ANTIGENIC DIVERSITY OF *PLASMODIUM VIVAX* AND THEIR GEOGRAPHIC DISTRIBUTION IN THAILAND

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Abstract: Fifty-eight monoclonal antibodies (MAbs) raised against the erythrocytic stages of *Plasmodium vivax* were selected for typing of 501 *P.vivax* isolates from different geographic locations throughout Thailand. Based on their reactivities in the indirect fluorescent antibody test, these MAbs were classified into five groups: group I MAbs showing generalized staining of all blood stages; group II MAbs reacting with merozoites and their organelles; group III MAbs reacting with the surface membrane of merozoites; Group V MAbs reacting with the surface membrane of trophozoites and schizonts; and group VII MAbs reacting with internal components of the parasites. Sixteen MAbs reacted with more than 95% of the isolates; the epitopes recognized by these MAbs were considered as being invariant. The remaining MAbs reacted with 30-90% of the isolates, and the epitopes recognized by these MAbs were regarded as being variable. The variant epitopes were associated with > 200-, 135-, and 100-kilodalton (kDa) molecules of all blood stages, the 95-kDa molecule on merozoite organelles, the 200-kDa molecule on the surface of trophozoites and schizonts, and the 85-kDa molecule of the parasite internal components. Antigenic diversity occurred among the *P. vivax* population in the endemic areas of Thailand and was shown to vary from place to place and was highest in the area with the highest rate of transmission along the Myanmar border in western Thailand and along the Cambodian border in eastern Thailand, including Trat (48.4%), Tak (41.7%), Chantaburi (36.5%), and Mae Hong Son (36.4%). Demonstration of antigenic diversity of *P. vivax* parasites signals a note of caution in the development of vaccines for vivax malaria. The vaccines should be directed against protective, conserved and not against variant epitopes.

INTRODUCTION

Antigenic diversity on malaria parasites are important factors in the epidemiology of malaria as well as implications for the development of acquired immunity and for antimalarial vaccine research. Strategies on antigenic variation and antigenic diversity are critical to a parasite's ability to evade the host immune response and infect previously exposed individuals (Reeder and Brown, *et al,* 1996). Infections of human erythrocytes with the mature asexual blood stages of *Plasmodium falciparum* result in antigenic changes in host cell membrane that, by virtue of their position, length of exposure and closed association with functional changes critical to pathogenesis, are a potentially important target for host effector mechanisms. These parasite-induced antigens expressed on the surface of infected erythrocytes have been shown to exhibit considerable polymorphism (Riggione *et al,* 1996). Polymorphism may be artificially produced through splenectomy of the host, prolonged parasite culture, or immune pressure following vaccination. In these regards, the daily dynamics of these parasite subpopulations were investigated in asymptomatic children in rural Tanzania as shown by genotyping of *P. falciparum* done by using nested polymerase chain reaction (PCR) assay targeting polymorphic regions on the merozoite surface protein-1 (MSP-1), MSP-2 and glutamine-rich protein (GLURP) genes (Farnert *et al,* 1997; Brown, 1993; Putaporntip *et al,* 1997).

Studies on antigenic diversity among the blood stages of different isolates of *P.vivax* are relatively limited primarily due to the inability to maintain *P.vivax* in long-term *in vitro* culture (Brockelman *et al,* 1985; Golenda *et al,* 1997). The parasites used in the study were therefore obtained from infected blood of humans or other primates. Compared with *P. falciparum, P.vivax* causes much less severe illness and death is infrequent. Thus, hosts can mount accumulated immune responses that will exert immune pressure for parasite growth develop-

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ment. The parasites then have to find means to evade the hostile environment in the host, one of which is to change their antigenicity.

We raised and characterized a panel of monoclonal antibodies (MAbs) directed against the blood stages of *P. vivax* to determine the magnitude of antigenic polymorphism of the species and their geographic distribution in Thailand.

MATERIALS AND METHODS

Plasmodium vivax

The asexual blood stage parasites of *P. vivax* were obtained from 501 isolates from both patients at the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok and infected individuals who visited a malaria clinic, the Malaria Eradication Center Unit 2, Ministry of Public Health (Chieng Mai, Thailand). From clinical histories as well as the most likely place where malaria was contacted, these isolates were from 49 provinces throughout Thailand.

Preparation of *P. vivax* antigens

Antigens were enriched from infected blood using the SEC-G-25-Percoll method (Tharavanij *et al*, 1987). Briefly, 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 of 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 the 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 the 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.

In vitro culture of *P. vivax*

The method previously described (Brockelman *et al*, 1985) for short-term culture of the parasite was adopted and applied only to some hospital-derived blood samples in which ring stage parasites were dominant so that late blood stage parasites were available for antigen preparations and for monoclonal antibody (MAb)-based indirect immunofluorescent antibody test (IFAT).

Production of MAbs

Six-to-eight-week-old BALB/c mice were immunized intraperitoneally at two-week intervals with 5 x 10^7-1 x 10^8 pooled parasites from various individuals composed of ring, trophozoite, and schizont stages of *P. vivax* emulsified initially in an equal volume of Freund's complete adjuvant and subsequently in incomplete Freund's adjuvant. Three days after the fifth immunization, spleens were removed and the spleen cells fused with the mouse myeloma cell line Sp2/0, a non-immunoglobulin secretor (Khusmith *et al*, 1984). The culture supernatants of growing hybrids were screened for anti-blood stage antibodies by the IFAT. The positive hybrids were cloned using the limiting dilution technic to obtain a cell line derived from a single hybrid cell or clone (Khusmith *et al*, 1984).

Preparation of ascitic fluids

Approximately 1 x 10^7 hybridoma cells were inoculated intraperitoneally into each of several BALB/c mice earlier primed intraperitoneally with 0.5 ml of pristane (2,6,10,14-tetramethydecanoic acid) (Aldrich Chemical Company, Milwaukee WIS, USA) for 10 days. The ascitic fluids were collected after 1-2 weeks and centrifuged at 300*g* for 10 minutes to remove cell debris and stored at -70°C until use.

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 tested 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 1 hour. Slides were washed, air-dried, and mounted in 0.1 M-Tris buffer containing 4.5% N-propyl-gallate and glycerol and examined under a fluorescence microscope.

Possible inter-observation error during the course of fluorescence microscopic scoring was obviated by having one person read all IFAT slides. The validity of the results was ensured by the following practices throughout the study: 1) a single batch of all MAbs was used during the entire experiment, 2) at least four different isolates was processed simultaneously in the IFAT, and 3) the quality control of the assay was monitored by including a IFAT-positive MAb control and a PBS control.

Species specificity

The species specificity of the MAbs was assessed by IFAT against eleven strains of *P.falciparum.* The parasites were cultured *in vitro* according to the method described elsewhere (Trager *et al,* 1976)

Isotype determination

Classes and subclasses of anti-blood stage MAbs were determined by ELISA using isotyping reagent set (Dakopatts, Denmark).

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) by the procedure previously described (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 of the hybridoma culture supernatants or with a 1:100 dilution of ascitic fluid in a diluting buffer. The strips were washed three more 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, New York) for three days at -70°C before the films were developed.

RESULTS

Immunofluorescent antibody (IFA) reactivity of anti-blood stage MAbs

Fifty of 58 MAbs were classified into groups I (21), II (17), III (5), and V (7) previously described for *P.falciparum* and eight others exhibited staining patterns different from those previously reported (Khusmith *et al,* 1984, 1987) and were therefore designated group VII. These characteristic IFA patterns were indicative of stage specificity of the IFA reaction and possible locating of the reactive antigen(s) either on the parasites or on the infected erythrocytes membrane (Fig 1).

Characterization of MAbs

Isotyping of these MAbs showed that 43, three, three, and nine were IgG1, IgG2a, IgG3, and IgM, respectively. No MAbs cross-reacted by IFAT with asexual stages of *P. falciparum.*

Reaction of primary isolates with MAbs

The number of IFAT-positive and IFAT-negative parasitized erythrocytes were invariably present in the same slide preparation; therefore, the percentage of parasites reacting with a given MAb was determined by counting the number of IFAT-positive and IFAT-negative parasitized erythrocytes.

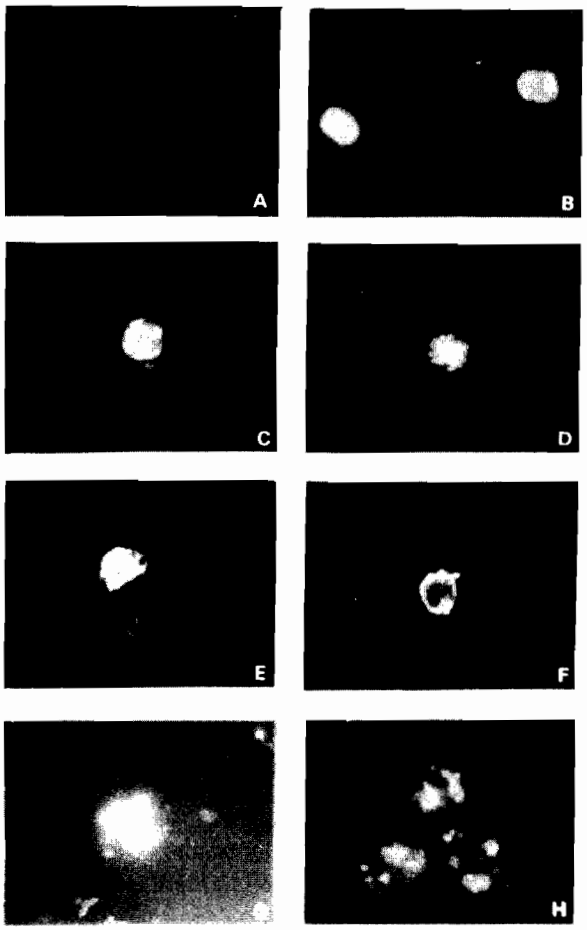

Fig 1–Different patterns of immunofluorescent antibody staining produced by monoclonal antibodies (MAbs) to *Plasmodium vivax* blood stages. A, trophozoites and B, schizonts with generalized staining by group I MAbs. C and D, punctate staining of merozoites by group II MAbs. E, staining of the surface membrane of schizont by group III MAbs. F, schizonts and H, trophozoites stained with group V MAbs. Note the intense staining around the surface of schizonts and trophozoites. G and H, trophozoites stained with group VII MAbs. Note the strong irregular staining of the internal components of trophozoites .

For each MAb tested, % reactivity was calculated using the following formula: (number of IFA-positive parasitized erythrocytes/number of erythrocytes containing pigment granules) x 100.

The IFAT-negative parasitized erythrocytes were those containing malaria pigment granules observed with light microscopy. The results of the percent reactivities of primary isolates with 58 MAbs (Fig 2).

Distribution of antigenically diverse parasites

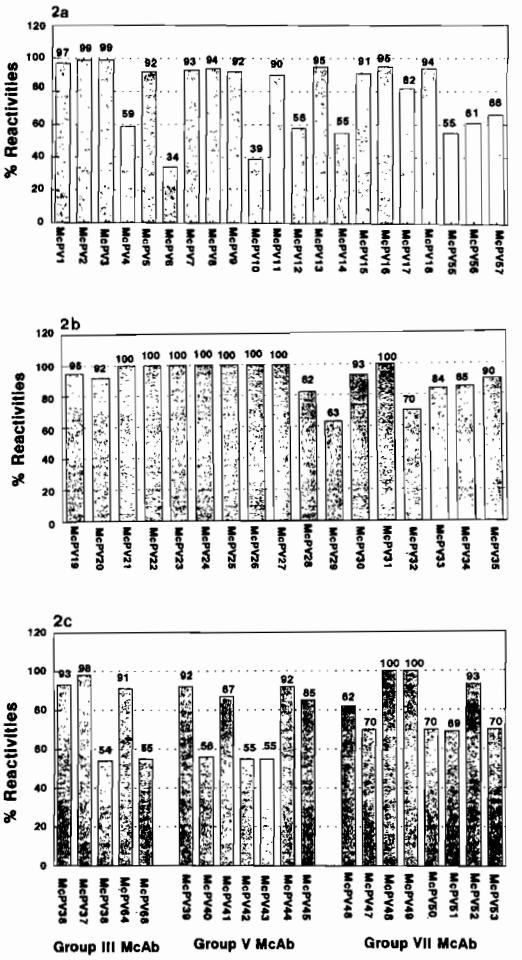

Fig 2–Antigenic diversity of *Plasmodium vivax*. Percent reactivities of primary isolates with a, group I monoclonal antibodies (MAbs), b, group II MAbs, and c, group III, V, and VII MAbs. For each MAb tested, the percent reactivity was calculated using the formula: (number of indirect immunofluorescent antibody test-positive parasitized erythrocytes/number of erythrocytes containing pigment granules) x 100.

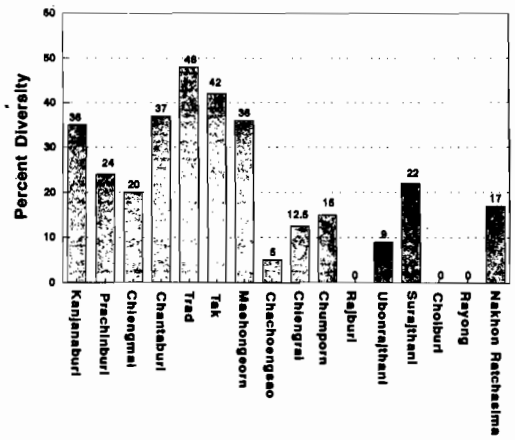

Fig 3–Diversity of *Plasmodium vivax* isolates from different provinces in Thailand. The percentage of *P. vivax* isolates that gave different reactivities with a panel of monoclonal antibodies (MAbs) tested was calculated using the formula: (number of *P. vivax* isolates with different immunofluorescent antibody reactions to the MAbs tested/total number of the parasite isolates from the same geographic location) x 100.

and their geographic location

Five hundreds and one *P. vivax* isolates were identified to originate from 49 provinces around Thailand. The percentage of *P. vivax* isolates that gave different reactions with a panel of MAbs tested was calculated using the following formula: (number of *P. vivax* isolates with different IFA reactions to the MAbs tested/total number of the parasite isolates from the same geographic location) x 100.

The antigenic diversity was shown to vary throughout Thailand from place to place and was highest in the area with the highest rate of transmission (Fig 3). The high rates of diversity were observed in areas along the Myanmar border in western Thailand and along the Cambodian border in eastern Thailand, including Trat (48.4%), Tak (41.7%), Chantaburi (36.5%), and Mae Hong Son (36.4%), where the highest malaria incidence was detected (Ministry of Public Health report, unpublished data) . Lower rates of diversity were observed in Chumphon (15%), Chiang Rai (12.5%), Ubon Ratchathani (9%), and Chachoengsao (5%). The antigenic diversity of less than 5% (1-4%) was observed in other 20 provinces, while no antigenic diversity was found in the other 11 provinces in which only 2-5 cases were collected.

DISCUSSION

Monoclonal antibodies from 58 hybridoma clones were selected from characterizations based on the uniqueness of their immunochemical properties. These MAbs were different from each other by IFA staining patterns, immunoglobulin isotypes, antigen molecular size, and strain distribution.

The population of *P. vivax* in various regions of Thailand is antigenically heterogeneous. The major proteins with apparent molecular weights of 30, 35, and 85 kDa recognized by MAbs McPV1, McPV2, McPV3, and McPV13 were located throughout the cytoplasm of all blood stages in almost all isolates of the parasite tested (> 95%). Thus, these parasite molecules may represent common antigens of this parasite species and as a consequence the recognizing MAbs may be of value in the development of an immuno-diagnostic test for vivax malaria. With group II MAbs, the merozoite-associated molecules of 41, 67, 75, 90, 115, 200 kDa from all isolates tested were recognized by four MAbs (McPV22, McPV23, McPV24, and McPV25) and might therefore represent a common antigen in *P. vivax* merozoites. Furthermore, MAbs McPV32, McPV29, and McPV26 reacting with merozoites in the IFA showed discrete puncture dots suggesting that the antigens recognized may be associated with rhoptries.

The MAbs McPV36, McPV37, McPV54, and McPV58 of group III reacted with the 200- and 195-kDa antigens of 91-98% of the isolates tested. The IFA reactions of these MAbs to mature segmenters assumed a cluster of grape-like appearance indicating that they reacted with the surface membrane of merozoites. These 200- and 195-kDa proteins recognized by the MAbs tested would thus be analogous to the 200- and 170-kDa proteins shown by electron microscopy (Barnwell, 1986) localized on the surface of merozoites in mature segmented schizonts of *P. vivax* and on the Pf190-Pf205 of *P. falciparum.*(Freeman *et al,* 1983). Recently, the merozoite of *P. vivax* was shown to possess a high molecular mass surface protein called Pv-merozoite

surface protein 1, PvMSP-1, exhibits antigenic diversity among isolates (Putaporntip *et al,* 1997). In this study Group V MAbs, gave a speckled immunofluorescent staining pattern over the entire infected erythrocyte especially trophozoites and schizonts at 115-, 95-, 56-, and 39-kDa antigens. The precise location of these antigens are at present unknown. They might be associated with the surface of infected erythrocytes or with the surface membrane of trophozoites or schizonts. However, the 115- and 95-kDa antigens were present in 86.8% and 92.3% of parasite isolates, respectively. This indicated that these antigens have been evolutionarily highly conserved and appeared to be analogous to an antigens complex of 105-, 95-, 55-, and 28-kDa proteins that have been shown to be associated with the caveola-vesicular complexes (Barnwell *et al,* 1990; Matsumoto *et al,* 1988). Future electron microscopic studies may unravel this problem.

In addition, the 30-kDa antigen recognized by group VII MAbs that reacted strongly with internal component antigens was evolutionarily highly conserved in all parasite isolates tested. None of the 501 primary isolates were negative when tested with all MAbs. Approximately 43 Mabs with different IFA patterns reacted with epitopes that were represented in more than 70% of the parasite isolates. The remaining MAbs, especially those of groups I, V, and VII, were shown to be highly variable in that their IFA reactivities varied from 34% to 66% of the isolates tested.

Antigenic diversity occurred among the *P. vivax* population in the endemic areas of Thailand and was shown to vary throughout Thailand from place to place and was highest in the area with the highest rate of transmission. The high rates of diversity were observed in areas along the Myanmar border in western Thailand and along the Cambodian border in eastern Thailand, including Trat (48.4%), Tak (41.7%), Chantaburi (36.5%), and Mae Hong Son (36.4%), where the highest malaria incidence was detected (Ministry of Public Health report, unpublished data). These areas have been known to be a major foci of transmission, as well as a foci of multidrug-resistant *P.falciparum* (Ministry of Public Health report, 1997). Our finding of antigenic diversity in *P.vivax* confirmed the similar results from Sri Lanka previously reported (Udagama *et al,* 1987). The diversity observed could be due to immune pressure, drug pressure, or different genetic constituents. 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 several 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. However, recent advances in the understanding of the underlying molecular mechanisms of antigenic variation are being examined and questions posed for future research.

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