Abstract. Cytoadherence of Plasmodium falciparum-infected erythrocytes to the microvascular endothelium is believed to be a key factor in the development of cerebral malaria. Erythrocyte rosette formation has been correlated with malaria severity in studies from east and west Africa. We cultured fresh isolates from Malawian children with severe (n = 76) or uncomplicated (n = 79) malaria to pigmented trophozoite stage and examined rosette formation and adherence to CD36, intercellular adhesion molecule-1 (ICAM-1), chondroitin sulfate A (CSA), and thrombomodulin (TM). Most (126 of 148) isolates bound to CD36, and 76 of 136 bound to ICAM-1. Fewer bound to CSA (40 of 148) or TM (23 of 148). After controlling for parasitemia, there was an inverse association between binding to CD36 (P = 0.004) or ICAM-1 (P = 0.001) and disease severity. Parasites from children with severe malaria anemia bound least to CD36, whereas ICAM-1 binding was lowest in children with cerebral malaria. There was no difference in rosette formation between any of the groups. In Malawian children, there was no evidence of a positive association between adherence to any of the receptors examined and disease severity. The negative association found raises the possibility that adherence to certain receptors could instead be an indicator of a less pathogenic infection.

Erythrocytes infected with mature stages of Plasmodium falciparum sequester in the deep circulation and are rarely found in the peripheral blood. At post-mortem examination, parasitized red blood cells (PRBC) containing trophozoites or schizonts are found in capillaries and postcapillary venules of the brain and other organs of individuals who die of cerebral malaria.1 This sequestration is believed to be necessary, but not sufficient, for the development of cerebral malaria.2,3

In vitro systems have shown that erythrocytes parasitized with mature stage parasites may adhere to receptors found on the endothelial cell surface. Such receptors include CD36,4 intercellular adhesion molecule-1 (ICAM-1),5 chondroitin sulfate A (CSA),6 E-selectin and vascular cell adhesion molecule-1 (VCAM-1),7 thrombomodulin (TM),8 platelet endothelial cell adhesion molecule-1 (PECAM-1),9 and P-selectin.10 A key question is whether the ability of PRBC to adhere to these receptors is a risk factor for severe disease. Previous studies found no association between cerebral malaria and parasite adherence, but many have had inadequate sample sizes.11–14 In one of these studies, there was an association between adherence to C32 melanoma cells and severe (but not cerebral) malaria in Thai adults.12 A recent large study from Kenya, using cryopreserved and thawed isolates, showed a trend to more binding to ICAM-1 by isolates from children with cerebral malaria than from children with uncomplicated or asymptomatic disease.15 In contrast, formation of erythrocyte rosettes (a trophozoite-infected PRBC with two or more uninfected RBC adherent to it) is the only parasite adherence property to have been repeatedly associated with disease.16,17

We have examined the adherence profiles of PRBC cultured from the blood of Malawian children with severe or uncomplicated malaria. We have measured binding to CSA and TM (not previously evaluated as a correlate of disease severity in children), CD36, and ICAM-1 and formation of erythrocyte rosettes in 155 children.

METHODS

The study took place at the Malaria Research Project and Wellcome Trust Centre (MRP), Queen Elizabeth Central Hospital in Blantyre, Malawi between January and June 1997. Venous blood samples collected into tubes containing lithium heparin or EDTA were obtained from children with severe malaria defined according to World Health Organization criteria,18 and from children with uncomplicated disease. Informed consent for venesection was obtained from the parents or guardians of all children enrolled in the study. The study was approved by the Malawi National Health Sciences Research Committee. Cerebral malaria was defined as a Blantyre coma score ≤ 2 in the absence of other apparent cause, and severe anemia was defined as a hematocrit < 15%. Patients with uncomplicated disease were from several sources: 1) ambulant children screened for enrollment in studies of novel antimalarial therapy at Ndirande Health Centre, Blantyre, 2) ambulant controls attending the MRP, 3) patients discharged from the MRP who were significantly parasitemic but without any malarial complications at a follow-up visit 2 weeks to 4 months later, and 4) patients admitted to the MRP ward with a final diagnosis of uncomplicated malaria. Controls had no history of convulsions or coma and a Blantyre coma score of 5 of 5. The numbers of isolates successfully grown and tested from children from each group are shown in Table 1. Clinical data, including basic demographics, conscious state, history of convulsions, history of prior drug treatment, and age, hematocrit, and outcome of infection were recorded for all patients.

Plasma was removed from the sample, and RBC were
washed three times in phosphate-buffered saline (PBS) with buffy coat depletion. When parasitemia was at least 1%, an aliquot of washed RBC was cultured in RPMI 1640 medium (Life Technologies, Ltd., Paisley, United Kingdom) supplemented with 25 mM HEPES, gentamicin (10 μg/ml) (Life Technologies, Ltd.), NaHCO3 (2 mg/ml), and 10% human serum albumin in PBS for 30–60 min and washed with RPMI 1640 medium-HEPES until no non-adherent RBC were visible by inverted microscopy. Numbers of mature stage parasites adherent to each receptor were determined by counting 25 fields using a 10× eyepiece and a 100× objective under oil immersion in a standardized manner. Numbers of parasites bound were standardized for a parasitemia of 1% by dividing PRBC count by parasitemia. Significant binding was defined as ≥ 5 PRBC/mm2 and was calculated both at binding parasitemia and after parasitemia had been adjusted to 1%.

Rosette formation was measured on triplicate aliquots of parasite culture (15 μl) added to 7–10 μl of acidine orange on a glass slide. After placing a 22 × 22 mm cover slip over the slide, the sample was examined by fluorescence and direct light microscopy, and the proportion of trophozoite-infected cells in rosettes was measured for each sample as previously described. The isolates from children with uncomplicated malaria resulting in hospital admission were recruited from the following sources: antimalarial drug trial (39 children), ambulant hospital controls (9 children), follow-up patients (21 children), and uncomplicated malaria resulting in hospital admission (10 children).

RESULTS

Parasites from 76 children with severe malaria and 79 children with uncomplicated malaria were cultured in RPMI 1640 medium-HEPES supplemented with 25 mM HEPES, gentamicin (10 μg/ml) (Life Technologies, Ltd.), NaHCO3 (2 mg/ml), and 10% human serum albumin in PBS for 30–60 min and washed with RPMI 1640 medium-HEPES. Red blood cells were centrifuged and resuspended in 5% AB serum in RPMI 1640 medium-HEPES, pH 6.8, at a 1% hematocrit, and 1.7 ml was added to each dish. Assays were incubated at 37°C for 60 min with gentle swirling every 15 min. Dishes were gently washed with RPMI 1640 medium-HEPES until no non-adherent RBC were visible by inverted microscopy, and bound cells were fixed with 2% glutaraldehyde (Agar Scientific, Stansted, United Kingdom) in PBS, stained with Giemsa (Merck, Ltd., Poole, United Kingdom), and counted by light microscopy. Numbers of mature stage parasites adherent to each receptor were determined by counting 25 fields using a 10× eyepiece and a 100× objective under oil immersion in a standardized manner. Numbers of parasites bound were standardized for a parasitemia of 1% by dividing PRBC count by parasitemia. Significant binding was defined as ≥ 5 PRBC/mm2 and was calculated both at binding parasitemia and after parasitemia had been adjusted to 1%.

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Data were expressed as the mean of triplicate measurements for most data points. A shortage of ICAM-1 meant that only duplicates were performed in some assays, and technical problems with fixing in single replicates led to these being excluded in a small proportion of cases.

Data were entered into Microsoft (Redmond, WA) Access® version 7.0 and analyzed using a combination of EpiInfo version 6.0 (Centers for Disease Control and Prevention, Atlanta GA), Microsoft Excel®, and SPSS version 7.5 (SPSS Institute, Chicago, IL). Because the data were not normally distributed, the Mann-Whitney U test and the Kruskal-Wallis test were used for comparing means, unless otherwise indicated. A P value = 0.05 was considered significant for this analysis.

RESULTS

Parasites from 76 children with severe malaria and 79 children with uncomplicated malaria were grown successfully to the trophozoite stage and binding was measured in at least one assay. Children with severe malaria had cerebral malaria (38), severe malaria anemia (22), or both (16) and were similar to controls in age (mean ± SD = 38.9 ± 24.9 versus 32.5 ± 16.7 months) and sex distribution (48.7% versus 49.4% males). Two of the controls had a history of antimalarial treatment (which was always sulfadoxine-pyrimethamine) in the past week, whereas 15 children with severe malaria had this treatment. Children with severe malaria anemia were significantly younger (P = 0.001) than children with cerebral malaria or controls, consistent with previous observations. The characteristics of these patients and their parasitemias are shown in Table 1.

Table 2 shows the comparison of mean (range) parasitemia-adjusted binding (PRBC/mm²) for isolates from all children with severe malaria, subsets of severe malaria cases, and the isolates from children with uncomplicated malaria. Figure 1 shows the percentage of isolates binding at significant levels (≥ 5 PRBC/mm²) to each receptor. There was no evidence of a higher prevalence of significant binding or of greater numbers of PRBC bound when isolates from patients with severe malaria were compared with those with uncomplicated disease. Indeed, for all receptors, the trend was in the opposite direction (Figure 2), and reached statistical significance for both CD36 (P = 0.004) and ICAM-1 (P = 0.001). The binding of ICAM-1 was significantly lower (P = 0.011) for isolates from children with severe malaria even when raw (non-parasitemia-adjusted) data were used.
Few isolates bound to CSA or TM (which contained a CSA chain). These results were not significantly different when follow-up samples from patients previously admitted were excluded.

There was consistently lower binding to all receptors in the group of children with severe anemia compared with controls. An inverse relationship between hemoglobin concentration and receptor binding was demonstrated by comparing the binding levels of CD36 and ICAM-1 and patient hematocrit measurements of the total study population (Figure 3). The binding of isolates to CD36 was significantly different at each hemoglobin level; however, the ICAM-1 binding was only significantly different between the < 25% and ≥ 25% categories.

There was no significant difference in mean rates of rosette formation between PRBC from children with severe malaria, subsets of severe malaria, and uncomplicated malaria (Table 1 and Figure 4). Only three isolates failed to form rosettes, two from children with severe malaria and one from a child with mild disease.

Correlations between binding of ICAM-1 and CD36 and rosette formation were examined. A significant correlation from a child with mild disease.

We have examined cytoadherence and rosette formation in 155 patient isolates cultured to the trophozoite stage. We found no correlation between rosette formation and disease severity, and no evidence of a predisposition for isolates from children with more severe or complicated malaria to bind at higher rates to any of the purified receptors used. After standardizing for parasitemia, parasite isolates from children with uncomplicated disease bound at higher levels to all receptors, significantly so for ICAM-1 and CD36. There was a significant association between hematocrit and parasite adhesion, with lower binding to CD36 and ICAM-1 by isolates from children with severe anemia than children with mild or no anemia.
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malaria-free and antimalarial-free blood donors in Malawi,
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 umes, whereas the current study used fresh patient isolates. Individual patient isolates may be com-
population there may be multiple variant antigenic types (VATs) with different adherence profiles.22 Genotyping by the polymerase chain reaction and phenotyping with antibodies to merozoite surface protein-1 (MSP-1) and MSP-2 2 showed that almost all infections in these children are composed of multiple clones (Dobaño C, unpublished data). To avoid a possible selective loss of some VATs over others with cryopreservation, we performed studies on site. Changes in dominant antigenic types in a sample following cryopreservation are reported, as reflected by the agglutination profile obtained using semi-immune adult sera.23 Examining patient isolates placed directly into culture minimizes but does not eliminate the risk of selecting some variant antigenic types from a mixed population for binding.
Due to the difficulty of getting fresh erythrocytes from malaria-free and antimalarial-free blood donors in Malawi, we did not dilute high-binding parasitemia to a standard level. We may therefore have underestimated binding from some isolates.15 However, we found no difference in the relationship between groups in mean binding when we compared only isolates with a binding parasitemia < 10%, which has previously been found to be in the range giving linear binding with parasitemia (Beeson J, unpublished data). Similar to findings from Kilifi,15 binding to CD36 was lowest in children with severe malaria anemia; unlike their results, we found lower levels of binding to ICAM-1 in severe disease in general, rather than specifically severe malaria anemia.
Since there are potential problems examining parasites from children with severe malaria anemia or cerebral malaria together, we have analyzed the results both together and separately. Their pathogenesis may be quite different,24 and our finding of a correlation between patient hematocrit and parasite binding (Figure 3) is consistent with this hypothesis (although cytoadherence may be implicated in the development of both cerebral malaria and severe malaria anemia).
Rosette formation was very frequent in our series: all but three isolates tested formed rosettes to some degree. Studies from east and west Africa have found a high prevalence of non-rosette-forming parasite isolates from individuals with uncomplicated malaria (from 30% to 45%) with higher prevalences of rosette formation in children with severe malar-
ia.15,16,17,25 In contrast, in a large study from Papua New Guinea,26 all isolates tested formed rosettes. The reasons for this difference may include host genetic differences in the prevalence of rosette formation receptors, such as complement receptor 1 polymorphisms;27 differences in heparan sulfate molecule type or density on the uninfected erythrocyte surface, or differences in prevalence of other blood group determinants.28 We examined a larger number of trophozoite-infected erythrocytes per sample than other studies reported. In the Papua New Guinean study, there was no difference in rosette formation between isolates from different patient groups, a finding borne out in the current study. It will be important to examine the prevalence of rosette formation receptors in different populations in relation to the frequency and intensity of rosette formation and in relation to the associations between rosette formation and disease severity in those populations.
Our study did not set out to examine the associations between PRBC binding and prior drug treatment or disease outcome. Examining the results in relation to these variables, we found no evidence for impaired cytoadherence by isolates from children with prior treatment within the previous week with pyrimethamine-sulfadoxine, as has been described in in vitro and in vivo drug exposed parasites for quinine and artemisinins.29 We also found no association between disease outcome (death or sequelae versus recovery)
and binding (Table 3). Again, the trends were in the opposite direction.

What might be the explanation for the negative association between cytoadherence and disease severity? It is unlikely, given the level of significance, that this is due to chance alone, and the same findings were observed on several preliminary examinations of the data. A negative association between CD36 or ICAM-1 binding and disease severity could suggest that PRBC binding to those receptors is in fact more prone to cause less severe disease. Such parasites could sequester in less harmful parts of the body. The population in the patient’s peripheral blood represents the progeny of PRBC that were sequestered throughout the body on the previous parasite cycle, only a proportion of them in the brain. Almost all of these will have the same adherence type and VAT as their parents, and so reflect a population previously sequestered in a heterogeneous range of sites, including notably organs such as the gut, brain, skin, and lung. Endothelium differs between tissues, and it is highly likely that different adherence mechanisms may predominate in different tissues. Thus the circulating population may contain a relatively small proportion of PRBC capable of cerebral sequestration (and thus facilitating the development of cerebral malaria) and a larger proportion that were previously sequestered in other non-cerebral sites, whose adherence profile obscures that of the more pathogenic minority.

Alternatively, if an infection contains two highly synchronous broods of parasites, it is possible that the brood sequestered in the brain and initiating the events leading to cerebral malaria differs in adherence behaviour or genotype from the brood circulating at the time of admission, again obscuring a pathogenic parasite characteristic. It is known that populations of parasites can change rapidly in asymptomatic individuals in endemic areas, and similar dynamic processes may be occurring in these symptomatic children. In the subset of children who died, we are comparing MSP-1 and MSP-2 phenotypes of circulating and sequestered parasites using monoclonal antibody typing (Dobaño C, unpublished data).

Several of our isolates showed no binding to any of the receptors tested, although all isolates with no binding still formed rosettes to some extent. These parasites must be able to sequester in the host, and their failure to do so in vitro could reflect a failure of our system. We did not examine binding to PECAM-1, P-selectin, VCAM-1, or E-selectin. Given the findings from Kilifi of very low levels of adherence of cultured parasites to PECAM-1, VCAM-1 and E-selectin, it suggests either that there are other as yet unidentified receptors that are playing a critical role in the sequestration process, or that cooperation between receptors is essential.

Our findings could have implications, for example, for development of vaccines aimed at blocking adherence to CD36. Such a vaccine, if successful, could select positively for more pathogenic variants that use alternative sequestration receptors expressed on cerebral endothelium. Further evaluation of vaccine candidates derived from sequesterin or P. falciparum erythrocyte membrane protein 1 will need to take this possibility into account.

There is clearly a great diversity of cytoadherence profiles from patient isolates from many places in the world that is explained in part by the variety of different assay systems used. For results to be comparable between settings, standardization of techniques should be encouraged as much as possible. It is clear that most or all PRBC bind to CD36, and that binding to ICAM-1 is also common, whereas a minority of isolates bind to other receptors identified.

Examination of adherence of PRBC to isolated purified receptors in static in vitro systems may be a poor reflection of what happens in vivo. The proportion of PRBC introduced into the assay that remain attached to the receptor is far lower than the proportion of PRBC of similar stages that sequester in vivo, and how representative the bound population is of the total population is unknown. Flow systems have been proposed as a more physiologic method. As well as the effects of flow, cooperation between receptors or a multiple-step process of sequestration such as that described for leukocytes may be of importance in malaria. It will be important to develop systems that more closely resemble in vivo conditions that incorporate endothelial cell lines and parasites under physiologic flow.

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