INTRODUCTION

Malaria is a major health problem in sub-Saharan Africa but also in parts of Asia and Latin America, and it is estimated that about 1 million children die every year because of Plasmodium falciparum malaria.1 Despite major efforts, there is still no vaccine for malaria, and there is a great need for new effective therapies, especially against severe malaria. A striking difference between P. falciparum and other human Plasmodium species is the capacity of the P. falciparum parasitized erythrocytes to uninfected erythrocytes (rosetting) is associated with severe malaria. The glycosaminoglycan heparan sulfate is an important receptor for rosetting. The related glycosaminoglycan heparin was previously used in treatment of severe malaria, although abandoned because of the occurrence of severe bleedings. Instead, low anticoagulant heparin (LAH) has been suggested for treatment. LAH has successfully been evaluated in safety studies and found to disrupt rosettes and cytoadherence in vitro and in vivo in animal models, but the effect of LAH on fresh parasite isolates has not been studied. Herein, we report that two different LAHs (DFX232 and Sevuparin) disrupt rosettes in the majority of fresh isolates from Cameroonian children with malaria. The rosette disruption effect was more pronounced in isolates from complicated cases than from mild cases. The data support LAH as adjunct therapy in severe malaria.

Rosettes in Fresh Clinical Isolates

Plasmodium falciparum Rosettes Disrupts Plasmodium falciparum Rosettes in Fresh Clinical Isolates

Anna M. Leitgeb,† Karin Blomqvist,† Fidelis Cho-Ngwa, Moses Samje, Peter Nde, Vincent Titanji, and Mats Wahlgren*
Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institutet, Stockholm, Sweden; Dilafor AB, Solna, Sweden;
Biotechnology Unit, Faculty of Science, University of Buea, Buea, Cameroon

Abstract. The binding of Plasmodium falciparum parasitized erythrocytes to uninfected erythrocytes (rosetting) is associated with severe malaria. The glycosaminoglycan heparan sulfate is an important receptor for rosetting. The related glycosaminoglycan heparin was previously used in treatment of severe malaria, although abandoned because of the occurrence of severe bleedings. Instead, low anticoagulant heparin (LAH) has been suggested for treatment. LAH has successfully been evaluated in safety studies and found to disrupt rosettes and cytoadherence in vitro and in vivo in animal models, but the effect of LAH on fresh parasite isolates has not been studied. Herein, we report that two different LAHs (DFX232 and Sevuparin) disrupt rosettes in the majority of fresh isolates from Cameroonian children with malaria. The rosette disruption effect was more pronounced in isolates from complicated cases than from mild cases. The data support LAH as adjunct therapy in severe malaria.
children with malaria. We report that LAH disrupts rosettes of 42 of 47 fresh isolates obtained from Cameroonian children with mild or complicated malaria, and we state that rosettes from children with complicated malaria are more sensitive to the LAH than are rosettes of children with mild malaria.

MATERIALS AND METHODS

Sample collection in Cameroon. The study was conducted in Buea, a city with 150,000 inhabitants in the southwestern part of Cameroon. Malaria transmission takes place throughout the year but peaks during the rainy season that lasts from April to October. The entomological inoculation rate (EIR) has been shown to be 0.56 infected Anopheles mosquito bites per person per night in this area during the rainy season. The field-work was performed between March and May 2007 at three different health centers and four different hospitals in the Buea area. The study included malaria-infected children between 6 months and 14 years of age. Informed consent was given by the guardian. The diagnosis was based on laboratory tests (Giemsa-stained blood smears) and clinical examination. Hemoglobin levels in whole blood were measured using HemoCue Hb 201+ (Hemocue, Ängelholm, Sweden). Complicated *P. falciparum* malaria was defined as a patient requiring hospital admission and quinine or artemether infusion because of anemia, hyperparasitemia (parasitemia > 5%), or severe symptoms including hyperpyrexia, seizures, prostration, and/or vomiting. Cases with a positive blood smear for *P. falciparum* without complicating manifestations were classified as mild malaria and treated as outpatients with treatment *per os*. Ethical permissions for the study were obtained both in Cameroon and Sweden (numbers G379/900 and 2006/1323-31, respectively).

*P. falciparum* clinical isolates used in the study. Blood was withdrawn from patients with parasitemia above 10,000 pRBC/μL blood and collected in ethylene diamine tetraacetic acid (EDTA) tubes. The blood samples were depleted of leukocytes by treatment with polymorph preparation (Axis-Shield, Oslo, Norway) according to the manufacturer’s instructions within 2 hours of collection. Briefly, 2–5 mL whole blood were carefully layered over 5 mL polymorph preparation and centrifuged at 500 × g for 5 min to separate RBCs, leukocytes, and plasma. The plasma and leukocyte band were removed, and separated packed RBCs were washed three times with RPMI (Roswell Park Memorial Institute) 1640 medium (Sigma Aldrich, St. Louis, MO). A total of 200 μL packed RBCs was transferred to 4.0 mL malaria culture medium supplemented with 10% inactivated human AB+ non-immune Swedish serum and placed at 37°C for maturation of ring-stage parasites to trophozoites using standard methods. The parasitemia was counted, and the rosetting rate was determined by calculating the number of trophozoite pRBCs within rosettes relative to the total number of trophozoite pRBCs present in the culture. A rosette was defined as at least two uninfected RBCs bound to one pRBC.

Glycosaminoglycans used in the study. LAHs are heparin derivatives prepared from porcine intestinal heparin. LAH was chemically generated by periodate oxidation of the heparin at the antithrombin (AT) binding sequence. This process led to splitting of C2–C3 bonds of all non-sulfated hexuronic acid residues and subsequent cleavage of the heparin chain at these sites. Because the AT binding pentasaccharide sequence contains a non-sulfated glucuronidic acid unit, this structure and hence, the anticoagulant activity were eliminated. A first LAH batch, DFX232, was prepared by oxidizing heparin followed by a reduction. The second LAH batch, Sevuparin, was prepared in the same manner but included an acidic hydrolysis step. The preparations were dried, and the anticoagulant activities of the two LAH batches were determined by measuring the anti-IIa and anti-Xa activities. The assays were performed according to the Ph Eur procedure for low molecular mass heparin (monograph 0828) in the European Pharmacopoeia. The anticoagulant activity of LAH was strongly reduced compared with heparin and LMWH. The final products DFX232 and Sevuparin had molecular weights of 10.1 and 7.4 kDa, respectively.

Rosette disruption assay. Rosette disruption assays were performed on pRBC cultures harboring trophozoites 20–24 h post-invasion. LAH was diluted in RPMI, and samples were analyzed in triplicates as previously described. Briefly, parasite cultures were concentrated two times from a hematocrit of 5–10%, and 25 μL culture was added to 25 μL LAH in RPMI, giving final concentrations of 1,000 and 100 μg/mL. After incubation of samples at 37°C for 30 minutes, the parasites were stained with acridine orange, and the rosettes were counted. For each sample, 25 fields, equivalent to approximately 3,500–4,000 RBCs, were counted. In parallel, mock-treated samples were analyzed in the presence of RPMI alone.

Statistical analysis. Before statistical analysis, the rosetting rates for the isolates were converted into relative rosetting rates by calculating the proportion of remaining rosettes after treatment compared with the mock-treated erythrocytes. The statistical analysis was carried out with Prism version 5.0 for Windows (Graphpad Software, San Diego, CA). Student paired *t* test or Wilcoxon signed rank test (when the samples were not normally distributed) was used. For the comparisons between groups with mild and complicated disease, unpaired *t* test and Mann–Whitney *U* test, respectively, were used. Pearson’s *χ*² test was used when the data were binary. As estimated in a previous study, rosette disruption of ≥ 15% was regarded as significant. Where applicable, data are presented as mean and standard error of mean (SEM).

RESULTS

Characteristics of the study sample. In all, 1,079 children between 6 months and 14 years of age were screened for malaria, and 63% were found to be infected (682 of 1,079). When the parasite density was ≥ 10,000 pRBC/μL blood, a venous blood sample was collected, which resulted in a total of 144 samples (Table 1). Of the 144 samples, 140 samples were cultivated, and 112 isolates (80%) successfully grew into trophozoites. Forty-seven isolates showed a rosetting phenotype, with a rosetting rate that ranged from 2.1% to 79% (Figure 1 and Table 1). Of these, 20 samples were from children diagnosed with complicated malaria, and the other 27 samples were from children with mild malaria. There was a significant difference in the number of isolates that formed rosettes between the mild and complicated malaria groups (*P* = 0.0009) (Table 1), because rosettes were present in 22% of the samples in the mild group and 50% of the samples in the complicated group. There was a significant difference in parasitemia between the
392 LEITGEB, BLOMQVIST AND OTHERS

groups, with a mean parasitemia of 3.4% and 6.1% for the mild and complicated groups, respectively ($P = 0.04$). However, there were no significant differences in rosetting rates or hemoglobin levels between the mild and complicated groups.

**LAH disrupt rosettes from fresh isolates.** To examine the activity of LAH against a large sample size of fresh patient samples collected from malaria-infected children, 47 rosetting isolates were tested for sensitivity to two different batches of LAH (DFX232 and Sevuparin). Two concentrations, 100 and 1,000 μg/mL, were used, and results were compared with mock-treated samples prepared in RPMI. Rosette disruption of ≥15% (defined as significant) was shown in 42 of 47 (89%) samples with DFX232 at a concentration of 1,000 μg/mL, 39 of 47 (83%) samples with Sevuparin at a concentration of 1,000 μg/mL, 34 of 47 samples with DFX232 at 100 μg/mL, and 36 of 47 samples with Sevuparin at 100 μg/mL (Figures 1 and 2). Both DFX232 and Sevuparin caused disruption of ≥50% of the rosettes in 32 of 47 (68%) samples at the higher concentration. At the lower concentration, the number of highly reacting samples was 18 of 47 for DFX232 and 25 of 47 for Sevuparin (Figure 2A and B). Only three samples showed no significant effect with either of the two substrates used at any of the concentrations. Relative rosetting rates (RR; the proportion of remaining rosettes after treatment compared with the control-treated erythrocytes) ranged from 36% (Sevuparin, higher concentration) to 61% (DFX232, lower concentration) (Figure 3A). Statistically significant rosette disruption compared with the control was seen in all groups, with $P < 0.0001$ (Figure 3A). There was no significant difference in rosette disruption effect between the two batches.

**Rosettes in complicated malaria isolates are more sensitive to LAH than rosettes in mild malaria isolates.** When comparing samples from mild and complicated malaria, a

<table>
<thead>
<tr>
<th>Field isolate</th>
<th>Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of children tested for <em>Plasmodium falciparum</em></td>
<td>1,079</td>
</tr>
<tr>
<td>No. of children infected with <em>P. falciparum</em></td>
<td>682 (63%)</td>
</tr>
<tr>
<td>No. of children included in the study (≥10,000 parasites/μL blood)</td>
<td>144 (12%)</td>
</tr>
<tr>
<td>No. of cases of complicated malaria</td>
<td>54 (38%)</td>
</tr>
<tr>
<td>No. of cases of mild malaria</td>
<td>90 (62%)</td>
</tr>
<tr>
<td>No. of isolates successfully matured to trophozoites</td>
<td>112 (80%)*</td>
</tr>
<tr>
<td>No. of rosetting isolates (mild/complicated)</td>
<td>42% † (27/20)</td>
</tr>
<tr>
<td>Percent rosetting isolates (mild/complicated)</td>
<td>22%/50% ‡</td>
</tr>
<tr>
<td>Mean rosetting rate (range)</td>
<td>20.42%/2.79%</td>
</tr>
<tr>
<td>Mean rosetting rate (mild/complicated)</td>
<td>20.9%/19.7%</td>
</tr>
<tr>
<td>Mean parasitemia (mild/complicated)</td>
<td>3.6%/2.7%/5.3% ‡</td>
</tr>
<tr>
<td>Mean parasitemia in rosetting isolates (mild/complicated)</td>
<td>4.5%/3.4%/6.1% ‡</td>
</tr>
<tr>
<td>Mean hemoglobin level (mild/complicated)</td>
<td>90.2 g/L (95/82) §</td>
</tr>
<tr>
<td>Mean hemoglobin level in rosetting isolates (mild/complicated)</td>
<td>90.9 g/L (92/89)</td>
</tr>
</tbody>
</table>

*One hundred twelve of one hundred forty isolates grew to the mature trophozoites stage in culture.
†Forty-seven of one hundred twelve isolates showed a rosetting phenotype.
‡$P < 0.05$.
§$P < 0.0001$.

![Figure 1. Rosetting rates before and after the addition of LAHs in the 47 isolates. The mean rosetting rate ± SEM is shown. Rosetting rate before addition of LAH (black bars), DFX232 (dark grey bars), or Sevuparin (light grey bars). *Not done. (A) DFX232 and Sevuparin at concentrations of 100 μg/mL. (B) DFX232 and Sevuparin at concentrations of 1,000 μg/mL.](image-url)
difference in rosette disruption effect by the LAHs was revealed (Figure 3B and C). Both DFX232 and Sevuparin had a greater rosette disruption effect on the isolates from the complicated malaria cases compared with the mild cases, with an LAH concentration of 100 μg/mL. Thus, after treatment with 100 μg/mL DFX232, the remaining relative RR was 70% (range = 0–115%) in the mild malaria isolates and 49% (range = 14–94%) in the complicated malaria isolates (P = 0.03) (Figure 3B). With Sevuparin at 100 μg/mL, the figures were 62% (range = 0–104%) in the mild cases and 41% (range = 13–78%) in the complicated cases (P = 0.02) (Figure 3B). A similar trend but no statistically significant difference was seen between the groups when the higher concentrations of both compounds were used (Figure 3C). No correlation was found between sensitivity to the LAH and parasitemia or RR (data not shown).

**DISCUSSION**

The present investigation shows that LAH effectively disrupts rosettes in fresh clinical isolates. We compared two different preparations of LAH (DFX232 and Sevuparin) at two different concentrations, 100 and 1000 μg/mL, on 47 fresh isolates from Cameroonian children with mild or complicated malaria. The parasites were selected based on previously reported studies performed in vitro. The concentrations are in the range of the doses of LAH evaluated in a finalized Phase I study. In the Phase I study, no severe adverse events were reported, and the drug candidate was proven to be safe and well-tolerated (unpublished data).

A majority, 68% (32/47), of the samples showed a rosette disruption effect ≥ 50% when the compounds were analyzed at the higher concentration. In all, 89% (42/47) of the isolates were sensitive (≥ 15%) to rosette disruption by any of the LAH studied (Figures 1 and 2). We further found a difference in rosette disruption effect by LAH depending on the severity of the malaria infection. Both LAH compounds, when analyzed at the lower concentration, had a significantly stronger rosette disruption effect (P < 0.05) in samples from complicated malaria cases than in samples from mild cases (Figure 3B). The difference in rosette disruption effect was about 40% for both compounds between the mild and complicated isolates (Figure 3B). For the higher concentration, the effect was also more pronounced in the complicated cases than in the mild cases, although not significant (Figure 3C). These results are in line with previous findings showing that binding of soluble heparin to the surface of pRBC is significantly associated with severe disease. The parasites from the mild and...
complicated groups may use different rosetting receptors. One can speculate that the use of CR1 or blood group A as a rosetting receptor may be more frequent in the mild compared with the complicated group, making these rosettes less sensitive to LAH treatment.

Rosette disruption by heparin, HS, and LAH and the binding of GAGs to the DBL1α domain of PfEMP1 are well-described in several studies. When comparing rosette disruption data for heparin or HS with the data for LAH, the present study might indicate a stronger rosette disruption effect by DFX232 and Sevuparin. Carlson and others reported that 30% (16/54) of the samples collected in Gambia showed ≥ 50% rosetting disruption effect of heparin, and 50% (27/54) of the samples showed ≥ 15% disruption at a concentration of 650 μg/mL. In the present study, we found ≥ 50% rosetting disruption effect in 53% (25/47) of the samples and ≥ 15% disruption effect in 77% (36/47) of the samples at the concentration 100 μg/mL. Three rosetting isolates (6%) in our study showed no sensitivity to either of the two LAH preparations tested. This finding is not surprising but in concordance with the results of previous studies where it has been shown that some isolates are insensitive to heparin, heparin derivatives, and other rosette-disrupting sulfated glycoconjugates, even at very high concentrations.

In the present study, there was a statistically significant difference (P < 0.05) between the complicated and mild groups, with a higher parasitemia and more isolates forming rosettes in the complicated malaria cases than in the mild malaria cases. Another observation is that the isolates that showed a rosetting phenotype in the mild group were of higher parasitemia and lower hemoglobin levels compared with the isolates in the same group that did not show any rosetting. There was a trend but no statistical difference in hemoglobin levels between the mild and complicated groups, which may be because the complicated group includes children with malaria with diverse symptoms such as hyperpyrexia, anemia, hyperparasitemia, seizures, prostration, and vomiting. The children with severe anemia, therefore, belong to a subgroup within the complicated group. The rosetting rate did not significantly differ between the mild and complicated groups, although a significantly higher number of isolates formed rosettes in the complicated group than in the mild group. The similarity in rosetting rates in the mild versus complicated group may be a consequence of a different prevalence of hemoglobinopathies, such as sickle cell anemia or thalassemia, in the mild and complicated groups, because these diseases have been shown to impair the ability to form rosettes instead creating small and weak rosettes. Indeed, both sickle cell anemia and thalassemias are common in Cameroon and surrounding areas.

In this report, we only used fresh parasitized blood samples collected from malaria-infected children. The samples were directly analyzed without freezing and thawing. The aim was to obtain parasite samples as close to in vivo conditions as possible. In some rosette disruption studies, frozen and thawed parasite samples or laboratory strains have been used. In a recent study, it was found that the dominant var gene coding for PfEMP1 changed before and after cryopreservation in a number of isolates, which was analyzed by quantitative polymerase chain reaction (Q-PCR) using strain and stage-specific primers. By using fresh isolates, the risk of changes in antigens present on the erythrocyte surface, including expression of PfEMP1, is limited. Hence, the use of entirely fresh blood samples may present a more correct picture of the infected cells’ surfaces in vivo than frozen and thawed blood samples.

Another sulfated glycoconjugate that has been shown to be effective in rosette disruption and inhibition of parasite invasion of RBCs is curdlan sulfate (CRDS). This compound has been reported to be effective in vitro at the concentration 50 μg/mL in the majority of 18 P. falciparum isolates from Kenyan children. The mechanism of rosette disruption...
by CRDS is unclear; hence, it is not known if the mechanism of action of CRDS is a specific binding to the PFEMP1 protein. Two clinical trials with a small number of severe malaria patients have shown that fever clearance was shortened in the group receiving CRDS, but the study was unable to show any difference in mortality, parasitemia, or coma resolution after treatment with CRDS.17

LAH is being documented as adjunct treatment of severe malaria. This includes a recent clinical phase I safety study conducted in healthy male volunteers (unpublished data). No severe adverse effects were reported, and the candidate drug (Sevuparin) was proven to be safe and well-tolerated (unpublished data). Here, we show that LAH affects the majority of fresh rosetting clinical isolates analyzed without any manipulation of the parasites. In addition, LAHs have earlier been shown to inhibit cytoadhesion and merozoite invasion in vitro and block and reverse sequestration of pRBCs in vivo in rats and monkeys.18 By inhibiting the three processes rosetting, cytoadherence, and merozoite invasion with a receptor analogue like LAH, a change in the sequestered parasite load and parasitemia may also be achieved in humans. One can speculate that this may reestablish the microcirculation and release bound pRBCs into the circulation; this renders the latter more available for an immune attack on passing through the spleen. We conclude that drugs reducing the sequestration of pRBCs, such as LAH, have a potential as adjunct treatment of severe malaria.

Received May 4, 2010. Accepted for publication December 10, 2010.

Acknowledgments: The authors thank the children and their parents who participated in the study. They are grateful to the staff at the University of Buea. This work was supported by Dilafor AB, Erik and Edith Fernströms stiftelse, and the Swedish National Board of Health and Welfare.

Disclaimer: The authors declare competing financial interest. A.M.L. and M.W. hold stock and/or stock options in Dilafor AB, which owns the intellectual property of the substances generated in this manuscript. A.M.L. has received research funding from Dilafor AB. The remaining authors have no known conflict of interest.

Authors’ addresses: Anna M. Leitgeb, Dilafor AB, Solna, Sweden, E-mail: anna.leitgeb@dilafor.com. Karin Blomqvist and Mats Wahlgren, Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet (MTU), Stockholm, Sweden, E-mails: karin.blomqvist@ki.se and mats.wahlgren@ki.se. Fidelis Ch-Ngwa, Moses Samje, Peter Nde, and Vincent Titanji, University of Buea, Biotechnology Unit, Faculty of Science, Buea, Cameroon, E-mails: chongwa_ub@yahoo.co.uk, msamje@yahoo.com, ndepf@yahoo.com, and vpk.titanji@yahoo.com.

REFERENCES


