

PD-AAX-388

54796.

PROGRESS REPORT TO USAID

PROGRESS REPORT NO. 4

"ANTIGENIC DIVERSITY OF P. VIVAX"

A RESEARCH PROJECT

USAID/PSTC PROGRAM

GRANT NO. 936-5542-G-00-5078-00

SUBMITTED BY

SRISIN KHUSHITH

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

FACULTY OF TROPICAL MEDICINE

MAHIDOL UNIVERSITY

BANGKOK 10400

THAILAND

- / -

Progress report to USAID

Contract No. 936-5542-G-00-5078-00

Title of project : Antigenic diversity of Plasmodium vivax

Institute : Department of Microbiology and Immunology

Faculty of Tropical Medicine

Mahidol University

420/6 Rajvithi Road, Bangkok 10400

Thailand

Tel. 2460056, 2460058, 2461278, 2461272-3

Telex 84770 UNIMAH TH

Chief Scientific Investigators :

Srisin Khusmith, B.Sc., M.Sc., D.Sc.

Savanat Tharavanij, M.D., Ph.D.

Investigators :

Pramuan Tapchaisri, B.Sc., M.Sc., Ph.D.

Thutwadee Ratanaprapa, B.Sc.

Srisurang Tuntrakul

Kingkarn A. Indravijit

Danai Bunnag, F.R.C.S., D.T.M. & H.

Time period covered :

March 1987 - August 1987

Scientific progress

- 1) Investigation of antigenic heterogeneity in P. vivax isolated from different parts of Thailand using monoclonal antibodies.
- 2) Characterization of P. vivax antigen to which monoclonal antibodies react.
- 3) Determination of the immunoglobulin class of monoclonal antibodies by a dot immunobinding assay.
- 4) Demonstration the species specific monoclonal antibodies with P. falciparum isolate.
- 5) Preparation of parasite antigens from vivax malaria patients for Western blot analysis.
- 6) Preparation of P. vivax antigen slides from vivax malaria patients for use in the indirect fluorescent antibody test.

1) Demonstration of antigenic diversity in P. vivax

Monoclonal antibodies against blood forms of Plasmodium vivax were used to demonstrate considerable antigenic diversity in this species. Different isolates from different parts of Thailand were distinguished by their ability to react with certain antibodies by using immunofluorescent antibody test (IFA)

The indirect fluorescence using acetone-fixed infected blood as antigen was used (Khusmith et al. 1984, Asian Pacific J. Allerg. Immunol., 2:91-95). Blood smears were made from washed infected blood of patients with P. vivax and stored at -70° C in sealed containers with Silica gel until required. The IFA was carried out at 37° C in a moisture chamber. After fixation in acetone (-20° C) each well was treated with 10 µl of listed monoclonal antibodies for 2 hours at 37° C, the slides were washed vigorously and reacted for a further 1 hr with fluorescein isothiocyanate conjugated goat anti-mouse IgG, A, M (Pasteur Institute, France). After washing and drying the slides were mounted in a mounting medium containing 4.5% N propyl gallate in 0.2 M Tris in glycerol and examined under a fluorescent microscope.

More than 54 monoclonal antibodies have been tested with more than 30 isolates of P. vivax. The data are currently under analysis. Our preliminary result showed that different parasite isolates reacted differently to monoclonal antibodies of different or even the same group, suggesting the existence of antigenic diversity in different isolates.

2) Characterization of monoclonal antibodies by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Electrophoresis was carried out in a vertical slab gel apparatus (Bio-Rad, U.S.A.) 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. vivax* blood stages 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. Each lane contained 5×10^6 infected red blood cells. The electrophoresis was made at 30 mA per slab gel for 3-4 hours until the tracking dye reached the bottom of the gel. After electrophoresis the gels were cut and subjected to protein staining, or electroblotted onto a 0.45 μ m nitrocellulose membrane (Bio-Rad Laboratories, California, U.S.A.) according to the method of Towbin et al., (1979). After blotting the unreacted sites on the membranes were blocked by soaking the strips in phosphate buffered saline (containing 3% gelatin and 0.02% sodium azide (PBS-GEL)) at 4° C for 18 hours. The blots were treated with 2 ml of monoclonal antibody or supernatant of positive clones 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 anti-mouse immunoglobulin (Amersham, England) ($1 - 5 \times 10^5$ cpm/ml in PBS-BSA) at 26° C for 30 minutes with rocking. The nitrocellulose strips were washed as above, dried and autoradiographed on Kodak X-Omat RP films (Eastman

Kodak Co., N.Y., U.S.A.) at -70° C.

Results of the Western blot analysis of some monoclonal antibodies of different IFA patterns are shown in table 1.

Table 1. Western blot analysis of the molecules of P. vivax antigen reacting with anti P. vivax monoclonal antibodies.

Monoclonal antibodies	Number of monoclonal antibodies tested	Molecule of <u>P. vivax</u> antigens in kilodalton to which monoclonal antibodies react
Group I	18	PV30, PV36, PV39, PV85, PV95, PV100, PV115, PV200, PV>200
Group II	9	PV35, PV100, PV135
Group III	2	PV190
Group V	7	PV115, PV95, PV85, PV39, PV36
Group VII	8	PV30, PV85

3) Determination of the immunoglobulin class of anti P. vivax antibodies

A technique modified from Beyer (1984) (J. Immunol. Methods, 67:79-87) was used. Briefly, strips (9.5 x 0.6 cm) of nitrocellulose membrane (SS BA88, Schleicher & Schuell, Dassel, West Germany) were soaked in Tris buffered saline pH 7.5 (TBS) and dried on absorbent paper for 5 min. Three μ l of monoclonal antibodies was spotted to each strip and dried. The strips were incubated together in 100 ml of TBS containing 0.2% gelatin for 30 min, rinsed twice in TBS and each strip was individually incubated for two hours in five ml of appropriately diluted anti-human IgG1, IgG2a, IgG2b, IgG3, IgG4 and IgM (Serotec, Blackthorn, Becester, England). After incubation, the strips were rinsed in TBS and then incubated for one hour in appropriately diluted peroxidase-protein A conjugated solution (Sigma Chemicals, St. Louis, Missouri). After washing in TBS, the strips were transferred to a solution of 4-chloro 1-naphthol (Sigma) and incubated further for 10-30 min. The strips were rinsed in water to stop the reaction and the result read with naked eyes.

Results of the isotyping of monoclonal antibodies to blood stages of P. vivax are shown in table 2.

Table 2. Isotyping of the *P. vivax* monoclonal antibodies

Immunofluorescent pattern	Number of monoclonones tested	Isotypes				
		IgG1	IgG2	IgG3	IgG4	IgM
Group I	18	12	-	1	-	5
Group II	17	17	-	-	-	-
Group III	2	1	-	-	-	1
Group V	8	6	-	2	-	-
Group VII	7	-	-	-	-	-

4) Demonstration the species specific monoclonal antibodies with P. falciparum isolate

The SO strain of P. falciparum was used (Tharsvanij et al., 1981. Southeast Asian J. Trop. Med. Pub. Hlth. 12:518). The parasites were grown in RPMI-1640 medium in the presence of 10% AB serum using a candle jar technique of Trager and Jensen. (1976) (Science, 193:673-675).

The species specificity of monoclonal antibodies was determined by an indirect fluorescent antibody test using acetone-fixed infected blood as antigen (Khusmith et al., 1984, Asian Pacific J. Ailerg. Immunol., 2:91-95). Blood smears were made from unwashed asynthronously grown SO strain of P. falciparum and stored at -70^o C in sealed containers with Silica gel until required. The results are shown in table 3.

Table 3. Reactivities of strains and species specific monoclonal antibodies with different strains of *P. vivax* and *P. falciparum* SO strain.

Monoclonal antibodies to <i>P. vivax</i> (MPV)	IFA pattern with <i>P. vivax</i>	Strain distribution of MPV No positive strains/ Total strains tested	IFA reaction with <i>P. falciparum</i> SO strains
MPV1-MPV18	Group I	7/16 - 16/16	Neg
MPV19-MPV36	Group II	3/20 ^a	Neg
MPV37, MPV38, MPV54	Group III	3/20 ^a	Neg
MPV39-MPV45	Group V	12/16 - 16/16	Neg
MPV46-MPV53	Group VII	6/10 - 10/10	Neg

a = only 3 isolates of *P. vivax* tested have schizont stage

5) Preparation of *P. vivax* antigens for fusion and Western blot analysis

Enrichment of *P. vivax* infected RBCs was achieved by using SEC-G-25-Percoll method described recently (Tharavanij et al., Southeast Asian J. Trop. Med. Hyg., 18:39-43) from 54 vivax malaria patients (PV087-PV140) admitted to the Hospital for Tropical Diseases during February 1987 and August 1987. The specimens were either processed immediately or stored overnight at 4° C.

A one ml aliquot of heparinized whole blood was passed through a column an equal volume of sulfoethyl cellulose and Sephadex G-25 previously equilibrated with phosphate buffer pH 7.5. The column was then washed with approximately 40 ml of the buffer until the column was free from RBCs. The eluate was centrifuged, the supernate removed and the cell sediment restored to 50% hematocrit. The cell suspensions from several tubes were pooled and two ml each was layered on top of discontinuous gradients comprising successive layers of two ml each of 75%, 60%, 50% and 40% of Percoll in the same buffer. After centrifugation at 2,600 rpm for 10 min at 20° C. Two and sometime three bands were obtained. The first and second bands were localized in the layers of 50% and 60% Percoll and the third band in the interface of 60% and 75% Percoll. The cells in the first and second bands were pooled and called "upper band", whereas the third band was called "lower band". The smears were made from the blood prior to passage through the column and after Percoll gradient centrifugation, stained with Giemsa and the

result examined by light microscopy.

Cells in the upper and lower consisted of both trophozoites and ring stages with more mature trophozoites and a relatively higher percentage of trophozoites in the upper band. Enrichment of *P. vivax* infected red blood cells from the blood of 54 malaria patients were summarized in table 4.

Table 4. Parasite yields after enrichment

Patient	Parasite count ($\times 10^7$) after enrichment		
	Upper band	Lower band	Total
PV087	0.69	4.90	5.80
PV088	1.17	10.00	11.17
PV089	55.00	25.00	80.00
PV090	0.25	TB	0.25
PV091	0.70	0.50	1.20
PV092	0.65	1.07	1.72
PV093	0.51	0.27	0.78
PV094	0.64	10.40	11.04
PV095	5.40	40.00	45.40
PV096	2.00	1.70	3.70
PV097	3.00	43.00	46.00
PV098	0.26	0.41	0.67
PV099	1.80	2.43	4.23
PV100	ND	ND	ND
PV101	1.00	1.04	2.04
PV102	TB	0.72	0.72
PV103	23.00	24.00	47.00
PV104	25.00	53.00	78.00
PV105	3.40	2.70	6.10
PV106	0.11	1.10	1.21
PV107	0.35	0.24	0.59
PV108-PV111	36.00	16.80	52.80
PV112-PV114	39.00	17.00	56.00
PV115-PV118	8.20	8.40	16.60
PV117-PV119	6.10	18.00	24.10
PV120-PV121	1.10	2.00	3.10
PV122	TB	0.43	0.43
PV123-PV124	12.00	18.00	30.00
PV125	TB	8.70	8.70
PV126-PV127	2.00	5.00	7.00
PV128 and PV130	5.60	5.60	11.20
PV129	3.20	5.20	8.40
PV131 and PV132	7.80	26.00	33.80
PV133-PV135	0.43	5.20	5.63
PV136-PV140	37.00	40.00	77.00

ND = not done

TB = tube was broken during experiment

6) Preparation of *P. vivax* antigen slides for indirect fluorescent antibody test (IFA)

P. vivax infected cells obtained from 54 patients with vivax malaria (PV087 - PV140) admitted to the Hospital for Tropical Diseases, Bangkok, the blood from patients was washed 5 times with RPMI-1640 without serum and the cell sediment re-suspended in the same medium, followed by smear on clean glass slides, quickly dried in air, kept for a day at room temperature, placed in a sealed plastic envelop together with silica gel and stored at -70° C until used. One day-old culture of the parasites from some patients were also used to prepare the antigen slides. These *P. vivax* antigen slides were used for screening of monoclonal antibodies and for the study of the antigenic diversity of *P. vivax*.

Conclusion

We are now in the stage of determining whether there is antigenic diversity among different strains of *P. vivax* using an indirect fluorescent antibody test and monoclonal antibodies. A panel of monoclonal antibodies will be chosen then for investigation further whether distribution of different antigenic types is related to their geographic locations as well as to demonstrate whether the strain recrudescent after anti-malaria drug therapy is the same or different from these causing initial clinical symptoms finally.