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SUMMARY

Knowledge of antigenic diversity among populations of malaria parasites has important implications to understand the immune evasion mechanism of the parasites and for vaccine research. A panel of 58 monoclonal antibodies (McAbs) against asexual erythrocytic stages of Plasmodium vivax was established and used to demonstrate antigenic diversity of this species. At least 35 different antigenic polypeptides of P. vivax reacted with these McAbs were characterized by sodium dodecylsulphate polyacrylamide gel electrophoresis and Western blot analysis. The possible location of these antigens as well as their stage and species specificity were determined. Based on the indirect fluorescent antibody (IFA) staining patterns produced, these McAbs could be classified into five groups. Group I McAbs gave bright, generalized staining of all blood stages and reacted with proteins of 30 kd, 36kd, 85 kd, 95 kd, 100 kd, 115 kd, 135 kd, 200 kd and >200 kd. Group II McAbs reacted strongly with merozoites or their organelles and were specific for 35 kd, 41 kd, 75 kd, 90 kd, 110 kd and 200 kd proteins. Group III McAbs reacted with 85 kd, 195 kd and 200 kd molecules on the surface of merozoites and their reactions to the intraschizont merozoites took a form of a cluster of grapes. Group V McAbs reacted with the proteins of Mr 39 kd, 56 kd, 95 kd and 115 kd on the surface of trophozoites and schizonts. Group VII McAbs reacted with 30 kd, 85 kd and 95 kd proteins of the internal components of the

parasites. No cross-reactivity of these anti-P. vivax McAbs with asexual blood stages of Plasmodium falciparum was found. Cross reactivity against Plasmodium malariae or Plasmodium ovale has not been determined, mainly because of the rarity of infection with these two species in Thailand.

Immunoflourescence performed with 58 McAbs on 606 different isolates obtained from patients revealed a high rate of antigenic diversity of this parasite. About 16 McAbs of different IFA patterns reacted with epitopes that were represented in more than 95% of the parasite isolates, and therefore, appeared to be relatively conserved among different isolates. The remaining McAbs especially those of groups I, V and VII were shown to be highly variable in that their IFA reactivities varied from 30% to 90% of the isolates tested. It should be brought to attention that none of 606 primary isolates was negative for all McAbs tested.

The reactions of McAbs on different isolates of P. vivax were shown to be related to the molecules to which these recognized. Two major proteins (Pv30 and Pv80) with apparent molecular weight of 30 kd and 85 kd respectively were recognized by McAbs of Group I. These molecules were located throughout the cytoplasm of the parasites and were present in all blood stages in almost all isolates (>90%) of the parasites tested. Thus, they may represent common antigens of this parasite species and as a consequence may prove to be of value in the development of immunodiagnostic test for this parasitic infection. The

protein, Pv200, which migrated as a major band close to 200 kd was strongly expressed in merozoites and recognized by several McAbs of group II in all isolates tested (100%) and might therefore represent a common antigen in P. vivax merozoites. In addition, McAbs against Pv200 and Pv195 antigens on the surface membrane of merozoites has also indicated limited polymorphism (reacted with 90-98% of the isolates tested). The Pv115 and Pv95 antigens recognized by group V McAbs (84.6-92.3% of the isolates tested) have been evolutionally highly conserved and appeared to be associated with caveola-vesicular complexes. The Pv30 antigen recognized by group VII McAbs (69.4-100% of the isolates tested) and associated with internal components of trophozoites has been evolutionally conserved while Pv85 and Pv95 antigens recognized by the same group of McAbs has been variable.

The distribution of antigenically-diversed parasites and their geographical location was determined in 466 and 6 P. vivax isolates originated from 49 provinces in Thailand and Burma, respectively. Antigenic diversity was shown to vary from place to place and was high in the areas with high rate of transmission along the Burmese border in the west and along the Kumpuchean border in the east. The highest rates of diversity were observed in Trad (48.4%), Tak (41.7%), Chantaburi (36.5%) and Maehengsorn (36.4%) where the highest malaria incidence was detected. These areas have been known to be major foci of transmission as well as foci of P. falciparum multidrug resistances. High rate of diversity was likely to occur in some areas in the South

especially in Krabi, Ranong and Suratthani but the result has to be interpreted with caution because of the small number of P. vivax isolates tested. The lower rates of diversity (<20%) were observed in Chumporn (15.4%) Chiengrai (12.5%), Ubonrajthani (9.1%) and Chachoengsao (5.3%). The populations in these areas were perhaps more static than those in the areas with high transmission.

The antigenic disparity of P. vivax parasites causing initial clinical symptoms and those causing relapses were also determined. In this study, the initial and relapse strains from three out of five patients (R3, and R4 and R5) exhibited similar reactivities with all McAbs tested. In contrast, the initial and relapse isolates from the other two relapse cases (R1 ad R2) showed different pattern of reactivity. The observed difference could be attributable to the multiplicity of mosquito bites and the immune evasion mechanism of the parasites. The parasites in patients received multiple mosquito bites would be more heterogeneous and so would be the hypnozoites causing relapses. It was also speculated that under immune pressure, the parasites would generate antigenically diverse strain which would be different from the strain responsible for the initial infection.

The demonstration of diverse and conserved antigens of P. vivax will significantly contribute to the future development of vivax malaria vaccine as well as in the development of the immunological assay to detect low grade parasites. The vaccine development as well as the McAbs to be selected for use in the

immunoassay should be directed towards the conserved proteins of the parasites.

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INTRODUCTION

Malaria remains prevalent throughout tropical and sub-tropical areas and almost one-third of the world's population is exposed to the risk of infection. The number of estimated malaria cases is 150 millions per year. The mortality and morbidity directly attributable to malaria constitute major constraints on economic progress in the tropical and subtropical area of the world (1). Failure of global eradication is attributable primarily to the practical difficulties of implementing an appropriate programme in countries that have relatively lack of public health facilities. These practical problems have been compounded by the spread of insecticide resistance in mosquitoes, by alterations in vector behaviour and expecially by the widespread appearance of chloroquine resistant strains of malaria parasites (2).

Another factor which could contribute to the maintenance of malaria transmission in the endemic areas is the carrier. Such carriers can be found in both Plasmodium falciparum and Plasmodium vivax infection. Vivax primary attacks, if untreated, may last for three weeks to months or longer. As the attack wanes, paroxysms may become less severe and are irregular in periodicity. In perhaps half of all cases, relapses occur following a period of weeks, months or even years without

symptoms causing a latent infection and persisting low grade parasitaemia. This asymptomatic state may have following untowards effects including maintenance of transmission in the endemic area, and development of recrudescence when the patients becomes debilitated, perhaps from intercurrent infection or immunosuppression. Besides, if at any time blood from such a patient is used for transfusion, the recipient will unfortunately experience a typical attack of quartan malaria (3).

At present, little is known about the nature of the relapse. One theory is that malaria parasites occur as two populations, one developing in the liver to produce merozoites which invade the blood after the normal prepatent period of 7-8 days, and the remaining the parenchymal cells for periods in a form of arrested development, the so called "hypnozoites", which ultimately complete their development and cause relapses (4). It is not clear whether these two forms are antigenically different, and if this is the case, it should be possible to distinguish between these strains by typing with a panel of McAb the stains causing initial parasitaemia and the strains causing recrudescence.

OVE' ALL OBJECTIVES

1. To raise and characterize McAb against blood stages of P. vivax.

2. To determine whether there is antigenic diversity among different strains of P. vivax using an indirect immunofluorescent antibody test and a panel of McAb.

3. To investigate whether distribution of different antigenic types is related to their geographical locations.

4. To demonstrate whether there is any antigenic disparity between parasites causing initial clinical symptoms and those causing recrudescence.

If it is shown that antigenic diversity of P. vivax does exist, and their antigenic types vary according to geographical locations, such findings will have following contributions:-

1. If a vaccine is to be developed in the future possibly through a recombinant DNA technology, the vaccine should be active against all invariants. In other words, the gene(s) to be cloned should encode for the invariant "protective" antigens and not against variant antigens.

2. A panel of McAb (McAbs) can be established for strain typing, and this will be useful for strain characterization especially in relation to their biological features including drug sensitivity, ability to grow in animal hosts particularly in the squirrel monkeys. Strain typing will also be useful for the

epidemiological studies of vivax malaria.

3. The success of a search for McAb reacting with invariant antigen(s) will help in the development of a highly sensitive technique such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) for the detection of a low grade parasitaemia in carriers or in patients after treatment with anti-malarial drugs. Such approach will help in predicting recrudescence before the parasites appear in the peripheral blood, so that appropriate treatment can be given even before the development of clinical recrudescent malaria.

4. Monoclonal antibodies which have properties to kill or inactivate the parasites in vitro (either by working alone or working in combination with other effector cells such as natural killer cells, K cells, macrophages or polymorphonuclear leucocytes) will help in the identification of "protective" antigen(s). This information is highly valuable in the development of a vaccine against vivax malaria.

MATERIALS AND METHODS

1. Parasites

1.1 *P. vivax*

P. vivax asexual-stage parasites were obtained from both infected individuals admitted to the Hospital for Tropical Diseases, Bangkok, Thailand (TMPV 051 - TMPV 540) and individuals who visited the outpatient clinic of the Malaria Eradication Center Unit 2 of the Ministry of Public Health (CMPV 001 - CMPV 116) during June 1985 and December 1988.

The numbe of isolates and their origins were shown in table 1. Bloo . ected in heparinized tubes (15 IU heparin/ml) were either processed immediately or stored overnight at 4°C. Thick and thin blood films were made and kept at -70°C until use.

Table 1. *P. vivax* isolates and their origins

Source	No. of isolates
1. Kanjanaburi	86
2. Prachinburi	66
3. Chiengmai	55
4. Chanthaburi	52
5. Trad	31
6. Tak	24
7. Maehongsorn	22
8. Chachoengsao	19
9. Chiengrai	16
10. Chumporn	13
11. Rajburi	11
12. Ubonrajthani	11
13. Surat Thani	9
14. Chonburi	9
15. Rayong	6
16. Nakhon Ratchasima	6
17. Nakhorn Nayck	5
18. Prachuap Khiri Khan	5
19. Phetburi	5
20. Buriram	5
21. Suphanburi	4
22. Nan	3
23. Phitsanulok	3

Table 1. (cont.)

Source	No. of isolates
24. Yala	3
25. Krabi	3
26. Udornthani	2
27. Nakhornsawan	2
28. Samutprakarn	2
29. Ranong	2
30. Sakolnakorn	2
31. Nongkhai	1
32. Samutsakorn	1
33. Srisakate	1
34. Aranyaprathade	1
35. Uthaithani	1
36. Phetchabun	1
37. Lampang	1
38. Chaiyapum	1
39. Lopburi	1
40. Narathiwat	1
41. Uttraradit	1
42. Lamphun	1
43. Pranburi	1
44. Mahasarakam	1
45. Angthong	1
46. [illegible]korn Srithammarat	1

Table 1. (cont.)

Source	No. of isolates
47. Trang	1
48. Surin	1
49. Leue	1
50. Burma	6
51. No exact origins	99

1.2 *P. falciparum*

Eleven isolates of *P. falciparum* were obtained from acutely infected individuals and those with cerebral malaria. These isolates were cryopreserved in our laboratory since 1980 as shown in table 2.

Table 2. Eleven isolates of *P. falciparum*

Isolate	Clinical symptoms	Date obtained
SO	acute malaria	February 18, 1980
H5	acute malaria	June 28, 1983
H11	acute malaria	November 7, 1983
K1	acute malaria	July 7, 1983
BW2	acute malaria	-
SN3-5	acute malaria	-
I1	cerebral malaria	November 7, 1983
R5	cerebral malaria	July 8, 1983
R1	cerebral malaria	May 5, 1983
R6	cerebral malaria	July 11, 1983
QD7	cerebral malaria	November 8, 1983

The cryopreserved parasites was thawed out immediately at 37°C for 30 sec. The parasites were centrifuged at 500 x g for 5 min at room temperature and the supernatant was discarded. The cell sediment was washed with an equal volume of saline solution (3.5% NaCl) and then washed at least twice with RPMI 1640 medium. The parasites were recultured by adding washed uninfected AB erythrocytes and RPMI-1640 containing 10% AB serum to obtain a final concentration of 5% haematocrit, 0.1% parasitaemia and then incubated in a candle jar at 37°C (5).

2. *P. vivax* antigen preparation

Two methods of enrichment comprising CF-Percoll and SEC-G25-Percoll methods were established.

2.1 CF-Percoll method

Twenty ml of heparinized blood from patients was first centrifuged at 500xg at 4°C for 7 min, the plasma removed and the cell sediment resuspended to its original volume with RPMI-1640 containing 10% AB serum. The blood cell suspension was passed through a column of five ml of Whatman CF-11 cellulose (W & R Balston Ltd., England) packed in a 20 ml-syringe column plugged at the outlet with nylon wool (LP-1 Leuko-Pak Leukocyte filter, Fenwal Laboratories, Illinois, U.S.A.) and was pre-equilibrated with warm RPMI-1640 medium with 10% AB serum. The column was then washed with warm, serum-free RPMI-1640, the eluate was

collected and subjected to centrifugation at 500xg at 4°C for 10 min. The supernate was then removed and two ml of packed cells was mixed in a 16x150 mm screw-capped tube with three ml of 90% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) in phosphate buffered saline pH 7.2 (PBS), above which a successive layers of three ml each of 75% Percoll, 60% and two ml of PBS were made. The tubes were centrifuged at 250xg for 5 min and at 1,000xg for another 10 min. Bands of cells were seen, each of which was harvested, washed once with PBS, resuspended in the same solution and their numbers counted in a haemacytometer. Thereafter the cell suspension was centrifuged, the sediment was smeared on a glass slide, stained with Giemsa and the number of parasites counted with a light microscope.

2.2 SEC-G25-Percoll Method

One ml aliquot of heparinized whole blood was passed through a column of 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 red blood cells (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 thrid 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.

3. *In vitro* culture of *P. vivax*

Human blood infected with *P. vivax* was collected from 10 patients admitted to the Hospital for Tropical Diseases, Bangkok. Five ml of each heparinized sample was obtained. Thick and thin blood films were made, stained with Giemsa and examined for stages of parasites.

3.1 Culture medium

Two types of culture medium were used: TMMI 900B prepared by supplementing of reconstituted RPMI-1640 as described by Trager and Jensen (5) with 0.55 M $CaCl_2$, 0.0018 M of $CaCl_2$ and $5x10^{-2}$ mM hypoxanthine, SCMI 612 prepared by mixing five different sterile solutions as described by Brockleman *et al*. (6) as follows: to each 90 ml of balanced salt solution, 1 ml of vitamin solution, 6 ml of amino acid mixture, and 1 ml of hypoxanthine solution were added. Osmolarity of the medium was

adjusted by adding 0.2 ml of 3.5% NaCl solution. Prior to use, 4.2 ml of 5% $NaHCO_3$ solution and 15 ml of human serum group AB were added to make complete SCMI medium with serum.

3.2 Culture method

The blood was centrifuged at 400xg at 4°C for 10 min. After the plasma and the buffy coat were removed, the cells were resuspended with 2 volumes of RPMI medium distributed in three ml portion into two tubes and recentrifuged for 5 min. The RPMI medium was removed and the erythrocytes in each of the two tubes were washed twice by centrifugation, each time with TMMI900B (culture A) or SCMI612 (culture B) media. After the final washing, an equal volume of the corresponding culture medium, supplemented with 15% human AB serum, was added to the packed erythrocytes in each tube to make culture materials consisting of 50% erythrocytes. This starting materials was further diluted with the corresponding culture medium (1:6) (v/v) and dispensed into 4 culture dishes (15 mm diameter) and incubated in a candle jar at 37°C for 48 h, unless otherwise specified. Culture media were changed manually at 44 h and every 20 h thereafter (6).

3.3 Evaluation

Thick and thin films were made from the starting materials (time 0) and after 24, 48, 72 h until 216 h of incubation starting. They were stained with Giemsa and examined microscopically for morphologic transformation of the parasites. Productivity of cultures was recorded by differentially counting

the number and stages of parasites per 10,000 erythrocytes on a thin film. The effects of media on parasite growth were evaluated by determining the percentage of parasites in the mature schizont stage (having 12-16 merozoites) among 200 counted.

4. Indirect immunofluorescent assay (IFA)

The indirect immunofluorescence using acetone-fixed infected blood as antigen was done according to the technique previously described (7). Smears were made from either washed infected blood of patients with P. vivax or purified parasites (ring, trophozoites, schizonts, gametocytes) and from washed, asynchronously grown P. vivax mostly in schizont and late trophozoites. The smears were air dried, 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 McAb 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.

Smears made from asynchronously culture of P. falciparum

using the method of Trager and Jensen (5) were used to investigate the species cross-reactivity.

5. Murine monoclonal antibody production

5.1 Immunization of mice

The eight week old BALB/c mice were immunized with three intraperitoneal injections of washed $5x10^7$ - $1x10^8$ parasites composing of ring and trophozoites of P. vivax emulsified in an equal volume of Freund's complete adjuvant initially and incomplete adjuvant subsequently at an interval of 1-2 weeks. A week after the third immunization, the antibody responses were evaluated by indirect immunofluorescent test (IFA) as described previously. Because of the initial low antibody titer, the mice were again boostered with two to three other intraperitoneal injections of the same amount of parasites in the presence of an equal volume of Freund's incomplete adjuvant at the interval of 1-2 weeks. Antibody responses were assayed again one week later in order to choose the best responder as the immune spleen cell donor for fusion experiments. Three days prior to fusion, the best responder was boostered intraperitoneally with $1x10^8$ washed parasites in physiological saline.

5.2 Myeloma cells

The immunoglobulin non-producer myeloma cell line, Sp2/0 was used. The cells were grown in RPMI-1640 medium (Flow

Laboratories) supplemented with 10% heat inactivated foetal bovine serum (FBS) (Flow Laboratories), 15 mM HEPES buffer (Sigma, U.S.A.) and $6x10^{-3}$ mM 8-azaguinine (8-Ag) (Sigma, U.S.A.) to select against any HGPRT+ revertant myeloma cells. Cell viability determined at the time of fusion by the trypan blue exclusion test was more than 95 per cent.

5.3 Hybridoma formation and selection

The general method of Kohler and Milstein was used (8). Three days after the last immunization, the mice were sacrified and the spleen was removed. Spleen cells were fused with Sp2/0 myeloma cells with spleen cell to myeloma ratio of 10:1 using polyethylene glycol (PEG, Sigma) with the molecular weight of 4,000 according to the technique of Galfre and Milstein (9). After a brief PEG treatment (90 seconds), the cell suspension was diluted with warm RPMI-1640 without serum followed by centrifugation at 200xg for 10 min at room temperature. The supernatant was then removed and the cell sediment resuspended in a RPMI-1640 that contained 20% fetal calf serum, 15 mM HEPES, $5x10^{-2}$ mM hypoxanthine (Sigma), $1x10^{-2}$ mM azaserine (Sigma), 100 units/ml of Penicillin (Dumex), 100 ug/ml of Streptomycin (Dumex), 100 ug/ml of Kanamycin, 30% glucose and 100 mM pyruvate (selection medium) so that the final cell concentration was $1.0x10^{6}$/ml. Two hundred and twenty ul of the cell suspension was then dispensed into each well of 96 well tissue culture plates (Costar, Mass., U.S.A.) and the cells were incubated at 37°C in

5% CO_2 in air in a humidified CO_2 incubator.

After 7-10 days, the wells were examined for hybridomas. Supernatants from the culture of growing hybridomas were removed and tested for anti-malarial antibody using the IFA. Cell populations positive for P. vivax antibody were then expanded by transferring into 24 well tissue culture plates intially and tissue culture bottles subsequently (Costar, U.S.A.) and further incubated. All positive were further subcloned twice by the limiting dilution method, followed by further propagation and the clone product analyzed by the IFA test. Positive clones will be cryopreserved in liquid nitrogen until further use.

5.4 Large scale production of McAb in mice

Since large amounts of McAb were needed for the later experiments, hybridomas were grown in ascites form in histocompatible mice to allow production of much higher concentration of McAb than culture supernatants. Adults BALB/c mice were injected intraperitoneally with 0.5 ml Pristane (2,6,10,10-titramethyl pentadecane, Aldrich Chemical Company, Milwaukee, WI) 7-15 days prior to injection of hybridoma clones. The hybridoma clones were cultured in mid-log phase in RPMI-1640 supplemented with 10% heat inactivated FBS. The healthiest cultures were selected and washed with serum free RPMI medium to minimize the serum concentration. Finally, the cell density was then adjusted such that 0.5 ml contained 5.0×10^6 - 2×10^7 viable cells. Two to four pristane-primed mice were injected

intraperitoneally with each individual clone. Ascites formation usually was evident 2-4 weeks after injection. The injected mice were observed daily starting 1 week after injection. When mice showed apparent abdominal distension, the ascites fluid was collected and centrifuged at 300xg for 10 min to remove the ascites cells. The ascites from each technique of the same or different individual mice were kept separately at -20°C. The IFA was performed to confirm the specificity and the titer of McAb.

6. Characterization of McAb

6.1 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 (10). 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 min 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 um nitrocellulose membrane (Bio-Rad Laboratories,

California, U.S.A.) according to the method of Towbin et al. (11). 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 x 10^5 cpm/ml in PBS-BSA) at 26° C for 30 min 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.

6.2 Dot immunobinding assay

A technique modified from Beyer (12) 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 ul of McAb 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 Chemical, St. Louis, Missouri). After washing in TBS, the strips were transferred to a solution of 4-chloro-1-naphthol (Sigma, USA) 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/DISCUSSIONS

Enrichment of P. vivax-infected red blood cells

Enrichment of P. vivax infected red blood cells was achieved in the beginning of the present study using CF-Percoll and SEC-G-25-Percoll methods. Compared results of these two methods can be found in the reprint of the paper entitled "Enrichment of Plasmodium vivax-infected red blood cells from patients with vivax malaria" enclosed herewith.

Though the magnitude of parasite enrichment was not much different (Fig. 1A, B, and C), the SEC G-25-Percoll method (Fig. 2A, B, C, D) was superior to the CF-Percoll method (Fig. 3A, B, C) for two reasons: it had higher recovery rate (Fig. 4A, B) and was least contaminated with leukocytes (Fig. 5A, B). The lower recovery rate with the CF Percoll method was partly due to the retention of infected red blood cell to the CF-11 column and this could be eluted with buffer containing EDTA (13). This method of elution is unlikely to reduce the high rate of leucocyte contamination. Though our recovery rate with the SEC-G-25-Percoll appears at first glance to be lower than that reported by Andrysiak et al. (14). This is likely to be deceptive. It should be pointed out that only buffy coat-deprived blood samples were used in the Andrysiak's method, and some parasitized infected cells localized near the buffy coat layer could be inevitably removed resulting in the initial loss of infected red

blood cells prior to the Percoll gradient centrifugation. It has not been assessed as to how much the parasite loss would be, and if this initial loss is taken into account, the recovery rate with Andrysiak's technique would probably be comparable to our SEC-G-25-Percoll method. With respect to leucocyte contamination, the SEC-G-25-Percoll method appears to be superior to that of Andrysiak et al. (14) with the average of 20 and <2 parasitized cells per 10^4 red blood cells with Andrysiak's and SEC-G-25-Percoll method respectively. The SEC-G-25 Percoll method is therefore used for parasite antigen preparation throughout the studies.

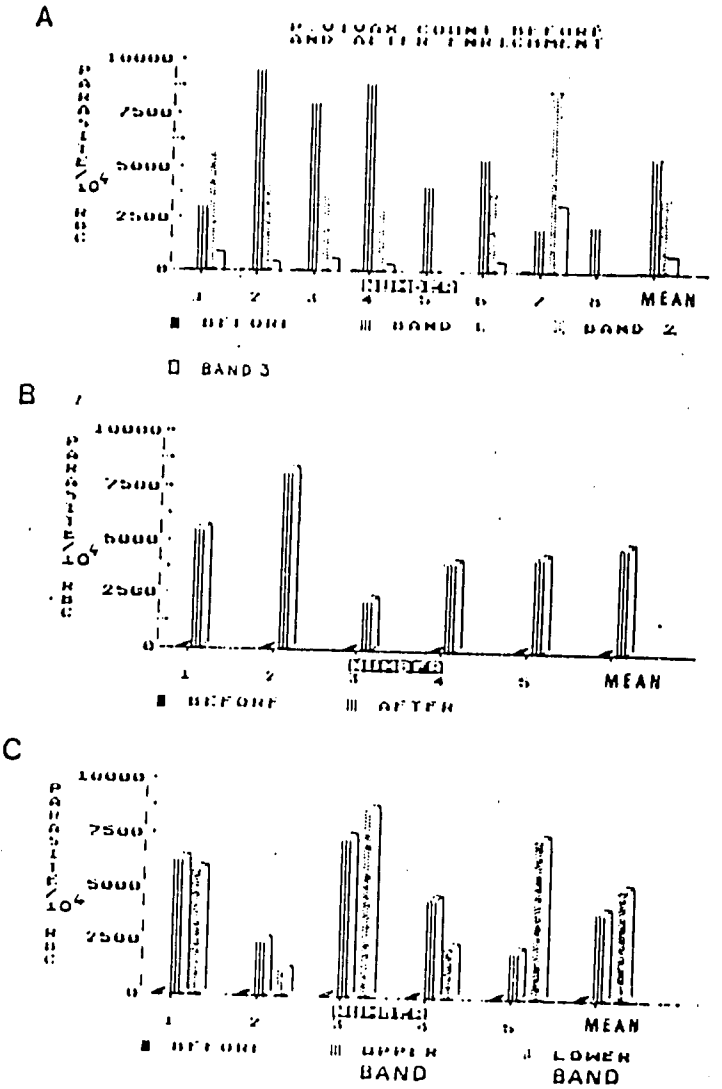
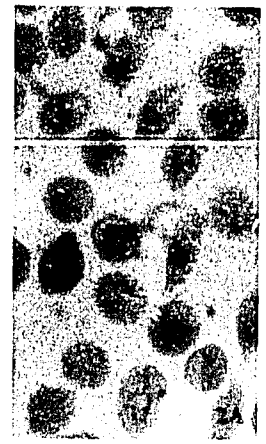


Fig. 1 Concentration of Plasmodium vivax-infected red blood cells from peripheral blood of patients with vivax malaria using a CF-Percoll method (A) and a SEC-G-25-Percoll method in which the upper and lower bands were pooled (B) and separated (C). Each column represents one patient.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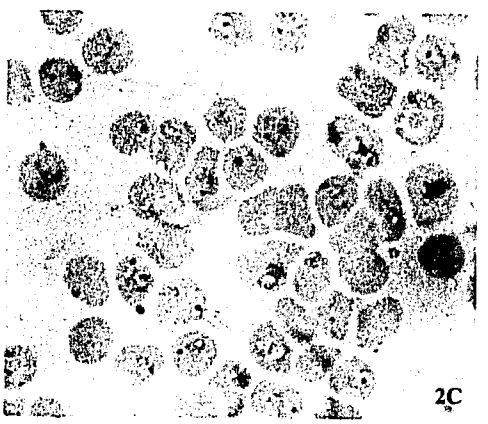
3B

3A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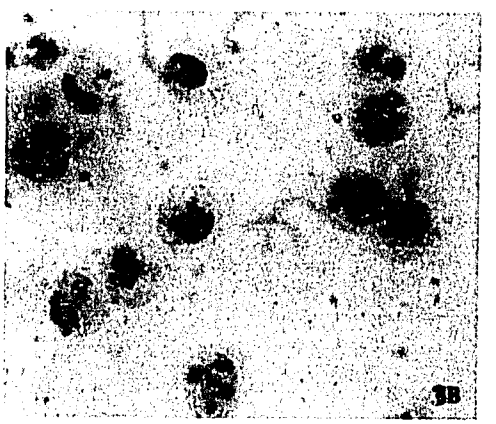
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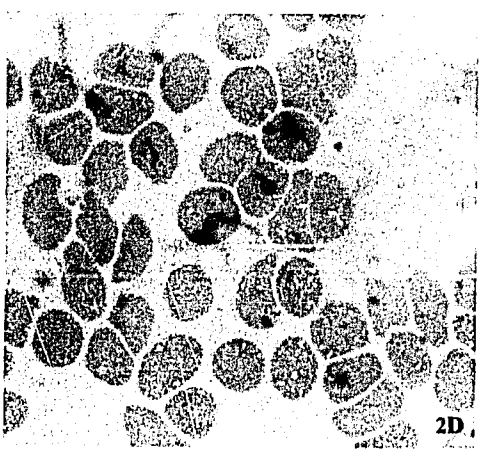
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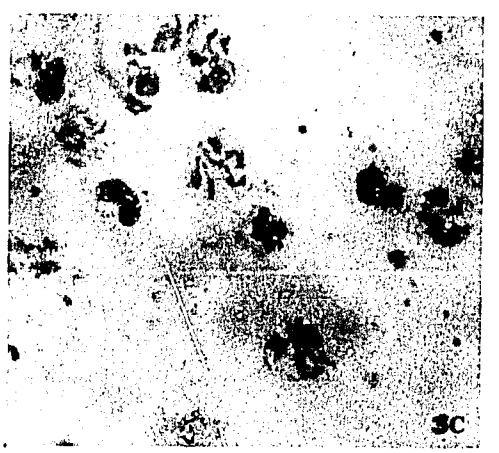

3C

Fig. 2 Photographs of Giemsa-stained Plasmodium vivax-infected red blood cells. 2A. Cells from peripheral blood before passing though CF-11 column. 2B. Cells from Band 1 comprising a mixture of trophozoites, schizonts, rings and contaminating lymphocytes. 2C. Parasited cells from Band 2 composing predominantly of ring stages. 2D. Cells from Band 3 comprising almost entirely with ring forms but with lower percentage of parasitised cells.

Fig. 3 Photographs of Giemsa-stained parasitized red blood cells concentrated by a SEC-G-25-Percoll method. 3A. Before passage to a mixture of sulfoethyl cellulose and Sephadex G-25 column. 3B. Cells from the "upper band" showing mostly growing trophozoites. 3C. Cells from the "lower band" showing growing trophozoites and rings, but the trophozoites were less mature than those in the upper band.

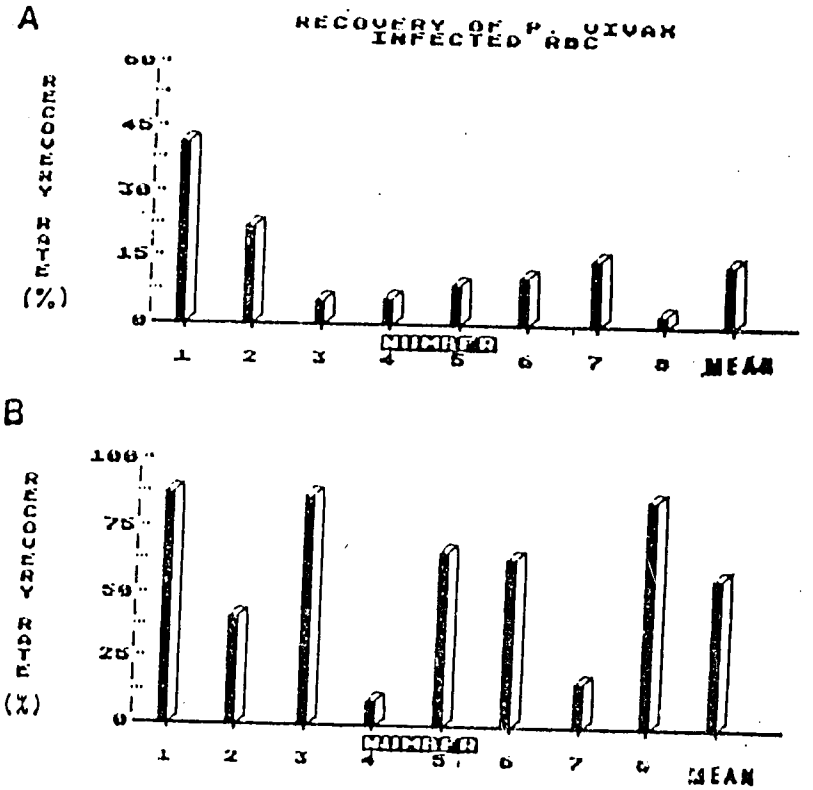


Fig. 4 Recovery rate of Plasmodium vivax-infected red blood cells from peripheral blood of patients with vivax malaria using a CF-Percoll method (eight patients, fig. 4A) and a SEC-G-25-Percoll method (eight patients, fig. 4B).

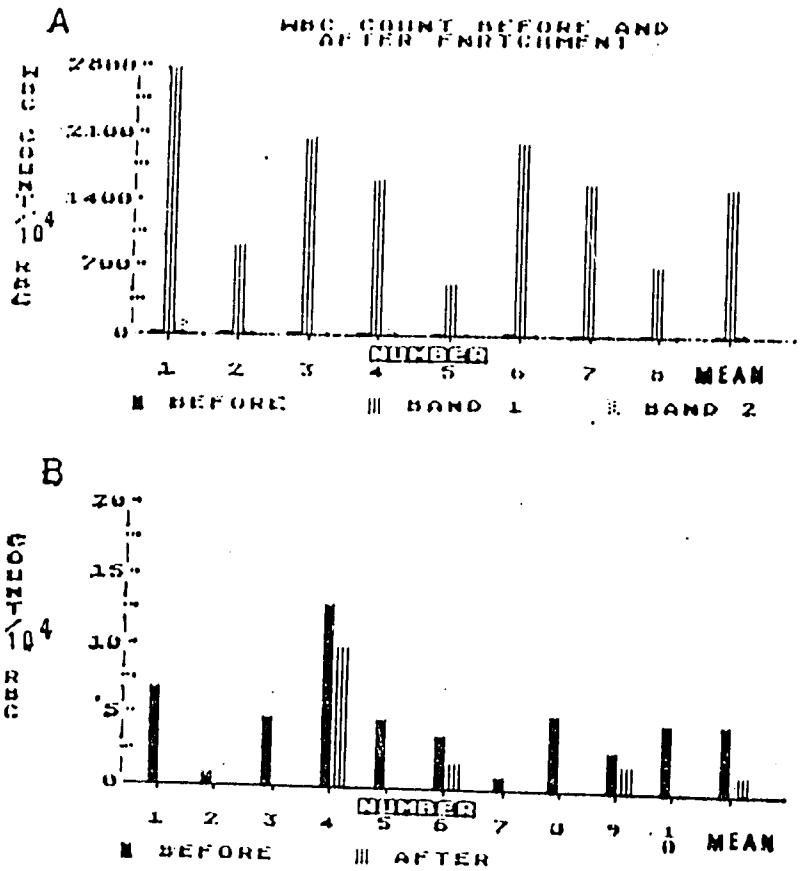


Fig. 5 Leucocyte contamination before and after enrichment with CF-Percoll method (A) and with SEC-G-25-Percoll method (B).

In vitro culture of *P. vivax*

The continuous supply of *P. vivax* infected red blood cells is most needed in this study to be used in the monoclonal antibody production and for their screening and characterization especially by the immunofluorescent and immunoblot technique. Though the continuous *in vitro* culture technique for *P. vivax* has not been established, short term cultivation has been successfully developed in several laboratories. In Thailand, Brockelman *et al*. (6) have recently devised a new culture medium so called SCMI 612 in which as high as 99% of ring-infected red blood cells developed synchronously to segmenters. With this lead, we therefore attempted to culture this parasites using initially the SCMI 612 medium. We shall also attempt to divise other culture medium, the TMMI 900B by supplementing the RPMI medium with high salt concentration particularly for $MgCl_2$ and $CaCl_2$ which yielded a large population of healthy parasites (15) and hypoxanthine which is preferentially incorporated by the material parasite for use in the biosynthesis of nucleic acid (16).

In ten patients in whom the culture in SCMI 612 and TMMI 900B have been attempted, it was found that in 8 patients the parasites developed up to day 6 after completing only one cycle of the *in vitro* growth and then started to die on day 9 (table 3). Within 48 hours of incubation the number of mature schizonts containing 12-24 merozoites. In two other parasites (TMPV045 and

TMPV046) the parasites had maturation arrested and died on day 2 and day 4 respectively. Cultivation of P. vivax isolate in SCMI 162 and TMMI 900B revealed that schizogony was most successful in both medium (Fig. 6).

However, reinvasion of merozoites in all media was poor, and the cultures died off after day 7 to day 9, despite careful subculturing on days 4 or day 6 by adding fresh, non-infected erythrocytes. This failure was possibly due to the specific requirement of P. vivax for immature erythrocytes (17), which normally constitute only 1-2% of the total erythrocyte population.

Table 3. Growth of *P. vivax* parasites examined at different days of culture in SCMI 612 medium

Isolates	Number of parasites per 10,000 erythrocytes examined at days							
	d1	d2	d4	d5	d6	d7	d8	d9
TMPV044	30	30	60	-	70	18	2	die
TMPV056	30	35	70	-	80	-	10	die
TMPV057	30	80	112	98	96	-	20	2
TMPV058	54	54	88	122	86	-	32	4
TMPV059	25	50	112	130	95	52	24	die
TMPV060	20	46	118	198	108	68	27	9
TMPV061	20	20	74	136	92	48	10	die
TMPV064	80	99	225	161	102	63	31	10
PV045	70	40	10	2	die	die	die	die
PV046	10	2	die	die	die	die	die	die

d1 = arrival of the blood specimens

d2 = 24 h *in vitro* cultivation

d3 = 48 h *in vitro* cultivation

d4 = 72 h *in vitro* cultivation

d5 = 96 h *in vitro* cultivation

d6 = 120 h *in vitro* cultivation

d7 = 144 h *in vitro* cultivation

d8 = 168 h *in vitro* cultivation

d9 = 192 h *in vitro* cultivation

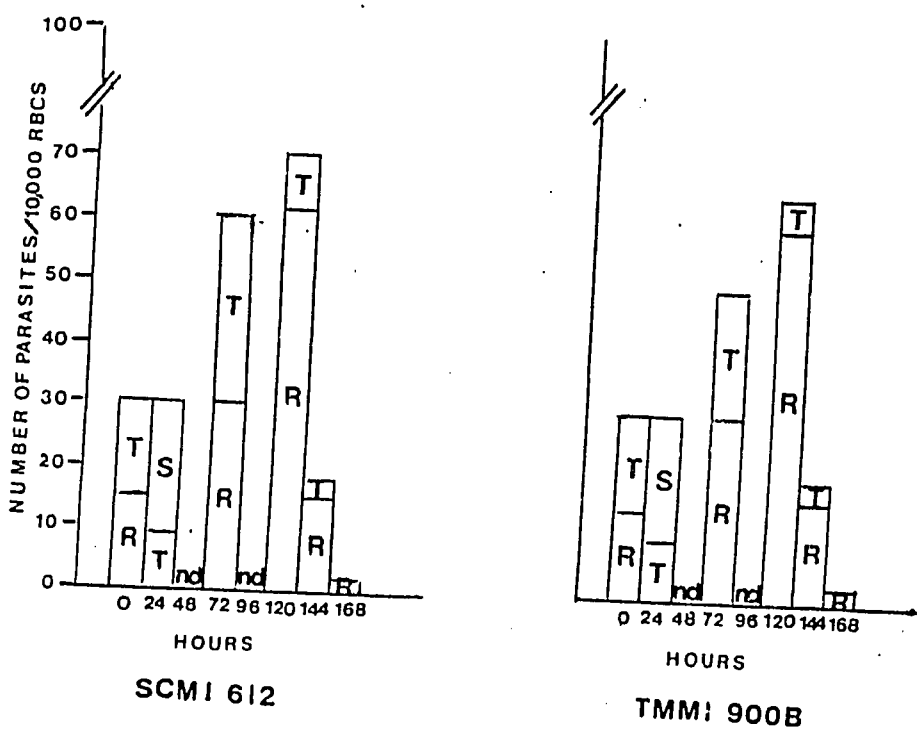


Fig. 6 Development of P. vivax, isolate PV044, in two types of medium (SCMI 612, TMMI 900B) examined at 0, 24, 48, 72, 96, 120, 144, and 168 h in vitro cultivation. Histograms show number of in vitro per 10,000 erythrocytes. Abbreviation: R, ring; nd, not done; T, trophozoite; S, schizont

Patterns of immunofluorescence

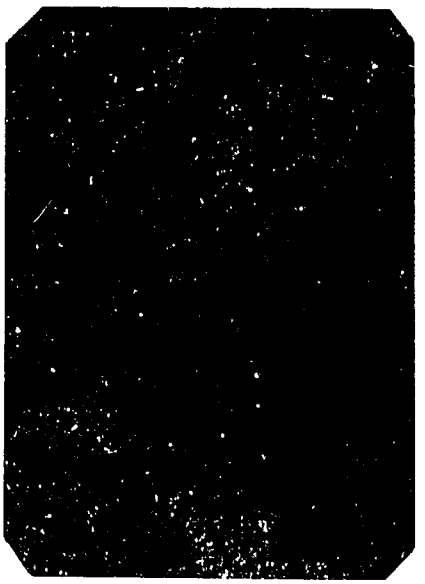
Hybridoma produced from splenic lymphocytes of BALB/c mice immunized with a schizont-merozoite antigens were screened by indirect immunofluorescence (IFA) to detect those producing antimalarial antibody. In three fusions using various isolates of P. vivax from different geographical locations in Thailand, 1175 hybrid cultures were obtained of which 494 secreted antiplasmodial antibodies. Since the hybridomas were raised against a mixture of development stages of the parasite, the antibodies produced by them were expected to recognize a range of different antigens.

A number of different indirect immunofluorescence assay (IFA) reactivity patterns were distinguished during the primary screening, and 155 hybrids producing antibodies representing five patterns were successfully cloned (Table 4). The pattern produced by any given antibody was the same on all parasite isolates which reacted with that McAb regardless of whether culture supernatants or ascitic fluids were used.

Based on the immunofluorescence reactivities demonstrated with P. falciparum (7). Five staining patterns were recognized and the antibodies were classified into five corresponding groups (Table 4, Fig. 7). These characteristic IFA patterns are indicative of stage specificity of the IFA reaction and possible location of the reactive antigen(s) either on the parasite or on or in the infected erythrocytes.

Table 4. Monoclones and their IFA staining patterns from three fusions

Group	Staining patterns	Number of monoclones
I	Bright generalized staining of all blood stages	67
II	Strong reactions with merozoites as well as organelles	21
III	Strong reactions with the surface membranes of merozoites with cluster of grape appearance	5
V	Strong reactions with the surface of schizont and trophozoites	16
VII	Strong irregular staining of internal components of trophozoites	46



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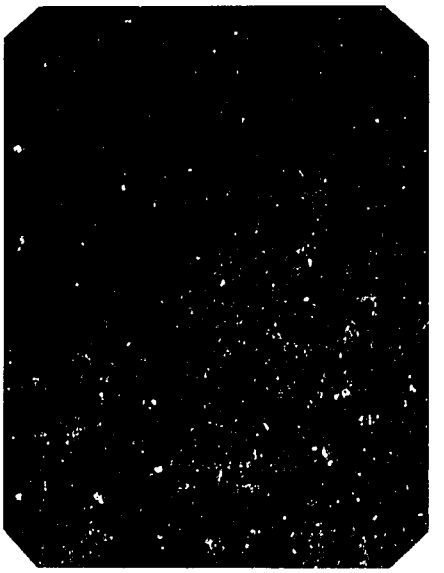

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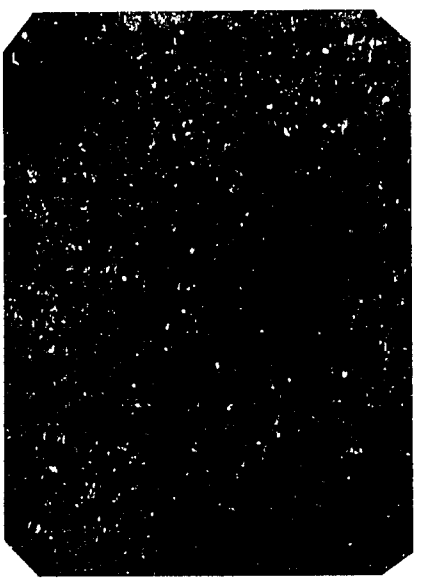

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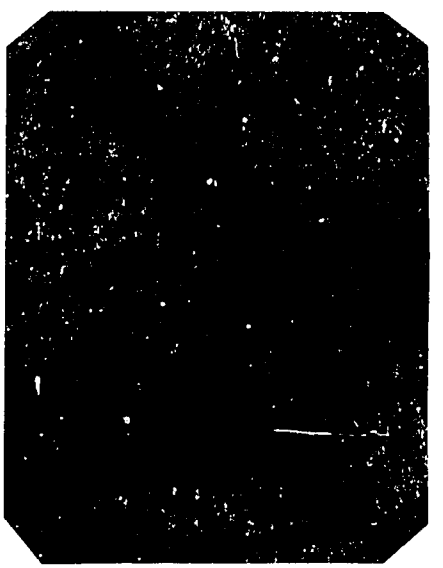

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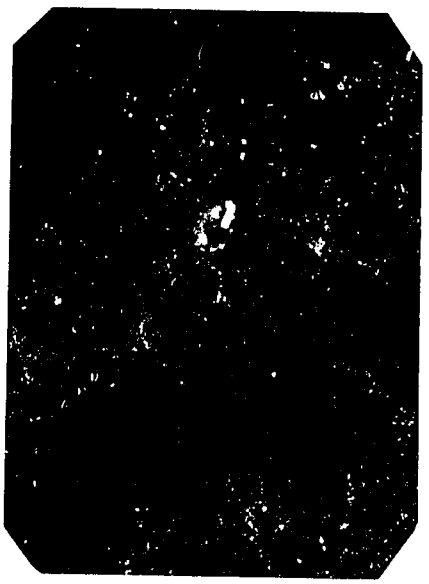

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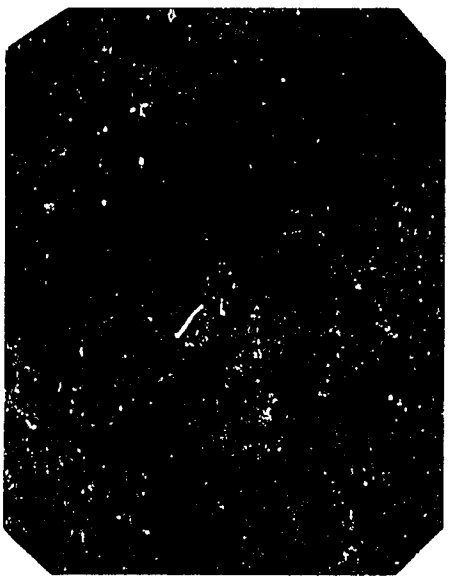

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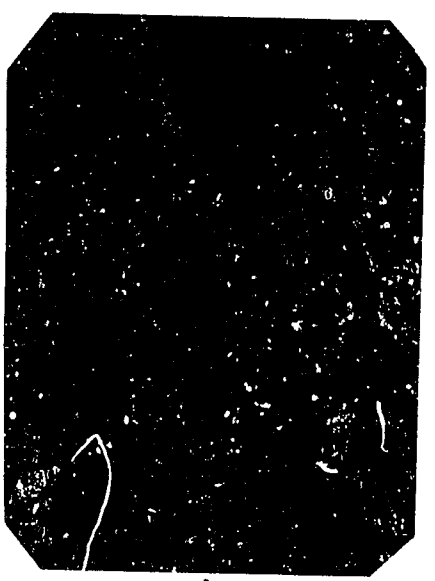

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Fig 7. Different patterns of IFA staining produced by McAb to P. vivax on ring, trophozoite, mature schizonts and merozoites. Acetone-fixed blood films of patients infected with vivax malaria were stained for fluorescence microscopy. (a) Trophozoites and (b) schizonts stained by group I antibodies; these McAb also reacted with all other asexual blood forms (not shown). (c, d) Schizont and merozoites stained by group II antibodies. Note localization of the most intense staining to a small area of the merozoites, indicating that the antigen or antigens concerned may be associated with an organelle (c). These McAb also reacted with whole merozoites (d). (e) Schizonts and merozoites stained with group III antibodies. Note the concentration of staining around the perimeter of individual merozoites. (f) Schizont and (h) Trophozoite stained with group V antibodies. Note the intense staining around the surface of schizonts and trophozoites. (g, h) trophozoites stained with group VII antibodies. Note the strong irregular staining of internal components of trophozoites.

Molecular size and relative abundancies of P. vivax blood stage proteins

By SDS-polyacrylamide gel electrophoresis under reducing conditions, P. vivax antigens containing ring, trophozoite and schizont stages exhibited a characteristic polypeptide, size and abundance distribution as shown in Fig. 8. There were at least 25 protein bands with the relative molecular weight ranging from 15 kd to >200 kd. Based on the intensity of staining, the prominant bands were 15 kd, 18 kd, 20 kd, 21 kd, 23 kd, 30 kd, 42 kd, 52 kd, 57 kd and >200 kd whereas the additional fainted bands were 24 kd, 25 kd, 26 kd, 28 kd, 39 kd, 47 kd, 49 kd, 61 kd, 68 kd, 75 kd, 85 kd, 95 kd, 115 kd, 175 kd, 195 kd and 200 kd. However, the bands of 15 kd, 26 kd, 30 kd and 75 kd were also prominent in the erythrocyte control (Figure not shown here).

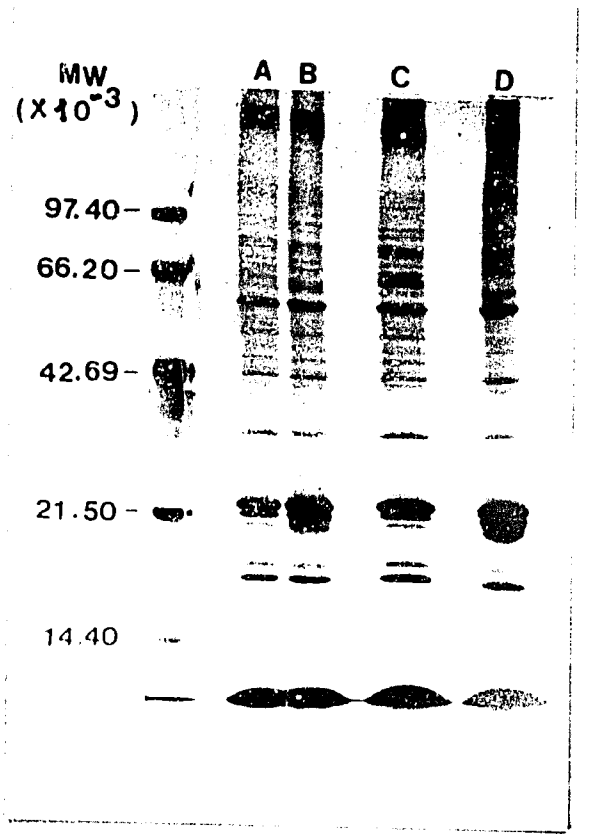


Fig 8. Analysis of P. vivax antigens. Coomasie blue stained SDS-PAGE profiles of pooled antigens of PV066, 067, 107 (lane A), PV084, 085, 086 (lane B), PV 093, 094, 097 (lane C), PV101, 104, 044 (lane D) and protein M.W. standards. Molecular weights in kilodaltons are shown on the left.

Monoclonal antibody characterization of P. vivax antigens

A panel of 58 McAb was established against asexual erythrocytic stages of P. vivax by IFA and the Western blot technique (Table 5). Twenty one McAb described here produced uniformly bright generalized staining of all blood stages and reacted with a series of antigen molecule of 30 kd, 36 kd, 85 kd, 95 kd, 100 kd, 115 kd, 135 kd, 200 kd and >200 kd. Seventeen McAb reacted strongly to 35 kd, 41 kd, 75 kd, 90 kd, 110 kd and 200 kd molecules on merozoites and to 30 kd, 39 kd, 50 kd, 56 kd, 68 kd, 85 kd, 95 kd, 115 kd, 140 kd and 200 kd on merozoite organelles. Five McAb reacted to 85 kd, 195 kd, and 200 kd molecules on the surface of merozoites and their reactions to the intraschizont merozoites took a form of cluster of grapes. Seven McAb reacted to the proteins of Mr. 39 kd, 56 kd, 95 kd, and 115 kd on the surface of trophozoites and schizonts. Eight McAb reacted to 30 kd, 85 kd and 95 kd irregularly with parasites and internal components.

Classes and subclasses of immunoglobulin antibodies were also determined. Forty two McAb belonged to IgG1, three to IgG2a, three to IgG3 and nine to IgM.

Table 5. Characteristics of P. vivax asexual erythrocytic stages monoclonal antibodies (McAbs)

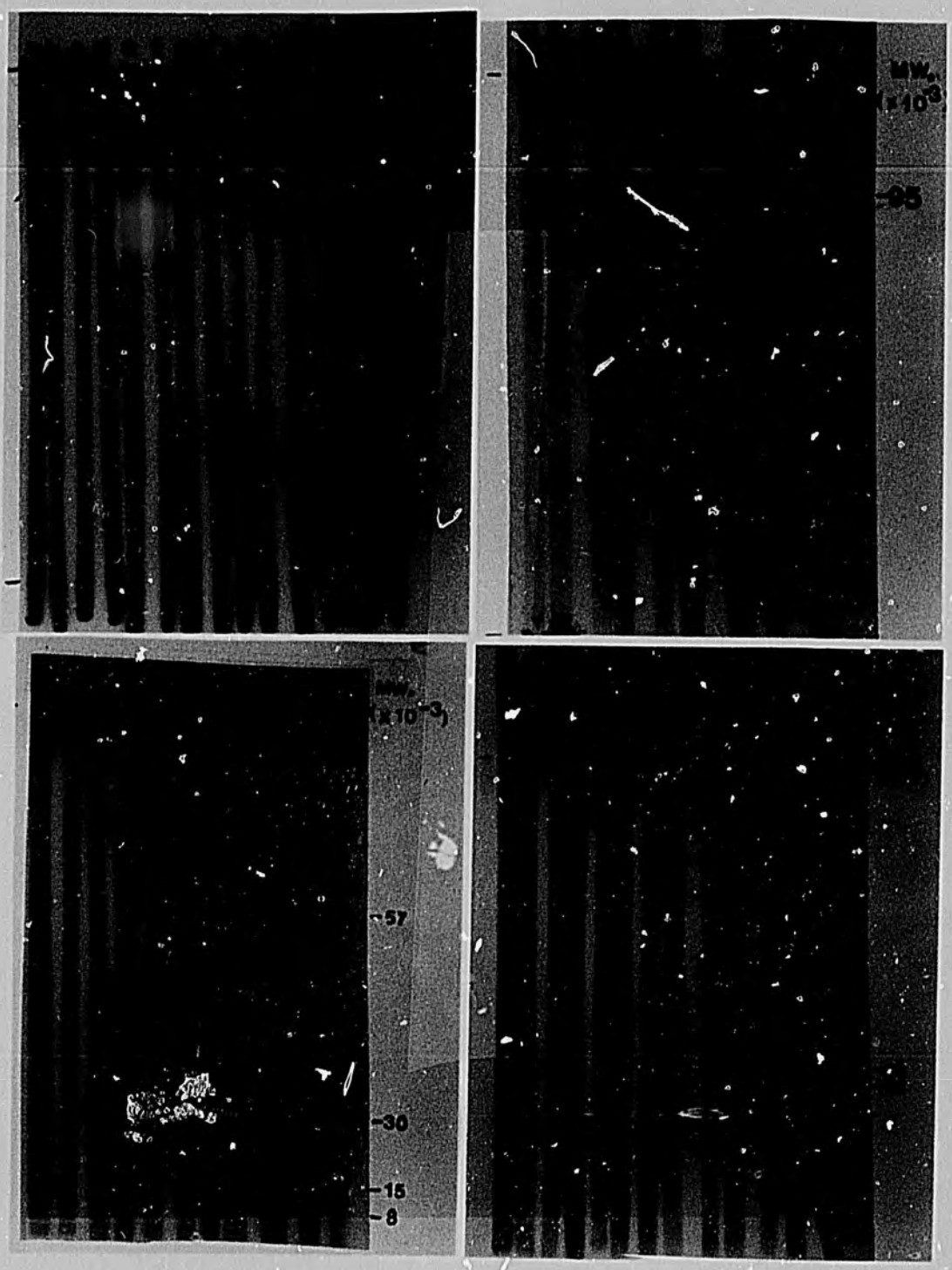
McAb	Isotype	IFA pattern	Mr ($x10^{-3}$)
McPV1	IgG1	I	30, 85
McPV2	IgG1	I	30, 85
McPV3	IgG1	I	30
McPV4	IgG3	I	100
McPV5	IgG1	I	30
McPV6	IgG1	I	100
McPV7	IgG1	I	30
McPV8	IgG1	I	30
McPV9	IgG1	I	30
McPV10	IgM	I	>200
McPV11	IgG1	I	36, 39, 85, 95, 115
McPV12	IgG1	I	100, 135, >200
McPV13	IgM	I	30
McPV14	IgM	I	135, 200
McPV15	IgM	I	35, 85
McPV16	IgM	I	100, 135, >200
McPV17	IgM	I	36, 39, >200
McPV18	IgG1	I	>200
McPV55	IgG2a	I	15, 28
McPV56	IgG2a	I	28
McPV57	IgG1	I	200
McPV19	IgG1	II	67, 75
McPV20	IgG1	II	35, 41

Table 5. (cont.)

McAb	Isotype	IFA pattern	Mr ($x10^{-3}$)
McPV21	IgG1	II	41, 67, 75, 90
McPV22	IgG1	II	115, 200
McPV23	IgG1	II	200
McPV24	IgG1	II	90, 115, 200
McPV25	IgG1	II	200
McPV26	IgG1	II	140
McPV27	IgG1	II	30, 56
McPV28	IgG1	II	39
McPV29	IgG1	II	95
McPV30	IgG1	II	115
McPV31	IgG1	II	30
McPV32	IgG1	II	56
McPV33	IgG1	II	30, 50, 85
McPV34	IgG1	II	50, 85
McPV35	IgG1	II	115, 200
McPV36	IgG2a	III	30, 68, 200
McPV37	IgG1	III	195
McPV38	IgM	III	200
McPV54	IgG1	III	85, 200
McPV58	IgG1	III	200
McPV39	IgG1	V	95
McPV40	IgG1	V	56
McPV41	IgG1	V	115
McPV42	IgG3	V	39

Table 5. (cont.)

McAb	Isotype	IFA pattern	Mr ($x10^{-3}$)
McPV43	IgG3	V	39
McPV44	IgG1	V	95
McPV45	IgG1	V	115
McPV46	IgG1	VII	95
McPV47	IgG1	VII	85
McPV48	IgG1	VII	30
McPV49	IgG1	VII	30
McPV50	IgGM	VII	85
McPV51	IgGM	VII	85
McPV52	IgG1	VII	30
McPV53	IgG1	VII	85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-30
-15
-8

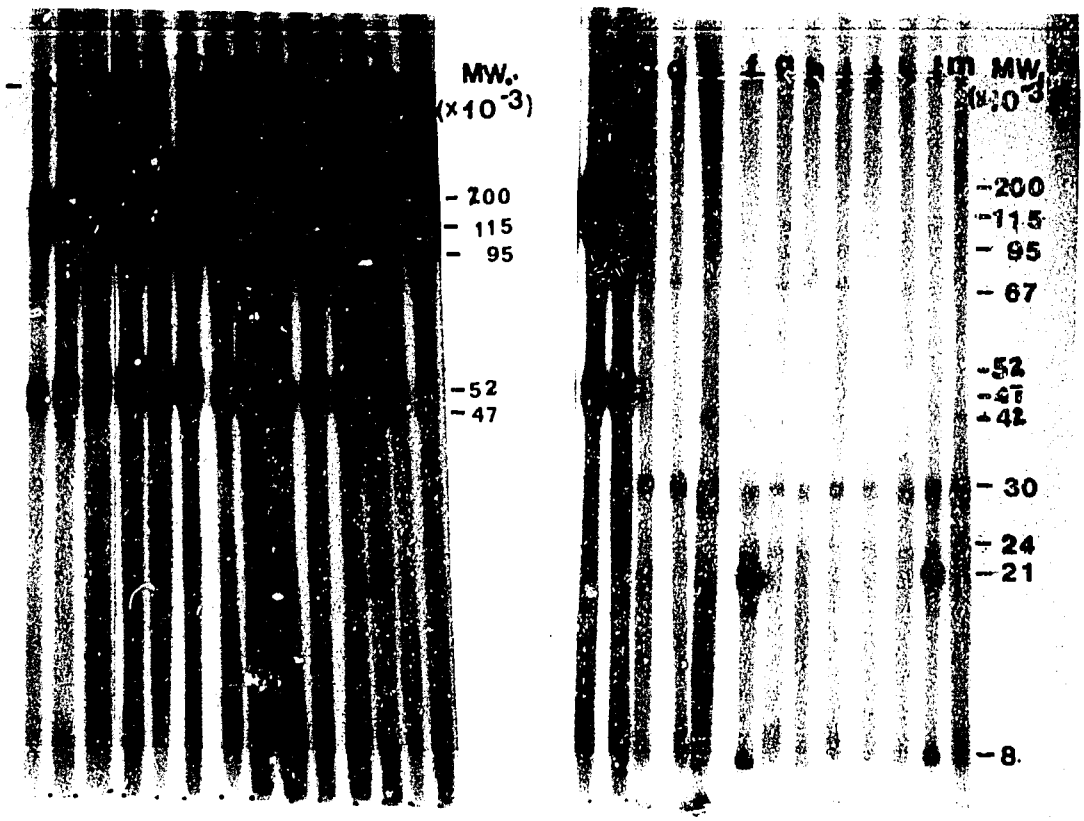

Fig. 9 Western blot analysis of stage-specific P. vivax antigens detected with monoclonal antibodies. Antigens were analyzed on a 10% separating gel in SDS-PAGE, electroblotted onto Ba88 nitrocellulose membrane and detected with monoclonal antibodies.

Species cross reactivity

The species cross reactivity of these monoclonal antibodies with asexual stages of P. falciparum was investigated by indirect immunofluorescent assay using acetone-fixed infected blood as antigens. The results are shown in table 6.

Table 6. Reactivities of strains and species specific monoclonal antibodies with different strains of P. vivax and P. falciparum

Anti-P. vivax Monoclonal antibodies P. vivax (McPV)	IFA patterns with P. vivax	Strain distribution of McPV	Cross reactivities with P. falciparum
		No.positive strains/ total strains tested	No.positive strains/ total strains tested
McPV1-McPV18, McPV55-McPV57	Group I	90/300 - 299/300	0/11
McPV19-McPV35	Group II	37/74 - 74/74[a]	0/11
McPV36, McPV38, McPV54, McPV58	Group III	30/74 - 68/74[a]	0/11
McPV39-McPV45	Group V	149/285 - 160/285[b]	0/11
McPV46-McPV53	Group VII	171/285 - 285/285[b]	0/11

[a] Only 74 isolates of P. vivax tested have schizont stage

[b] Only 285 isolates of P. vivax tested have trophozoite and schizont stages

Reaction of primary isolates with monocloanal antibodies (McAbs)

To evaluate the extent of antigenic diversity of P. vivax, 600 primary isolates of P. vivax collected from 49 provinces in Thailand and additional 6 other isolates from Burma were tested by IFA against 58 McAb.

Since in several occasions, the number of IFA-positive and IFA-negative parasitized erythrocytes were invariably present in the same slide preparation, the percentage of parasites reacting with a given McAb was therefore determined by counting the number of IFA-positive and the IFA-negative parasitized erythrocytes and the IFA reactivities were classified into three groups comprising those with $\geq$50%, <50% and 0% reactive. The IFA-negative parasitized erythrocytes were those containing malaria pigment granules observed with light microscopy. In discussing the staining reactivity of a given McAb, attention would be focussed on those with $\geq$50% reactive.

To obviate inter-observer error during the course of fluorescence microscopic reading, one person read all the IFA slides. Positive reactivities of these McAb with a given isolate were scored, according to the intensity of the fluorescence, as 1+ to 4+ and in each case the percentage of IFA-positive parasites was also recorded.

The steps taken to ensure the validity of the results :

1. A single batch of all McAb was used during the entire inves-

tigation.

2. At least 4 different isolates were processed simultaneously in the IFA assay.
3. The quality control of the assay was monitored by including a IFA-positive McAb control and a PBS control.

The results shown in Table 7 demonstrated that the population of P. vivax in various regions of Thailand is antigenically heterogeneous. With group I McAb, four of 21 McAb (19%) comprising McPV1, McPV2, McPV3 and McPV13 reacted to ≥95% of 606 isolates. The antigens recognized by these four McAb were thus invariant, and should be considered as target molecules for the development of enzyme immunoassay (EIA) or immunoradiometric assay (IRMA) for the detection of P. vivax antigen. With group II McAb, determination of IFA reactivities was limited in only 100 of 606 isolates. Such a limit was imposed by the absence of merozoite-containing schizonts in the slide preparations to react with McAbs tested. It should be emphasized that group II McAbs reacted with merozoite-associated molecules. Nine of 17 (53%) of McAb of this group (McPV21, McPV22, McPV23, McPV24, McPV25, McPV26, McPV27, McPV31, and McPV19) reacted ≥95% of 100 isolates. One of five (20%) McAb of group III (McPV37) and two of eight (25%) McAbs of group VII (McPV48 and McPV49) reacted to >95% of 100 and 571 isolates respectively. All seven McAbs of group V reacted to less than 95% of 571 isolates. It should be brought to attention that none of 606 primary isolates was negative for

all McAbs. McAbs especially those of group I, V and group VII were found to be highly variable in that their IFA reactivities varied from 30% to 90% of the isolates tested.

In addition to culture supernatants, several McAbs prepared as ascites fluid were tested at various dilutions against a series of parasite isolates. It was shown that the result of testing against the same isolates with McAb from culture supernatant and ascites fluid were qualitatively comparable, except that higher titer up to 1:131,072 could be seen with ascites fluid but not with culture supernatant. When a culture supernatant of a McAb failed to react, the ascites fluid would also be non-reactive even when it was used at a dilution of 1:2.

Table 7. Primary isolates of *P. vivax* reacting with 58 monoclonal antibodies (McAbs)

McAb	Total examined	No. and (%) isolates classified		
		50% reactive	<50% reactive	0% reactive
McPV1	606[a]	590 (97.35)	16 (2.65)	0 (0)
McPV2	606	602 (99.33)	4 (0.67)	0 (0)
McPV3	606	604 (99.67)	2 (0.33)	0 (0)
McPV4	606	356 (58.75)	215 (35.48)	35 (5.77)
McPV5	606	556 (91.75)	50 (8.25)	0 (0)
McPV6	606	205 (33.83)	199 (32.84)	202 (33.33)
McPV7	606	561 (92.57)	45 (7.42)	0 (0)
McPV8	606	568 (93.73)	38 (6.27)	0 (0)
McPV9	606	556 (91.75)	50 (8.25)	0 (0)
McPV10	606	236 (38.94)	69 (11.38)	301 (49.66)
McPV11	606	540 (89.11)	66 (10.89)	0 (0)
McPV12	606	350 (57.76)	130 (38.94)	20 (3.30)
McPV13	606	578 (95.38)	28 (4.62)	0 (0)
McPV14	606	336 (55.45)	202 (33.33)	68 (11.22)
McPV15	606	551 (90.92)	55 (9.08)	0 (0)
McPV16	606	573 (94.55)	33 (5.45)	0 (0)
McPV17	606	498 (82.18)	74 (12.21)	34 (5.61)
McPV18	606	567 (93.56)	39 (6.44)	0 (0)
McPV19	100[b]	95 (95.00)	5 (5.00)	0 (0)
McPV20	100	92 (92.00)	8 (8.00)	0 (0)

Table 7. (cont.)

McAb	Total examined	No. and (%) isolates classified		
		50% reactive	<50% reactive	0% reactive
McPV21	100	100 (100)	0 (0)	0 (0)
McPV22	100	100 (100)	0 (0)	0 (0)
McPV23	100	100 (100)	0 (0)	0 (0)
McPV24	100	100 (100)	0 (0)	0 (0)
McPV25	100	100 (100)	0 (0)	0 (0)
McPV26	100	100 (100)	0 (0)	0 (0)
McPV27	100	100 (100)	0 (0)	0 (0)
McPV28	100	82 (82.00)	18 (18.00)	0 (0)
McPV29	100	63 (63.00)	20 (20.00)	17 (17.00)
McPV30	100	93 (93.00)	7 (7.00)	0 (0)
McPV31	100	100 (100)	0 (0)	0 (0)
McPV32	100	70 (70.00)	22 (22.00)	8 (8.00)
McPV33	100	84 (84.00)	16 (16.00)	0 (0)
McPV34	100	85 (85.00)	15 (15.00)	0 (0)
McPV35	100	90 (90.00)	10 (10.00)	4 (5.40)
McPV36	100	93 (93.00)	7 (7.00)	0 (0)
McPV37	100	98 (98.00)	2 (2.00)	0 (0)
McPV38	100	54 (54.00)	26 (26.00)	10 (10.00)
McPV39	571[c]	527 (92.30)	40 (7.00)	4 (0.70)
McPV40	571	321 (56.21)	40 (7.00)	210 (36.78)
McPV41	571	496 (86.87)	69 (12.08)	6 (1.05)

Table 7. (cont.)

McAb	Total examined	No. and (%) isolates classified		
		50% reactive	<50% reactive	0% reactive
McPV42	571	312 (54.65)	54 (9.45)	205 (35.90)
McPV43	571	314 (54.99)	52 (9.11)	205 (35.90)
McPV44	571	526 (92.12)	28 (4.90)	17 (2.98)
McPV45	571	484 (84.76)	82 (14.36)	5 (0.88)
McPV46	571	467 (81.79)	48 (8.41)	56 (9.80)
McPV47	571	397 (69.53)	106 (18.56)	68 (11.91)
McPV48	571	571 (100)	0 (0)	0 (0)
McPV49	571	571 (100)	0 (0)	0 (0)
McPV50	571	398 (69.70)	140 (18.21)	69 (12.08)
McPV51	571	396 (69.35)	105 (18.39)	70 (12.26)
McPV52	571	529 (92.64)	42 (7.36)	0 (0)
McPV53	571	400 (70.05)	102 (17.86)	69 (12.08)
McPV54	100	91 (91.00)	9 (9.0)	0 (0)
McPV55	606	332 (54.79)	59 (9.73)	215 (35.48)
McPV56	606	368 (60.73)	70 (11.55)	168 (27.72)
McPV57	606	398 (65.68)	172 (28.38)	36 (5.94)
McPV58	100	94 (94.00)	6 (6.00)	0 (0)

a All isolates

b Only 100 isolates of P. vivax tested have schizont stage

c Only 571 isolates of P. vivax tested have trophozoite stage with and without schizont stage

P. vivax asexual erythrocytic-stage antigens recognized by McAbs

A panel of 58 McAb which reacted to asexual erythrocytic stages was used to characterize at least 35 different antigenic polypeptides of this parasite by SDS-PAGE and Western blot analysis. The association between the reaction of McAb on different isolates of P. vivax and the molecules to which these McAb recognized is shown in Table 8.

Two major proteins (PV30 and PV85) with apparent molecular weights of 30 kd and 85 kd respectively were recognized by McAb of group I. These molecules were located throughout the cytoplasm of the parasite and were present in all blood stages in almost all isolates (>90%) of the parasites tested. Thus, they may represent common antigens of this parasite species and as a consequence may prove to be of value in the development of immunodiagnostic test for this parasitic infection.

The protein, PV200, which migrated as a major band close to 200 kd was strongly expressed in merozoites, were recognized by several McAb of group II (McPV22, McPV23, McPV24, McPV25) in all isolates tested and might therefore represent a common antigen in P. vivax merozoites. On the other hand, some McAb (McPV32, McPV29 and McPV26) reacted with merozoites in IFA showed discrete punctate dots suggesting that the antigens recognized may be associated with the rhoptry.

Several group III McAb (McPV35, McPV36, McPV54, McPV58) reacted to 200 kd antigen (Pv200) of more than 90% of the iso-

lates tested. Another McAb (McPV37) was shown to react with the 195 kd antigen (Pv195) in 98% of the isolates tested. The IFA reactions of these McAb to mature segmenters assumed a cluster of grape appearance indicating that they reacted to the surface membrane of merozoites. The 200 kd and 195 kd proteins recognized by our McAb would thus be analogous to the 200 kd and 170 kd proteins shown by electron microscopy by Barnwell et al., (18, 19) to be localized to the surface of merozoites in mature segmented schizonts of P. vivax and to the Pf190-Pf205 of P. falciparum (20, 21). Recently, a genomic DNA expression library of squirrel monkey-adapted Lelem strain of P. vivax constructed in λgtll was screened with a McAb reacted to a polymorphic 200 kd antigen. A clone expressing a large β-galactosidase fusion protein (190 kd) was isolated and their secretory product purified by SDS-PAGE followed by electroelution and used to immunize rabbits and mice. The immune sera obtained was shown to recognize the 200 kd antigen on Western blots and produced a cluster of grape-like IFA pattern with segmenters of P. vivax (22).

Group V McAb which gave a speckled immunoflourescent staining pattern over the entire infected erythrocyte especially trophozoites and schizonts were shown by Western blot analysis to react to 115 kd , 95 kd, 56 kd and 39 kd antigens. The precise location of these antigens are at present unknown. They may be associated with the surface of infected erythrocytes or with the surface membrane of trophozoites or schizonts. Further electron microscopic study would in the future unravel this problem. McAb

to 115 kd, 95 kd molecules (McPV41, McPV45 and McPV39, McPV44) were shown to react by IFA to 84.6% to 92.3% of parasite isolates. This indicates that these antigens have been evolutionally highly conserved and appear to be analogous an antigen complex of 105 kd, 95 kd, 55 kd, 28 kd proteins which have been shown by Barnwell et al. (18) to be associated with the caveola-vesicular complexes.

In addition to these surface-oriented trophozoite and schizont antigens, three other antigens comprising 30 kd, 85 kd and 95 kd protein were identified by group VII McAb, which produced strong irregular staining of internal components in trophozoites. McAb to 30 kd protein was shown to react by IFA to 69.4% to 100% of 571 isolates. As with group V McAb, this finding was interpreted to mean that antigens of internal components of trophozoites are evolutionally highly conserved.

Table 8. Distribution of blood stage antigens in *P. vivax* isolates tested

McPVa	IFA pattern	Mr ($\times 10^{-3}$)	% reactive with isolates
McPV1	I	30, 85	97.35
McPV2	I	30, 85	99.33
McPV3	I	30	99.67
McPV4	I	100	58.75
McPV5	I	30	91.75
McPV6	I	100	33.83
McPV7	I	30	92.57
McPV8	I	30	93.73
McPV9	I	30	91.75
McPV10	I	>200	38.94
McPV11	I	36, 39, 85, 95, 115	89.11
McPV12	I	100, 135, >200	57.76
McPV13	I	30	95.38
McPV14	I	135, 200	55.45
McPV15	I	35, 85	90.92
McPV16	I	100, 135, >200	94.55
McPV17	I	36, 39, >200	82.18
McPV18	I	>200	93.56
McPV55	I	15, 28	54.79
McPV56	I	28	60.73
McPV57	I	200	65.68

Table 8. (cont.)

McPVa	IFA pattern	Mr ($\times 10^{-3}$)	% reactive with isolates
McPV19	II	67, 75	95.00
McPV20	II	35, 41	92.00
McPV21	II	41, 67, 75, 90	100
McPV22	II	115, 200	100
McPV23	II	200	100
McPV24	II	90, 115, 200	100
McPV25	II	200	100
McPV26	II	140	100
McPV27	II	30, 56	100
McPV28	II	39	82.00
McPV29	II	95	63.00
McPV30	II	115	93.00
McPV31	II	30	100
McPV32	II	56	70.00
McPV33	II	30, 50, 85	84.00
McPV34	II	50, 85	85.00
McPV35	II	115, 200	90.00
McPV36	III	30, 68, 200	93.00
McPV37	III	195	98.00
McPV38	III	200	54.00
McPV54	III	85, 200	91.00
McPV58	III	200	94.00

Table 8. (cont.)

McPVa	IFA pattern	Mr ($\times 10^{-3}$)	% reactive with isolates
McPV39	V	95	92.30
McPV40	V	56	56.21
McPV41	V	115	86.87
McPV42	V	39	54.65
McPV43	V	39	54.99
McPV44	V	95	92.12
McPV45	V	115	84.76
McPV46	VII	95	81.79
McPV47	VII	85	69.53
McPV48	VII	30	100
McPV49	VII	30	100
McPV50	VII	85	69.70
McPV51	VII	85	69.35
McPV52	VII	30	92.64
McPV53	VII	85	70.05

Distribution of antigenically-diversed parasites and their geographical location

Reactivities of strain-specific McAb with P. vivax isolates from different regions were determined. Of 606 P. vivax isolates used in this study, 466 and 6 were originated from 49 provinces in Thailand and Burma, respectively, whereas the exact origin of the remaining 99 isolates were not known. Antigenic diversity was shown to vary from places to places and was high in the areas with high rate of transmission (Table 9). Thus high rates of diversity were observed in areas along the Burmese border in the west e.g. Kanjanaburi, Maehongsorn, Tak, Chumporn etc.; and along the Kumpuchean border in the east e.g. Trad, Chanthaburi, and Prachinburi. In this study, the highest rates of diversity were observed in Trad (48.4%), Tak (41.7%), Chanthaburi (36.5%), and Maehongsorn (36.4%), where the highest malaria incidence was detected (reported by Ministry of Public Health). These areas have been known to be major foci of transmission as well as foci of P. falciparum multidrug resistances. The areas of Chanthaburi and Trad were very fertile to which many laborers from the northeast migrated to work in the fruit orchard, in the surgar cane, casava and rubber plantation and to mine natural gems. High rate of diversity was likely to occur in some areas in the South especially in Krabi, Ranong and Suratthani but this result has to be interpreted with caution because of the small number of isolates tested. The reason for the possible high attributable to migration of laborers from elsewhere to coffee

plantations, new settlements of rubber plantation and a high rate of refusal to DDT spraying. The lower rates of diversity (<20%) were observed in Chumporn (15.4%), Chiengrai (12.5%), Ubonrajthani (9.1%), and Chachoengsao (5.3%). The populations in these areas were perhaps more static than those in the areas with high transmission.

Table 9. Distribution of antigenically-diversed parasites and geographic locations

Source	Total examined	% *P. vivax* isolates that gave different reaction with McAb
1. Kanjanaburi	86	34.88
2. Prachinburi	66	24.24
3. Chiengmai	55	20.00
4. Chanthaburi	52	36.53
5. Trad	31	48.38
6. Tak	24	41.66
7. Maehongsorn	22	36.36
8. Chachoengsao	19	5.26
9. Chiengrai	16	12.50
10. Chumporn	13	15.38
11. Rajburi	11	0
12. Ubonrajthani	11	9.09
13. Surat Thani	9	22.22
14. Chonburi	9	0
15. Rayong	6	0
16. Nakhon Ratchasima	6	16.66
17. Nakhorn Nayok	5	20.00
18. Prachuap Khiri Khan	5	0
19. Phetburi	5	0
20. Buriram	5	0
21. Suphanburi	4	0

Table 9. (cont.)

Source	Total examined	% *P. vivax* isolates that gave different reaction with McAb
22. Nan	3	0
23. Phitsanulok	3	0
24. Yala	3	0
25. Krabi	3	33.33
26. Udornthani	2	0
27. Nakhornsawan	2	0
28. Samutprakarn	2	0
29. Ranong	2	50.00
30. Sakolnakorn	2	0
31. Nongkhai	1	-
32. Samutsakorn	1	-
33. Srisakate	1	-
34. Aranyaprathade	1	-
35. Uthaithani	1	-
36. Phetchabun	1	-
37. Lampang	1	-
38. Chaiyapum	1	-
39. Lopburi	1	-
40. Narathiwat	1	-
41. Uttraradit	1	-
42. Lamphun	1	-
43. Pranburi	1	-
44. Mahasarakam	1	-

Table 9. (cont.)

Source	Total examined	% *P. vivax* isolates that gave different reaction with McAb
45. Angthong	1	-
46. Nakorn Srithammarat	1	-
47. Trang	1	-
48. Surin	1	-
49. Leue	1	-
50. Burma	6	16.66
51. No exact origins	99	-

Antigenic diversity of parasites causing initial clinical symptoms and those causing relapsed

A panel of 58 McAb against asexual erythrocytic stages of Plasmodium vivax was tested by IFA to determine the antigenic disparity of the parasites causing initial clinical symptoms and those causing relapses. The results are shown in table 10. The initial and relapse strains from three patients (R3, R4 and R5) exhibited similar reactivities with all McAbs tested. In contrast, isolates from two relapse cases (R1, R2) showed different patterns of reactivity. In R1, the different reactivities were observed with McPV1, McPV2, McPV11, McPV15, McPV39, McPV40, McPV41, McPV42, McPV43, McPV46, McPV47 and McPV53. Nine of these McAbs (McPV1, McPV2, McPV11, McPV39, McPV41, McPV42, McPV43, McPV46, and McPV53) reacted with the initial (RPV261) but not the relapse (RPV393) isolates. Three other McAb (McPV15, McPV40 and McPV47) reacted only to the relapse isolate and not to the initial isolate. Since McPV39, McPV40, McPV41, McPV42 reacted to the proteins with 95 kd, 115 kd and 39 kd which were shown by IFA to be associated with the trophozoite surface antigen, it is therefore likely that expression of these molecules, under the immune pressure developed during the course of infection, were suppressed. The absence of some epitopes on this molecule may endow the parasites to evade from the host immune attacks. The emergence of new antigenic epitope on the proteins recognised by McPV15, McPV40 and McPV47 could be interpreted to indicate the variable nature of these antigens and could be responsible for

the antigenic variation in P. vivax.

As regards the second relapse case (R2), in whom two relapses occurred, the cytoplasmic antigens of Mr 15, 28 and 36 kd recognized by group I McAb (McPV11, McPV55 and McPV56) and Mr 115, 95 and 39 kd molecules recognized by McPV39, McPV41, McPV44, McPV45, McPV46 of group V McAb were present only in the initial isolate (RPV182) and the isolate from the first relapse (RPV240) but not in the isolate from the second relapse (RPV300). In contrast, the Mr 200 kd recognized by McAb McPV14 and McPV57 of group I was present only in the initial isolate. An epitope on the molecule recognized by McAb McPV15 was variably present on the parasite surface membrane as judged by its presence in the initial isolate, absence in the first relapse isolate and reappearance in the second relapse isolate. Similar finding was observed with the molecule of Mr 57 kd recognized by McPV40.

The changes in the expression of parasite antigens could reflect the nature of immune responses in patients during initial infections and relapses. Thus the more diverse the parasites during relapse, the more variation in the nature of the molecules recognized by the relapse sera. In R3 and R4 cases, in whom no alteration in reactivities of relapse parasites to a panel of McAb was observed, there was no difference in the spectrum of antigens recognized by antibodies from initial and relapse sera (Table 11, Figs. 10, 11). In contrast, when reactivities to a panel of McAb of relapse parasites were altered as exemplied in R1 and R2 patients, the molecules reognized by antibodies in

initial and relapse sera were markedly altered. In R5 relapse case, no alteration of the relapse parasites was detected, small difference of molecules recognised antibodies in initial and relapse sera was observed. It is likely that this represent an early event of antigenic switch whereby the parasite population with antigenic changes constitute only a small proportion to be detected by a panel of McAb used. This alteration, even in a small degree, was sufficient to stimulate the host to alter the immune response to the altered antigen even before the replacement of the immune-susceptible parasites with the antigenically-altered parasite population.

Another explanation for the antigenic difference in initial and relapse strains is related to the hetergeneity of the parasites infected. The patients may have variability in receiving infective mosquito bites. Those who receive only one bite would in therory be different from those who receive several bites. It follows that the hypnozoites in the liver of a patient who receive one mosquito bite would be antigenically more homogenous than those receiving several bites. Under the pressure of anti-malarial drugs, the parasites in the circulation will be completely eliminated. After certain period of time, the parasites will emerge from hypnozoites and cause relapse. The antigenicity of the relapse parasites from a patient who receives only one bite would in theory be the same as those causing initial infections whereas the parasite antigenicity in the patient who receives multiple bites would be different.

Table 10. Indirect immunoflourescence reactivities of the panel of 58 anti-*P. vivx* monoclonal antibodies (McAbs) with 11 parasite isolates causing initial clinical symptoms and causing relapse

McAb	Case	Test result for parasite isolate										
		R1		R2			R3		R4		R5	
	isolate	RPV261	RPV393	RPV182	RPV240	RPV300	RPV324	RPV411	RPV72	RPV272	RPV52	RPV57
McPV1		+	-	+	+	+	-	-	+	+	+	+
McPV2		+	-	+	+	+	-	-	+	+	+	+
McPV3		+	+	+	+	+	+	+	+	+	+	+
McPV4		-	-	-	-	-	-	-	-	-	-	-
McPV5		+	+	+	+	+	+	+	+	+	+	+
McPV6		-	-	-	-	-	-	-	-	-	-	-
McPV7		+	+	+	+	+	+	+	+	+	+	+
McPV8		+	+	+	+	+	+	+	+	+	+	+
McPV9		+	+	+	+	+	+	+	+	+	+	+
McPV10		+	+	-	-	-	+	+	-	-	-	-
McPV11		+	-	+	+	-	+	+	+	+	+	+
McPV12		+	+	-	-	-	+	+	-	-	-	-
McPV13		+	+	+	+	+	+	+	+	+	+	+
McPV14		+	+	+	-	-	+	+	-	-	-	-
McPV15		-	+	+	-	+	-	-	+	+	+	+
McPV16		+	+	-	-	-	+	+	-	-	-	-
McPV17		+	+	-	-	-	+	+	+	+	-	-
McPV18		+	+	-	-	-	+	+	-	-	-	-
McPV55		+	+	+	+	-	+	+	+	+	+	+

Table 10. (cont.)

McAb	Case isolate	Test result for parasite isolate										
		R1		R2			R3		R4		R5	
		RPV261	RPV393	RPV182	RPV240	RPV300	RPV324	RPV411	RPV72	RPV272	RPV52	RPV57
McPV56		-	-	+	+	-	+	+	+	+	+	+
McPV57		+	+	+	-	-	+	+	-	-	-	-
McPV19		NS	NS	NS	+	NS	NS	NS	NS	NS	NS	NS
McPV20		NS	NS	NS	-	NS	NS	NS	NS	NS	NS	NS
McPV21		NS	NS	NS	+	NS	NS	NS	NS	NS	NS	NS
McPV22		NS	NS	NS	-	NS	NS	NS	NS	NS	NS	NS
McPV23		NS	NS	NS	-	NS	NS	NS	NS	NS	NS	NS
McPV24		NS	NS	NS	-	NS	NS	NS	NS	NS	NS	NS
McPV25		NS	NS	NS	-	NS	NS	NS	NS	NS	NS	NS
McPV26		NS	NS	NS	-	NS	NS	NS	NS	NS	NS	NS
McPV27		NS	NS	NS	+	NS	NS	NS	NS	NS	NS	NS
McPV28		NS	NS	NS	-	NS	NS	NS	NS	NS	NS	NS
McPV29		NS	NS	NS	-	NS	NS	NS	NS	NS	NS	NS
McPV30		NS	NS	NS	-	NS	NS	NS	NS	NS	NS	NS
McPV31		NS	NS	NS	-	NS	NS	NS	NS	NS	NS	NS
McPV32		NS	NS	NS	+	NS	NS	NS	NS	NS	NS	NS
McPV33		NS	NS	NS	+	NS	NS	NS	NS	NS	NS	NS
McPV34		NS	NS	NS	+	NS	NS	NS	NS	NS	NS	NS
McPV35		NS	NS	NS	-	NS	NS	NS	NS	NS	NS	NS
McPV36		NS	NS	NS	-	NS	NS	NS	NS	NS	NS	NS
McPV37		NS	NS	NS	+	NS	NS	NS	NS	NS	NS	NS

Table 10. (cont.)

McAb	Case	Test result for parasite isolate										
		R1		P2			R3		R4		R5	
	isolate	RPV251	RPV393	RPV182	RPV240	RPV300	RPV324	RPV411	RPV72	RPV272	RPV52	RPV57
McPV38		NS	NS	NS	-	NS	NS	NS	NS	NS	NS	NS
McPV54		NS	NS	NS	+	NS	NS	NS	NS	NS	NS	NS
McPV58		NS	NS	NS	-	NS	NS	NS	NS	NS	NS	NS
McPV39		+	-	+	+	-	+	+	-	-	+	+
McPV40		-	+	+	-	+	-	-	+	+	-	-
McPV41		+	-	+	+	-	+	+	-	-	+	+
McPV42		+	-	-	-	-	-	-	+	+	-	-
McPV43		+	-	-	-	-	-	-	+	+	-	-
McPV44		-	-	+	+	-	+	+	-	-	+	+
McPV45		-	-	+	+	-	+	+	-	-	+	+
McPV46		+	-	+	+	-	+	+	-	-	+	+
McPV47		-	+	+	+	+	-	-	+	+	+	+
McPV48		+	+	+	+	+	-	-	+	+	+	+
McPV49		+	+	+	+	+	-	-	+	+	+	+
McPV50		-	-	+	+	+	-	-	+	+	+	+
McPV51		-	-	+	+	+	-	-	+	+	+	+
McPV52		+	+	+	+	+	-	-	+	+	+	+
McPV53		+	-	+	+	+	-	-	+	-	+	+

Table 11. Spectrum of *P. vivax* antigens recognized by antibodies from initial and relapse sera

Case	Probable origin	Species and strains	Molecule of *P. vivax* antigens in kilodalton to which the antibody react
R1	Kanjanaburi	RPV261	21, 25, 31, 39, 42, 61, 95, 115, 200, >200
		RPV393	21, 24, 31, 35, 57, 75, 200, >200
R2	Prachinburi	RPV182	23, 30, 52, 57, 68, 75, 85, 95, 115, 195
		RPV240	23, 30, 52, 85, 95, 115,
		RPV300	30, 57, 68, 75, 85, 195
R3	Chiangmai	RPV324	15, 18, 20, 23, 25, 28, 41, 49, 75, 95, 115, 175, 200, >200
		RPV411	15, 18, 20, 23, 25, 28, 41, 49, 75, 95, 115, 175, 200, >200
R4	Prachinburi	RPV72	18, 23, 26, 30, 39, 42, 47, 52, 57, 68, 75, 85
		RPV272	18, 23, 28, 30, 39, 42, 47, 52, 57, 68, 75, 85
R5	Kanjanaburi	RPV52	18, 23, 26, 30, 52, 68, 85, 95, 115
		RPV57	18, 23, 26, 30, 47, 52, 68, 85, 95, 115

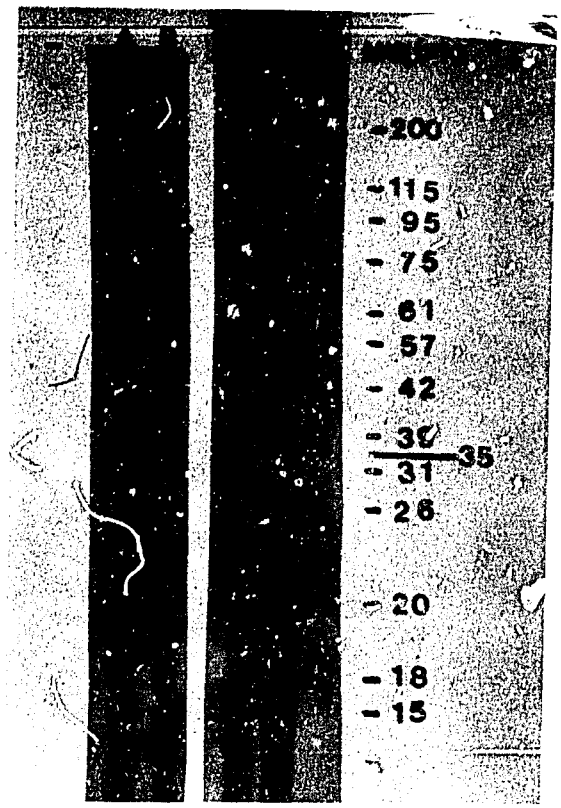

Fig. 10 Western blot analysis of P. vivax antigens detected with initial and relapse sera of case R1 and R3. Antigens were analyzed on a 10% separating gel in SDS-PAGE, electroblotted onto BA88 nitrocellulose and detected with serum samples from relapsed cases. Serum samples were (A) RPV261, (B) RPV393, (C) RPV324, (D) RPV411. The parasite antigens were prepared from various isolates.

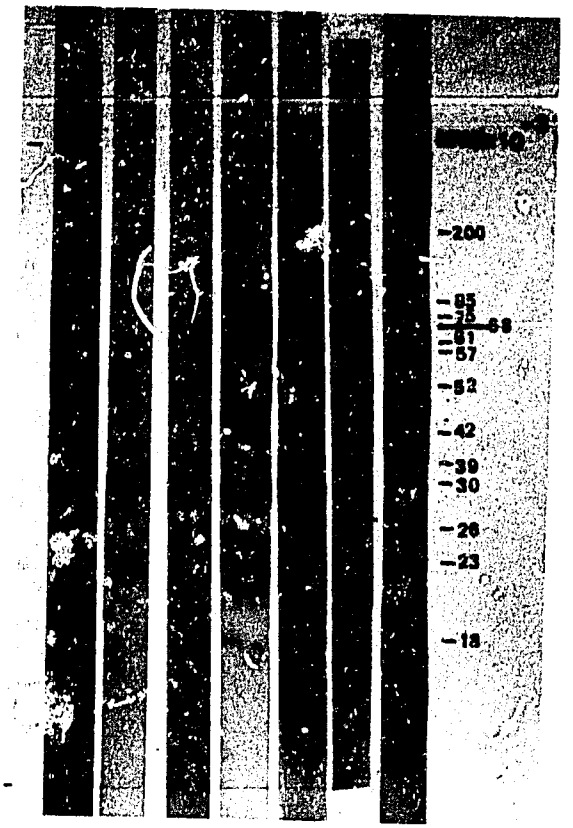

Fig. 11 Western blot analysis of P. vivax antigens detected with initial and relapse sera of case R2, R4 and R5. Antigens were analyzed on a 10% separating gel in SDS-PAGE, electroblotted onto BA88 nitrocellulose and detected with serum samples from relapsed cases. Serum samples were (A) RPV182, (B) RPV240, (C) RPV300, (D) RPV72, (E) RPV272, (F) RPV52, (G) RPV57. The parasite antigens were prepared from various isolates.

CONCLUSIONS

Our results provide evidence of considerable antigenic diversity in the human malaria parasite P. vivax. Different isolates were distinguished by their ability to react with certain antibodies. The diversity among the antigens was shown to vary from place to place and was high in the areas with high rate of malaria transmission. Furthermore, the antigenic disparity of P. vivax isolates causing initial clinical symptoms and those causing relapses were also demonstrated. The difference observed could be attributed to the multiplicity of mosquito bites and the immune mechanism of the parasites. The parasites in patients received multiple mosquito bites would be more heterogeneous as would be the hypnozoite causing relapses. It was also speculated that under immune pressure, the parasites would generate antigenically diverse strain which would be different from the strain responsible for the initial infection.

The demonstration of diverse and conserved antigens of P. vivax will significantly contribute to the future development of vivax malaria vaccines as well as to the development of highly sensitive technique for detection of low grade parasitaemia. The vaccine to be developed possibly through a recombinant DNA technology and the monoclonal antibodies to be selected for use in the immunoassay should be active against all invariant antigens of the parasites.

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