ANTIGENIC DISPARITY OF *PLASMODIUM VIVAX* CAUSING INITIAL SYMPTOMS AND CAUSING RELAPSE

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Abstract. Relapse infections are an important obstacle to the successful treatment and control of *Plasmodium vivax* malaria, but little is known about the nature of the relapse. To provide insight into the antigenic disparity of the parasites causing initial clinical symptoms and causing relapse, a panel of 58 monoclonal antibodies (MAbs) against erythrocytic stages of *Plasmodium vivax* was tested by indirect fluorescent antibody test in five relapse cases. The initial and relapse strains from three patients (R3, R4, and R5) exhibited similar IFA reactivity with all MAbs tested, whereas the isolates from two relapse cases (R1 and R2) showed different patterns of reactivity and were seen only with 15 MAbs In case R1, different IFA reactivities were observed with 12 MAbs, nine of which reacted with the initial (RPV261) but not the relapse (RPV393) isolates, whereas the other three MAbs reacted only with the relapse isolates. With regards to the second relapse case (R2) in whom two relapses occurred, different IFA reactivities were demonstrated with seven MAbs that reacted only with the initial isolate (RPV 182) and with the isolate from the first relapse (RPV 240) but not with the isolate from the second relapse (RPV 300). The antibody responses from patients who developed primary clinical symptoms and relapse were detected by Western immunoblotting. In cases R3, R4 and R5, there was no difference in the spectrum of antigens from initial and relapse sera recognized by the antibodies. In contrast, in cases R1 and R2, the molecules recognized by antibodies in initial and relapse sera were markedly altered. In case R1, the series of molecules of *P. vivax* antigens recognized by initial (RPV 261) and relapse (RPV 393) sera were 21, 25, 31, 39, 42, 61, 95, 115, 200, > 200 kDa and 21, 24, 31, 35, 57, 75, 200, > 200 kDa, respectively. In case R2, the initial serum (RPV 182) recognized *P. vivax* antigens with molecular weights of 23, 30, 52, 57, 68, 75, 85, 95, 115, and 195 kDa while the first relapse (RPV 240) and the second relapse sera recognized *P. vivax* antigens with molecular weights of 23, 30, 52, 85, 95,115 kDa and 30, 57, 68, 75, 85,195 kDa, respectively.

INTRODUCTION

Plasmodium vivax causes much less severe illness and death is infrequent when compared with *P. falciparum*. The factor which could contribute to the maintenance of malaria transmission in the endemic areas is the carrier. Such carriers can be found both in *P. falciparum* and *P. vivax* infections. 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 parasitemia. 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. Recently, there was a report of *P. vivax* malaria late-forms more than twenty years after a stay in endemic area (Maslin *et al*,1997). Therefore, if at any time blood from such patient is used for transfusion, the recipient will unfortunately experience a typical attack of quartan malaria.

Relapse infections are an important obstacle to the successful treatment and control of *P. vivax* malaria, but 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 in parenchymal cells for periods in a form of arrested development, the so called "hypnozoites", which ultimately complete their development and cause relapses. It is not clear

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whether these two forms are antigenically different. The objective of this study was to determine the antigenic disparity of *P. vivax* strains causing initial clinical symptoms and those causing relapse by using a panel of monoclonal antibodies (MAbs).

MATERIALS AND METHODS

Blood and serum samples

Eleven blood smears and sera were obtained from eleven samples of five patients with *P. vivax* infection admitted to the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Bangkok during initial clinical symptoms and relapses. These patients has been exposed to vivax malaria in malaria endemic areas of Thailand (Kanchanaburi, Prachin Buri, and Chiang Mai Provinces).

Panel of MAbs

A panel of 58 MAbs against asexual erythrocytic stages of *P. vivax* previously described (Khusmith *et al,* 1984, 1987) was used to test the antigenic disparity of the parasite isolates causing initial clinical symptoms and causing relapses in five patients. Based on their reactivities in the indirect fluorescent antibody test, these MAbs were classified into five groups: group I MAbs (21 MAbs) showing generalized staining of all blood stages and reacted with a series of antigen molecule of 30, 36,85, 95, 100, 115, 135, 200 and >200 kDa; group II MAbs (17 MAbs) reacting with merozoites at the Mr 35, 41, 75, 90, 110 and 200 kDa and their organelles at the Mr 30, 39, 50, 56, 68, 85, 95, 115, 140 and 200 kDa; group III MAbs (5MAbs) reacting with the surface membrane of merozoites and schizonts with Mr 39, 56, 95 and 115 kDa; and group VII MAbs (8 MAbs) reacting with internal components of the parasites at the Mr 30, 85, and 95 kDa.

Indirect immunofluorescent antibody test (IFAT)

The IFAT was performed using acetone-fixed infected blood as antigen. (Khusmith *et al,* 1984). Smears made from either washed *P. vivax*-infected blood of patients or enriched parasite preparations and from washed, asynchronously grown *P. vivax*, mostly in schizonts and late trophozoites, were air-dried and stored at -70°C until use. After fixation with acetone (-20°C), each well partition of the smear was treated with 10 µl of each MAb for 2 hours at 37°C, and washed vigorously twice with phosphate-buffered saline (PBS), pH 7.2. Fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG), IgA, and IgM (Pasteur Institute, Paris, France) was added and the mixtures were incubated for an 1 hour. Slides were washed, air-dried, and mounted in 0.1 M Tris containing 4.5% N-propyl gallate and glycerol and examined under a fluorescence microscope.

Preparation of *P. vivax* antigens

The pooled *P. vivax* antigens for dodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared from both enriched infected blood using the SEC-G-25-Percoll method (Tharavanij *et al,* 1987) and from short term *in vitro* cultures. For enrichment of the parasite, a 1-ml aliquot of heparinized whole blood was passed through a column of an equal volume of sulfoethyl cellulose and Sephadex G-25 (Uppsala, Sweden) previously equilibrated with phosphate buffer, pH 7.5. The column was then washed with approximately 40 ml of phosphate buffer, pH 7.5, until the column was free from red blood cells. The eluate was centrifuged, the supernatant was removed, and the cell sediment was restored to a 50% hematocrit. The cell suspensions from several tubes were pooled and 2 ml each was layered on top of discontinuous gradients composed of successive layers of 2 ml each of 75%, 60%, 50%, and 40% Percoll in the same buffer. After centrifugation at 1,000*g* for 10 minutes at 20°C, two and sometimes three bands were obtained. The first and the second bands were localized in the layers of 50% and 60% Percoll that contained mostly growing trophozoites and schizonts and the third band at the interface of 60% and 70% Percoll contained trophozoites and rings but fewer trophozoites than in the first and second bands. The smears were made from the blood prior to passage through the column and after Percoll gradient centrifugation, stained with Giemsa, and examined by light microscopy.

The short-term culture technic of *P. vivax* previously described (Brockelman *et al,* 1985) was modified and applied for blood samples in which ring stage parasites were dominant so that late blood stage parasites were available for antigen preparation.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunodetection

The method of Laemmli (1970) was used for SDS-PAGE of pooled *P. vivax* blood stage antigens

with a 10% polyacrylamide gel and a 4% stacking gel. The antigens were transferred from the gel onto a sheet of nitrocellulose (Bio-Rad, California, USA) using the method described elsewhere (Towbin *et al,* 1979). The electrophoretic transfer was carried out at 64 volts for 14-16 hours at 4°C in a Trans-Blot cell (Bio-Rad, California, USA). The non-reactive sites on the nitrocellulose were blocked by submerging it in PBS containing 3% gelatin and 0.02% sodium azide at 4°C for 18 hours with continuous agitation and the sheet was washed twice in PBS - 0.05% Tween. The sheet was cut into small strips, and each of these was incubated for 2 hours at room temperature with each serum appropriately diluted in a diluting buffer. The strips were washed three times in PBS-Tween and incubated for 30 minutes with ^{125}I-labeled goat anti-mouse immunoglobulin (Amersham, Buckinghamshire, UK) at a concentration of 1 x 10^5 counts per minute/ml. The excess labeled antibodies were removed by washing the strips with PBS-Tween. The strips were air-dried and exposed to X-Omat RP films (Eastman Kodak, Rochester, NY) for three days at -70°C before the films were developed.

RESULTS

Antigenic disparity of *P. vivax* parasites causing initial clinical symptoms and causing relapses

A panel of 58 MAbs against asexual erythrocytic stages of *P. vivax* was tested by IFA to determine the antigenic disparity of the parasite isolates causing initial clinical symptoms and causing relapses in five patients. The parasites causing initial symptoms and those causing relapses could be followed up only in five cases. The initial and relapse strains from three patients namely R3, R4, and R5, exhibited similar IFA reactivities with all MAbs tested, whereas the isolates from two relapse cases (R1 and R2) showed different patterns of reactivity and were seen only with 15 MAbs (McPV1, McPV2, McPV11, McPV15, McPV56 of group I MAbs; McPV39, McPV40, McPV41, McPV42, McPV43 of group II MAbs; McPV44 of group V MAbs; McPV46, McPV47, McPV53, McPV55 of group VII MAbs). In case R1, different IFA reactivities were observed with 12 MAbs (McPV1, McPV2, McPV11, McPV15, McPV39, McPV40, McPV41, McPV42, McPV43, McPV46 McPV47, and McPV53), nine of which (McPV1, McPV2, McPV11 McPV39, McPV41, McPV42, McPV43, McPV46, and McPV53) reacted with the initial (RPV261) but not the relapse (RPV393) isolates, whereas the other three MAbs (McPV15, McPV40, and McPV47) reacted only with the relapse isolate. With regard to the second relapse case (R2) in whom two relapses occurred, different IFA reactivities were demonstrated with seven MAbs (McPV11, McPV55, McPV56 of group I MAbs; McPV39, McPV41, McPV44 of group V MAbs; McPV46 of group VII MAbs) that reacted only with the initial isolates (RPV 182) and with the isolate from the first relapse (RPV 240) but not with the isolate from the second relapse (RPV 300).

Analysis of *P. vivax* antigens

The molecular sizes and relative abundancies of pooled *P. vivax* blood stage protein containing ring, trophozoite and schizont stages are shown in Fig 1. There are at least 25 protein bands with the relative molecular weight ranging from 15 kDa to >200 kDa. Based on their intensity of staining, the prominent bands were 15, 18, 20, 21, 23, 30, 42, 52, 57, and >200 kDa whereas the additional fainted bands were 24, 25, 26, 28, 39, 41, 47, 49, 61, 68, 75, 85, 95, 115, 175, 195, and 200 kDa (Fig 1).

Antibody response in *P. vivax* relapse cases

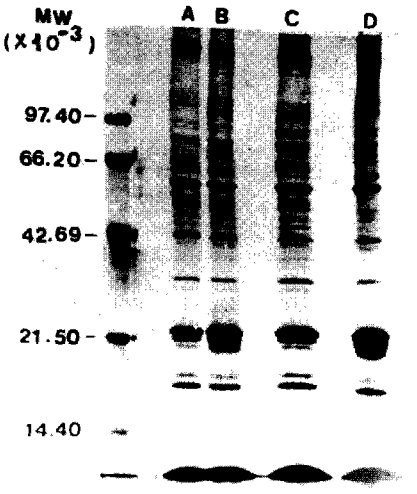

Fig 1–Analysis of *P. vivax* antigens. Coomasie blue stained SDS-PAGE profiles of pooled antigens of *P. vivax* isolates: pooled PV066, PV067 and PV107 (lane A); pooled PV084, PV085 and PV086 (lane B), pooled PV093, PV094 and PV097 (lane C); pooled PV 101, PV104 and PV044 (lane D). Protein molecular weight standards were shown on the left lane.

Table 1

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

Relapse case	Origin	Isolates	Molecules (kDa) recognized
R1	Kanchanaburi	RPV261[a]	21, 25, 31, 39, 42, 61, 95, 115, 200, >200
		RPV393[b]	21, 24, 31, 35, 57, 75, 200, >200
R2	Prachin Buri	RPV182[a]	23, 30, 52, 57, 68, 75, 85, 95, 115, 195
		RPV240[b]	23, 30, 52, 85, 95, 115
		RPV300[c]	30, 57, 68, 75, 85, 195
R3	Chiang Mai	RPV324[a]	15, 18, 20, 23, 25, 28, 41, 49, 75, 95, 115, 175 200, >200
		RPV411[b]	15, 18, 20, 23, 25, 28, 41, 49, 75, 95, 115, 175, 200, >200
R4	Prachin Buri	RPV72 [a]	18, 23, 26, 30, 39, 42, 47, 52, 57, 68, 75, 85
		RPV272 [b]	18, 23, 26, 30, 39, 42, 47, 52, 57, 68, 75, 85
R5	Kanchanaburi	RPV52 [a]	18, 23, 26, 30, 52, 68, 85, 95, 115
		RPV57 [b]	18, 23, 26, 30, 47, 52, 68, 85, 95, 115

[a] initial isolates, [b] first relapse, [c] second relapse
Underlined: the different molecules recognized by the initial sera and relapse sera.

The antibody response from patients who developed primary clinical symptoms and relapse were detected by Western immunoblotting. An SDS-PAGE was performed using solubilized pooled *P. vivax* blood stage antigens followed by reacting with initial and relapse sera obtained from five successful follow-up cases. As shown in Table 1, Fig 2, there was no difference in the spectrum of antigens from initial and relapse sera recognized by the antibodies in cases R3, R4, and R5. In contrast, in cases R1 and R2, the molecules recognized by antibodies in initial and relapse sera were markedly altered. In case R1, the series of molecules of *P. vivax* antigens recognized by initial (RPV 261) and relapse sera (RPV 393) were 21, 25, 31, 39, 42, 61, 95, 115, 200, > 200 kDa and 21, 24, 31, 35, 57, 75, 200, > 200 kDa, respectively. In case R2, the initial serum (RPV 182) recognized *P. vivax* antigens with molecular weight of 23, 30, 52, 57, 68, 75, 85, 95, 115, 195 kDa while the first relapse (RPV 240) and the second relapse sera (RPV300) recognized *P. vivax* antigens with molecular weights of 23, 30,

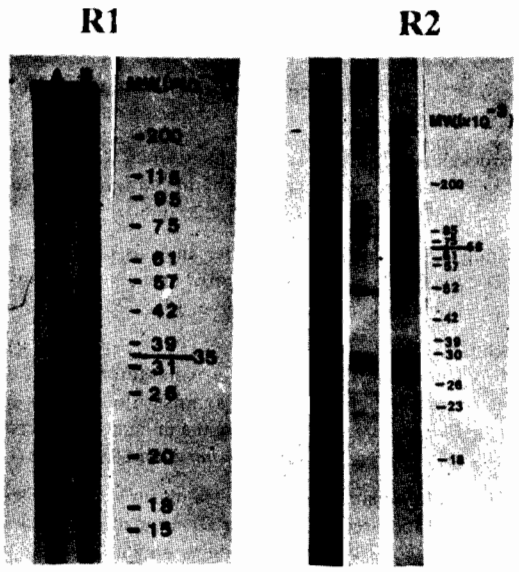

Fig 2–Western blot analysis of *P. vivax* detected with initial and relapse sera of cases R1 and R2 showed different patterns of reactivity. Each case was performed separately. Case R1: (A), RPV261, initial serum; (B), RPV393, relapse serum. Case R2: (A), RPV182, initial serum; (B), RPV 240, the first relapse serum; (C); RPV300, the second relapse serum.

52, 85, 95, 115 kDa and 30, 57, 68, 75, 85, 195 kDa, respectively.

DISCUSSION

The antigenic disparity of *P. vivax* was shown in isolates causing initial clinical symptoms and those causing relapses. The isolates from two of the five relapse cases showed different IFAT reactivities with a panel of MAbs. In case R1, the different IFA reactivities were observed with twelve MAbs in which nine of these MAbs reacted with initial isolate (RPV261), but not with relapse-isolate (RPV393), while the other three MAbs reacted only to the relapse isolate but not the initial isolate. Therefore, it is likely that expression of these molecules under the immune pressure developed during the course of infection was suppressed. The absence of some epitopes on this molecule may allow the parasites to evade host immune attacks (Mendis *et al*,1991) as shown in *P. falciparum* in that antigenic variation and antigenic diversity are critical to a parasite's ability to evade the host immune response (Reeder and Brown, 1996; Riggione *et al*, 1996). The emergence of a new antigenic epitope on the proteins recognized by MAbs McPV15 (30, 85 kDa), McPV40 (56 kDa), and McPV47 (85 kDa) could indicate the variable nature of these epitopes and could be responsible for antigenic variation in *P. vivax*.

With regards to the second relapse case (R2) in whom two relapses occurred, the cytoplasmic antigens of the 15-, 28-, and 36-kDa molecules recognized by MAbs McPV11, McPV55, and McPV56 of group I and the 115-, 95-, and 39-kDa molecules recognized by MAbs McPV39, McPV41, McPV44, McPV45, and McPV46 of group V were present only in the initial isolate (RPV182) and the isolate from the first relapse (RPV300). In contrast, the 200-kDa protein recognized by MAbs McPV14 and McPV57 of group I was present only in the initial isolate, while the 30- and 85-kDa molecules recognized by MAb McPV15 and the 56-kDa protein recognized by MAb McPV40 were variably present on the parasite surface membrane as judged by their presence in the initial isolate, absence in the first relapse isolate, and reappearance in the second relapse isolate. The changes in the expression of the parasite antigens could reflect the nature of immune responses in patients during initial infection 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 cases R3, R4, and R5, in whom no alteration in reactivity of relapse parasites to a panel of MAbs was observed, there was no difference in the spectrum of antigens recognized by antibodies from initial and relapse sera. In contrast, when reactivities of the relapse parasites to a panel of MAbs were altered as exemplified in cases R1 and R2, the molecules recognized by antibodies in primary and relapse sera were markedly altered. It is likely that this represents an early event in antigenic changes that constitute only a small proportion of those events that can be detected by the panel of MAbs used. This alteration, even to 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.

These results indicated that the most relapses seem to be caused by the same parasite populations that circulated during the primary infection and did not rise from antigenic distinct population. This finding supported the recent study on the molecular analysis of strains of *P. vivax* from paired primary and relapse isolates by single-strand conformational polymorphism (SSCP) and sequence analysis of the circumsporozoite and merozoite surface protein-1 genes which showed that 5 of 6 relapse isolates were identical to or were clones of their matched primary isolates (Craig *et al,* 1996). The results indicated that most relapses were caused by the same parasite populations that circulated during the primary infection and did not arise from a genetically distinct subpopulation.

In this study, however, it is not possible to draw a definite conclusion since many factors could be involved: 1) the heterogeneity of the parasites infected, 2) the parasite population injected during by the bite of the mosquito, and 3) the number of infected mosquito bites. The patients may show variability in the number of infective mosquito bites they received. Those who received only one bite would in theory be different from those who received multiple bites. It follows that the hypnozoites in the liver of a patient who received one mosquito bite would be antigenically more homogenous than those who received several bites. Under the pressure of antimalarial drugs, the parasites in the circulation will be completely eliminated. After a 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. It is 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.

ACKNOWLEDGEMENTS

We thank the staff of the Hospital for Tropical Diseases for their help in blood collection. This work was supported by the USAID Program on Science and Technology Cooperation (grant no. 936-5542-G-00-5078-00).

REFERENCES

Brockelman CR, Tan-Ariya P, Laovanitch R. Observation on complete schizogony of *Plasmodium vivax in vitro*. *J Parasitol* 1985; 32: 76-80.

Craig AA, Kain KC. Molecular analysis of strains of *Plasmodium vivax* from paired primary and relapse infections. *J Infect Dis* 1996; 174: 373-9.

Khusmith S, Tharavanij S, Patarapotikul J, Koonrangsesomboon D. Characterization of monoclonal antibodies against blood form of *Plasmodium falciparum* by the immunofluorescent test. *Asian Pac J Allergy Immunol* 1984; 2: 91-4.

Khusmith S, Tharavanij S, Chongsa-nguan M, Tapchaisri, P, Sabchareon A, Bunnag D. Human monoclonal anti- *P. falciparum* antibodies produced by stable EBV-transformed lymphocytes from patients with falciparum malaria. *Southeast Asian J Trop Med Public Health* 1987; 18: 24-31.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-5.

Maslin J, Cuguillre A, Bonnet D, Martet G. Malaria attack: a very late relapse due to *Plasmodium vivax*. *Bull Soc Pathol Exot* 1997; 90: 25-6.

Mendis KN, David PH, Carter R. Antigenic polymorphism in malaria: is it an important mechanism for immune evasion? *Immunol Today* 1991; 12: A34-7.

Reeder JC, Brown GV. Antigenic variation and immune evasion in *Plasmodium falciparum* malaria. *Immunol Cell Biol* 1996; 74: 546-54.

Riggione F, Pulido M, Noya O. *Plasmodium falciparum*: surface modification of infected erythrocytes from clinical isolates. Evidence of antigenic diversity using Venezuelan human malarial sera. *Parasitol Res* 1996; 82: 490-6.

Tharavanij S, Mahakunkitcharoen Y, Khusmith S, Bunnag D. Enrichment of *Plasmodium vivax* infected red blood cells from patients with vivax malaria. *Southeast Asian J Trop Med Public Health* 1987; 32: 39-43.

Towbin H, Stachelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedures and some application. *Proc Natl Acad Sci USA* 1979; 76: 4350-4.