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Cytokine modulation of human blood viscosity from vivax malaria patients

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ABSTRACT

Malaria is a major infectious disease in several countries and is caused by protozoa of the genus *Plasmodium*. In vivax malaria patients, inflammatory processes occur, as well as changes in cytokines and blood flow. The present study analyzed the cytokine modulation of blood viscosity from patients infected with *Plasmodium vivax* (*P. vivax*). Blood samples were collected from 42 non-infected individuals (control group) and 37 individuals infected with *P. vivax*. The IL-2, IL-4, IL-6, IL-10, TNFα, TGF-β and IL-17 cytokine concentrations in the serum were assessed, and the blood rheological properties were determined. The analysis of blood viscosity for shear rates revealed that the blood viscosity of the infected patients was significantly greater than that of the non-infected individuals. The viscosity of the blood was greater in the infected individuals than in the non-infected subjects. The serum from individuals with *P. vivax* infections exhibited higher IFN-γ and IL-17 concentrations and lower TGF-β levels. Incubation of the blood from infected individuals with IL-17 or IL-17 associated with IFN-γ reduced the viscosity to rates equivalent to the blood from non-infected individuals. Independently of cytokine modulation, no correlation was found between the parasitemia and blood viscosity of the infected patients. These data suggest that the alterations of blood viscosity are relevant as an auxiliary tool for the clinical diagnosis of disease. In malaria, erythrocytes are more sensitive to osmotic shock, and the reduction of viscosity by IL-17 may be related to a possible immunomodulator agent during infection.

1. Introduction

Malaria is a serious health problem worldwide (Murray et al., 2012). Epdemiological studies estimate that malaria is responsible for approximately 584,000 deaths. The highest indexes occur in the African Region, where an estimated 90% of all malaria deaths occur, and in children aged younger than 5 years, who account for 78% of all deaths (WHO, 2014). Malaria, caused by protozoa of the genus *Plasmodium*, is endemic (Sagaki et al., 2013) and considered to be re-emerging in several countries. *Plasmodium vivax* is the most prevalent species and is responsible for the most cases of malaria (Gething et al., 2012; Li et al., 2010), including in the Brazilian Amazon region (Honorio-França et al., 2011; Aguiar et al., 2014).

In malaria, the development of intracellular parasites is accompanied by a series of structural, biological, chemical, and functional changes in the erythrocytes, as evidenced by changes in the cell membrane, which are responsible for the clinical and pathological symptoms. Changes in the adhesion and rheological properties of erythrocytes are very important because these traits are directly linked to the increased destruction of these cells, leading to anemia and the sequestration of cells (Mohandas and An, 2012). Due to erythrocyte tropism and their modifications to the structure of the infected cells, *Plasmodium* spp. infections play an important role in blood flow, particularly in the vascular system.

Blood rheology is influenced by the viscosity of the plasma, the erythrocytes, and the deformation of erythrocyte aggregation (Piagnerelli et al., 2003), which are primarily the blood alterations

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caused by infections such as malaria. The role of blood viscosity is to maintain the systemic blood pressure and blood gases (Cabrales et al., 2005), and experimental studies showed that restoration of homeostatic conditions is required for the restoration of blood rheological properties (Cabrales et al., 2007).

The blood from individuals infected with *Plasmodium falciparum* shows changes in the conformational and rheological properties of blood cells, resulting in the obstruction of capillary vessels and hampering the flow of erythrocytes (Fedosov et al., 2011) Studies also report that in cases of severe malaria, erythrocyte modifications that may enhance the inflammatory response occur, resulting in changes in blood flow (Mohandas and An, 2012; Cromer et al., 2013), thrombocytopenia (Cox and Mcconkey, 2010; Lacerda et al., 2011), and activation and/or apoptosis of platelets (Coelho et al., 2013).

Studies on hemorheological alterations in malaria vivax are controversial. Some authors reported the high deformability of *P. vivax*–infected red blood cells (Handayani et al., 2009), whereas others showed a reduction of aggregation and deformability of *P. vivax*–infected red blood cells associated with an increased level of parasitemia (Jayavanth et al., 2004), suggesting that rheological parameters are linked to the evolution of infections.

An inflammatory process occurs in malaria that can lead to cytokine release (Chen and Liu, 2009). It is known that an overproduction of pro-inflammatory cytokines occurs during infection with *Plasmodium* spp. (Franklin et al., 2009). During infection, these cytokines can control the growth of the parasite, as well as exacerbate the pathology (Kabyemela et al., 2013). Some studies verified the importance of cytokine profiles and their interactions in the immune response of the malaria parasite *P. vivax* (Zeyrek et al., 2006; Sortica et al., 2012 Sortica et al., 2012). Parasitemia and the immune response during the course of disease are factors that determine its severity (Zeyrek et al., 2006). High levels of Th1 and Th2 cytokine profiles, such as IFN-γ and IL-4, respectively, are associated with disease severity, and the immunomodulatory cytokine TGF-β plays a key role in limiting the pathology of malaria (Omer et al., 2000). However, the balance between pro-inflammatory and anti-inflammatory cytokines is not fully understood, and some studies suggest the participation of both anti- and pro-inflammatory cytokines (Sortica et al., 2012).

By contrast, the effect of cytokines on rheological parameters during *P. vivax* infection has not yet been elucidated. It is possible that alterations in the cytokine concentrations in the blood of patients with malaria vivax can modify the blood viscosity. Therefore, the objective of this study was to verify the cytokine modulation of blood viscosity in individuals infected with *P. vivax*.

2. Materials and methods

2.1. Subjects

The human blood flow and levels of cytokines were evaluated in a cross-sectional study. The study was carried out in the Brazilian Amazon endemic region at the Health System Program of Porto Velho, Rondônia State, Brazil, and Hospital Juruá—Malaria Center, Acre State, Brazil. Symptomatic patients diagnosed with malaria infection were asked to participate in the study. The samples were collected prior to starting treatment for malaria. The study included patients with confirmed diagnosis of *P. vivax* with negative serology for hepatitis, HIV, syphilis and dengue. The control group was composed of healthy individuals that were negative for malaria parasites as determined thick blood smear and had not reported any malaria episodes for at least one year (Rodrigues-Da-Silva et al., 2014). Blood samples were collected from 42 individuals with negative for *P. vivax* and 37 individuals with *P. vivax*. This study was approved by the institutional Research Ethics Committee of the Campus of Araguaia of Federal University of Mato Grosso, Brazil (Protocol Number CAAE: 14793413.2.0000.5587) and all the subjects gave informed written consent before entering the experimental protocol.

2.2. Determination of parasitemia

Parasitemia was evaluated by a semiquantitative analysis using blood smear microscopy. Thick and thin blood films were stained with Giemsa. The *Plasmodium* species were identified, and parasitemia was determined by microscopic examination. All slides were examined by two well-trained microscopists from the Brazilian Ministry of Health. Parasitemia levels were estimated by counting the number of parasites (all species and stages) per 200 white blood cells (WBC) on blood films. If fewer than nine parasites were detected, 300 additional leucocytes were counted to obtain more precise results (Rodrigues-Da-Silva et al., 2014).

2.3. Blood rheological parameters

The rheological parameters were measured using the Modular Compact Rheometer—MCR 102 [Anton Paar® GmbH, Ostfildern, Germany] according to França et al. (2014). For all experiments, 600 μL of blood treated with or without cytokines was applied to the surface of a plate reader, followed by the removal of excess sample. The readings were obtained with a permanent control of gap measurements with TruGap™ in 0.099-mm increments and the measuring cell Toolmaster™ CP 50. Precise temperature control was achieved using T-Ready™ and the software Rheoplus V3.61. The graphics were obtained using Rheoplus. For the flow curves and viscosity, established parameters were based on the control of shear stress [τ] to 0–5 Pa for the upsweep and 5–0 Pa for the downward curves. The tests were conducted under isothermal conditions at 37 °C, with 75 readings analyzed.

For the viscosity curve under a temperature scan, established parameters were based on the fixed control shear stress [τ] to 1 Pa with a variation of temperature from 25 ± 0.1 to 45 ± 0.1 °C and a heating rate of 1 °C/min. The parameters were recorded every 0.5 ± 0.1 °C, with 41 readings analyzed.

2.4. Serum sampling and quantification of cytokines

We collected 8 mL of blood from each individual in tubes without anticoagulant. We centrifuged the blood samples at $300 \times g$ for 15 min, until serum separation. Serum samples were stored individually at −80 °C for further cytokines determination.

The serum samples were thawed, and cytokines IL-2,IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17 were measured using a cytometric bead array (CBA, BD Biosciences) according to the manufacturer's procedures. A flow cytometer was used for these analyses (FACSCalibur, BD Biosciences, USA). The data were analyses using the software FCAP Array 1.0.

The TGF-β concentrations were analyzed using an ELISA kit from Enzo® Life Sciences (United Kingdom). The reaction rates were measured by absorbance in a spectrophotometer with a 450 nm filter. The results were calculated using the standard curve and shown in pg/dL.

2.5. Treatment of blood with cytokines

Samples of 8 mL of blood from each individual in tubes with anticoagulant were collected for rheological analysis. To assess the effect of cytokines TGF-β, IFN-γ and IL-17 on human blood flow, a 580 μL sample of blood were incubated with 20 μL of cytokines [Sigma St. Louis, USA; final concentration 100 pg/mL] for