Immune mimicry in malaria: *Plasmodium falciparum* secretes a functional histamine-releasing factor homolog *in vitro* and *in vivo*

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The *Plasmodium falciparum* translationally controlled tumor protein (TCTP) is a homolog of the mammalian histamine-releasing factor (HRF), which causes histamine release from human basophils and IL-8 secretion from eosinophils. Histamine, IL-8, and eosinophils have been reported to be elevated in patients with malaria. This study was undertaken to determine whether malarial TCTP is found in the plasma of malaria-infected patients and to determine whether it has HRF biologic activity. Malarial TCTP was found in lightly infected human volunteers and in heavily infected Malawian children, but not in uninfected patients. Recombinant malarial TCTP, like HRF, stimulated histamine release from basophils and IL-8 secretion from eosinophils *in vitro*. Whereas malarial TCTP was less active than HRF, the concentrations that were effective *in vitro* could be achievable *in vivo*. These data suggest that malarial TCTP, present in human plasma during a malarial illness, may affect host immune responses *in vivo*.

Each year, 300–500 million people have a malarial illness, and as many as 2.7 million individuals, mostly African children, die (1). *Plasmodium falciparum* is the parasite responsible for the majority of fatal malarial infections. Malaria infections can cause fever, severe anemia, coma, and renal failure in children and adults (2) and poor birth outcomes in pregnant women (3).

The pathogenesis of malaria is complex. The immune system may mediate both protection from malaria and the development of disease. Elevations in immune mediators such as IL-1, IL-6, IL-8, tumor necrosis factor-α, and nitric oxide have been associated with disease severity in numerous studies (4–10). Eosinophils, basophils, and mast cells also seem to play important roles. Increases in plasma and tissue histamine, derived from basophils and mast cells, have been associated with disease severity in human *P. falciparum* infections and in several animal malarias (11–14). In addition, elevated plasma levels of IgE, which binds to basophils and mast cells, have been associated with severity of *P. falciparum* infection (15). Furthermore, increased eosinophil counts have been associated with recovery from infection (16–18). Despite these observations, little is known about the relationship between these cell types and disease.

Histamine-releasing factor (HRF) is a peptide described in mice and humans that causes the release of histamine, IL-4, and IL-13 from basophils (19, 20). More recently, HRF was shown to promote IL-8 secretion and a calcium response in purified human eosinophils (21). Thus, HRF plays an important role in regulating basophils and eosinophils.

HRF belongs to a class of proteins called the translationally controlled tumor protein (TCTP) homologs. Recently, a *P. falciparum* TCTP was identified (22). This protein has a high homology to human HRF; their amino acid sequences are 33% identical and 54% similar.

In view of the homology between human HRF and malarial TCTP, we investigated whether malarial TCTP may be important during malaria infections. In this article, we measured malarial TCTP in the plasma of *P. falciparum*-infected patients and show that it has *in vitro* HRF biological activity.

Methods

Plasma from Nonimmune Adult Volunteers and Malawian Children Infected with Malaria. Plasma was obtained from three individuals previously enrolled in the placebo arm of a malaria prophylaxis study. This study investigated healthy adult volunteers with no history of exposure to malaria who were treated with atovaquone or placebo, then challenged by the bite of *P. falciparum*-infected mosquitoes (23). Blood was taken before challenge and at frequent intervals after challenge to monitor for parasitemia. To detect parasites, whole blood was analyzed at once by microscopy (limit of detection, 4 parasites per μl blood), and stored samples were subsequently analyzed by PCR (limit of detection, 2 parasites/ml blood). Three placebo recipients developed erythrocytic infections and were successfully treated with chloroquine. Plasma samples were obtained from these patients before infection and 1–2 days after treatment was initiated. This study was approved by the Johns Hopkins University Institutional Review Board.

Plasma was also obtained from patients with malaria on admission to the Queen Elizabeth Central Hospital in Blantyre, Malawi. The patients were children ranging in age from 8 months to 12 years (mean age 44 months). Twenty-nine of these patients had severe malaria infections consisting of either severe anemia and/or cerebral malaria; 15 had uncomplicated malaria (24). Additionally, from 10 of these patients, plasma was available 1 month after treatment when patients were no longer infected. All studies were approved by the Research Committee of the University of Malawi College of Medicine, the Johns Hopkins University Institutional Review Board, and/or the University of Michigan Institutional Review Board.

Malarial TCTP Secretion and Measurements. *P. falciparum* was cultured, synchronized (>95%), and isolated from erythrocytes as previously described (22, 25). Isolated parasites were homogenized by sonication (Branson model W140D) in 50 mM Tris-HCl, pH 7.4, containing 1 mM phenylmethylsulfonyl

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Abbreviations: HRF, histamine-releasing factor; TCTP, translationally controlled tumor protein.

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fluoride for 5 s. Culture medium was concentrated by centrifugation through Centricon-10 filters (Millipore). Parasite homogenate (1–10 μg of protein) and medium (100 μg of protein) were loaded onto 10% NuPAGE gels (NOVEX, San Diego), electrophoresed, and blotted onto polyvinylidene difluoride according to the manufacturer's directions. The blots were then incubated with antibody to recombinant *P. falciparum* TCTP (25), developed by electrochemifluorescence (Amersham Pharmacia), and scanned by using a Storm 860 FluorImager with IMAGEQUANT software (Molecular Dynamics). For every experiment, control lanes containing known amounts of recombinant malarial TCTP (22) were used to construct a standard curve. All samples were measured in triplicate, and the results are presented as means ±SD.

To quantitate malarial TCTP in plasma samples, plasma aliquots (200 μg of protein) were loaded onto gels and quantitated as above.

Recombinant Malarial TCTP and HRF Production. Recombinant *P. falciparum* TCTP was produced in *Escherichia coli* and purified as described previously (22). Some incubations with basophils used recombinant His-tagged *P. falciparum* TCTP (a generous gift from Dr. Ian MacReadie, Commonwealth Scientific and Industrial Research Organization, Melbourne). HRF fusion protein was excision cloned from the PGEX-2T vector used in the *E. coli* production (19), including the 5′ end glutathione *S*-transferase tag, into the baculovirus vector pVL1393. Plasmid DNA was transfected into Sf9 cells for viral isolation and amplification according to the company's specifications (Invitrogen). Subsequently, the Sf9 insect cells were grown commercially on a large scale in serum-free media (Paragon Biotechnology). The cell pellet from 8 liters of Sf9 cells was dissolved in 800 ml of NBB buffer (20 mM sodium phosphate/500 mM sodium chloride, pH 7.8) to which 800 μl of PharMingen inhibitor mixture was added. The suspension was freeze thawed three times and centrifuged at 9000 rpm for 20 min. Purification of the supernatant, which contained the glutathione *S*-transferase–HRF fusion protein, was accomplished by affinity chromatography on immobilized glutathione (26) and confirmed by SDS/PAGE and Western blotting by using a polyclonal anti-HRF antibody generated against the recombinant material produced in *E. coli* (24). Protein concentration was determined by using the Bio-Rad protein assay and was found to be 160 μg/ml HRF. HRF was dialyzed against physiologic 1 × Pipes for use in all assays. Both malarial TCTP and HRF were found to contain single bands on SDS/PAGE and were judged to be greater than 95% pure.

Endotoxin Removal. Because malarial TCTP was produced in *E. coli*, endotoxin levels were measured by the limulus amebocyte lysate kit (BioWhittaker) before and after removal of endotoxin by using Detoxi-Gel endotoxin removal gel (Pierce). Both endotoxin measurements and removal were performed according to the manufacturers' specifications.

Basophil Histamine Release. After informed consent, peripheral blood was obtained from normal allergic donors by venipuncture. Mixed leukocytes containing basophils were prepared by dextran sedimentation as previously described (27). Histamine release was assayed by a standard method (19) and was measured by the automated fluorometric method (28).

Eosinophil Purification and Culture. Human granulocytes were isolated from EDTA-anticoagulated venous blood of mildly allergic donors by gradient centrifugation in isotonic Percoll (1.090 g/ml). Red blood cells were removed by hypotonic lysis followed by removal of neutrophils with an anti-CD16 antibody by using an immunomagnetic bead technique (29). Eosinophils were differentiated by light microscopy at high power magnification after staining with the Diff-Quik stain kit (Dade, Dudingen, Switzerland). Eosinophil purity was 98–100%.

Purified eosinophils were resuspended in medium M199 (GIBCO) containing 20% FCS (Sigma) and were incubated in 96-well plates (Costar) with various concentrations of malarial TCTP and HRF. The final concentration of eosinophils was 2×10^6 cells/ml in 0.25 ml total volume for each culture condition. The cell supernatants were harvested by extracting the suspension from each well, centrifuged at 500 × *g*, and the cell-free supernatant was removed. The supernatants were stored at −20° until ELISA analysis.

IL-8 Determination. Supernatants from stimulated eosinophil cultures and plasma samples were assayed with an IL-8-specific ELISA (BioSource International, Camarillo, CA) according to the manufacturer's specifications. The threshold for cytokine detection was 15.6 pg/ml.

Statistical Analysis. Means and standard deviations were calculated by using MICROSOFT EXCEL 97.

Results

Detection of Malarial TCTP in Cultured Parasites and Supernatants. Malarial TCTP was measured by quantitative immunoblotting by using polyclonal antibodies to recombinant *P. falciparum* TCTP. This antibody did not crossreact with human HRF (data not shown). Intact *P. falciparum* erythrocytes (synchronized as trophozoites) contained 87 ± 21 μg of TCTP/10^9 infected cells. Synchronized cultures were also harvested after schizogony (host red cell lysis), at which point the culture supernatants contained 44 ± 6 μg of TCTP/10^9 infected cells. Therefore, about half of the total malarial TCTP may be released during schizogony.

Detection of Malarial TCTP in Patients. Malarial TCTP was detected in two groups of patients from different clinical settings. For three nonimmune patients with PCR-positive but subpatent infections (<4 per microliter), plasma *P. falciparum* TCTP concentrations were 0.60 ± 0.44 μg/ml. The plasma TCTP concentrations were also measured in 44 Malawian children with mean parasitemias of 253,000 ± 266,000 per microliter. This is a population living in an area of high malaria transmission with relatively high levels of immunity. Plasma samples from this group contained 1.41 ± 1.44 μg/ml TCTP (mean ± SD). Plasma TCTP levels ranged from 0 to 6.9 μg/ml, with the top quartile containing TCTP levels between 2.4 and 6.9 μg/ml. Thus, malarial TCTP is present in the plasma of patients with *falciparum* malaria, and the levels are similar in the two different clinical settings.

No TCTP was detected in the plasma of the three nonimmune patients before infection, nor in 10 Malawian malaria patients 1 month after treatment with antimalarials (data not shown; the lower limit of detection of TCTP is 50 ng/ml).

Human Basophils Secrete Histamine and Eosinophils Secrete IL-8 in Response to Malarial TCTP. Basophils were isolated from allergic donors as described in *Materials and Methods*. Recombinant *P. falciparum* TCTP was a potent secretagogue (Fig. 1), causing half-maximal secretion of histamine at ≈100 μg/ml. Malarial TCTP was a log less potent than its human counterpart, HRF.

To investigate the effect of endotoxin, levels were measured before and after treating malarial TCTP with Detoxi-Gel, which is polymixin B immobilized on agarose. Before treatment, there were 375,000 endotoxin units (EU); after treatment, there were 430 EU. This is a 900-fold purification. Histamine release dose-response curves were essentially the same when the two preparations were tested in the same experiment (data not shown).

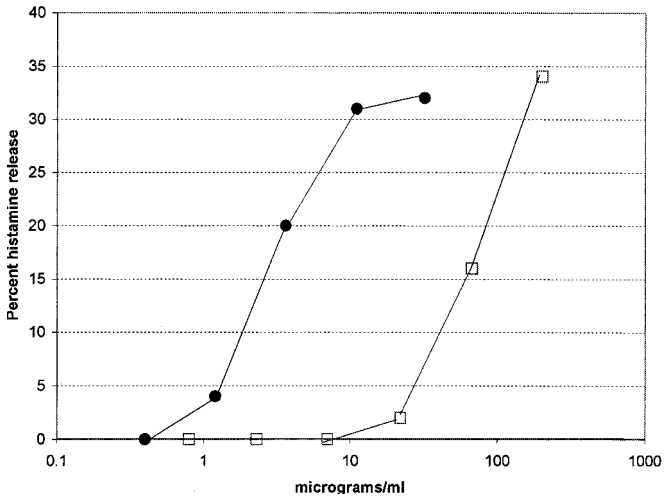

Fig. 1. Effects of malarial TCTP and HRF on basophil histamine release. Malarial TCTP (□) and HRF (●) induced basophil histamine release compared by using basophils from the same donor. Similar results were found by using basophils from three other donors (not shown).

Similar to the known action of HRF, malarial TCTP also induced IL-8 protein production from human eosinophils. Eosinophils were purified by negative selection from mildly allergic donors. Both recombinant molecules were able to induce IL-8 secretion over media alone in eosinophils from three separate donors (Fig. 2). Malarial TCTP induced IL-8 cytokine production at concentrations in the range of 10 μg/ml. Again, as was the case for histamine release, malarial TCTP was less potent than HRF in inducing eosinophil secretion.

Plasma concentrations of IL-8 in 10 Malawian patients with malaria were 43 $\pm$ 47 pg/ml. After treatment with antimalarials, IL-8 levels dropped by more than 80% in five of these patients and 15% to 55% in an additional three.

Discussion

We have demonstrated that TCTP, a parasite-produced homolog of HRF, is present in the plasma of *P. falciparum*-infected patients. Patients had plasma TCTP concentrations as high as 6.9 μg/ml. Recombinant *P. falciparum* TCTP has HRF activity *in vitro* on basophils and eosinophils at concentrations of 10–100 μg/ml. Thus, TCTP concentrations in plasma approach levels that could affect the host eosinophil and basophil responses to malaria.

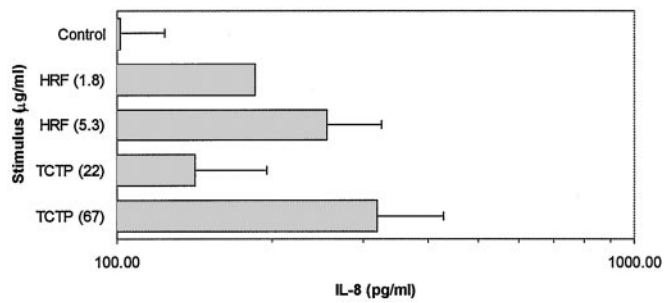

Fig. 2. IL-8 production from human eosinophils by malarial TCTP and HRF. Eosinophils were cultured in media with different concentrations of malarial TCTP or HRF, and IL-8 was measured in the supernatants. Each bar represents the average of three experiments (each using cells from a different donor), except for HRF (1.8), which represents the average of two experiments. Error bars represent standard deviations.

Plasma TCTP concentrations may reflect total parasite burdens rather than peripheral parasitemias. Volunteers in the prophylaxis study had subpatent parasitemias (PCR-positive, but less than four per microliter). Yet, their TCTP levels were not significantly different from Malawian patients who had much higher parasitemias (10^3–$10^6/\mu$l). Furthermore, in the Malawian patients, there was no correlation by regression analysis between TCTP levels and peripheral parasitemias (data not shown). Thus, plasma concentrations of *P. falciparum* TCTP do not reflect circulating parasitemias. However, they might reflect total parasite burdens, which are quite different from peripheral parasitemias, as the majority of *P. falciparum* parasites sequester in capillaries of internal organs. Unfortunately, there is currently no effective measure of total body parasite burden.

Because *P. falciparum*-infected erythrocytes are predominantly sequestered, some of the effects of released TCTP may be local rather than systemic. Heavily infected individuals may harbor as many as 10^{10} parasites per milliliter of blood (2). Based on our *in vitro* culture data, $\approx$400 μg of malarial TCTP could be released per milliliter of blood during one 48-h life cycle. Thus, in patients with large numbers of sequestered parasites, malarial TCTP could be produced locally at levels that would have profound effects on basophils and eosinophils.

Recombinant *P. falciparum* TCTP *in vitro*, like its human homolog, HRF, causes histamine release and IL-8 production from human basophils and eosinophils, respectively. The malarial TCTP is only partially homologous to HRF and is $\approx$10-fold less potent than HRF in terms of its ability to induce histamine secretion from basophils and IL-8 secretion from eosinophils. This difference might be due to inherent differences between the proteins or to the fact that the recombinant malarial TCTP was produced in *E. coli*, whereas the human HRF was produced in baculovirus. Further studies with recombinant products made in the same vectors are needed.

The malarial TCTP is a functional homolog of an immune mediator produced by a eukaryotic parasite. A number of viral pathogens have previously been found to contain genes for cytokine, chemokine, and cytokine receptor homologs such as the Epstein–Barr virus (IL-10), HHV-8 (IL-6, MIP-1α; refs. 30 and 31), orf virus (IL-10; ref. 32), myxoma virus (tumor necrosis factor-α; ref. 33), and vaccinia virus (IL1β and IFN receptors; ref. 34). A gene homologous for TGF-β was identified in filarial nematodes (35). The production of these homologs is thought to provide an evolutionary advantage to the pathogens, but the mechanisms by which this happens are still unknown. In the case of malarial TCTP, one could speculate that the vasodilatory effects of histamine might permit the parasites to circulate more readily through narrow blood vessels. Alternatively, histamine might increase endothelial cell-surface expression of thrombomodulin (36), which is both a tissue anticoagulant and a receptor for parasitized erythrocyte sequestration. So histamine may modulate some of the pathologic changes seen in malaria, prevent a disseminated intravascular coagulopathy, and possibly also increase expression of a sequestration receptor. In addition, it is possible that HRF, like other immune mediators, has other yet undiscovered functions that may be of relevance.

In any event, further work is needed to understand the effects of the malarial TCTP on the host immune system and whether it plays a role in the pathogenesis of severe malaria. A prospective study of infected patients is warranted.

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