000 parasites) blotted onto the nitrocellulose sheets were allowed to react with the homologous hyperimmune mouse serum or serum from an AM patient (F5) who was IFA and CSP positive against the parasites. Three defined bands of antigens with molecular weight (MW) of approximately 65,000 (Pf65), 60,000 (Pf60), and 58,000 (Pf58) daltons were shown to react with both sera with similar pattern (Fig. 1A and 1B). Another faint band of antigen with MW of 67,000 (Pf67) daltons was also found especially in samples containing 5,000 parasites or more. When MAB 2A10 was used, it bound to all four antigens of the parasites with pattern identical to those of immune sera indicating that these antigens were antigenically related (Fig. 1C).

In another experiment when high amount of sporozoites (up to 40,000 parasites) was used to react with serum from another AM patient (M6), a very faint band of antigen with MW of approximately 80,000 (Pf80) daltons was identified (Fig. 2). This minor antigen was not clearly seen when lower amount of the sporozoites, i.e., 20,000 or less, was used.

Normal salivary glands of *Anopheles balabacensis* (*A. dirus*) were used as the antigen control. None of the two immune sera and the MAB showed any band against the salivary glands of the mosquitoes (Fig. 2).

Due to the limitation of the sporozoite antigens, an amount of approximately 10,000

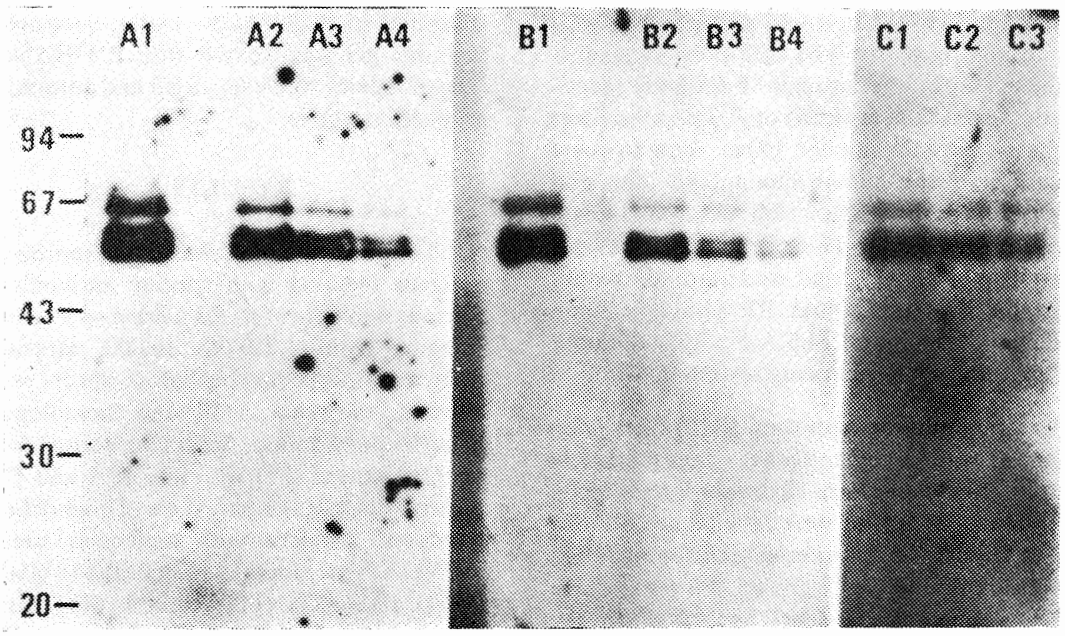

Fig. 1—Reactivities of serum antibodies from hyperimmune mouse (A1-A4), acute uncomplicated falciparum malaria (B1-B4) and monoclonal antibody 2A10 (C1-C3) against various numbers of *P. falciparum* sporozoites (A1 and B1 = 10,000, A2, B2 and C1 = 5,000, A3, B3 and C2 = 2,500 and A4, B4 and C3 = 1,250 parasites). On left molecular weight standards given in kilodaltons.

sporozoites was selected for further tests throughout this study.

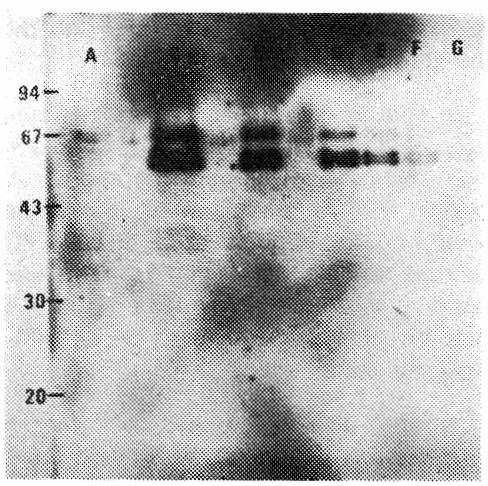

Fig. 2—Reactivities of serum antibodies from a patient with acute uncomplicated falciparum malaria against various numbers of *P. falciparum* sporozoites (A = normal salivary glands of *A. balabacensis*, B = 40,000, C = 20,000, D = 10,000, E = 5,000, F = 2,500, and G = 1,250 parasites).

Species and stage specificities of *P. falciparum* sporozoite antigens : *P. falciparum* sporozoite antigens failed to react with the MAB 2F2 and hyperimmune mouse serum specific against *P. vivax* sporozoites. When *P. vivax* sporozoites were used as the antigen, however, the MAB 2F2 reacted with 3 bands of antigens with MW of approximately 51,000 (Pv51), 49,000 (Pv49), and 45,000 (Pv45) daltons. It was further found that the MAB 2A10 had no reactivity against *P. vivax* sporozoites (data not shown).

The stage specificity of Pf67, Pf65, Pf60, and Pf58 was shown by reacting the serum from the patient (F5) who was also positive (IFA titer of 1:1,280) against the blood stages of *P. falciparum* with antigens obtained from each stage of the parasites. Approximately 30 bands of blood stage antigens were identified. These blood stage antigens had MW range from 200,000 to 22,000 daltons with the prominent bands of 200,000, 88,000,

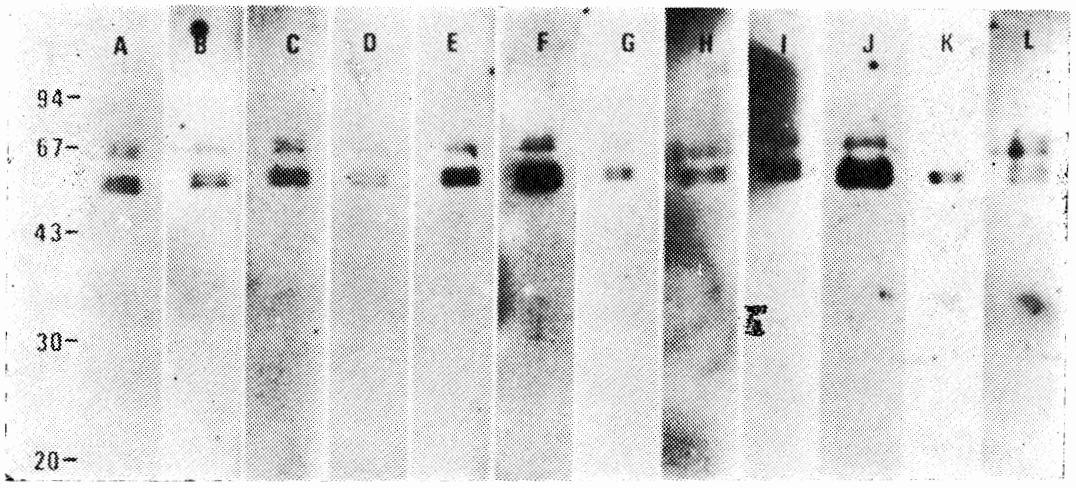

Fig. 3—Reactivities of serum antibodies from individuals living in a malaria endemic area (A-D), patients with acute falciparum malaria (E-H), and patients with cerebral malaria (I-L) against *P. falciparum* sporozoites.

79,000, 51,000, 44,000, 24,000 and 22,000 daltons (data not shown). Pf67, Pf65, Pf60, and Pf58 were absence in the blood stage parasites. MAB 2A10 also failed to react with the blood stage antigens of the parasites.

Antibody responses to *P. falciparum* sporozoite antigens : Antibodies against Pf60 and Pf58 were found in all ten individuals living in a malaria endemic area. Antibodies against Pf65 were found in 7 individuals of whom three were positive for antibodies against Pf67. The autoradiograms of the antibody reactivities against *P. falciparum* sporozoites in 4 individuals representing those who lived in the endemic area are shown in Fig. 3.

The pattern of antibody responses to *P. falciparum* sporozoite antigens in AM patients and CM patients were similar to those of the individuals living in the malaria endemic area. Antibodies against Pf65, Pf60 and Pf58 were found in all 10 AM patients and in 10 CM patients. Representative autoradiograms from AM and CM patients are shown in Fig. 3. Two patients (one in AM patient group and another in CM patient group) had also reactivities against two bands of the antigens with MW approximately 71,000 and 63,000 daltons (e.g., those show in Fig. 3, Lane L). However, these two antigens were not specific for *P. falciparum* sporozoites since they were also found in salivary glands of normal *A. balabacensis*.

It was found that all 10 normal individuals had no serum reactivity against *P. falciparum* sporozoites (data not shown).

RIP of *P. falciparum* sporozoite antigens : Due to limited ^{35}S-methionine metabolically-labeled *P. falciparum* sporozoite antigens, only sera from mice immunized against either *P. falciparum* or *P. vivax* sporozoites, an AM patient (F5), a CM patient (70), and an individual living in the endemic area (63) and the MABs were tested. It was found that *P. falciparum* sporozoite-immunized mouse serum immunoprecipitated 5 bands of antigens, namely, Pf80, Pf67, Pf65, Pf60 and Pf58 (Fig. 4). Another band of antigen with MW of 73,000 daltons was also precipitated but this antigen was not recognized by human immune sera. Sera from AM and CM patients also precipitated the five antigens except that Pf60 was not clearly seen. Serum from individual living in the endemic area showed reactivity against Pf80, Pf67 and probably

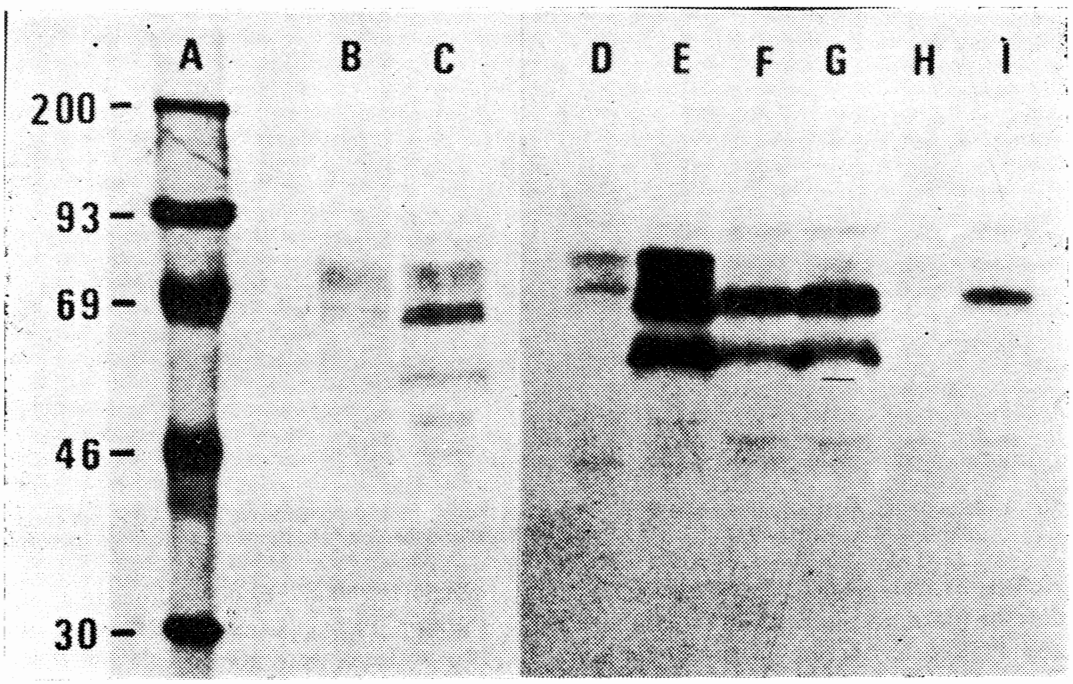

Fig. 4—Radioimmunoprecipitation of *P. falciparum* sporozoite antigens using sera and monoclonal antibodies (A = molecular weight kilodaltons, B = monoclonal antibody 2F2, C = monoclonal antibody 2A10, D = *P. vivax* sporozoite-immunized mouse serum, E = *P. falciparum* sporozoite-immunized mouse serum, F = acute uncomplicated falciparum malaria serum, G = cerebral malaria serum, H = normal serum, and I = sera from individual in malaria endemic area.

Pf60. MAB 2A10 was found to specifically immunoprecipitated Pf67 and Pf58 (Fig. 4). Normal serum and MAB 2F2 failed to react specifically with the *P. falciparum* sporozoite antigens but *P. vivax* sporozoite-immunized mouse serum showed cross-reaction against Pf80 (Fig. 4).

DISCUSSION

The present study indicated that CS proteins were the major antigenic component responsible for the induction of antibody responses in individuals naturally infected with *P. falciparum* sporozoites. This finding was similar to those previously described in animals experimentally immunized with malaria sporozoites which included *P. berghei* in mice (Yoshida *et al.*, 1980) and *P. knowlesi* in monkeys (Cochrane *et al.*, 1983) and also in volunteers immunized by the bites of irradiated mosquitoes infected with *P. falciparum* and *P. vivax* sporozoites (Nardin *et al.*, 1982). The high immunogenicity of the CS proteins has been attributed to the presence of repeating epitopes within their molecules which were shown in *P. knowlesi* (Ozaki *et al.*, 1983) and *P. falciparum* sporozoites (Dame *et al.*, 1984; Enea *et al.*, 1984).

It was found in the present study that CS proteins of *P. falciparum* exhibited four different molecular weights, i.e., Pf67, Pf65, Pf60 and Pf58. Interestingly, Pf65, Pf60 and Pf58 were commonly seen by the Western blot analysis whereas Pf67, Pf65 and Pf58 were usually found by the RIP. The main difference of the two assays is that the former detects the malarial antigens already present

whereas the latter detects those newly synthesized during *in vitro* incubation of the sporozoites in the presence of radioacitve ^{35}S-methionine. It is likely that Pf67 and Pf65 are the precursors of Pf60 and Pf58 although the actual demonstration of the relationship between these proteins has never been clearly demonstrated. Nardin *et al.*, (1982) found that serum from *P. falciparum* sporozoite-immunized volunteer and MAB against CS proteins of the parasites consistently immunoprecipitated two bands of antigens, namely, Pf67 and Pf58. However, Pf67 appeared as a doublet and was equivalent to Pf67 and Pf65 found by Santoro *et al.*, (1983) and in the present study. The same group of investigators have subsequently analysed by RIP using MABs, two-dimensional gel electrophoresis and tryptic peptide analysis the various CS proteins from different species of malaria parasites including *P. falciparum* and concluded that Pf67 indeed consisted of two polypeptides (Pf67 and Pf65) which had identical retention time in the reverse-phase high performance liquid chromatography with Pf58 (Santoro *et al.*, 1983). This reflects the structural homology between these proteins of the parasites. They have speculated that Pf67 and Pf65 were intracellular proteins and that Pf67 was processed by two sequential cleavages into the surface membrane protein, Pf58 (Santoro *et al.*, 1983). The present finding that Pf58 consistently yielded two bands (60,000 and 58,000 daltons) was interesting and raised the possibility that CS proteins of *P. falciparum* existed in two different variants, that Pf67 and Pf65 were the precursors of Pf60 and Pf58, respectively, and the generation process of Pf60 and Pf58 was derived from a single cleavage. However, pulse-chase experiment similar to those performed in *P. knowlesi* (Cochrane *et al.*, 1983) was not carried out due to limited *P. falciparum* sporozoites.

The present study indicated that CS proteins were stage specific and were not present in the blood stage parasites. CS proteins were found uniformly distributed on the surface membrane of the mature salivary gland sporozoites as demonstrated in *P. berghei* (Aikawa *et al.*, 1981) and *P. knowlesi* (Fine *et al.*, 1984). CS proteins of *P. falciparum* are not strain specific since cross reactivity occurred between MAB 2A10 which was raised against a West African isolate of *P. falciparum* sporozoites and the local strains of the parasites. Furthermore, CS proteins exhibited species specificity, i.e., no cross reactivity was observed between CS proteins of *P. falciparum* and MAB specific against those of *P. vivax* and *vice versa*. These findings are consistent with the previous findings of the characteristics of humoral immune response and protective immunity to malaria sporozoites (Cochrane *et al.*, 1980).

In addition to the CS proteins of *P. falciparum*, another antigen (Pf80) was recognized by sera from mice immunized against *P. falciparum* or *P. vivax* sporozoites, AM and CM patients and individual living in malaria endemic area but not from normal individual (Figs. 2 and 4). This antigen was present in a minute amount as shown by Western blot analysis (Fig. 2). Nardin *et al.* (1982) found that this antigen was probably not membrane associated, non-species specific and may not play a role in protective immunity.

Western blot analysis has been recently used for the detection of CS proteins in malaria infected mosquitoes (Cochrane *et al.*, 1984). Using ^{125}I-labeled MAB against *P. berghei* sporozoites and extract of the corresponding parasites, as few as 100 sporozoites could be detected by this assay. Although the sensitivity for the detection of CS proteins of *P. falciparum* has not been performed in the present study, it was found that our assay

could easily detect the CS proteins in extracts containing 1,250 parasites. However, the sensitivity of the assay will depend on several factors, e.g., the efficacy of protein transfer from the gel to the nitrocellulose membrane, the choice and the affinity of detecting reagent and/or the second antibody, etc. (Cochrane *et al.*, 1984).

Antibodies against CS proteins of several species of malaria sporozoites including *P. berghei* (Yoshida *et al.*, 1980), *P. knowlesi* (Cochrane *et al.*, 1983) and *P. falciparum* (Nardin *et al.*, 1982) have been associated with protective immunity against the parasites. It is very interesting that individuals living in malaria endemic area, AM and CM patients developed antibodies with identical patterns against CS proteins of *P. falciparum*. It has been speculated that these patients had been exposed to a large dose of infective parasites in a relatively short period of time and subsequently developed acute episode of malaria while the anti-sporozoite antibodies were being produced but were not yet effective in elimination of the parasites (Tapchaisri *et al.*, 1983). Studies in man of the protective immunity mediated by the anti-CS protein antibodies have been limited due to the lack of simple and appropriate animal or laboratory models. Recently, Hollingdale *et al.* (1984) demonstrated that MABs against CS proteins of *P. falciparum* and *P. vivax* could inhibit invasion of the homologous species of the parasites into cultured hepatoma cells. Sera from *P. falciparum* and *P. vivax* immunized volunteers and a few Gambian adults having antibodies against *P. falciparum* sporozoites also inhibited the *in vitro* penetration by the corresponding parasites into the cultured cells. It has been proposed that this sporozoite-neutralization test may represent the *in vitro* assay for protective antibodies (Hollingdale *et al.*, 1984). Further study using sera from the individuals living in malaria endemic area and patients with malaria in the *in vitro* sporozoite-neutralization assay would help defining the functional and the role of the anti-sporozoite antibodies induced by natural infection in the development of resistance to re-infection by *P. falciparum*.

SUMMARY

Sera from 10 individuals who lived in a malaria endemic area, 10 patients with acute uncomplicated falciparum malaria and 10 patients with cerebral malaria and hyperimmune mouse serum were tested for their reactivities against *Plasmodium falciparum* sporozoite antigens by Western blot analysis using ^{125}I-labeled staphylococcal protein A as the detecting reagent. These sera were shown by indirect immunofluorescence and/or circumsporozoite precipitation test to have antibodies reacting against the parasites. It was found that all serum antibodies from the three groups of individuals and the mouse serum reacted in a similar pattern with circumsporozoite (CS) proteins of *P. falciparum*. Ten sera from normal individuals were negative in all reactions. Monoclonal antibody (MAB) specific against CS proteins of the parasites showed that the proteins exhibited as four different molecular weight (MW) polypeptides, i.e., 67,000, 65,000, 60,000, and 58,000 daltons. These CS proteins of *P. falciparum* were found to be species and stage specific. Radioimmunoprecipitation using ^{35}S-methionine-labeled parasites and sera of individuals from the various categories or MABs gave a similar result.

Another protein antigen of *P. falciparum* sporozoites had a MW of 80,000 daltons. This antigen was not species specific, probably not membrane associated and was present in a minute quantity in the parasite's extract.

ACKNOWLEDGEMENTS

The authors express sincere thanks to Professor Ruth S. Nussenzweig and Dr. Alan H. Cochrane for their help, advice and criticism. Specials thanks to Dr. Chaisit Dharakul, Director, and staff of the Department of Medicine, Phra Pokklao Hospital, and also Drs. D.A. Warrell and Sornchai Looareesuwan, Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University for their help in the collection of sera, and Dr. Supat Sucharit for providing facilities for the mosquitoes.

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