THE TIME COURSE OF CYTOADHESION, IMMUNOGLOBULIN BINDING, ROSETTE FORMATION, AND SERUM-INDUCED AGGLUTINATION OF PLASMODIUM FALCIPARUM–INFECTED ERYTHROCYTES

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Abstract. We describe morphologic characteristics of acridine orange–stained Plasmodium falciparum–infected erythrocytes and the sequential expression of several adhesion phenomena. In particular, we have studied when the adhesive and antigenic modifications appear on the infected erythrocyte surface that mediate binding to C32 melanoma cells (cytoadherence) or to erythrocytes (rosette formation) during a complete 48-hr life cycle of the parasite. The C32 melanoma cell binding started at about 12 hr and was seen during the whole life cycle with a peak around 28 hr (650 infected erythrocytes/100 C32 melanoma cells). Rosettes started to appear and immunoglobulin was found bound to the parasitized red blood cell (PRBC) somewhat later (16–20 hr). These adhesive events culminated at the mid-trophozoite/schizont stage (24–36 hr) with rosette formation and an immunoglobulin binding rate of about 50%, which decreased to about half of the peak values at the end of the life cycle. Serum-induced agglutination of the infected erythrocytes was also most extensive at 24–36 hr, but agglutination was seen with all late stage parasites, i.e., both trophozoites and schizonts at 24–48 hr of age. Taken together, adhesion to C32 melanoma cells starts prior to that of rosette formation, immunoglobulin binding, or serum-induced agglutination.

The incidence of Plasmodium falciparum malaria has been estimated to be 100 million new infections each year with 90% seen in tropical Africa. Estimates of malaria mortality vary from 1.5 to 2.7 million deaths annually affecting mainly children, with the two main causes of death being cerebral malaria and severe anemia. The current theories about the mechanisms leading to the development of cerebral and other forms of severe malaria are based on the fact that parasitized red blood cells (PRBC) sequester in the deep microvasculature of many organs and that excessive sequestration leads to vaso-occlusion, a histologic finding also seen at autopsy in the affected organs. Two forms of adhesive capacity have been identified that underlie the sequestration: cyto-adhesion to the vascular endothelium and rosette formation, the binding of uninfected red blood cells (RBC) to PRBC. An in vitro correlation has been found between binding of PRBC to CD36-expressing cells and pulmonary disease, and several studies have shown a strong association between the capacity in vitro to form rosettes and the severe forms of malaria. Antibodies that block cytoadherence or disrupt rosettes seem important for development of the immunity that protects against severe disease. Taken together, it appears that the causation of severe malaria is at least partly due to various adhesive events that are modulated by anti-adhesive antibodies.

In this study, two in vitro propagated strains of P. falciparum (FCR3S1 and TM284), selected either for rosette formation or cytoadherence to C32 melanoma cells, were investigated for their time course of adhesion. The parasites in culture were followed every 4 hr during a complete life cycle of 48 hr; binding of immunoglobulin, rosette formation, and C32 melanoma binding were assessed and the morphology was characterized after staining with Giemsa or acridine orange. The C32 melanoma binding assay was used as an in vitro model for the adherence of PRBC to endothelial cells. The C32 melanoma cells express mainly the P. falciparum receptor CD36, but intercellular adhesion molecule-1 (ICAM-1), thrombospondin (TSP), and chondroitin sulfate A (CSA) are also found at the surface. The in vitro agglutination of PRBC, using sera from semi-immune/immune donors, was also evaluated to study when the infected erythrocyte surface becomes antigenic.

MATERIALS AND METHODS

Plasmodium falciparum culture. Parasites were cultured according to standard procedures with 10% normal AB serum added to the buffered culture medium. Two strains were used: FCR3S1, a rosette-forming parasite cloned by limiting dilution from FCR3S obtained from the FCR3 strain originally isolated in The Gambia, and TM284, isolated in 1990 from a Thai patient with acute, severe malaria (provided by Dr. Sodsri Thaithong, Chulalongkorn University, Bangkok, Thailand). Parasite cultures were selected for either rosette formation or C32 melanoma adhesions and were grown to approximately 10% parasitemia and then cryopreserved. The receptor CD36 seems to be the main target on the C32 melanoma cells for the two strains tested (Fernandez V and others, unpublished data). Cultures were then thawed and expanded to sufficient amounts for the assays to be performed. It should be noted that the FCR3S strain in publications from our group has been referred to as Palo Alto (Uganda). However, molecular studies of the Palo Alto parasites have revealed that they are identical to parasites of the FCR3 lineage.

Assessment of rosette formation. An aliquot of the parasite culture was mixed with 0.001% AO, mounted on a glass slide, and 50 consecutive fields were counted with a Nikon (Tokyo, Japan) (Optiphoto2) light microscope using a 10× ocular and a 40× lens in incident UV light. The rate of rosette formation was calculated by counting the number of PRBC in rosettes relative to the total number of late stage PRBC (trophozoite and schizont) and expressed as a percentage.

Cytoadherence assay. The cytoadherence assay was performed as described elsewhere with slight modifications, using human amelanotic melanoma cells C32 (no. CRL...
Briefly, C32 melanoma cells were plated on 22-mm² glass coverslip. After 48 hr the cells were fixed with 1% formaldehyde for 1 hr and subsequently stored in phosphate-buffered saline (PBS), pH 7.4, at 4°C until use. The slides were equilibrated with preheated binding medium (RPMI 1640 medium, 25mM HEPES, 25 µg/ml of gentamicin, and 10% fetal calf serum, pH 6.8) for 20 min at 37°C prior to use and thereafter replaced with 1 ml of a suspension of RBC from the P. falciparum culture tested (2% hematocrit, 5–6% parasitemia). The plates were incubated at 37°C for 90 min, with gentle manual resuspension of the settled RBC every 15 min. After incubation, unbound RBC were rinsed off with binding medium and the PRBC bound to C32 melanoma cells were fixed with 1% glutaraldehyde in PBS, pH 7.4, and stained with 2% Giemsa. The number of bound PRBC per 100 C32 melanoma cells was determined using a Leitz (Wetzlar, Germany) light microscope with a 10× ocular and an oil-immersion lens with a magnification of 100×.

**Microagglutination assay.** The P. falciparum strain TM284, earlier selected for cytoadherence to C32 melanomas, was grown for several cycles until the culture was regarded as unsynchronous with all parasite-stages present. The assay was performed and scored according to a procedure modified from Aguilar and others (Barragan A and others, unpublished data). Briefly, an aliquot was taken from the parasite culture, washed three times in RPMI 1640 medium–HEPES, and resuspended to a 20% hematocrit. Aliquots of 25 µl were added to polystyrene round-bottom tubes and 25 µl of the serum to be tested was added diluted in RPMI 1640 medium–HEPES at a concentration twice that of the final concentration. The mixture was slowly rotated for 1 hr (37°C) and a small amount (approximately 25 µl) was then taken and mixed with 0.001% AO, mounted on a glass slide and 50 consecutive fields were counted with a magnification of 40×. The rate of rosette formation was always scored in the sample as used for estimation of the fluorescence rate.

**Experimental set-up.** When the majority of the parasites of the selected P. falciparum culture were in the ring stage, they were frozen using the sorbitol/glycerol technique. Prior to use, the parasites were thawed using decreasing concentrations of sorbitol, which taken together with the freezing procedure, synchronized the parasites to be studied. The cultures were subsequently expanded to suffice for the assays. The experiment was started when the parasitemia reached 5–6% and > 80% of the parasites were in the early trophozoite stage. An aliquot was taken from the culture every 4 hr for 48 hr during which the PRBC were scored for rate of rosette formation, surface immunoglobulin fluorescence rate, and cytoadherence to C32 melanoma cells. In addition, staining with Giemsa and AO was performed to study the morphology of the internal parasites. The rate of rosette formation was assessed for strain FCR3S1 and the rate of rosette formation, cytoadherence, and SIF were assessed for strain TM284.

**RESULTS**

**Morphologic characteristics of AO- or Giemsa-stained parasites.** During the first 12 hr, the parasites were seen as classic ring forms. For the next 8–12 hr, the parasites became increasingly dense and pigment deposition could be demonstrated. Thereafter, the parasites enlarged with progressive accumulation of pigment. Nuclear division could be observed at 32–36 hr and it continued through the rest of the cycle together with an ongoing condensation of the pigment into dense granulae. A number of mature merozoites could be seen at 44–48 hr together with a number of newly infected RBC containing delicate ring forms (Figure 1). Staining with Giemsa and AO were equally good, although when the parasites were in the schizont stage it was slightly easier to see details if they were stained with Giemsa due to intense fluorescence of the AO-stained parasites.

**Adhesion to C32 melanoma cells.** Strain TM284, which was selected six times for C32 melanoma adhesion, was allowed to adhere to C32 melanoma cells through the life cycle (Figure 2B). Significant adhesion was seen at 12 hr (400 PRBC/100 melanoma cells), followed by a slow increase of adhesion seen with a peak at 28–32 hr (650 PRBC/100 melanoma cells). The adhesion was fairly stable with time, with only a slow decrease of adhering PRBC. The adhesion of PRBC to C32 melanoma cells at 48 hr returned a level lower than that observed at 12 hr.

**Rosette formation.** The two P. falciparum strains FCR3S1 and TM284 exhibited very similar patterns of rosette formation. Rosettes started to appear in significant numbers at 16–20 hr; thereafter, the frequency of rosette formation increased until a peak was reached at 32–36 hr (FCR3S1 = 58%, TM284 = 45%), and the level of rosette formation showed a stable level between 24 and 36 hr. After 36 hr, a significant decrease in level of rosette formation was observed and at 48 hr only a low number of rosettes were seen.

**Binding of nonimmune immunoglobulin.** Surface immunofluorescence was determined with the TM284 strain.
F I G U R E 1. Staining of the *Plasmodium falciparum* strain TM284 every four hours (h) with either acridine orange (AO) or Giemsa. (Magnification × 1,000.)
selected for rosette formation (Figure 2D). The immunoglobulin binding of this strain followed a pattern similar to that of rosette formation. Maximal immunoglobulin binding was seen somewhat later, between 36 and 40 hr, than the peak in rosette formation. A decrease in immunoglobulin binding was observed with increasing numbers of late-stage schizonts.

**Serum-induced microagglutination.** Strain TM284, which was selected six times for C32 melanoma binding, was grown for several weeks until the culture was judged to be fully unsynchronous, with approximately the same numbers of all parasite stages seen at one time point. When tested in the microagglutination assay, serum 93A011 was the most efficient microagglutinator (3+), while BD245 and 93AO39 were somewhat weaker (2+). The three sera showed approximately the same features (Figure 2E). Almost no ring-stage forms were included in the microagglutinates and only a low number of parasites was seen between 12 and 24 hr of maturation. Parasites in the stage between 24 and 36 hr dominated in the microagglutinates, while a relative lower number of parasites between 36 and 48 hr were agglutinated.

**Discussion**

In this study, we used two laboratory strains, FCR3S1 and TM284, to investigate four *in vitro* phenomena that are relevant to the pathogenesis of *P. falciparum* malaria. Three of these phenomena, rosette formation, cytoadherence to C32 melanoma cells, and binding of immunoglobulin to the surface of PRBC as detected by SIF, were studied in simultaneous assays; the fourth, microagglutination, was studied in a separate assay. The morphologic features of the parasites were assessed by staining with either AO or Giemsa. When stained with Giemsa, the parasites showed the same features as described previously. It was observed that rosette formation, cytoadherence and binding of immunoglobulin appeared in a similar pattern, but the ability to cytoadhere occurred somewhat earlier than the abilities to form rosettes or to bind immunoglobulin.

The various binding phenomena began between 12 and 16 hr after reinvasion and increased in a few hours close to peak adhesion. The adhesion was stable for at least 16 hr with maximum binding when the parasites were in the late trophozoite stage. When the parasites matured further and entered schizogony the binding diminished. After approximately 36 hr, all forms of binding decreased significantly. This may reflect the *in vivo* situation but also be due to methodologic difficulties in the assays. The morphologic features of the parasites may result in cell binding that is slightly weakened or disruption of the PRBC during the washing procedures in the assays. A similar phenomenon could be seen when the microagglutination was performed with a highly unsynchronous culture. Late-stage trophozoites dominated in the agglutinates while the presence of schizonts was relatively low.

The time course of C32 melanoma binding differs slightly from that of an earlier report in which the time course of a wild isolate from a Gambian patient was studied. It was observed that adhesion to C32 melanoma cells started at approximately 20 hr, but our findings are more consistent with a report by Gardner and others in which adhesion to CD36, TSP, and ICAM-1 of the parasite isolate A41+ appeared between 12 and 16 hr. They also described a time difference between the start of the several adhesive abilities, and adhesion to TSP occurred 2 hr earlier than adhesion to CD36 or ICAM-1. They proposed that this may be due to different molecules mediating the binding properties. We have studied the binding to C32 melanoma cells, a cell line that expresses several potential *P. falciparum* receptors. Even though CD36 seems to be one of the main receptors for the *in vitro* propagated strain TM284 (Fernandez V and others, unpublished data), TSP may be involved and have an influence on the start of adhesion. The assays of C32 melanoma cell binding, immunoglobulin binding, and rosette formation were performed in parallel to each other.
other and the results were reproducible, implicating that the different time points of interaction reported in this study might be true. However, differences in sensitivity between the various assays may have some bearing on the results. Taken together, our findings may be interpreted that sequestration could be divided in two phases; the parasite is first able to adhere to the endothelium of the microvascular system, followed by an increasing ability to adhere to noninfected RBC, thus forming erythrocyte rosettes.

It has been reported that preformed rosettes may adhere to the endothelium, and measurement of the adhesive forces between PRBC and endothelium respective RBC have revealed that the interaction of PRBC with RBC may be far stronger than that of PRBC with endothelium. However, this is dependent on host-receptor preferences of the parasite tested, i.e., ICAM-1 binding is weaker than the interaction with CD36. It has also been concluded that rosettes reform in the venules after the PRBC and RBC have transversed the narrow capillaries. However, whether PRBC already adhering to the endothelium may bind RBC under flow conditions thought to occur in the venular circulation has not yet been studied in detail. Parasites included in the microagglutinates showed the same distribution of maturity as the parasites involved in the adhesive interactions studied, again consistent with recent reports. This suggests that all four phenomena are to some extent associated with each other. Both adhesion to CD36 and RBC has been described to be mediated by P. falciparum erythrocyte membrane protein 1, but other molecules have also been attributed both to CD36 adhesion and rosette formation. A more extended study is necessary to determine whether the four characteristics are determined by the same molecular changes or if different molecules are involved.

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