PN-ABA-952

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON D.C. 20523

DATE: 9/28/88

MEMORANDUM

TO: AID/FPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose VO

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 5, 232
PR#6

Attachment

PN-ABH-752

5. 232

PROGRESS REPORT TO USAID

PROGRESS REPORT NO. 6

"ANTIGENIC DIVERSITY OF P. VIVAX"

A RESEARCH PROJECT

USAID/PSTC PROGRAM

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

SUBMITTED BY

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THAILAND

ANTIGENIC DIVERSITY OF *PLASMODIUM VIVAX*

Project Profile

Grant No. : 936-5542-G-00-5078-00

Program : Program on Science and Technology Cooperation

Project Title : Antigenic diversity of *P. vivax*

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Total Project Budget	:	U.S. $ 150,000
Project Duration	:	September 1985 - February 1989
Reporting Period	:	March 1988 - August 1988

Overall Objectives

1. To raise and characterize monoclonal antibodies against blood stages of *P. vivax*
2. To determine whether there is antigenic diversity among different strains of *P. vivax*
3. To investigate whether distribution of different antigenic types is related to their geographical locations
4. To demonstrate whether the strain recrudescent after anti-malaria drug therapy is the same or different from those causing initial clinical symptoms

Plans of work

1) Production of monoclonal antibodies against P. vivax
2) Characterization of monoclonal antibodies against P. vivax
   - 2.1 IFA
   - 2.2 SDS-PAGE and Western blot analysis
3) Preliminary screening of monoclonal antibodies and select them for establishment of a panel of monoclonal antibodies for strain specificities
4) Typing of P. vivax isolates using several monoclonal antiobodies and demonstrate their antigenic diversity
   - 4.1 Isolates from patients admitted to the Hospital for Tropical Diseases
   - 4.2 Isolates from P. vivax cases from malaria endemic areas in Thailand
5) Demonstration whether there is any antigenic disparity between parasites causing initial clinical symptoms and those causing recrudescence

Scientific Progress

Following progress has been made :

1. To explore the degree of polymorphism that prevailed in a series of *P. vivax* isolates obtained from infected patients.
2. Calculation the frequencies of several parasite antigens in the samples.
3. To examine the distribution of antigenic variation and geographic distribution.
4. Collection and preparation of *P. vivax* slides from vivax malaria patients attending malaria clinics in endemic areas.
5. Preparation of *P. vivax* antigens for immunofluorescent test and Western blot analysis.
6. Collection of *P. vivax* antigens from relapse cases.

1. Demonstration of antigenic diversity in *Plasmodium vivax*

A panel of 58 monoclonal antibodies were used to demonstrate considerable antigenic diversity in 300 primary isolates of *P. vivax* collected from 34 provinces around Thailand including one isolate from Burma using an immunoflourescent assay.

Samples :

Blood smears were made from :

1. *P. vivax* infected cells obtained from patients with vivax malaria (PV001-PV300) admitted to the Hospital for Tropical Diseases, Bangkok. These patients came from different areas of malaria including :

   1.1 Chanthaburi province

   1.2 Kanjanaburi province

   1.3 Trat province

   1.4 Tak province

   1.5 Rajburi province

   1.6 Chachoengsao province

   1.7 Prachin Buri province

   1.8 Chumporn province

   1.9 Prachuap Khiri Khan province

   1.10 Nakhorn Sawan province

   1.11 Chon Buri province

   1.12 Surat Thani province

   1.13 Nong Khai province

   1.14 Nan province

   1.15 Phet Buri province

1.16 Rayong province

1.17 Phitsanulok province

1.18 Samut Sakhon province

1.19 Nakhorn Ratchasima province

1.20 Nakhorn Si Thammarat province

1.21 Buriram province

1.22 Nakhorn Nayok province

1.23 Uthai Thani province

1.24 Phetchabun province

1.25 Chiangrai province

1.26 Ubonrajthani province

1.27 Krabi province

1.28 Lop Buri province

1.29 Narathiwat province

1.30 Kumphaeng Phet province

1.31 Udon Thani province

1.32 Uttaradit province

1.33 Lamphun province

1.34 Mae Hong Son province

1.35 Burma

The blood from patients was washed 5 times with RPMI-1640 without serum and the cell sediment resuspended 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 envelops together with silica gel and stored at -70°C until used.

Indirect immunofluorescent assay (IFA)

The indirect fluorescence using acetone-fixed infected blood as antigen was done according to the technique previously described (Khusmith et al., 1984, Asian Pacific J. Allerg. Immunol., 2:91-95).

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 humid chamber. After fixation in acetone (-20° C) each well was treated with 10 ul of listed monoclonal antibodies for 2 hours at 37° C, the slides were washed vigorously and reacted further for another hour 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.

The percentages of parasites reacting with particular monoclonal antibodies were estimated by assessing the proportions of malaria pigment granules (as observed by normal transmitted white light) which were associated with fluorescent parasites (as observed by incident UV light). To exclude inter-observer error one person read all the IFA slides. Positive reactivities of these monoclonal antibodies with an isolate were scored (+ to ++++) depending on the intensity of the fluorescence, and in each case the proportion of parasite giving a positive reaction was noted.

Steps taken to ensure the validity of results

1. A single batch of all monoclonal antibodies was used for the entire investigation.
2. At least 4 different isolates were processed for the IFA and assayed simultaneously.
3. The assay was standardized from one batch of isolates to another by including a previously tested isolate in each batch.

Reaction of primary isolates with monoclonal antibodies

The numbers and percentages of isolates which were classified as having >50%, <50% and those in which no parasites were reactive against the 58 monoclonal antibodies are shown in Table 1. For reasons of briefly, the reaction of each isolate to each monoclonal antibody is not presented.

The results shown in Table 1 demonstrated that the population of *P. vivax* in various regions of Thailand is antigenically heterogenous. All isolates reacted to some degree with group II and group III monoclonal antibodies McPV19, McPV20, McPV21, McPV22, McPV23, McPV24, McPV25, McPV26, McPV43, and McPV49. With established isolates, these monoclonal antibodies always gave the same reaction and were thought to be directed against the same antigenic determinant. For monoclonal antibodies McPV1, McPV2, McPV3 and McPV27, 95-99% of the isolates were classified as containing >50% fluorescent schizont and merozoites. In the present study no isolate from a sample of 300 primary isolates were negative for all monoclonal antibodies. The remaining monoclonal antibodies especially group I, V and

group VII was found to be highly variable in that the monoclonal antibodies reacted with only 30% to 90% of the isolates tested.

In addition to culture supernatants, several monoclonal antibodies were tested as ascites fluid at various dilutions with a series of parasite isolates. When a culture supernatant of an antibody reacted with an isolate, the ascites fluid of that monoclonal antibodiy also reacted with that isolate up to a dildution of 1:131072. When a monoclonal antibody as culture supernatant did not react with an isolate, it failed to react even as ascites fluid at a dilution of 1:2.

Table 1. Primary isoaltes of P. vivax reacting with 58 monoclonal antibodies

Monoclonal Abtibodies	Total examined	No. and (%) isolates classified		
		50% reactive	<50% reactive	0% reactive
McPV1	300	295 (98.33)	5 (1.66)	0 (0)
McPV2	300	297 (99.00)	3 (1.0)	0 (0)
McPV3	300	299 (99.66)	1 (0.33)	0 (0)
McPV4	300	120 (40.00)	160 (53.33)	20 (6.66)
McPV5	300	270 (90.00)	30 (10.00)	0 (0)
McPV6	300	90 (30.00)	100 (33.33)	110 (36.66)
McPV7	300	274 (91.33)	26 (8.66)	0 (0)
McPV8	300	275 (91.66)	25 (8.33)	0 (0)
McPV9	300	274 (91.33)	26 (8.66)	0 (0)
McPV10	300	99 (33.00)	19 (6.33)	182 (60.66)
McPV11	300	255 (85.00)	45 (15.00)	0 (0)

Monoclonal Abtibodies	Total examined	No. and (%) isolates classified		
		50% reactive	<50% reactive	0% reactive
McPV12	300	150 (50.00)	130 (43.33)	20 (6.66)
McPV13	300	280 (93.33)	20 (6.66)	0 (0)
McPV14	300	165 (55.00)	100 (33.33)	35 (11.88)
McPV15	300	255 (85.00)	45 (15.00)	0 (0)
McPV16	300	281 (93.66)	29 (9.66)	0 (0)
McPV17	300	244 (81.33)	120 (40.00)	34 (11.33)
McPV18	300	285 (90.00)	25 (8.33)	0 (0)
McPV19	74	74 (100)	0 (0)	0 (0)
McPV20	74	74 (100)	0 (0)	0 (0)
McPV21	74	74 (100)	0 (0)	0 (0)
McPV22	74	74 (100)	0 (0)	0 (0)
McPV23	74	74 (100)	0 (0)	0 (0)
McPV24	74	74 (100)	0 (0)	0 (0)
McPV25	74	74 (100)	0 (0)	0 (0)
McPV26	74	74 (100)	0 (0)	0 (0)
McPV27	74	74 (95.94)	0 (0)	0 (0)
McPV28	74	53 (79.72)	15 (20.27)	0 (0)
McPV29	74	37 (50.00)	20 (27.07)	17 (22.97)
McPV30	74	67 (90.54)	7 (9.45)	0 (0)
McPV31	74	74 (100)	0 (0)	0 (0)
McPV32	74	44 (59.45)	22 (29.72)	8 (10.81)
McPV33	74	60 (81.08)	14 (18.91)	0 (0)
McPV34	74	63 (85.13)	11 (14.86)	0 (0)
McPV35	74	60 (81.08)	10 (13.51)	4 (5.40)
McPV36	74	67 (90.54)	7 (9.45)	0 (0)

Monoclonal Abtibodies	Total examined	No. and (%) isolates classified		
		50% reactive	<50% reactive	0% reactive
McPV37	74	70 (94.59)	4 (5.40)	0 (0)
McPV38	74	30 (40.00)	34 (45.94)	10 (13.51)
McPV39	285	241 (84.56)	40 (14.03)	4 (1.40)
McPV40	285	149 (52.28)	20 (7.01)	116 (40.70)
McPV41	285	210 (73.68)	69 (24.21)	6 (2.10)
McPV42	285	160 (56.14)	28 (9.82)	97 (34.03)
McPV43	285	172 (60.35)	38 (13.33)	75 (26.31)
McPV44	285	240 (84.21)	28 (9.82)	17 (5.96)
McPV45	285	198 (69.47)	82 (28.77)	5 (1.75)
McPV46	285	181 (63.50)	48 (16.84)	56 (19.64)
McPV47	285	191 (67.01)	45 (15.78)	49 (17.19)
McPV48	285	285 (100)	0 (0)	0 (0)
McPV49	285	285 (100)	0 (0)	0 (0)
McPV50	285	190 (66.66)	46 (16.14)	49 (17.19)
McPV51	285	171 (60.00)	64 (22.45)	50 (17.54)
McPV52	285	258 (90.52)	42 (14.73)	0 (0)
McPV53	285	186 (65.26)	49 (17.19)	40 (14.03)
McPV54	74	66 (90.00)	8 (10.81)	0 (0)
McPV55	300	122 (40.66)	49 (16.33)	129 (43.00)
McPV56	300	135 (47.36)	52 (17.33)	113 (37.66)
McPV57	300	201 (67.00)	68 (22.66)	31 (10.33)
McPV58	74	68 (91.89)	6 (8.10)	0 (0)

2. Distribution of blood stage antigen in *P. vivax* isolates tested

The association between the reaction of monoclonal antibodies on different isolates of *P. vivax* and the molecules that these monoclonal antibodies reacted is shown in Table 2.

Table 2. Distribution of blood stage antigens in *P. vivax* isolates tested

McAb[a]	Mr (10^3)	No. of positive isolates (%)
McPV1	30, 85	295 (98.33)[b]
McPV2	30, 85	297 (99.00)[b]
McPV3	30	299 (99.66)[b]
McPV4	100	120 (40.00)[b]
McPV5	30	270 (90.00)[b]
McPV6	100	90 (30.00)[b]
McPV7	30	274 (91.33)[b]
McPV8	30	275 (91.66)[b]
McPV9	30	274 (91.33)[b]
McPV10	>200	99 (33.00)[b]
McPV11	36,39,85,95,115	255 (85.00)[b]
McPV12	100,135,>200	150 (50.00)[b]
McPV13	30	280 (93.33)[b]
McPV14	135,200	165 (55.00)[b]
McPV15	35,85	255 (85.00)[b]

a = monoclonal antibodies antibodies
b = 300 isolates were initially tested
c = only 74 isolates have schizont stages
d = only 285 isolates have trophozoites and schizont stages

McAb[a]	Mr (10^3)	No. of positive isolates (%)
McPV16	100,135,>200	281 (93.66)[b]
McPV17	36,39,>200	244 (81.33)[b]
McPV18	>200	285 (90.00)[b]
McPV19	67,75	74 (100)[c]
McPV20	35,41	74 (100)[c]
McPV21	41,68,75,90	74 (100)[c]
McPV22	110,200	74 (100)[c]
McPV23	200	74 (100)[c]
McPV24	4,90,110,200	74 (100)[c]
McPV25	200	74 (100)[c]
McPV26	110	74 (100)[c]
McPV27	30,50	74 (95.94)[c]
McPV28	39	59 (79.72)[c]
McPV29	95	37 (50.00)[c]
McPV30	115	67 (90.54)[c]
McPV31	30	74 (100)[c]
McPV32	36	44 (59.45)[c]
McPV33	30,50,85	60 (81.08)[c]
McPV34	50,85	63 (85.13)[c]
McPV35	115,200	60 (81.08)[c]
McPV36	30,68,100	67 (90.54)[c]
McPV37	95	70 (94.59)[c]

a = monoclonal antibodies antibodies
b = 300 isolates were initially tested
c = only 74 isolates have schizont stages
d = only 285 isolates have trophozoites and schizont stages

McAb[a]	Mr (10^3)	No. of positive isolates (%)
McPV38	100	30 (40.00)[c]
McPV39	85,95	241 (84.56)[d]
McPV40	36	149 (52.28)[d]
McPV41	115	210 (73.68)[d]
McPV42	39	160 (56.14)[d]
McPV43	39	172 (60.35)[d]
McPV44	85,95	240 (84.21)[d]
McPV45	115	198 (69.47)[d]
McPV46	85	181 (63.50)[d]
McPV47	85	191 (67.01)[d]
McPV48	30	285 (100)[d]
McPV49	30	285 (100)[d]
McPV50	85	190 (66.66)[d]
McPV51	85	171 (60.00)[d]
McPV52	30	258 (90.52)[d]
McPV53	85	186 (65.26)[d]
McPV54	85	66 (90.00)[c]
McPV55	15,28	122 (40.66)[b]
McPV56	28	135 (47.36)[b]
McPV57	200	201 (67.00)[b]
McPV58	30,41,68	68 (91.89)[c]

a = monoclonal antibodies antibodies
b = 300 isolates were initially tested
c = only 74 isolates have schizont stages
d = only 285 isolates have trophozoites and schizont stages

3. **Calculation the frequencies of several parasite antigens in the samples**

The data are currently under analysis.

4. **Distribution of antigenic variation and geographic distribution**

The data are currently under analysis. Our preliminary results seem to show that the antigenic diversity of *P. vivax* occurs even in a relative small area in Thailand.

5. **Preparation of *P. vivax* antigens for immunofluorescent test**

Enrichment of *P. vivax* infected RBCs was achieved by using SEC-G-25-Percoll method described recently. 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 160 malaria patients were summalized in Table 3.

Table 3. Parasite yields after enrichment

Patient	Parasite count (x 10^7)		
	Upper band	Lower band	Total
PV301-PV302	1.90	4.50	6.40
PV303	ND	ND	0.50
PV304-PV307	4.50	17.00	21.50
PV308-PV309	2.50	45.00	47.50
PV310-PV312	0.90	16.00	16.90
PV313	1.80	6.90	8.70
PV314-PV316	5.70	27.00	32.70
PV317-PV320	3.30	16.00	19.30
PV321-PV323	9.30	17.00	26.30
PV324	4.90	5.90	10.80
PV325-PV326	1.50	4.60	6.10
PV327-PV329	5.20	24.00	29.20
PV330	14.00	12.00	26.00
PV331-PV332	3.60	37.00	40.60
PV333-PV337	8.50	61.00	69.50
PV338-PV341	3.00	19.00	22.00
PV342-PV343	16.00	24.00	40.00
PV344-PV347	3.80	19.00	22.80
PV348-PV349	6.80	53.00	59.80
PV350-PV351	S	S	S
PV352-PV353	2.30	18.00	20.30

*Most of the parasites are degenerated

S = smear
L = lysis
ND = not done
TB = tube was broken during experiments

Patient	Parasite count (x 10^7)		
	Upper band	Lower band	Total
PV354-PV356	0.44	0.45	0.89
PV357-PV358	2.50	12.00	14.50
PV359	24.00	3.40	27.40
PV360-PV362	1.50	7.00	8.50
PV363-PV364	2.80	13.00	15.80
PV365-PV366	2.60	17.00	19.60
PV367-PV368	S	S	S
PV369	S	S	S
PV370-PV371	S	S	S
PV372	S	S	S
PV373	S	S	S
PV374-PV377	TB	17.00	17.00
PV378-PV380	2.50	18.00	20.50
PV381-PV382	15.00	21.00	36.00
PV383-PV385	3.70	9.90	13.60
PV386-PV387	1.90	7.40	9.30
PV388-PV389	3.80	35.00	38.80
PV390-PV392	2.10	L	2.10
PV393-PV394	4.00	4.90	8.90
*PV395	4.40	3.50	7.90
PV396-PV397	3.40	3.60	7.00

*Most of the parasites are degenerated

S = smear
L = lysis
ND = not done
TB = tube was broken during experiments

6. Collection of *P. vivax* antigens from relapse cases

P. vivax infected cells obtained from relapse cases admitted to the Hospital for Tropical Diseases, Bangkok, are now being collected. 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). Thick smears on clean glass slides were made from these concentrated parasites, 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.

Conclusion

Reactivities of a panel of 58 monoclonal antibodies with 300 different *P. vivax* isolates obtained from patients from 42 provinces in Thailand including one isolate from Burma revelaed a high level of antigenic diversity of this parasite. About 14 monoclonal antibodies reacted with epitopes that were represented in more than 95% of the parasite isolates, and therefore, appeared to be relatively conserved among different isolates. Most of the strain specific determinants identified in this work were associated with the merozoites or their surface. It will be of interest to ascertain the function of these antigens in protective immunity. The remaining monoclonal antibodies reacted with only 30% to 90% of parasite isolates.

A given IFA patterns could not be related to a given geographical origin of the parasite However it seems that antigenic diversity of *P. vivax* could occur in some area in

Thailand. However, a better information of the relationship between the antigenic difference and the geographic distribution of some of these specificities among the *P. vivax* isolates is now being investigated. The antigens from relapse cases of *P. vivax* are now being collected for further investigation whether there is any antigenic disparity between parasites causing initial clinical symptoms and these causing recrudescence.