

## *Plasmodium falciparum* Rosetting Is Associated with Malaria Severity in Kenya

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**Rosette formation in 154 fresh *Plasmodium falciparum* isolates from Kenyan children with mild ( $n = 54$ ), moderate ( $n = 64$ ), or severe ( $n = 36$ ) malaria was studied to determine whether the ability to form rosettes in vitro is correlated with malaria severity. There was a wide distribution of rosette frequencies within each clinical category; however, a clear trend towards higher rosette frequency with increasing severity of disease was seen, with the median rosette frequency of the mild-malaria group (1%; range, 0 to 82%) being significantly lower than those of the moderate-malaria group (5%; range, 0 to 45%; Mann-Whitney U test,  $P < 0.02$ ) and the severe-malaria group (7%; range, 0 to 97%; Mann-Whitney U test,  $P < 0.003$ ). Within the severe-malaria category there was no difference in rosetting among isolates from cerebral malaria patients or those with other forms of severe malaria. We also examined the ABO blood groups of the patients from whom isolates were obtained and found that isolates from group O patients (median rosette frequency, 2%; range 0 to 45%) rosetted less well than those from group A (median, 7%; range 0 to 82%; Mann-Whitney U test,  $P < 0.01$ ) or group AB (median, 11%; range 0 to 94%; Mann-Whitney U test,  $P < 0.03$ ). We therefore confirm that rosetting is associated with severe malaria and provide further evidence that rosetting is influenced by ABO blood group type. Whether rosetting itself plays a direct role in the pathogenesis of severe malaria or is a marker for some other causal factor remains unknown.**

*Plasmodium falciparum* malaria remains a common cause of morbidity and mortality throughout the tropical regions of the world and is estimated to cause 1 million deaths a year in Africa alone (6). Although infection with malaria parasites is common, only 1 to 2% of infections lead to severe life-threatening disease characterized by a range of clinical features, including unrousable coma (cerebral malaria), severe anemia, metabolic acidosis, and multiorgan failure (25). The majority of malarial deaths in Africa occur in children under 5 years of age, as with increasing age and recurrent exposure to malaria a nonsterile immunity develops (14). It remains unclear why some children develop the severe manifestations of disease while others suffer only mild symptoms or remain asymptomatic.

The pathogenesis of malaria is incompletely understood, but severe disease is thought to be related to the sequestration of parasitized erythrocytes in vascular beds within the brain and other vital organs (13). Sequestration occurs by means of cytoadherence to endothelial cells, and several receptors have been identified which may mediate this interaction, including CD36 (18), thrombospondin (22), the intercellular adhesion molecule 1 (2), the vascular cell adhesion molecule, and E-selectin (17). It remains unclear how and if adhesion to any of these receptors is related to the development of severe malaria, as studies with field isolates have to date been inconclusive (10, 15, 16).

Another adhesion property demonstrated by some *P. falciparum* isolates which has been associated with severe malaria is rosette formation. Rosetting is the spontaneous binding of uninfected erythrocytes to erythrocytes infected with mature asexual parasites (5). Two studies in The Gambia have shown that parasite isolates from children with cerebral malaria have

a higher mean rosette frequency than those from children with mild malaria and that children with cerebral malaria are less likely to have rosette-disrupting antibodies, suggesting that rosetting may contribute to the pathogenesis of cerebral malaria (3, 23). A subsequent study of Thai adults showed a higher median rosette frequency in isolates from cerebral cases than in isolates from other severe malarial and nonsevere controls; however, the numbers in this study were small and the differences were not statistically significant (9). Another small study in Madagascar showed that the median rosette frequencies were higher in cases of cerebral and other severe malarial than in cases of uncomplicated malaria (21).

We therefore examined rosetting in 154 isolates from patients in Kenya with severe, moderate, and mild malaria to see if rosetting was associated with disease severity in East Africa and to determine whether rosetting was specifically associated with cerebral malaria or whether it was also seen with other forms of severe malaria.

### MATERIALS AND METHODS

**Patients.** Blood samples from children with a parasitemia level of 0.3% or higher were collected at Kilifi District Hospital, Kilifi, Kenya. Three groups of children were studied: those with mild, moderate, and severe malaria. Children with mild malaria exhibited an acute febrile illness but no features of severe malaria and were treated as outpatients. Children with moderate malaria were admitted to the pediatric ward but did not fulfill the criteria for admission to the high-dependency unit (i.e., they showed no disturbance of consciousness, they were not prostrated [unable to maintain a sitting posture], and they did not have respiratory distress). All children from the mild malaria and moderate malaria groups made an uneventful recovery on oral therapy. Children with severe malaria were admitted to the high-dependency unit for parenteral therapy. These fell into two broad groups: (i) those with cerebral malaria (i.e., children in a coma and unable to localize a painful stimulus) and (ii) fully conscious children who were either prostrated or in respiratory distress (having abnormally deep breathing with intercostal or subcostal recession). The majority of the latter group were severely anemic (hemoglobin level,  $<5$  g/dl), severely acidotic (bicarbonate level,  $<15$  mmol/liter), or both.

**Parasite culture.** One-milliliter blood samples were collected into 0.15 ml of acid-citrate-dextrose. It has been shown previously that the type of anticoagulant

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TABLE 1. Characteristics of patients with mild, moderate, or severe malaria

Clinical category of disease	No. of cases	Mean age (SD)	Mean hemoglobin level (g/dl) (SD)	Mean parasite density (SD) <sup>a</sup>
Mild	54	3 yr 5 mo (27 mo)	8.6 (2.0)	50,476 (40,314)
Moderate	64	1 yr 6 mo (20 mo)	7.0 (2.1)	239,832 (300,352)
Severe	36	2 yr 9 mo (20 mo)	6.9 (2.3)	361,245 (425,986)

<sup>a</sup> Number of parasites per microliter of blood.

used to collect samples (acid-citrate-dextrose or heparin) did not influence the final rosette frequency (22a). Isolates were spun through Lymphoprep, washed twice to remove leukocytes, and then cultured by standard methods with RPMI medium supplemented with 25 mM glucose, 37.5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 2 mM glutamine, 25 µg of gentamicin per ml, and 10% human AB serum, with the pH of the final solution adjusted to between 7.2 and 7.4 with 1 M NaOH. Cultures were gassed with a mixture of 94% nitrogen, 5% carbon dioxide, and 1% oxygen. Parasites were grown for at least 16 h in vitro, and maturity was assessed with Giemsa-stained thin films.

**Assessment of rosette frequency.** Rosette frequency was assessed when the majority of parasites in a culture had reached the pigmented trophozoite or schizont stage. Of 184 isolates, 154 grew to maturity. Of the 30 isolates which failed to grow, 9 were from severe cases, 14 were from moderate cases, and 7 were from mild cases. To assess rosette frequency a sample of parasite culture at 1 to 2% hematocrit was stained with 20 µg of ethidium bromide per ml, and a wet preparation of the culture was viewed under fluorescence. Two hundred cells infected with mature parasites were counted, with the binding of two or more uninfected erythrocytes constituting a rosette. The rosette frequency is the percentage of mature-parasite-infected cells found in rosettes. Assessment of rosette frequency was made without knowledge of the clinical category of all samples.

**Assessment of antirosetting antibodies.** Isolates with rosette frequencies of >20% were tested with plasma from the same patient for the presence of rosette-disrupting antibodies. One hundred microliters of parasite culture was incubated for 30 min at 37°C at a final plasma dilution of 1/5 before the assessment of rosette frequency and was compared with a control incubated with a 1/5 dilution of European AB plasma. Plasma samples from all patients were stored at -20°C and transported to the United Kingdom, where they were later tested at a 1/10 dilution for their ability to disrupt a laboratory rosetting clone, R29, and compared with a European plasma control. R29 has a baseline rosette frequency of 50 to 80%.

**Blood grouping.** The patient's ABO blood group was determined for each isolate by a standard slide agglutination technique.

**Statistics.** Correlations were assessed by the method of least squares, and medians were compared by the Mann-Whitney U test.

## RESULTS

Isolates which grew to maturity were obtained from 54 mild, 64 moderate, and 36 severe malaria cases. The characteristics of the three groups are shown in Table 1. There was no correlation between rosette frequency and hemoglobin, parasite density, or age in any group. The distribution of rosette frequencies in the three groups is shown in Fig. 1. Rosettes failed to form with 20 of 54 (37%) of the isolates from mild cases, 11 of 64 (17%) of the isolates from moderate cases, and 4 of 36 (11%) of the isolates from severe cases. In each group there was a broad scatter of rosette frequencies, with the distribution being skewed towards lower values. The median rosette frequency of the mild-malaria group (median, 1%; range, 0 to 82%) was lower than those of the moderate-malaria group (median, 5%; range, 0 to 45%; Mann-Whitney U test,  $P < 0.02$ ) and the severe-malaria group (median, 7%; range, 0 to 97%; Mann-Whitney U test,  $P < 0.003$ ). The difference between the moderate- and severe-malaria groups was not statistically significant (Mann-Whitney U test,  $P < 0.15$ ). Within the severe-malaria group there were 21 cases of cerebral malaria, 12 cases of prostration, and 3 cases of respiratory distress. As shown in Fig. 2, there was no difference in rosetting between the cerebral malarias (median, 6%; range, 0 to 94%) and the other severe malarias (median, 7%; range, 0 to 97%; Mann-Whitney U test,  $P < 0.4$ ).

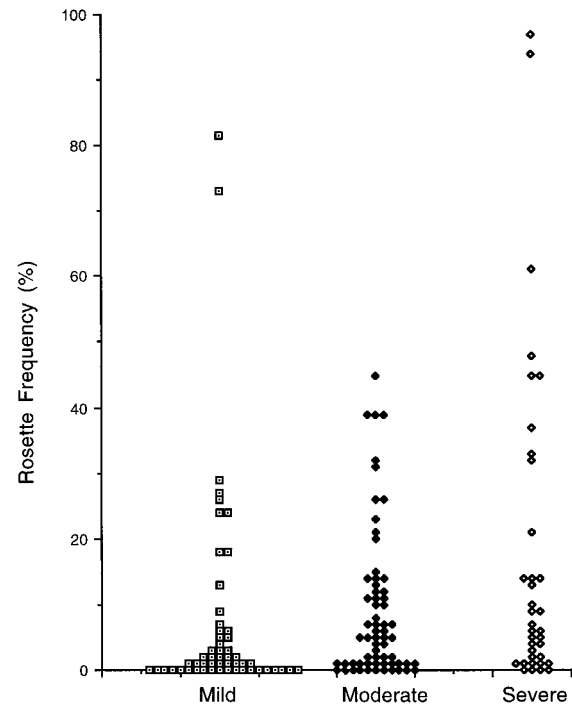


FIG. 1. Rosette frequencies of isolates from children with mild, moderate, or severe malaria.

Twenty isolates (six from severe, eight from moderate, and six from mild cases) with rosette frequencies of >20% were tested with autologous plasma at a 1/5 dilution for the presence of rosette-disrupting antibodies. No rosette disruption was detected in any sample. However, in 8 of 20 isolates (4 from severe, 2 from moderate, and 2 from mild cases), microagglutination of uninfected and infected erythrocytes was seen.

Plasma samples from the first 150 patients were tested at a 1/10 dilution against a laboratory rosetting clone, R29. Only three of these plasma samples caused >50% rosette disruption of R29.

The first 116 isolates were tested for the patient's ABO blood group, and the rosette frequencies for each blood group type are shown in Fig. 3. Isolates from blood group O patients ( $n = 51$ ; median, 2%; range, 0 to 45%) rosetted less well than isolates from blood group A patients ( $n = 30$ ; median, 7%; range, 0 to 82%; Mann-Whitney U test,  $P < 0.01$ ) or blood group AB patients ( $n = 16$ ; median, 11%; range, 0 to 94%; Mann-Whitney U test,  $P < 0.03$ ). Isolates from blood group B patients ( $n = 19$ ; median, 4%; range, 0 to 27%) also rosetted less well than isolates from blood group A and AB patients, but this difference was not statistically significant.

## DISCUSSION

In this study we have examined rosetting in a large group of Kenyan children with mild, moderate, or severe malaria, and here we show that there is an increase in rosetting with increasing severity of disease. Previous studies in the Gambia have shown that rosetting is more common in isolates from children with cerebral malaria than in isolates from children with uncomplicated malaria (3, 23), and it has been suggested that rosetting may play a direct role in the pathogenesis of cerebral malaria by causing obstruction to blood flow in the microvasculature (11). However, rosette-like aggregates of

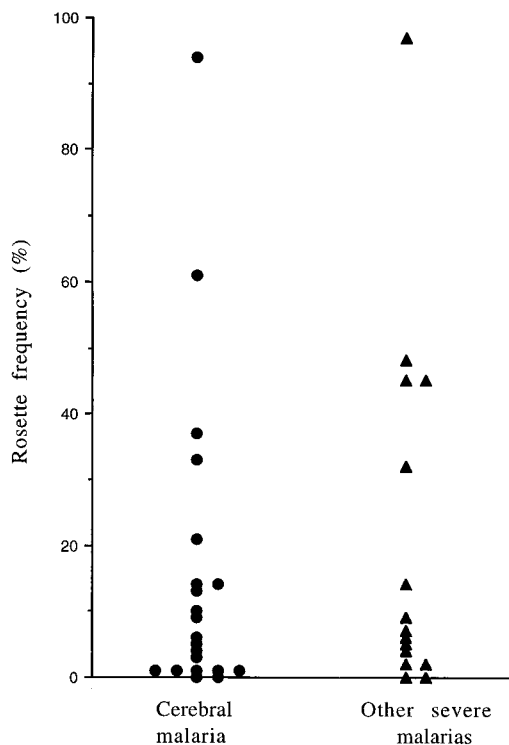


FIG. 2. Rosette frequencies of isolates from children with cerebral malaria or other forms of severe malaria.

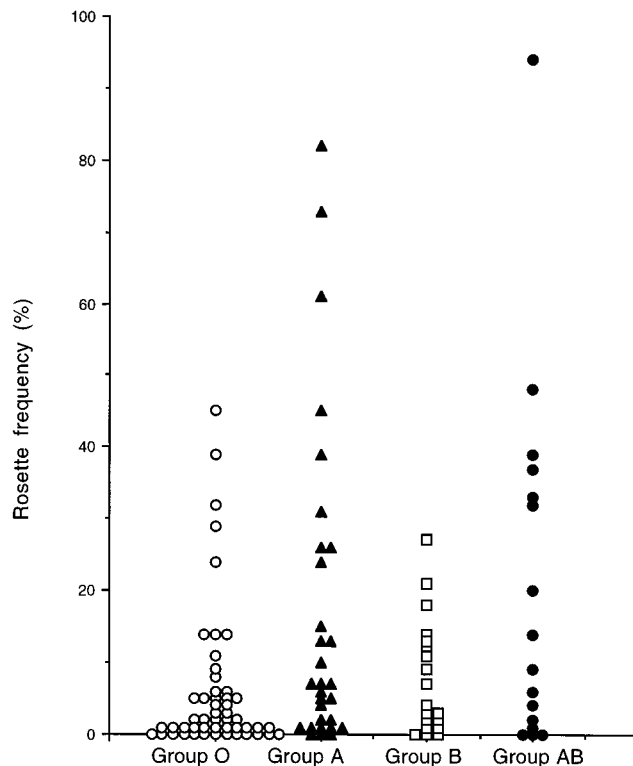


FIG. 3. Rosette frequencies of isolates by patient ABO blood group.

cells have only rarely been described in postmortem studies on malaria (7, 19, 20), and it is not even certain that rosettes actually form *in vivo*. The present study suggests that rosetting is not related specifically to cerebral malaria, as there was no difference in rosetting between the cerebral cases and other forms of severe malaria. The largest differences seen were between the mild-malaria group (fever but no other complications) and both the moderate- and severe-malaria groups. The median rosette frequency in the severe malaria cases was higher than that in the moderate cases, but this difference was not statistically significant. It seems, therefore, that rosetting is associated with increasing severity of malaria in a general way rather than with the extreme symptoms of the disease or with any particular clinical syndrome.

It remains unclear whether rosetting itself is important in the pathogenesis of malaria or whether its association with disease reflects some other unidentified causal factor. Ringwald et al. (21), in a small study of wild isolates from Madagascar, showed that rosetting is correlated to the plasma level of tumor necrosis factor (TNF), which has itself previously been shown to be associated with cerebral malaria and poor outcome (12). Another study linking rosetting and the level of TNF showed that in a cloned rosetting line, subcultures selected for high rosetting frequency gave significantly higher levels of TNF stimulation than did subcultures with low rosetting frequencies (1). The relationship between rosetting and TNF stimulation remains unexplained, and their precise roles in malaria pathogenesis remain uncertain.

If rosetting is directly involved in pathogenesis, it needs to be explained why some children are infected with parasites which rosette very well *in vitro* yet have only mild malaria. One explanation is that these children might have gone on to develop more severe symptoms if they had not received treat-

ment in time. It is also possible that the size and strength of rosettes are important in determining clinical outcome, with large rosettes or those which are particularly resistant to physiological shear forces being more likely to result in microvascular obstruction and disease. Another possibility is that antibodies which inhibit rosetting may modulate clinical outcome. In previous studies in the Gambia, rosette-disrupting antibodies were commonly found in the plasma of children with uncomplicated malaria but not in the plasma of children with cerebral malaria (3, 23). In the present study we were unable to detect autologous rosette-disrupting antibodies in any of 20 high-rosetting-frequency isolates studied (6 from severe, 8 from moderate, and 6 from mild cases of malaria). We did, however, detect microagglutination of uninfected and infected erythrocytes in 8 of 20 cases, as described previously (23). Whether such microagglutination contributes to microvascular obstruction and disease pathogenesis *in vivo* is unknown. We also tested plasmas against a laboratory rosetting clone, R29, and only rarely detected rosette-disrupting antibodies (3 of 150 samples).

The molecular mechanisms of rosette formation remain unknown, but there is good evidence that the ABO blood group affects the ability of uninfected erythrocytes to form rosettes with parasitized cells. By competition experiments with erythrocytes of different ABO types, it has been shown that blood group O cells rosette less readily than do cells of other blood groups (4, 24). The present study examined for the first time the relationship between blood group and rosetting in a large group of field isolates and showed that the rosetting frequency is lower in isolates from blood group O patients than in isolates from blood group A or AB patients. If rosetting is directly involved in the pathogenesis of malaria, one might expect that blood group O children would be relatively protected from

severe disease. In the Gambia, an association between blood group O and protection from cerebral malaria has been demonstrated (7a, 8), but in Kenya, where the present study was undertaken, blood groups O and A seem to confer some protection (13a); however, in both cases the effects are small.

There is now mounting evidence that rosetting is related to the severity of clinical disease in malaria, but it remains unclear whether rosetting itself is important in pathogenesis or is simply a marker for some other factor which mediates the disease process. It is possible that rosetting is a manifestation of cytoadherence to an as-yet-uncharacterized endothelial receptor which also happens to be expressed on erythrocytes, but this remains speculative until the precise receptor-ligand interactions which mediate rosette formation have been identified.

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