Complement Activation Correlates With Disease Severity and Contributes to Cytokine Responses in Plasmodium falciparum Malaria

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The impact of complement activation and its possible relation to cytokine responses during malaria pathology was investigated in plasma samples from patients with confirmed Plasmodium falciparum malaria and in human whole-blood specimens stimulated with malaria-relevant agents ex vivo. Complement was significantly activated in the malaria cohort, compared with healthy controls, and was positively correlated with disease severity and with certain cytokines, in particular interleukin 8 (IL-8)/CXCL8. This was confirmed in ex vivo–stimulated blood specimens, in which complement inhibition significantly reduced IL-8/CXCL8 release. P. falciparum malaria is associated with systemic complement activation and complement-dependent release of inflammatory cytokines, of which IL-8/CXCL8 is particularly prominent.

Keywords. malaria; complement activation; inflammation; cytokines; hemozoin; heme; IL-8/CXCL8; C5a/C5aR1; Plasmodium falciparum.

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Plasmodium falciparum malaria is a major cause of mortality in sub-Saharan Africa. The disease is inflammatory, and the consequence of inflammation is double-edged. A balanced inflammatory response promotes elimination of the infectious agent and contributes to tissue resolution, whereas a greater than necessary inflammatory response fuels tissue damage, further morbidity, and possibly death.

The complement system is part of innate immunity, acting as a surveillance system that quickly can be activated by sensing danger to the host by both sterile and nonsterile stimuli and thereby contributing to maintain tissue homeostasis and prevent tissue repair. On the other hand, undesired or uncontrolled complement activation can induce tissue damage and organ dysfunction in the host, as in septicemia and autoimmune disorders. Studies have shown extensive complement activation during malaria [1]. However, data on terminal complement cascade activation are scarce, and it is still not clear how malaria parasites induce complement activation and how complement activation can activate downstream inflammatory pathways during malaria.

An important stimulus in malaria immunopathology is crystalline hemozoin, produced as a heme detoxification product after hemoglobin catabolism during the intraerythrocytic phase of the Plasmodium life cycle and released together with the parasite and hemoglobin upon rupture of infected erythrocytes [2]. Ferric heme (hemin) is liberated from hemoglobin as a result of oxidation of the heme moiety within hemoglobin [3]. Hemozoin crystals and monomeric hemin have been proposed as modulators of inflammation in malaria pathology and have been shown to induce leukocyte activation with generation of inflammatory cytokines [2, 4]. However, it remains unclear whether hemein and hemozoin directly induce complement activation and whether complement activation is crucial in cytokine response following hemozoin/hemin release.

The aim of this study was to elucidate the role of complement activation during P. falciparum infection. By combining patient data with findings of ex vivo analyses of human whole-blood specimens, we were able to document a relation between complement activation in P. falciparum malaria and subsequent release of cytokines, in particular interleukin 8 (IL-8)/CXCL8.

MATERIALS AND METHODS

Descriptions of the whole-blood sampling protocols, ex vivo incubation of whole-blood specimens, preparation of hemin and...
hemozoin, complement inhibitors, complement assays, and cytokine assays are given in the Supplementary Materials.

Study Design and Participants
The study population has previously been described [5]. Briefly, during 2 malaria seasons, clinical data and blood samples were collected from all adult nonpregnant patients admitted to the Central Hospital of Maputo, Mozambique, with an axillary temperature of ≥38°C and/or suspected malaria. P. falciparum malaria was found in 131 patients, of whom 70 were coinfection with human immunodeficiency virus type 1 (HIV). Severe and very severe malaria were defined as the presence of >1 and ≥3 malaria severity criteria, respectively, modified from the World Health Organization [5]. Healthcare workers, their family, and their friends were included as controls (n = 52). All included controls tested negative for P. falciparum. More-detailed descriptions of study participants and sample preparation are provided in the Supplementary Materials.

Ex Vivo Incubation of Whole-Blood Specimens With Hemin and Hemozoin
The inflammatory response in blood from nonimmune healthy adult volunteers was studied by incubating hemin (1000 µg/mL) and hemozoin (10 µg/mL) in lepirudin-anticoagulated whole-blood specimens in round-bottomed sterile polypropylene tubes (Nalgene NUNC). To characterize the cytokine response and to study the complement dependency thereof, blood specimens were supplemented with the complement inhibitors compstatin Cp40 analogue (20 µM), eculizumab (100 µg/mL), and C5aR1 antagonist PMX53 (20 µM). The inhibitors were preincubated in whole-blood specimens for 5 minutes prior to the addition of hemin or hemozoin.

Statistical Analysis
Differences between the patient groups were analyzed by the χ² test, for categorical variables, and the Mann–Whitney U test, for continuous data. Correlations between sC5b-9 and cytokines were investigated using Spearman rank order correlation. Results from the ex vivo experiments are presented in scatterplots as mean values and 95% confidence intervals. Values from hemin and hemozoin were compared to those for buffer controls, and statistical significance was calculated using a paired t test. If significantly separated (P < .05), hemin and hemozoin were further compared to hemin and hemozoin supplemented with complement inhibitors, using 1-way analysis of variance (ANOVA) followed by the Dunnett multiple comparisons test, in GraphPad Prism, version 6.0d for MAC OS X (GraphPad Software, La Jolla, California). When pairing was significantly effective (P < .05), statistical significance was calculated using repeated-measurements ANOVA with the Dunnett post hoc test and corrected for unequal sphericity (Geisser-Greenhouse correction).

Ethics Statement
This study was designed and performed according to the Helsinki Declaration from the 59th World Medical Association General Assembly, Seoul, Republic of Korea, 2008. Blood sampling, export of samples, and subsequent analyses of blood specimens from adults with malaria and healthy controls were approved by the National Bioethical Committee at the Ministry of Health in Mozambique (ethics ID 374/CNBS/10) and the Regional Ethical Committee in Eastern Norway (ethics ID 2010/1347). We obtained informed written consent or, in cases of illiteracy, fingerprint consent from all included subjects either directly or, if the patient was unconscious or confused, from their next of kin. For the ex vivo analysis, a whole-blood specimen was collected from healthy blood donors. Informed written consent was obtained from every donor. The study was approved by the Regional Ethical Committee of South-Eastern Norway Regional Health Authority (ethics ID S-04114) and by the Uppsala ethical review board (Reference Number 2008/264).

RESULTS

In Vivo Complement Activation During P. falciparum Infection
Patients with P. falciparum malaria had markedly raised plasma levels of soluble terminal complement complex (sC5b-9), compared with healthy controls, with increasing levels according to disease severity (Figure 1A). Patients with the most-severe malaria had significantly raised sC5b-9 levels, not only compared with healthy controls (P < .001), but also compared with patients with less severe disease (P < .001; Figure 1A). Seventy of the patients were coinfected with HIV, and we have previously shown that these patients were characterized by more-severe malaria [5]. HIV-coinfected patients had a moderately higher mean sC5b-9 level (±SD) than the other patients with malaria (4.4 ± 5.4 complement-activation units [CAU]/mL vs 2.5 ± 3.5 CAU/mL; P = .003; data not shown).

Correlation Between Complement Activation and Cytokines
We have previously published data on cytokine levels in these patients with malaria [6], and the level of sC5b-9 was significantly correlated with the levels of the following 4 cytokines that were raised in patients with malaria, compared with controls: IL-8/CXCL8 (rₛ = 0.52), interleukin 6 (IL-6; rₛ = 0.63), monocyte chemotactic protein 1 (MCP-1)/CCL2 (rₛ = 0.62; Figure 1B–D), and macrophage inflammatory protein 1β (MIP-1β)/CCL4 (rₛ = 0.62; Supplementary Figure 1) (P < .01 for all comparisons). Levels of interleukin 1 receptor antagonist, interleukin 9, interleukin 10, eotaxin/CCL11, and interferon-inducing protein 10/CXCL10 were also increased in the patients [6] but did not correlate with the sC5b-9 level.

Hemin- and Hemozoin-Induced Inflammatory Response in Human Whole-Blood Specimens Ex Vivo
The ability of hemin and P. falciparum hemozoin to induce complement activation was examined in whole-blood specimens
obtained from healthy donors. Incubation of hemin (1000 µg/mL) and hemozoin (10 µg/mL) caused significant (P < .01) complement activation detected as generation of sC5b-9, which was abolished by C3 and C5 blockage (Figure 2A and 2B). Also, levels of C3bc and C5a rose significantly after hemin stimulation (Supplementary Figure 2A and 2B). Hemin induced a robust release of IL-8/CXCL8 (Figure 2C), IL-6, and MCP-1/CCL2 (Supplementary Figure 2C and 2D) in whole-blood specimens after culturing for 4 hours. Similar findings were also found with a lower dose of hemin (250 µg/mL; Supplementary Figure 3A–D). Hemozoin induced a significant (P < .05) increase in the IL-8/CXCL8 level (Figure 2D). The levels of all of these cytokines were significantly lower in the presence of all tested complement inhibitors (P < .01). No significant differences were observed between the respective inhibitors, implying that downstream C5αR1 inhibition was as effective as upstream C3 inhibition. We have previously shown a particularly strong association of IL-8/CXCL8 and disease severity in these patients with malaria [6], and, of note, the hemin- and hemozoin-induced IL-8/CXCL8 response was particularly prominent and substantially counteracted by complement inhibition (Figure 2C and 2D). P. falciparum hemozoin carries parasitic DNA [7], but degradation of DNA in the hemozoin preparation or incubation with β-hematin (synthetic hemozoin) did not reduce sC5-9 or IL-8/CXCL8 levels (data not shown).

**DISCUSSION**

We demonstrate here that patients with *P. falciparum* malaria have an increased level of sC5b-9, which correlates with elevated...

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**Figure 1.** Complement activation as measured by sC5b-9 and its relation to malaria severity score and interleukin 8 (IL-8)/CXCL8, interleukin 6 (IL-6), and monocyte chemotactic protein 1 (MCP-1)/CCL2 in patients with malaria. **A**, Plasma samples from patients with *Plasmodium falciparum* malaria were analyzed for the complement activation product sC5b-9. Patients were divided into 2 groups on the basis of malaria severity scores (MSS) of <3 (n = 114) or ≥3 (n = 15) and compared to healthy controls (HC; n = 52). Levels of sC5b-9 are presented as a box and whisker plot displaying the 25th–75th percentiles (box) and 5th–95th percentiles (whiskers). Points plotted individually lie outside the 5th–95th percentiles. Statistical difference was analyzed with the Mann–Whitney U test. **B–D**, The correlation between the sC5b-9 level and cytokine levels in plasma samples obtained from patients with malaria (n = 129) was tested using Spearman rank order correlation on logarithmic values. Significant (P < .01) positive correlation was found between sC5b-9 and each of the following variables, with r_s = 0.63 for IL-8/CXCL8 (**B**), r_s = 0.52 for IL-6 (**C**), and r_s = 0.62 for MCP-1/CCL2 (**D**). Linear values are plotted in graphs on a logarithmic scale. Abbreviation: CAU, complement activation units.
levels of certain inflammatory cytokines in these patients. We document a causal association between complement activation and 3 of these cytokines in ex vivo experiments, in which hemin- and hemozoin-induced cytokine release, and in particular the IL-8/CXCL8 level, was found to be complement mediated.

Increased levels of sC5b-9 in patients with malaria correlating to disease severity are in line with some previous reports [1]. In the present study, we extend these previous findings by examining a relatively large population of adult patients with *P. falciparum* infection and showing that a marked increase in the sC5b-9 level in these patients, along with disease severity, was significantly correlated with plasma levels of IL-6, IL-8/CXCL8, MCP-1/CCL2, and MIP-1β/CCL4. We cannot exclusively state that the elevated levels of these proinflammatory cytokines were a result of complement activation, but complement is likely among the factors that triggered the cytokine release. Induction of IL-8/CXCL8 regularly follows complement activation and is reduced in complement-deficient blood [8].

Our ex vivo findings further support a link between complement activation and induction of certain inflammatory cytokines during *P. falciparum* malaria. We show that hemin and hemozoin, which are released upon erythrocyte rupture, are both potent complement activators in human whole blood; that this activation subsequently leads to the generation of proinflammatory IL-6, IL-8/CXCL8, and MCP-1/CCL2 cytokines; and the generation of these cytokines is largely mediated by the interaction of C5a with C5aR1, because targeted inhibition of C3 and C5 activation was equally efficient to that of C5aR1 inhibition. However, hemin is hydrophobic and residual complement-independent immunostimulatory effect could be assigned to direct cytotoxic interaction with cell membranes [9].

Rupture of parasitized erythrocytes exposes the innate immune system to a variety of potential activating agents, which can contribute to the innate immune response. Hemin and hemozoin are released in large quantities and have been proposed as modulators of inflammation in malaria [2–4, 10]. The natural hemozoin tested

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**Figure 2.** Complement activation and cytokine release by hemin and hemozoin in whole-blood specimens ex vivo. Hemin or hemozoin were incubated for 4 hours in whole-blood specimens in the presence or absence of complement inhibitors at the level of C3 (compstatin), C5 (eculizumab), and C5aR1 (C5aR1ag). The degree of complement activation as measured by the sC5b-9 level in response to hemin (A) and hemozoin (B) is depicted. Further on, the effect of complement inhibitors on interleukin 8 (IL-8)/CXCL8 release induced by hemin (C) and hemozoin (D) was investigated. Zymosan served as a positive control for complement activation, and lipopolysaccharide (LPS) served as a positive control for IL-8/CXCL8. Data are presented as means and 95% confidence intervals (n = 6 in panels A and B; n = 3–5 in panels C and D). The paired t test was used to test the statistical difference between hemin/ hemozoin and buffer control, and 1-way analysis of variance followed by the Dunnett multiple comparisons test was performed to test the effect of the inhibitors. *P < .05, **P < .01, and ***P < .001. Abbreviations: CAU, complement-activation units; NS, nonsignificant; +, present; −, absent.
here was produced by *P. falciparum* in culture. Natural and synthetic hemozoin have been reported to induce somewhat different inflammatory response, which might be attributed to as hemozoin-associated biomolecules as DNA, peroxidized lipids such as the arachidonic acid derived 15-HETE [10], and proteins (primarily fibrinogen), originating from the parasite or the host. DNA-complexed hemozoin has, upon ingestion of phagocytes, been shown to activate Toll-like receptor 9 [11], an event also shown to prime cells to hemozoin-induced NLRP3 and AIM2 inflammasome activation [7]. Herein, however, a control experiment with DNase-treated hemozoin and with β-hematin indicated that the presence of DNA on hemozoin did not increase the inflammatory response with respect to sC5b-9 and IL-8/CXCL8 in our model.

Hemin, which represents the Fe3⁺-oxidized form of heme, is liberated extracellularly from the hemoglobin released upon erythrocyte rupture and is derived via reactive oxygen species [3]. Hemin has previously been shown to be a potent activator of complement [12]; this activity was reproduced here by adding hemin to human whole-blood specimens ex vivo. We used hemin derived from porcine blood in a fairly high concentration (250 μg/mL and 1000 μg/mL), representing rupture of <1% of the total erythrocyte count [12], which is in the range covering intravascular hemolysis of infected and primarily uninfected erythrocytes in malaria [13]. Hemin preparations are not completely pure, and one has to be aware of that impurities could influence the inflammatory response.

Interaction of C5a with C5aR1 seems to be the central mechanism for the induction of the cytokines that we found upregulated ex vivo. IL-8/CXCL8, the cytokine most strongly associated with disease severity in patients, was potently induced via C5aR1, a response initiated by both hemin and hemozoin. C5a has been implicated in many clinical contexts as being a main contributor to the complement-mediated inflammatory response. The impact of C5a in malaria has also been highlighted in murine experimental malaria [14], and C5a has been proposed as a mediator for cytokine release in placental malaria [15].

In conclusion, we postulate complement activation to be an important component in malaria pathophysiology by contributing to the initiation of a systemic inflammatory response and exacerbation of the disease. We show that hemozoin and hemin are potent complement activators in whole blood and that the resulting inflammatory response is largely dependent on the interaction of C5a with C5aR1. Translation of ex vivo findings to the in vivo situation should be made with caution. Still, our findings underscore C5a as a potential contributor to malaria pathogenesis and the interaction between C5a and C5aR1 as a potential target for the deleterious proinflammatory response in malaria.

**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 copublished. 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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