Paucity of *Plasmodium vivax* Mature Schizonts in Peripheral Blood Is Associated With Their Increased Cytoadhesive Potential

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Malaria in humans is caused by 5 species of parasites of the genus *Plasmodium* (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*). Malaria caused by *P. falciparum* is the most lethal form of disease and predominates in Africa. Although *P. vivax* infections are rarely fatal, this parasite is the most geographically widespread, with 2.48 billion people worldwide at risk [1].

The severity of *P. falciparum* is partially attributed to the ability of the mature asexual stages to cytoadhere to the microvasculature of several organs [2]. The absence of mature *P. falciparum* parasites in the peripheral blood circulation of patients is an unequivocal evidence of cytoadherence and parasite sequestration. Adhesion of *P. falciparum*-infected erythrocytes (Pf-iEs) is mediated by electron-dense protuberances on the surface Pf-iEs, named knobs, which contain several antigens, including *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is expressed at the cell surface [2]. PfEMP1 is directly involved in the adhesion of Pf-iEs to various host receptors [2]. Because *P. vivax*-infected erythrocytes (Pv-iEs) lack “sticky” knob structures and because all parasite forms can be observed in the peripheral blood of patients, it has become dogma that *P. vivax* lacks the ability to cytoadhere and therefore sequester.

Recently, we demonstrated that Pv-iEs are able to cytoadhere to endothelial cells expressing receptors known to mediate *P. falciparum* cytoadhesion, such as ICAM-1 and CSA [3]. The adhesive capacity of *P. vivax* was further confirmed by Chotivanich and collaborators, who showed that Pv-iEs adhere to CSA-coated plates and placenta sections via hyaluronic acid [4]. Moreover, although no PfEMP1 homolog has been found in *P. vivax*, variable protein members (VIR proteins) have been shown to play a role in adhesion. First, computational analysis using a *P. falciparum*-based algorithm revealed putative adhesive motifs in VIR proteins [5]. Second, antibodies toward VIR proteins partially prevented adhesion [3]. Finally, specific vir genes transfected into *P. falciparum* mediated specific adhesion to the ICAM-1 protein [6]. Here, we evaluated the potential differences in cytoadherance capability between the *P. vivax* mature forms, trophozoites and schizonts. In parallel, we determined the distribution of the different blood-stage forms (ring, trophozoite, and schizont) in freshly drawn peripheral blood from *P. vivax*-infected patients.

**METHODS**

**Ethics Statement**

All protocols and consent forms were approved by the Ethics Review Board of the *Fundação de Medicina Tropical*.
Dr. Heitor Vieira Dourado (FMT-HVD; approval CAAE 0044.0.114.000-11).

Study Area and Subjects
Patients were recruited and examined at FMT-HVD, a tertiary care center for infectious diseases in Manaus, Amazonas State, Brazil. Up to 7 mL of peripheral blood was collected immediately after confirmation of *P. vivax* infection by a thick blood smear (n = 50). Patients were then treated with chloroquine and primaquine according to the standard protocol recommended by the Brazilian Malaria Control Program. *P. vivax* monoinfection was subsequently confirmed by polymerase chain reaction (PCR) analysis as described elsewhere [7].

Parasitemia Determination
Blood samples from *P. vivax* patients were collected in sodium citrate tubes. Two thin blood smears were prepared and stained with Giemsa stain. The parasitemia was determined by calculating the number of parasites per 500 leukocytes (Supplementary Figure 1A). The parasite stages were determined for a minimum of 200 parasites and were separated into sexual (gametocytes) (Supplementary Figure 1B) and asexual forms (Supplementary Figure 1C). The asexual forms were further divided into the following 3 groups: (i) ring (<22 hours post-invasion); (ii) trophozoite, including late forms (23–40 hours post-invasion); and (iii) schizont (>40 hours post-invasion) (Supplementary Figure 1C).

Parasite Purification and Maturation
Samples with >50% asexual forms at the trophozoite stage were selected for the cytoadhesion assays (n = 8). First, the selected blood samples were filtered using a CF-11 column to remove white blood cells, as described elsewhere (Supplementary Figure 1D) [8]. Second, we performed parasite enrichment by Percoll 45% gradient as described by our group previously [3] (Supplementary Figure 1E). Half of the parasites obtained after Percoll enrichment were used immediately in cytoadhesion assays, the other half were cultured as described previously by Russell et al [8] for 18–22 hours for parasite maturation (Supplementary Figure 1F).

Cytoadhesion Assays
The cytoadhesion assays were performed as described elsewhere [3]. Briefly, human lung endothelial cells (HLECs) were grown to confluence on 8-well culture slides (BD), and then, 5 × 10^5* P.v-iE*, either in vitro matured or not matured, were added to each well in cytoadhesion medium (RPMI 1640 pH 6.8). The culture slides were incubated for 1 hour at 37°C and then extensively washed to remove any unbound parasites. The slides were then fixed in methanol and stained with Giemsa stain (Supplementary Figure 1G). The number of P.v-iEs adherent to the endothelial cell monolayer was determined, and cytoadhesion was expressed as the mean ± standard deviation of the number of erythrocytes per mm² observed in 3 wells.

Statistical Analysis
The Mann-Whitney test was used to compare 2 groups, and the Kruskal-Wallis test was used for comparisons between >2 groups. Correlations were analyzed using the Spearman correlation. Differences were considered statistically significant when *P* < .05. Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, CA).

RESULTS
To evaluate the capacity of *P. vivax* blood stages to cytoadhere, we performed cytoadhesion assays at 2 time-points for each of the 8 isolates: (i) immediately after blood withdrawal; parasites from these samples were Percoll-enriched purified and were primarily trophozoites; and (ii) after 18–22 hours of cultivation (in vitro maturation), in which the majority of parasites were schizonts. P.v-iE maturation increased the amount of schizonts from 7% to 59% (Figure 1A). Moreover, the adhesion assays performed with matured and nonmatured P.v-iEs showed that the matured parasites displayed a higher (3.5-fold) binding ability to HLECs than the nonmatured forms (Figure 1B). Additionally, we also performed adhesion assays using 3 other isolates containing a majority of parasites in ring stage and no adhesion was observed (data not shown). Importantly, we found a positive correlation between the schizont percentage and the cytoadhesion index (Figure 1C).

After demonstrating that the matured parasites have higher ability to cytoadhere and this higher ability is correlated to an increased proportion of schizonts in matching patient isolates, we wished to see if this result was reflected in a wider clinical survey of vivax malaria patients. We hypothesized that if the schizonts are more cytoadhesive they may "sequester", resulting in a lower than expected proportion of schizonts (relative to other asexual stages) in the peripheral circulation of patients. For this purpose, 50 patients were included in this study after being diagnosed with clinical *P. vivax* malaria by microscopy. *P. vivax* monoinfection was further confirmed with a sensitive PCR method [7]. No mixed infection was detected. Of these 50 patients, the majority (n = 27) had no schizonts in circulation at the time of blood withdrawal. Notably, in the remaining individuals who presented with schizonts in the peripheral circulation (n = 23), only a small proportion of this form was observed (Figure 1D). A predominance of parasite ring forms (68.09% ± 22.22%) compared to the other forms was observed (trophozoites, 28.82% ± 23.62%, and schizonts, 3.09% ± 7.87%; Figure 1D and 1E), indicating biased distribution of the asexual forms in the thin blood smears of all *P. vivax* patients analyzed.
DISCUSSION

In *P. falciparum*, the absence of mature trophozoites, schizonts, and developing gametocytes in the peripheral blood circulation of humans is clear evidence for the sequestration of these stages [2]. It has long been postulated that Pv-iEs do not sequester because all stages of this parasite can be observed in the peripheral blood of patients.

We have previously shown that Pv-iEs purified from patients’ blood cytoadhere ex vivo to endothelial cells and placental cryosections, most likely by binding to ICAM-1 and CSA host receptors [3]. This ability was further confirmed by an independent group that showed that Pv-iEs collected in Thailand can cytoadhere ex vivo to CSA and HA soluble receptors and placental cryosections [4]. Moreover, transfection of the nonadherent *P. falciparum* strain 3D7 with a *P. vivax* specific *vir* gene allowed this strain of *P. falciparum* to adhere to ICAM-1 under flow conditions [6]. Therefore, there is now a growing body of evidence supporting that *P. vivax* may have an adhesive phenotype, although this parasite does not express PfEMP-1 or generate knob structures at the surface of the iEs. Other *Plasmodium* species, including *P. knowlesi* and

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**Figure 1.** Distribution of *Plasmodium vivax* asexual stages in patients’ peripheral blood, schizont percentage, and Pv-iE cytoadhesion rate in HLEC before and after short-term in vitro maturation. The percentage of schizonts before and after short-term in vitro maturation (A). The number of Pv-iEs adhered per mm² in HLEC before and after short-term in vitro maturation (B). The correlation between the schizont percentage and the Pv-iE cytoadhesion rate to HLEC (C). The percentage of asexual Pv-iEs stages in the peripheral blood of each patient (D). The mean percentage of asexual Pv-iEs stages in 50 patients (E). In panel C, the squares represent Pv-iEs before maturation, whereas the triangles represent Pv-iEs after maturation. The statistical analyses were performed using the Mann Whitney test or Kruskal-Wallis test for comparisons between 2 or more groups, respectively. The correlational analyses were performed using the Spearman test. Abbreviations: HLEC, human lung endothelial cell; Pv-iE, *P. vivax*-infected erythrocyte.
In vivo, the sequestration of P. vivax schizonts cytoadhere to and to identify the parasite proteins involved in this phenomenon. These studies should include additional ex vivo studies using human target organs, which may allow a better understanding of P. vivax pathogenesis.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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