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In Vitro Inhibition of *Plasmodium falciparum* Rosette Formation by Curdlan Sulfate[∇]

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Spontaneous binding of infected erythrocytes to uninfected erythrocytes to form rosettes is a property of some strains of *Plasmodium falciparum* that is linked to severe complications of malaria. Curdlan sulfate (CRDS) is a sulfated glycoconjugate compound that is chemically similar to known rosette-inhibiting drugs such as heparin. CRDS has previously been shown to have antimalarial activity in vitro and is safe for clinical use. Here we show that CRDS at therapeutic levels (10 to 100 µg/ml) significantly reduces rosette formation in vitro in seven *P. falciparum* laboratory strains and in a group of 18 African clinical isolates. The strong ability to inhibit rosetting suggests that CRDS has the potential to reduce the severe complications and mortality rates from *P. falciparum* malaria among African children. Our data support further clinical trials of CRDS.

Despite decades of research, malaria still claims an estimated 1 million lives per year in sub-Saharan Africa, and severe complications, such as cerebral malaria, remain relatively common (19). Treatment for malaria involves the use of antimalarial drugs that kill the parasite, along with supportive measures, such as the provision of intravenous fluids and blood transfusion as required. Progress in understanding the pathogenesis of malaria has led to the possibility of designing therapies that target factors that contribute to severe disease in order to ameliorate the clinical complications and reduce the number of deaths from malaria.

Rosetting is the spontaneous binding of noninfected erythrocytes to erythrocytes infected with mature asexual blood-stage *Plasmodium* parasites (reviewed in reference 14). In *Plasmodium falciparum* malaria, rosetting is associated with high levels of parasitemia (16) and with disease severity in African children (13, 17), especially cerebral malaria (2). Other *Plasmodium* species also rosette, although it is only in *P. falciparum* that rosetting is linked to severe disease, probably because of the additional cytoadhesion properties that may act in concert with rosetting to cause microvascular obstruction (8, 14). Rosetting in *P. falciparum* is mediated by heterogeneous mechanisms involving specific members of the parasite variant antigen family PfEMP1 (15), which are expressed on the surface of infected erythrocytes and which binding to a variety of receptors on uninfected red cells, such as complement receptor 1 (CR1), heparan sulfate-like sugars, and ABO blood group oligosaccharides (reviewed in reference 14).

Previous work has shown that rosettes can be disrupted by

sulfated glycoconjugate compounds such as heparin and fucoidan (1, 12), but the anticoagulant effects of these drugs prevent their use as treatments for severe malaria (19). Curdlan sulfate (CRDS; a semisynthetic sulfated 1→3-β-D-glucan) has been shown to inhibit *P. falciparum* invasion in vitro (6) and is safe for use by adult patients with severe malaria (5). It was hypothesized that CRDS might be effective against *P. falciparum* rosetting (5) because of the chemical similarities between CRDS and heparin (7). The 10-fold lower anticoagulant effect of CRDS compared to the effect of heparin makes it a more suitable candidate than heparin as an adjunctive treatment for severe malaria (5). In this study we have tested the ability of CRDS to inhibit rosetting in *P. falciparum* laboratory strains and clinical isolates and show strong rosette-inhibiting activity at therapeutic doses of CRDS.

MATERIALS AND METHODS

Parasites. The laboratory strains studied were TM284, TM180, HB3R+, R29, Muz12R+, PAR+, and A4R. Apart from R29, A4R, and PAR+, these strains are genetically distinct; R29, A4R, and PAR+ are clones derived from strain IT/FCR3. The three IT/FCR3-derived clones express different PfEMP1 variants (15; J. A. Rowe, unpublished data) and therefore form rosettes via distinct molecular mechanisms and can thus be considered independent parasite lines. The parasites were cultured in RPMI 1640 medium plus 25 mM HEPES, 2 mM glutamine, 25 mM glucose, 25 µg/ml gentamicin, and 10% pooled human serum. Cultures were maintained at approximately 2% hematocrit in blood group O red blood cells with normal CR1 levels at 5 to 10% parasitemia and were incubated at 37°C and gassed with 3% CO₂, 1% O₂, and 96% N₂. The cultures were synchronized once a week by sorbitol lysis. Laboratory strains were maintained at a rosette frequency (RF; percentage of mature pigmented infected erythrocytes that bind to two or more uninfected erythrocytes) of 40% or higher by using Percoll or Plasmagel to enrich for rosetting parasites twice a week. Clinical isolates were collected from children with malaria at Kilifi District Hospital, Kenya, after informed consent was obtained from the patients' parents or guardians. Three clinical isolates were analyzed as fresh samples, and 15 clinical isolates were frozen (10 at the ring stage and 5 at the pigmented trophozoite stage). The fresh clinical isolates were cultured for at least 18 h in vitro to the mature pigmented trophozoite stage as described above, except that pooled human group AB serum was used. Frozen ring- and trophozoite-stage clinical

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isolates were cryopreserved in glycerolyte and were stored in liquid nitrogen before they were thawed for either immediate use (frozen trophozoites) or culture to the mature pigmented trophozoite stage (frozen rings). Cryopreservation of mature trophozoites does not affect the surface antigens of the trophozoite-infected erythrocytes (9), and an isolate's RF does not change significantly after freezing and thawing, as long as the sample is mixed thoroughly postthawing (data not shown). A summary of the clinical isolates used and their baseline RFs is as follows (Fzt, frozen trophozoites; Fzr, frozen rings; Fsh, fresh peripheral blood sample): 6394Fzt, RF 15%; 6399Fzt, RF 12%; 6457Fzt, RF 5%; 6542Fzt, RF 52%; 6566Fzt, RF 48%; 6794Fzr, RF 33%; 6908Fsh, RF 27%; 6921Fsh, RF 27.5%; 6962Fsh, RF 24.5%; KR1Fzr, RF 53.5%; KR7Fzr, RF 68%; KR9Fzr, RF 14%; KR10Fzr, RF 25%; KR11Fzr, RF 27%; KR14Fzr, RF 37.5%; KR17Fzt, RF 61%; KR18Fzt, RF 28%; and KR20Fzr, RF 63%.

Effect of CRDS on rosetting. All rosette disruption assays were performed with mature pigmented trophozoites, as determined by a Giemsa smear. The parasites were resuspended at 2% hematocrit in complete binding medium (sodium bicarbonate-free RPMI 1640 supplemented as described above for the culture medium). The culture suspension was prestained with ethidium bromide or acridine orange to identify infected erythrocytes. These staining methods do not alter the RF. CRDS was obtained from the Ajinomoto Co Inc., Tokyo, Japan (average molecular mass, 70 kDa; batch no. G2102; sulfur content, 14.6% [wt/wt]) and was dissolved in sterile phosphate-buffered saline (PBS). Two microliters of an appropriate dilution of the drug in PBS was added to 18 μ l of culture suspension to give final concentrations of 0, 10, 50, and 100 μ g/ml of CRDS. Final concentrations of 100 μ g/ml fucoidan and 100 μ g/ml hyaluronic acid (both from Sigma, United Kingdom) were used as positive and negative controls for rosette inhibition, respectively (12). The *P. falciparum* cultures were incubated with drug for 30 min before assessment of the RF. The laboratory strains were incubated at 37°C, and the cells were resuspended every 10 min by gently flicking the tubes. The clinical isolates were rotated for 30 min at room temperature. Rotation and room temperature incubation do not alter the RF, as tested with laboratory strain PAR+ (data not shown). Wet-preparation slides of the cultures were viewed with a fluorescence microscope. Two hundred infected erythrocytes were counted, and the percentage that formed rosettes was assessed; an infected erythrocyte that binds to at least two uninfected erythrocytes constitutes a rosette. All slides were counted blind. For laboratory strains, at least four independent experiments were carried out for each strain. The clinical isolates were tested in a single experiment in the first cycle of growth in vitro. The RF after addition of drug or PBS (control) was calculated as a proportion of the pretreatment RF, in order to allow comparisons across strains and to determine whether the addition of PBS (the buffer in which the drugs were solubilized) or the conditions of the experiment (temperature, agitation, etc.) had any effect on RF.

Effect of CRDS on platelet-mediated clumping. Parasite lines HB3, NF54, and IT/C10 were cultured by standard methods, as described above. Mature pigmented trophozoite-stage parasite cultures were incubated with 25 μ g/ml of ethidium bromide at 37°C for 5 min, washed once with RPMI 1640 medium, and finally suspended at 5% hematocrit in RPMI 1640 medium. Twenty microliters of platelet-rich plasma, obtained from group O-positive whole blood by centrifugation at 250 \times g for 10 min, was added to 200 μ l of the parasite suspension. The mixture was gently rotated for 1 h. The effect of CRDS on clumping was assessed by adding the drug either 30 min before or 30 min after the addition of the platelet-rich plasma (final CRDS concentration, 100 μ g/ml). Wet preparations of the different conditions were viewed under a UV microscope, and 750 infected red blood cells were counted and scored for clumping, with 3 or more infected cells adherent to each other constituting a clump. The clumping frequency (CF) was calculated as the percentage of infected erythrocytes in clumps of 750 infected erythrocytes counted.

Statistical analysis. Statistical analysis was carried out with Statview 5.0.1 software (SAS Institute Inc., Cary, NC). For both the laboratory strains and the clinical isolates, posttreatment RFs were first converted to a relative RF (calculated as a proportion of each isolate's own pretreatment RF), and then the effects of drug were compared to that of the PBS control (0 μ g/ml CRDS) by paired *t* test. Additional analysis by analysis of variance gave similar results (data not shown).

RESULTS AND DISCUSSION

The effect of CRDS on *P. falciparum* rosetting was tested with seven distinct laboratory parasite strains that had been selected for their high levels of rosetting (strains PAR+, A4R, R29, TM284, TM180, Muz12R+, and HB3R+). Three con-

centrations of CRDS (10, 50, and 100 μ g/ml) were used, alongside a PBS control (i.e., 0 μ g/ml CRDS). The plasma level of CRDS after treatment at therapeutic doses (4 mg/kg) is estimated to be approximately 50 to 60 μ g/ml (Investigator Manual, Aji Pharma, 1995, unpublished data). By combining the data for all seven parasite strains, a strong inhibitory effect of CRDS was seen at all three concentrations of drug (for the PBS control, the mean RF was 62.3% and the standard error [SE] was 2.8%; for 10 μ g/ml CRDS, the mean RF was 21.9% and the SE was 6.6%; for 50 μ g/ml CRDS, the mean RF was 14.6% and the SE was 4.6%; and for 100 μ g/ml CRDS, the mean RF was 8.7% and the SE was 2.7% [*P* < 0.005 for each concentration of drug compared to the results obtained with PBS, paired *t* test]). As expected (12), fucoidan provided a reliable positive control for rosette inhibition (mean RF, 5.4%; SE, 1.0%) and hyaluronic acid provided a reliable negative control (mean RF, 65.2%; SE, 2.8%).

By examining the data for each parasite strain independently, it was found that all seven strains responded to CRDS in a concentration-dependent manner, although there was some variation in the response between strains (Fig. 1A). Rosetting was significantly reduced (*P* < 0.05, paired *t* test) by CRDS at both 100 μ g/ml and 50 μ g/ml in all parasite strains (Fig. 1A). At 10 μ g/ml CRDS, RF was significantly reduced for five of seven parasite strains (Fig. 1A and B). It is unclear why some strains, such as PAR+, are very sensitive to CRDS, with complete inhibition of rosetting at 10 μ g/ml of drug (Fig. 1A and B), whereas others, such as TM284+, require higher doses for significant inhibition of rosetting (Fig. 1A). This may be due to heterogeneity in the molecular mechanisms of rosetting in distinct strains (reviewed in reference 14), resulting in differences in the strengths of the rosettes between strains. The mechanism of action of CRDS is unclear, that is, whether it inhibits rosetting by interacting nonspecifically with all uninfected red cells or whether it interacts specifically with infected erythrocytes. Further experiments investigating the mechanism of action of CRDS on rosetting may shed light on the reasons for the differences in strain sensitivity.

To test the speed of action of CRDS, PAR+ (mean RF, 70%; SE, 4.0%) was treated with 50 μ g/ml of drug and the RF was recorded over 2 h (at 0, 2, 5, 30, 60, and 120 min after drug addition). Rosettes were disrupted within 2 min and remained disrupted for up to 2 h after drug addition (Fig. 1A, PAR+ Kinetics). Similarly, with the least CRDS-sensitive strain TM284, the maximum effect occurred rapidly and was maintained for up to 2 h (Fig. 1A, TM284 Kinetics).

Rosette reformation assays were carried out with PAR+ parasites. After 30 min of CRDS treatment, the culture was washed with incomplete binding medium (as binding medium, but with no added serum) and then resuspended in fresh complete binding medium. The RF returned to 102% (SE, 6.5%) of the original RF within 2 h. This shows kinetics similar to that of the fucoidan-treated cultures (12), which return to 100% of the original RF (SE, 8.2%) after they are washed.

We also tested the ability of 100 μ g/ml of CRDS to inhibit another *P. falciparum* adhesion phenomenon, the platelet-mediated clumping of infected erythrocytes (11). By using laboratory parasite strain HB3 in three independent experiments, the mean CF (percentage of infected erythrocytes in clumps of three or more infected erythrocytes) in the PBS control culture

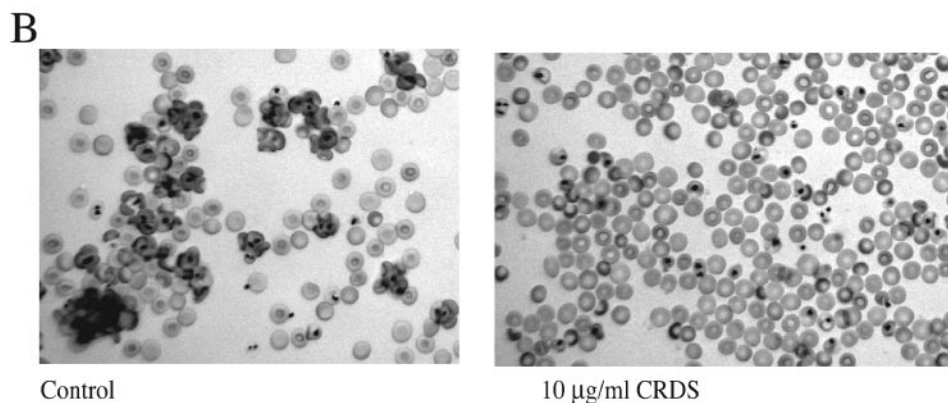
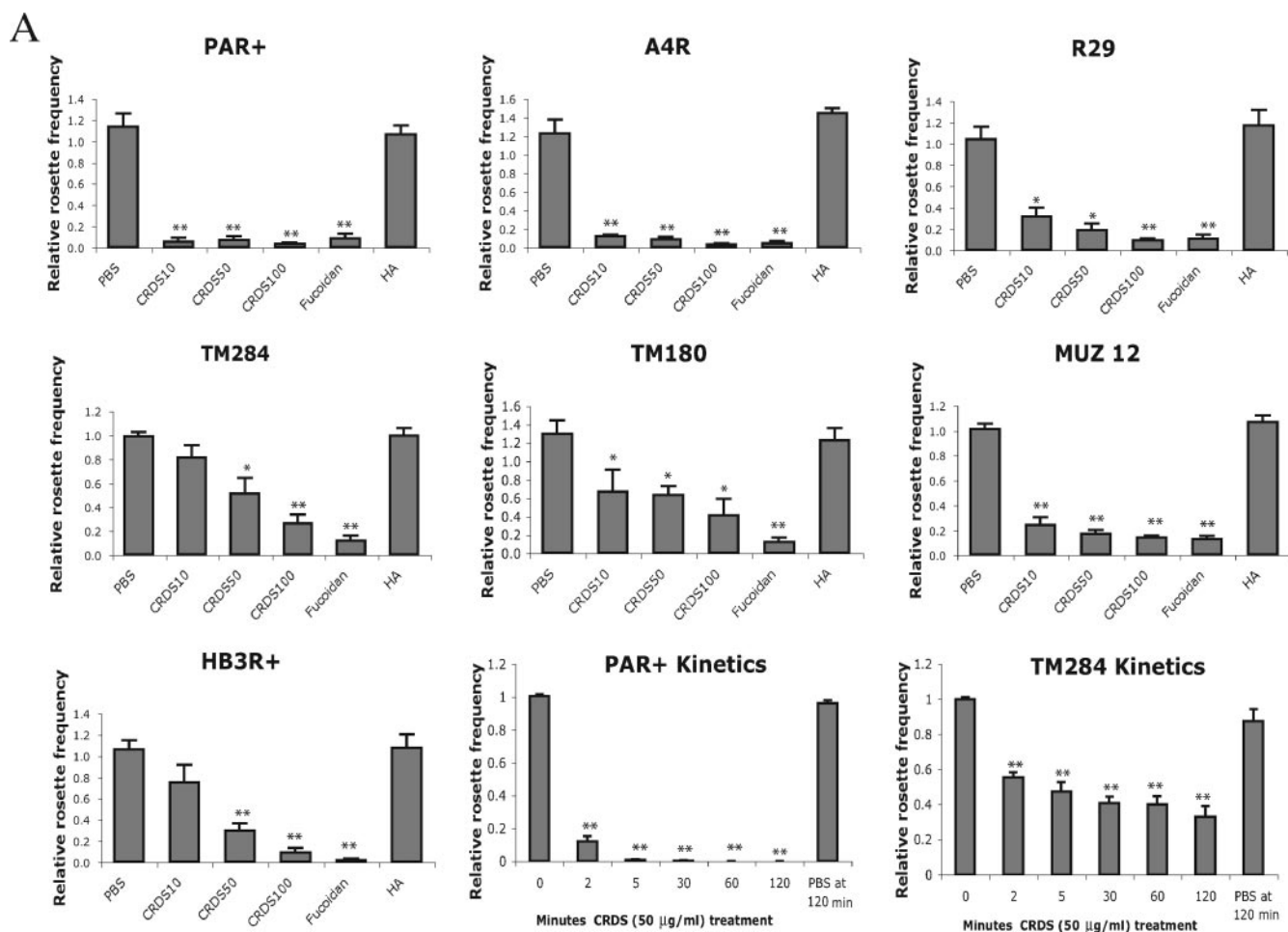


FIG. 1. Inhibition of rosetting by CRDS in laboratory strains of *P. falciparum*. (A) Parasite cultures were treated for 30 min with 0 µg/ml CRDS (PBS control), 10 µg/ml CRDS (CRDS10), 50 µg/ml CRDS (CRDS50), 100 µg/ml CRDS (CRDS100), 100 µg/ml fucoidan (positive control), or 100 µg/ml hyaluronic acid (HA; negative control). The parasite strain names are shown at the top of each box. Strains PAR+ and TM284 were also monitored over 120 min after treatment with 50 µg/ml CRDS or the PBS control to examine the kinetics of rosette inhibition by CRDS. Each graph shows the means ± SEs from at least four independent experiments for each parasite strain. The relative RF is calculated as a proportion of the pretreatment RF to allow comparison across strains and experiments. The pretreatment RF for each strain was stable on a given day but varied from 40 to 80% of infected erythrocytes in rosettes on different days, depending on factors related to different red blood cell donors and the timing of selection. *, $P < 0.05$ by paired t test compared to the result for the PBS control; **, $P < 0.005$ by paired t test compared to the result for the PBS control. (B) Complete inhibition of rosetting in *P. falciparum* laboratory strain PAR+ with 10 µg/ml CRDS. For the control culture (no drug), the RF was 62%; for the culture with 10 µg/ml CRDS, the RF was 0%. Live wet-preparation images, viewed with a ×40 objective lens under a bright-field microscope, are shown. Infected erythrocytes can be identified by the presence of black dots of malaria pigment inside the red blood cells.

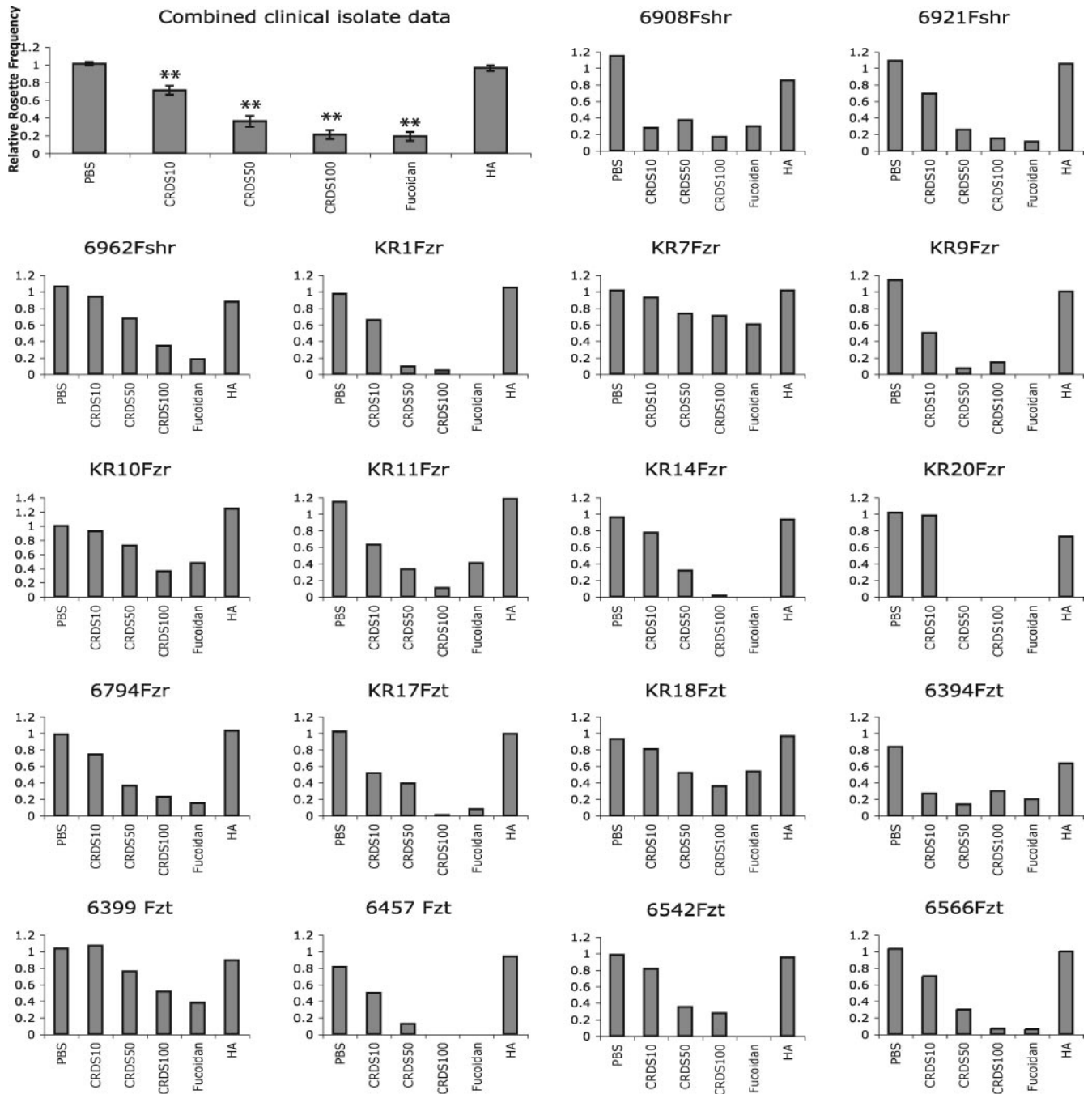


FIG. 2. Inhibition of rosetting by CRDS in *P. falciparum* clinical isolates from Kilifi, Kenya. Field isolates were treated with 0 $\mu\text{g/ml}$ CRDS (PBS); 10, 50, or 100 $\mu\text{g/ml}$ CRDS; 100 $\mu\text{g/ml}$ fucoidan (positive control); or 100 $\mu\text{g/ml}$ hyaluronic acid (HA; negative control). Data are from a single experiment in the first cycle of in vitro growth for each isolate. Data (means \pm SEs) are given for all 18 field isolates combined (**, $P < 0.001$ by paired t tests compared to the results for the PBS control) and for each isolate individually. The relative RF is calculated as a proportion of the pretreatment RF for each isolate. Isolate names are shown at the top of each box. Isolates were collected either as fresh rings (Fshr), frozen rings (Fzr), or frozen mature trophozoites (Fzt); however, the collection method did not affect sensitivity to CRDS (see the Materials and Methods and the Results for full details).

was 38.7% (SE, 8.9%). When CRDS was added after the platelets were added (see Materials and Methods for full experimental details), the mean CF was 38.3% (SE, 5.1%), and when CRDS was added before the platelets were added, the mean CF was 51.2% (SE, 8.8%) (the difference was not sig-

nificant by paired t tests compared to the results for the PBS control). Therefore, in contrast to the highly effective rosette disruption, we found that CRDS caused no disruption of preformed platelet-mediated clumps and was also unable to prevent clumping if it was added before the platelets were added.

Additional experiments with two other distinct laboratory parasite strains (strains NF54 and IT/C10) showed similar results, with no inhibition of platelet-mediated clumping by CRDS. Because clumping has previously been shown to be a phenomenon mediated at least in part by the platelet receptor CD36 (11), these data are consistent with the results of Evans et al. (3), who showed that CRDS has no effect on *P. falciparum* binding to platelets or CD36. Previous studies have suggested that platelet-mediated clumping might be another parasite adhesion phenotype, in addition to rosetting, that is associated with severe malaria (11). If clumping does contribute to the pathogenesis of severe malaria, the data shown here would suggest that CRDS would be useful only in cases of severe malaria associated with rosetting but not in cases associated with clumping. Our recent data from a case-control study in Mali suggest that clumping is primarily associated with high levels of parasitemia and not with disease severity (M. Arman et al., submitted for publication); therefore, further work will be required to determine the significance of clumping and the relevance of the resistance of clumping to CRDS in the treatment of severe malaria.

To investigate further the potential of CRDS as an antirosetting treatment for patients with malaria, we tested CRDS (0, 10, 50, and 100 µg/ml), alongside fucoidan and hyaluronic acid as positive and negative controls, respectively, with 18 clinical isolates collected from children with malaria at Kilifi District Hospital, Kenya. These were a mixture of fresh and frozen isolates, and they were cultured to the mature trophozoite stage in vitro (see Material and Methods). The RF was expressed relative to the pretreatment RF, so that all 18 clinical isolates could be compared. All isolates responded to CRDS; and there was a significant inhibition ($P < 0.001$) across all field isolates combined by treatment with CRDS at 10, 50, and 100 µg/ml (Fig. 2). As with the laboratory strains, there was evidence of high and low responders to CRDS, with some isolates reacting strongly to a low dose, whereas others required the highest dose for rosette disruption (Fig. 2). Of the 18 clinical isolates tested, 4 isolates showed $\geq 50\%$ rosette inhibition with only 10 µg/ml CRDS, whereas 10 required 50 µg/ml CRDS and 2 required 100 µg/ml CRDS for 50% rosette inhibition. The remaining two isolates (isolates 6399Fzt and KR7Fzr) showed 52% and 70% of the pretreatment RF, respectively, when they were treated with 100 µg/ml CRDS. Nine of the frozen isolates tested (those named KR) were collected in 1993, and the remaining isolates were collected in 2005 and 2006. CRDS was equally effective against both sets of isolates, showing that with time there is no change in the rosetting phenotype that leads to alterations in CRDS sensitivity. There were also no differences in the CRDS sensitivities of fresh isolates and frozen isolates.

These data show that CRDS has rosette-inhibiting activity comparable to that of fucoidan and is active against a broader range of isolates than heparin, which caused $\geq 50\%$ rosette inhibition in 6/10 isolates (12) and 16/54 isolates (1) in previous studies. Even at high doses (650 µg/ml), heparin had no inhibitory effect on rosetting in 23/54 clinical isolates (1). A recent paper suggested that heparin derivatives that lack anticoagulant activity might be useful as adjunctive therapies for severe malaria (18). However, because of strain variability in response to heparin, such heparin derivatives would need to show activ-

ity against a broader range of isolates than the parental compound to be of clinical use.

In conclusion, we have found that CRDS effectively and consistently disrupts rosetting in 7/7 laboratory strains and in a group of 18 rosetting field isolates at doses suitable for clinical use. High levels of rosetting are estimated to occur in approximately 25 to 50% of severe malaria cases in sub-Saharan Africa (14). Rosette-inhibiting drugs therefore have the potential to have a substantial impact on malaria mortality and morbidity in African children. The multiorgan failure type of severe malaria commonly seen in adults in Southeast Asia is not associated with high levels of rosetting (reviewed in reference 14); therefore, CRDS would not be expected to have a substantial clinical effect resulting from its rosette-inhibiting properties in this group of patients.

CRDS has already been shown to be safe and well tolerated in clinical trials for human immunodeficiency virus and malaria (4, 5). The drug has a short plasma half-life (2 to 3 h) and is given by intravenous infusion (4); therefore, it is suitable only for hospital use. A recent clinical trial found that CRDS was able to reduce the fever clearance time in patients with severe falciparum malaria in Thailand (5). CRDS is able to inhibit *P. falciparum* growth in vitro by inhibiting merozoite invasion (3, 6). In addition CRDS has also been shown to inhibit the production of cytokines, such as tumor necrosis factor alpha (10). Our data on rosette inhibition, together with data from previous reports, suggest that CRDS is a strong candidate for adjunctive therapy in patients with severe malaria, especially in sub-Saharan Africa, where rosetting is strongly associated with severe disease.

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I. Havlik holds a patent on the use of CRDS for malaria.

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