CHARACTERISTICS OF PLASMODIUM VIVAX-INFECTED ERYTHROCYTE ROSETTES

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Abstract. To investigate the rosette formation properties of Plasmodium vivax, blood was sampled from 26 adult Thai patients admitted with acute P. vivax malaria and a predominance of trophozoite and schizont stages in their peripheral blood smears. In each case, P. vivax-infected cells formed spontaneous rosettes with two or more uninfected red blood cells. Rosette formation of P. vivax was dependent on the divalent cations (Ca²⁺/Mg²⁺) and was highly sensitive to trypsin and heparin, but, unlike P. falciparum, rosettes of P. vivax did not reform after removal of heparin. Plasma taken from patients with either acute uncomplicated P. falciparum or P. vivax malaria reversed rosette formation of all P. vivax isolates whereas plasma from uninfected controls had no effect. There was a small but significant increase in rosette-reversing activity in plasma taken during the convalescent period (P < 0.001). The increment in reversal activity was significantly greater in plasma taken following recovery from P. vivax malaria compared with P. falciparum malaria. This suggests that P. vivax rosette reversal activity is antibody mediated and has both species-specific and cross-species components.

Plasmodium falciparum is the most virulent of all human malaria parasites. The high multiplication rate of the blood stage of this parasite and the adhesive properties of the infected red blood cells, which result in cytoadherence and rosette formation, both contribute to virulence, although the importance of these adhesive characteristics in determining disease manifestations has not been investigated thoroughly. The sequestration of P. falciparum-infected red blood cells in the microvasculature of vital organs that results from cytoadherence, is considered to result in dysfunction of these organs causing cerebral malaria, renal failure, and metabolic dysfunction. Studies to determine if there is an association between rosette formation of P. falciparum and cerebral malaria have produced conflicting results. The molecular mechanisms involved in rosette formation are still being elucidated. Plasmodium vivax is a major cause of morbidity in Asia and South America, but unlike P. falciparum malaria, infection with P. vivax malaria is seldom fatal. Nonetheless, P. vivax-infected red blood cells also readily form rosettes. We have compared the characteristics of rosette formation by P. vivax with those of P. falciparum and assessed the inhibitory effects of serum factors on P. vivax rosette formation.

MATERIALS AND METHODS

Patients. Patients admitted to the Hospital for Tropical Diseases, Bangkok, Thailand with an acute febrile illness caused by P. vivax and who had a predominance of trophozoite or schizont stages of parasite development on a peripheral blood smear were included in this study. The patients had not received any antimalarial drugs or other treatment before admission to hospital. All patients gave fully informed consent to donation of the blood samples used in this study. These studies did not interfere with routine clinical management and were part of chemotherapy evaluations approved by the ethics committee of the Faculty of Tropical Medicine, Mahidol University.

Parasites. Clinical isolates of P. vivax were obtained from patients on admission to the hospital. No anticoagulant was used. Blood specimens were prevented from clotting by defibrination using 33 glass beads/10 ml of blood. Blood group was determined by standard hemagglutination techniques using anti-A and anti-B antisera. Parasitemia and the stage distribution of parasite development were determined from microscopy of Giemsa-stained thin blood films.

Plasma samples. Defibrinated blood was collected from healthy donors (n = 3 for each blood group, A, B, and O) and from patients with acute uncomplicated P. falciparum (n = 3 for each blood group, A, B, and O) or P. vivax malaria (n = 3 for each blood group, A, B, and O) on admission (day 0), and from the same patients on days 7, 14, 21, and 28. These patients with P. falciparum and P. vivax malaria were all non-immune urban dwellers who had either not had malaria previously, or had one or two previous episodes. Plasma was separated, complement was inactivated at 56°C for 30 min, and the sample was then stored at -20°C until use.

Counting of rosettes. Rosette formation. Rosette formation was assessed by adding Giemsa solution (British Drug Houses, London, United Kingdom), final concentration 1:10, to whole blood diluted at a 5% hematocrit with phosphate-buffered saline. One drop of the stained blood was mounted on a glass slide with a cover slip. The number of rosettes per 200 infected red blood cells, and number of uninfected red blood cells in 100 rosettes were counted by microscopy (1,000 magnification). Rosette formation was expressed as the percentage of the infected red blood cells with two or more adherent uninfected red blood cells. The number of uninfected red blood cells per rosette was counted by microscopy (1,000× magnification). Rosette formation was expressed as the percentage of the infected red blood cells with two or more adherent uninfected red blood cells. The number of uninfected red blood cells per rosette was counted in each case. To determine intra-observation variation, the number of rosettes in a single sample was counted 10 times. In the in vitro studies, defibrination of whole blood using glass beads with gentle swirling did not cause hemolysis of the red blood cells. The Giemsa solution used to stain parasites in the culture also had no effect on the percentage of parasitized red blood cells forming rosettes or the number of adherent cells.

Reversion of P. vivax rosette formation. The patients were blood grouped on admission so that compatible sera could be tested. The patient’s defibrinated blood (25 μl) was mixed gently with an equal volume of blood group-matched sera from the healthy donors, and also from blood group-matched

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malaria patients in a 96-well microtiter plate and incubated at 37°C for 30 min. A control well containing patients’ blood without any serum was included in each case. Rosette formation of the *P. vivax*-infected red blood cells was then determined as described, and the reversion (%) of rosette formation was calculated using the following formula: (number of rosettes in a control well - number of rosettes in a tested well) / (number of rosettes in a control well) × 100.

**Heparin sensitivity of *P. vivax* rosettes.** Heparin (5,000 IU/ml) was diluted to 5, 10, 50, 100, 1,000 unit/ml, mixed with the patient’s defibrinated blood in a 24-well plate, and incubated at 37°C for 30 min. A well containing control parasitized red blood cells without heparin was also included. Rosette formation and the percentage of reversion was determined as described above. To see whether transient exposure to heparin inhibits the reformation of rosettes, heparin was removed by washing with RPMI 1640 medium, and the red blood cells were then incubated further in malaria culture medium at 37°C for 2 hr. The number of rosettes was then determined as described above.

**Trypsin sensitivity of *P. vivax* rosettes.** Trypsin (1:250) (Flow Laboratories, Thame, United Kingdom) was diluted in 10-fold dilutions in RPMI 1640 and mixed with the patient’s packed red blood cells in 24-well plates to a final hematocrit of 1% and then incubated at 37°C for 30 min. A control well without trypsin was included. The number of rosettes was counted in each well.

**Cation dependence of *P. vivax* rosettes.** Stock 1 M ethylene-bis (oxy-ethylenenitrito) tetra-acetic acid (EGTA) was diluted to 10, 5, 1, 0.1, 0.01 mM in veronal buffer, pH 7.2. The patient’s blood (25 µl) was mixed gently with equal volumes of EGTA in a 96-well plate, and incubated at 37°C for 30 min. The number of rosettes was counted as described above. A control rosette-forming parasite in veronal buffer without EGTA was included. To assess the effects of transient cation depletion on reformation of rosettes, blood samples with EGTA were washed with RPMI 1640 medium and pellets of the red blood cells were then resuspended in complete malaria culture medium for 2 hr at 37°C before the number of rosettes were counted.

**Statistical analyses.** The percentage of rosette formation reversal is dependent on the initial percentage of rosettes in each case. Therefore, all descriptive statistics and analyses were weighted by the percentage of individual’s rosettes. Non-normally distributed data were logarithmically transformed. To investigate the relationship between rosette reversion and the concentrations of EGTA and heparin, separate linear regressions were performed with adjustment for strain and weighting for admission percentage of rosettes.

To assess the effects of plasma from patients with *P. falciparum* and *P. vivax* on reversal of rosette formation, three summary measurements were calculated for each patient-serum combination. These were reversal (%) on admission, fractional change by day 28 compared with the admission value, and the slope calculated from the log-linear regression of reversal (%) versus time since admission. These data were compared by parametric and nonparametric tests as appropriate. All statistical calculations were performed using SPSS for Windows version 6.0 (SPSS Benelux, Inc., Gorinchem, The Netherlands). Because of the multiple comparisons made, the conservative choice of a *P* value < 0.01 was chosen for the level of statistical significance.

**RESULTS**

Twenty-six patients with acute *P. vivax* malaria were studied; all were febrile at the time of the study, all but two also complained of chills, and all made an uncomplicated recovery from their infection. The median (range) duration of preceding fever was three (1–5) days. The median (range) *P. vivax* parasitemia was 0.6% (0.1–2.1%). All patients’ samples showed rosette formation; the median (range) percentage of *P. vivax*-infected red blood cells forming rosettes was 52% (4–80%). The median (range) number of uninfected red blood cells in the rosettes was three (2–10) red blood cells. There was no significant difference in rosette formation between blood groups A, B, and O. The intra-observer coefficient of variation for the counting of rosettes was 2.5% (mean proportion of rosettes = 50%). Washing with RPMI 1640 solution and resuspension in malaria culture medium did not affect the number of rosettes formed.

**Effect of heparin and trypsin.** Heparin (*n* = 24) induced concentration-dependent reversal of *P. vivax* rosette formation in each case that was independent of blood group (*P* < 0.001). Rosette formation was inhibited completely by heparin at > 100 units (Figure 1). This effect of heparin could not be abolished by washing the red blood cells with RPMI 1640 medium containing 10% AB serum. *Plasmodium vivax* rosette formation was very sensitive to trypsin (*n* = 24); a concentration as low as 25 µg/ml produced a complete reversal of rosette formation.

**Effect of chelators.** When the chelating agent EGTA was added to *P. vivax*-infected blood, there was a significant relationship between dose and reversal (%) (*P* < 0.001) that was also independent of blood group. A 50% reversal of the rosette formation (EC50) was obtained at 5 mM EGTA (Figure 2), but complete reversal of *P. vivax* rosette formation by EGTA could not be obtained with concentrations of EGTA up to 100 mM. The rosettes could not be restored after removing the EGTA and reincubating in complete culture medium under malaria culture conditions.

**Reversal of rosette formation by acute and convalescent plasma.** Plasma from *P. vivax*- and *P. falciparum*-infected patients was investigated for a rosette-reversing effect with 17 of
the *P. vivax* isolates. The malaria-naive, healthy control plasma (n = 3) did not reverse rosette formation of any isolates. The geometric mean (range) reversal induced by the admission plasma was 21% (2–83%) from patients with uncomplicated *P. falciparum* malaria and 8% (0–40%) from patients with *P. vivax* malaria (*P* = 0.001) (Figure 3). When day 28 (convalescence period) plasma were compared with day 0 plasma (acute period), there was a small but significant increase in reversion of rosette formation by plasma from both *P. falciparum* and *P. vivax* patients (*P* < 0.001 in both cases). The median (range) fractional increase in reversal of rosette formation induced by *P. vivax* malaria plasma was significantly greater than that induced by *P. falciparum* malaria plasma: 1.75% (-0.06% to +39%) compared with 0.44% (-0.005% to +8.5%) (*P* = 0.001, by Wilcoxon signed rank test). There was a significant positive log linear trend in percentage reversion with time in convalescent plasma from both *P. falciparum* and *P. vivax* malaria patients. The slope for % reversal and day since admission for plasma from *P. vivax* malaria (mean = 0.03, range = 0.004–0.117) was steeper than for plasma from *P. falciparum* malaria (mean = 0.019, range = -0.001 to +0.065) (*P* < 0.001, by paired t-test).

Blood group antibodies precluded testing of unmatched sera. Blood group has also been identified as a confounder when assessing rosette formation. Therefore, for each isolate, six measurements were recorded with each of three different blood group–matched plasma samples from *P. falciparum*-infected patients and three different blood group–matched plasma samples from *P. vivax*-infected patients, i.e., a total of 102 measurements (blood group A = 36, blood group B = 42, blood group O = 24). To distinguish independent effects of blood group and plasma on the reversal of rosette formation, the Kruskal-Wallis test was performed to test for heterogeneity between the three plasma samples from each blood group. No significant differences were found within blood group A (*P* = 0.29; n = 6 for each plasma type), blood group B (*P* = 0.62; n = 7), and blood group O (*P* = 0.07; n = 4).

**DISCUSSION**

Rosette formation occurs with all mature *P. vivax* trophozoite- and schizont-infected erythrocytes we have tested. Some isolates of *P. vivax* also formed giant rosettes (>10 uninfected red blood cells bound to an infected red blood cell). Thus, rosette formation appears to be a consistent feature of *P. vivax* in Thailand, whereas not all Thai isolates of *P. falciparum* form rosettes (Angus B, Chotivanich K, Udomsangpet R, unpublished data). Otherwise, *Plasmodium vivax* rosette formation generally has similar properties to that observed with *P. falciparum*. Rosette formation occurs only with pigment-containing, mature stage parasites (trophozoites and schizonts). As for *P. falciparum*,*P. vivax* rosette formation was sensitive to trypsin and to heparin, but was reversed completely by standard heparin at a concentration of 100 units/ml, whereas clinical isolates of *P. falciparum* kept ex vivo under malaria culture conditions show >50% rosette disruption at this concentration of standard heparin, and some strains are even less sensitive to heparin. Also, unlike *P. falciparum* rosette formation, removal of heparin from the medium could not restore the rosettes of *P. vivax*. As shown in *P. falciparum*, the presence of divalent cations was essential for rosette formation of *P. vivax*. The chelator EGTA, which chelates calcium and magnesium ions from the culture medium, produces 100% reversal of *P. falciparum* rosette formation. EGTA (0.01–10 mM) also reversed *P. vivax* rosette formation but the maximum effect observed was 50% reversal, and this effect could not be reversed after replacing divalent cations in the culture medium.

Rosette formation has been implicated in the pathogenesis of severe falciparum malaria. In The Gambia, cerebral malaria in children was associated with rosette formation of the infecting parasite isolate, and absence of anti–rosette formation antibodies. The rosette-formation phenotype in *P. falciparum* has also been associated with disease severity in studies from Kenya, but not in studies from Thailand or Papua New Guinea. However, whereas only some isolates of *P. falciparum* show rosette formation, all *P. vivax* we have examined to date exhibit this property. However, *P. vivax* very rarely causes severe malaria. This suggests that rosette formation alone, in the absence of cytoadherence, is insufficient to cause severe disease.

In this study, acute plasma from both *P. falciparum*- and *P. vivax*-infected patients reversed *P. vivax* rosette formation, although the plasma from patients with *P. falciparum*...
malaria had a significantly greater effect. Plasma from healthy individuals who had never had malaria showed no reversal activity, which suggests that there is a disease- not species-specific component to this activity. The relative contributions of antibody and acute phase reactants were not determined. Serial plasma samples taken in convalescence from both malarial species showed a significant increase in reversal of rosette-formation activity, although the fractional increase was greater in patients who recovered from \textit{P. vivax} malaria. Homologous serum from patients with \textit{P. falciparum} malaria reverses \textit{P. falciparum} rosette formation, and this reversal activity also increases in convalescence. It suggests a predominantly species-specific, antibody-mediated effect. Further studies will be required to determine the nature and specificity of this antibody. \textit{Plasmodium vivax} rosette-reversing activity appears to have both species-trancending and more species-specific components.

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