CASE REPORT: DISSEMINATED INTRAVASCULAR COAGULATION COMPLICATED BY PERIPHERAL GANGRENE IN A RHESUS MACAQUE (MACACA MULATTA) EXPERIMENTALLY INFECTED WITH PLASMODIUM COATNEYI

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Abstract. We report the first case of disseminated intravascular coagulation (DIC) complicated by peripheral gangrene induced by Plasmodium coatneyi in rhesus monkeys. Ten days after experimental challenge, numerous petechiae were noted over the trunk and extremities, with polychromasias, severe anemia, thrombocytopenia, and moderate parasitemia. These changes were accompanied by elevated serum activity of blood urea nitrogen, creatinine, transaminases, and creatinine phosphokinase. The animal received intravenous fluid support, artemether, and blood transfusion. Three days after treatment, the platelet counts returned to normal, and parasitemia was abated. However, several areas of skin discoloration with gangrenous tissue in the hands and the tail were observed. Coagulation profile showed elevated D-dimers and elevated levels of fibrinogen/fibrin degradation products with low levels of protein S functional activity. DIC with peripheral gangrene is very rare in Plasmodium-infected individuals. Our results indicate that the experimental model of P. coatneyi infection of rhesus monkeys is important for studies of malarial anemia and coagulopathy.

INTRODUCTION

Plasmodium is responsible for > 500 million clinical cases of malaria annually around the world.1 Although natural human transmission of the simian malaria parasite Plasmodium knowlesi has been described,2–4 four species of Plasmodium are generally attributed to human infection: P. falciparum, P. vivax, P. ovale, and P. malariae. P. falciparum is responsible for most of the severe cases of malaria estimated in 10% of the total number of clinical cases. Fatal cases have been estimated between 1 and 3 million every year mainly among children younger than 5 years old. Ninety percent of these lethal cases are reported in sub-Saharan Africa.5–7 These numbers have been maintained without dramatic changes owing to the high prevalence of drug-resistant strains of parasites and failure of malaria control programs.1

The World Health Organization (WHO) has established criteria to classify severe malaria.8 The classification of clinical entities associated with the severity of the disease allows comparison in different epidemiologic settings. Severe malaria is defined by seven major syndromes: cerebral malaria, severe anemia, respiratory distress, renal failure, metabolic acidosis, hypoglycemia, and coagulopathy. Cerebral malaria and severe anemia are the most common complications in children and primigravidas.9–10 This is in contrast with the frequent expression of pulmonary edema and renal failure in severe malaria cases in adults.11 Parasite sequestration associated with a pro-inflammatory milieu is a critical pathogenic mechanism leading to multi-organ dysfunction in P. falciparum malaria.12 However, the precise molecular mechanisms associated with this and each of the individual syndromes of severe malaria are not fully understood.

Non-human primates (NHPs) have been extensively used in malaria research as experimental animal models for drug and vaccine development.13–16 The use of NHP for characterizing mechanisms associated with severe malaria is evident in recent studies.17–20 We used simian malaria parasites in rhesus macaques to explore several biologic aspects of the complex parasite–host interactions involved in the pathogenesis of multi-organ failure. P. coatneyi shares several morphologic and biologic features with P. falciparum.21,22 Experimental infections of rhesus monkeys with P. coatneyi reproduce several histopathologic findings reported in humans infected with P. falciparum.17,18,23–25 We describe here the first case of disseminated intravascular coagulation (DIC) and peripheral gangrene in a rhesus macaque experimentally infected with P. coatneyi. Although platelet dysfunction and increased pro-coagulant activity is a common finding in severe malaria, DIC complicated with symmetrical peripheral gangrene is very rare.26 The experimental design that we have used to follow-up infections provided us with a unique opportunity to explore hematologic and immunologic parameters associated with DIC.

MATERIALS AND METHODS

Study design and clinical laboratory assays. R1p-8, a 4-year-old male rhesus monkey (Macaca mulatta), was born and raised at the Yerkes National Primate Research Center. This animal was part of an experimental protocol designed to evaluate anemia induced by simian malaria parasite infections (unpublished data). Procedures used were approved by the Emory University’s Institutional Animal Care and Use Committee and followed accordingly. The animal was inoculated with fresh 2 × 10^6 P. coatneyi–infected erythrocytes/kg (corresponding to a total number of 1.77 × 10^8) obtained from a donor monkey previously infected with a cryopreserved stable. The monkey’s temperature was recorded every day after experimental challenge using a subcutaneous transponder chip (Bio Medic Data Systems, Seaford, DE). Capillary blood samples were obtained every day by ear prick and collected into EDTA-coated capillary tubes. Blood samples were used to determine hemoglobin concentration using a HemoCue photometer (HemoCue, Lake Forest, CA) and to quantify

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the parasite load using Giemsa-stained thin and thick smears. Reticulocyte counts were performed by the new methylene blue stain technique.\(^{27}\) Serum levels of fibrinogen degradation products were determined using a direct latex agglutination assay (Pacific Hemostasis, Middletown, VA). Serum levels of myoglobin were determined by ELISA using monkey-specific reagents (Life Diagnostics, West Chester, PA). On indicated dates, venous blood samples were obtained for a blood coagulation test and blood chemical analysis. Blood platelet counts were obtained by manual quantification using Giemsa-stained thin smears.

**Gross pathology and histopathology.** Immediately before necropsy, 14 days after experimental challenge, the monkey was sedated using intramuscular inoculation of Ketamine-HCl (15 mg/kg), and a complete physical exam was performed. The animal was euthanized by sodium pentobarbital overdose (Schering-Plough Animal Health, Union, NJ). Tissue samples from all organs including bone marrow and skin were placed in 10% normal buffered formalin and paraffin embedded. Sections (6 μm) were cut and stained with hematoxylin and eosin. To show polymerized fibrin, replicate sections were stained with phosphotungstic acid hematoxylin (PTAH) as described.\(^{28}\)

**Statistical analysis.** Linear correlation was used to evaluate the relationship between hemoglobin concentration, parasitemia, and reticulocyte counts. This approach was also used to evaluate the relationship between hemoglobin concentration and thrombocytopenia. \(P < 0.05\) was considered significant.

**RESULTS**

**Clinical evolution.** Parasites were identified in peripheral blood smears 48 hours after experimental challenge. Parasitemias followed the described pattern of alternating high and low values (Figure 1).\(^{29}\) During the follow-up period, sharp peaks of parasitemia were observed on days post-inoculation (DPI) 2, 4, and 6. The hemoglobin concentration exhibited a sustained decrease from the pre-challenge levels (18.2 g/dL) to reach 8 g/dL on DPI 5 (Figure 1A; Table 1). A small improvement in hemoglobin level was observed from DPI 6 to DPI 8. On DPI 9, the animal was reluctant to move and began to show signs of severe malaria that included anorexia and tachypnea. On examination, the animal was hypothermic (36.1°C) with mucocutaneous paleness. Blood tests showed thrombocytopenia (46,000 platelets/μL), mild anemia (hemoglobin level of 8.3 g/dL), and a parasitemia level of 356,868 parasites/μL that corresponded to 8.7% infected erythrocytes. In addition, the macaque exhibited a progressive decrease in platelet counts that reached a minimum of 46,000. A negative correlation between the number of platelets and parasitemia was found using linear correlation analysis \((r = -0.8, P = 0.028)\). The animal was treated with a single subcurative dose of artemether (2 mg/kg Artesian; Dafr Pharma, Turnhout, Belgium) to prevent life-threatening hyperparasitemia.

On DPI 10, the animal had a normal temperature (37.6°C) but showed severe mucocutaneous paleness, and numerous cutaneous petechiae over the trunk and extremities developed. Cardiopulmonary examination revealed holosystolic murmur grade 4 and tachypnea. Blood tests showed severe anemia (hemoglobin level of 4.4 g/dL), thrombocytopenia (67,000 platelets/μL), and parasitemia of 90,242 parasites/μL (2.2%). Thin blood smears revealed polychromasia and a strong medullary response with an erythroblastemia of 15%. However, reticulocyte counts did not correlate with peaks of parasitemia (Figure 1). Blood urea nitrogen (BUN) and creatinine values were increased (152 and 6.8 mg/dL, respectively). Elevated transaminase levels, glutamic oxaloacetic

**Figure 1.** (A) Course of parasitemia in rhesus macaque RIp-8 experimentally challenged with \(P.\) coatneyi infected erythrocytes. The values are plotted with hemoglobin levels determined using a hemoglobin meter and capillary samples obtained daily after infection. The kinetics of parasitemia were determined using Giemsa-stained thick smears and the Earle-Perez method\(^{15}\) and expressed as the number of parasites per microliter of blood (left y-axis). Hemoglobin concentrations are expressed as g/dL (right y-axis). ■, parasitemia levels; ◆, hemoglobin levels. (B) Number of platelets and proportion of reticulocytes in peripheral blood determined at different time-points after experimental infection. Arrow represents the day when the \(P.\) coatneyi-infected monkey received complete blood transfusion. ●, number of platelets in capillary blood; ▲, proportion of reticulocytes in red blood cell counts.

**Table 1**

<table>
<thead>
<tr>
<th>Laboratory parameter</th>
<th>RR</th>
<th>DPI 0</th>
<th>DPI 9</th>
<th>DPI 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (mg/dL)</td>
<td>11.2–13.7</td>
<td>12.6</td>
<td>8.3</td>
<td>7.4</td>
</tr>
<tr>
<td>Platelets (×10^3/μL)</td>
<td>109–597</td>
<td>410</td>
<td>460</td>
<td>600</td>
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<tr>
<td>Parasitemia (#/μL)</td>
<td>0</td>
<td>0</td>
<td>356,868</td>
<td>0</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>0.1–5.2</td>
<td>0.67</td>
<td>2</td>
<td>10.8</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>8–20</td>
<td>17</td>
<td>152</td>
<td>13</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.8–2.3</td>
<td>0.8</td>
<td>6.8</td>
<td>0.7</td>
</tr>
<tr>
<td>SGPT (U/L)</td>
<td>14–30</td>
<td>20</td>
<td>207</td>
<td>24</td>
</tr>
<tr>
<td>SGOT (U/L)</td>
<td>0–82</td>
<td>27</td>
<td>357</td>
<td>36</td>
</tr>
<tr>
<td>CPK (U/L)</td>
<td>0–1,680</td>
<td>744</td>
<td>2,340</td>
<td>439</td>
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</table>

RR, reference range; DPI 0, before experimental challenge; DPI 9, 9 days after experimental challenge; DPI 14, 14 days after experimental challenge and before necropsy.
(357 U/L), and glutamic pyruvic (207 U/L), were also associated with an increased level of creatinine phosphokinase (CPK, 2,340 U/L). Dipstick urinalysis revealed pigmenturia with hemoglobin recorded as 4+ and only four to six red blood cells per high-power field. The animal was hemodynamically stabilized, and a total volume of 100 mL of blood was transfused. Curative anti-malaria therapy was initiated using artemether at 4 mg/kg followed by 2 mg/kg/day for 4 days.

On DPI 11, the animal was more active, and the parasitemia was recorded as negative. However, the animal appeared reluctant to use the hands on DPI 12. Therefore, buprenorphine was initiated at a dose of 0.01 mg/kg every 6 hours. Discoloration of the digit tips and tail was noted, and the lesions deteriorated by self-inflicted bite injuries that required initiation of ceftriaxone sodium on DPI 13. At this time-point, a blood test showed that platelet counts had increased from 67,000 to 474,000 platelets/μL, and the hemoglobin concentration had increased from 4.4 to 9.0 g/dL. Nevertheless, deep red discoloration was detected on both hands and arms. On DPI 14, the animal’s hands and tail showed deep red tissue discoloration with evidence of gangrene at the margins. Blood tests on DPI 14 showed leukocytosis (absolute number, 21,800/μL) and normal platelet counts (359,000 platelets/μL). BUN, creatinine, CPK, and transaminase levels were normal. Coagulation tests showed elevated D-dimers (1,175 mg/L), elevated fibrinogen/fibrin degradation products (> 40 μg/mL), and decreased functional protein S levels (46%). Functional protein C levels, antithrombin III, and fibrinogen concentration were all within normal limits. Coagulation factors II, IX, and X were at normal levels. This is in contrast with elevated levels of coagulation factors V, VII, VIII, XI, and XII. The myoglobin concentration, determined by ELISA, was elevated (> 652 ng/mL). This clinical laboratory finding, along with evidence of pigmenturia and an elevated activity of serum CPK, reported on DPI 10, suggested acute rhabdomyolysis. In view of the clinical complications and the poor prognosis, euthanasia was elected.

**Gross pathologic findings.** On gross examination, the animal was in fair to poor body condition. The mucous membranes were pale and slightly yellow. The dorsal aspect of both hands was dark red and had multiple erosions. All of the fingertips and fingernails of both hands were black. The tips of the third, fourth, and fifth fingers of the right hand were ulcerated (Figure 2). The second and third toes of the left foot were dark red. The dorsal aspect of both feet was slightly reddened. Three fourths of the tail was dark red and covered with black spots (Figure 2). Both lungs were mottled and had a dark green discoloration. The liver was slightly enlarged and diffusely red with a green discoloration. There was mild splenomegaly. Both kidneys were diffusely pale with a green discoloration.

**Histopathologic findings.** Sections of haired skin ranged from diffusely necrotic to focal dermal necrosis with epidermal separation and cleft formation (Figure 3). The epidermis was mildly hyperkeratotic. There were multifocal areas of epidermal separation and cleft formation with occasional infiltration of moderate numbers of neutrophils. The superficial dermis had multifocal areas of collagen degeneration and necrosis intermixed with foci of pigmentary incontinence. The external, and occasionally the internal, root sheath of many hair follicles was necrotic. The deep dermis had multifocal to diffuse infiltration of neutrophils. Scattered in the deep dermis were multi-focal areas of hemorrhage (Figure 3). Occasional blood vessels contained fibrin thrombi. To identify fibrin deposits, we used PTAH staining (Figure 4). The renal tubules were markedly eosinophilic, and many contained hyaline casts. In the cortex, occasional tubules contained a basophilic material, consistent with mineral deposition. The medullary tubules had variable numbers of nucleated erythrocytes. Occasional pulmonary blood vessels contained fibrin thrombi. The alveolar spaces had variable numbers of alveolar macrophages. In some sections, the alveolar walls contained a brown-black pigment, consistent with hemoglobin deposition. The hepatic sinusoids contained large amounts of a black-brown pigment (hemozoin), free and within macrophages, intermixed with variable numbers of nucleated red blood cells. Scattered in the parenchyma and within sinusoids were small aggregates of extramedullary hematopoiesis (Figure 5).

**DISCUSSION**

Clinical syndromes associated with severe malaria are life-threatening entities that require aggressive clinical management. The WHO has defined severe malaria in patients with confirmed *P. falciparum* asexual parasitemia and the presence of one or more of the following clinical or laboratory features: prostration, impaired consciousness, respiratory distress, multiple convulsions, circulatory collapse, pulmonary edema, abnormal bleeding, jaundice, hemoglobinuria, or severe anemia. Subclinical evidence of hemostasis dysfunction
is common in *Plasmodium*-infected individuals, but severe coagulopathy with DIC and peripheral gangrene is not a common finding in severe cases of malaria. Only *P. falciparum* has been reported to be associated in rare instances with this clinical presentation. The prevalence of DIC can reach 10% of the total number of severe cases of malaria in endemic areas and 30% in non-immune patients with imported cases of *P. falciparum* malaria. Clinical expression of DIC in malaria patients is correlated with poor outcome. Although several host and parasite factors play a role in the pathogenesis of severe malaria, little is known about the molecular mechanisms involved in the physiopathology of the malarial hematologic complications. Novel experimental animal models are essential for defining the mechanisms involved in both coagulopathy and severe anemia.

*P. coatneyi* is a simian malaria parasite that displays several biologic and morphologic features in common with *P. falciparum*. Most relevantly, macaques experimentally infected with *P. coatneyi* exhibit clinical complications similar to severe cases of *P. falciparum* malaria in humans. Cerebral malaria, placental malaria, and metabolic complications of severe malaria have all been reported in macaques experimentally infected with *P. coatneyi*. We report here the clinical profile and pathologic findings of a rhesus macaque that developed severe coagulopathy compatible with DIC after experimental infection with *P. coatneyi*. To our knowledge, this is the first description of severe coagulopathy in a non-human primate model of malaria. We confirmed that vascular compromise mediated by DIC led to peripheral gangrene and probably acute rhabdomyolysis. The profound hematologic disturbances induced by *P. coatneyi* in rhesus macaques support the relevance of using this unique host–parasite combination to study the pathogenesis of severe malarial anemia and malarial coagulopathy.

Laboratory criteria pathognomonic for DIC include 1) evidence of procoagulant activation, thrombocytopenia, elevated prothrombin time, partial thromboplastin time or thrombin time, and decreased fibrinogen; 2) evidence of fibrinolytic activation, elevated fibrinogen/fibrin degradation products, and elevated D-dimer; 3) consumption of coagulation inhibitors, low levels of protein C, protein S, and anti-thrombin; and 4) biochemical evidence or organ damage. Clinical evidence from endemic areas of malaria indicates that severe malaria patients exhibit lower levels of protein C, protein S, and anti-thrombin III in comparison with patients with mild malaria. Consistent with the most common asso-
ciation of severe malaria cases with *P. falciparum* infection, individuals infected with *P. falciparum* exhibit lower levels of coagulation inhibitors in comparison with *P. vivax*-infected individuals. Decreased levels of protein C have also been correlated with elevated levels of tumor necrosis factor (TNF)-α in *P. falciparum* malaria. We found that thrombocytopenia and a high level of fibrin degradation products are a common finding in rhesus macaques experimentally infected with *P. coatneyi* (unpublished data). Coagulopathy disturbance in this experimental animal model can progress to DIC as reported here. Extensive fibrin thrombus formation progressed to peripheral gangrene. Laboratory findings compatible with DIC in the *P. coatneyi*-infected rhesus monkey reported here involved thrombocytopenia, elevated levels of D-dimer, increased levels of fibrinogen/fibrin degradation products, and decreased functional protein S levels.

The number of platelets dropped dramatically during the acute *P. coatneyi* infection. The origin of thrombocytopenia is multi-factorial in malaria-infected individuals. Reported mechanisms include immunologic platelet clearance mediated by auto-antibodies or enhanced destruction by macrophages. Platelet destruction can be mediated by elevated levels of macrophage-colony stimulating factor, clearance of parasite-infected thrombocytes, massive platelet pooling in an enlarged spleen, and oxidative stress that induces platelet lipid peroxidation. Severe thrombocytopenia seen in the *P. coatneyi*-infected rhesus monkey reported here is the result of platelet consumption by microthrombi during DIC.

*Plasmodium falciparum* malaria induces profound hemodynamic changes explained by vascular collapse or obstruction mediated by sequestration of infected erythrocytes and the increased adhesiveness of normal erythrocytes. The erythrocyte membrane is modified during malaria infection by several mechanisms that include expression of parasite antigens on the surface of infected erythrocytes, exposure of erythrocytic cryptic domains, and changes in phospholipid asymmetry. These erythrocyte membrane changes can result in the cytoadhesive interactions of *P. falciparum*-infected erythrocytes with endothelial receptors. The loss of phospholipid asymmetry, mediated by oxidative stress, causes the exposure of phosphatidylserine (PS) at the outer surface of the cell. PS-exposing erythrocytes cause endothelial activation, retraction, and subsequent exposure of the extracellular matrix. Activated endothelial cells can also upregulate the expression of tissue factor (TF) to modulate hemostasis through the activation of the extrinsic coagulation cascade. Histopathologic analysis using PTAH staining showed extensive intravascular fibrin thrombi in the *P. coatneyi* rhesus experimentally infected as a consequence of extensive activation of the coagulation cascade. Although sequestered infected erythrocytes were not identified in this case, cytoadherence of *P. coatneyi*-infected erythrocytes to endothelial cells have been reported in cerebral malaria. Our lack of observed sequestered parasites could be caused by the timing of the necropsy after anti-malaria treatment.

Systemic endothelial activation is a distinctive feature of *Plasmodium*-infected individuals. Endothelial cell activation causes the release of preformed procoagulant molecules such as the von Willebrand factor (vWF). vWF recruits platelets through its interaction with CD36, a process that results in the magnification of the endothelial perturbation. Platelets can also be activated through interaction with damaged endothelial cells. The activation of platelets gives rise to cell agglutination and the release of various vasoactive molecules from platelet granules. Endothelial activation by pro-inflammatory cytokines also promotes the adhesion of neutrophils. Neutrophil adhesion is followed by degradation and the release of enzymes such as myeloperoxidase and elastase that mediate local injury. These events facilitate the additional exposure of procoagulant molecules such as vWF, collagen, and fibrinectin. Activation of monocytes by pro-inflammatory cytokines also triggers the synthesis of TF, which can be released from the cell in microparticles to modulate the activation of the extrinsic coagulation cascade.

Criteria used to identify rhabdomyolysis include the clinical evidence of muscle damage associated with elevated creatinine kinase and pigmenturia, associated with the presence of myoglobin or hemoglobin in the urine. Myocyte injury induces the release of intracellular contents that include creatinine, urea, potassium, creatinine kinase, and other enzymes. Myoglobin can directly induce tubular necrosis that along with hypovolemia facilitated by massive intramuscular capillary destruction can be involved in renal failure. Skeletal muscle damage associated with increased serum levels of creatinine kinase and myoglobin have been reported in severe cases of malaria. Nevertheless, severe rhabdomyolysis with myoglobinuria and renal failure is rare. The information available for rhabdomyolysis in malaria infection indicate that there is a positive correlation between levels of creatinine and myoglobin. However, the most sensitive clinical laboratory parameter to detect rhabdomyolysis is the increased levels of creatinine phosphokinase. *P. coatneyi*-infected rhesus monkey reported here exhibited increase levels of creatinine phosphokinase and myoglobin with pigmenturia in the absence of red blood cells. These clinical features are suggestive of rhabdomyolysis. In severe cases of malaria with renal failure, hemoglobin casts have been reported. Although hyaline casts were seen histologically in the kidney of this rhesus monkey, special stains for hemoglobin were negative. In addition, reactivity with a myoglobin monoclonal antibody was also negative. The positive reactivity of blood in urine samples suggests the presence of hemoglobinuria and/or myoglobinuria. Although it has been proposed that parasite sequestration plays a leading role in the pathogenesis of rhabdomyolysis, we did not identify sequestered parasites in skeletal muscle tissue samples or histologic evidence of myocyte injury. The failure to show parasite sequestration can be explained by the presence of extensive fibrin thrombi in various stages of organization and anti-malarial treatment with an artemisinin derivative.

In this study, we used pathologic and hematologic laboratory studies to elucidate clinical features associated with hemostasis dysfunction in a rhesus macaque experimentally infected with *P. coatneyi*. To our knowledge, the case that we present here is the first evidence that this simian malaria parasite can induce DIC. The severe coagulopathy induced in a malaria naïve animal lead to peripheral gangrene. The high prevalence of coagulation dysfunction and the prominent clinical presentation seen in a rhesus macaque experimentally infected with *P. coatneyi* make this parasite–host combination a useful experimental animal model to evaluate the molecular mechanisms involved in the pathogenesis of severe malaria.
REFERENCES


