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Cytoadherence and Ultrastructure of *Plasmodium falciparum*-Infected Erythrocytes from a Splenectomized Patient

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In malarial infections of primates, the spleen has been shown to modulate parasite antigen expression on the surfaces of infected erythrocytes. The processes affected include cytoadherence, which is central to the pathophysiology of severe falciparum malaria, and the related phenomenon of rosette formation. In this study, the cytoadherence and rosette formation behaviors of *Plasmodium falciparum*-infected erythrocytes from a splenectomized patient were examined during the first erythrocytic cycle in vitro. Ultrastructural studies were also performed. Infected erythrocytes were found to cytoadhere to C32 melanoma cells via leukocyte differentiation antigen CD36 but not intercellular adhesion molecule 1. They also displayed on their surfaces electron-dense knobs similar in structure and density to those on infected erythrocytes from intact hosts. These findings may reflect a stable cytoadherent phenotype of the parasite isolate that is unaffected by the absence of the spleen. Alternatively, the modulating role of the spleen may have been assumed by other organs of the mononuclear phagocytic system in a previously infected individual. No rosette formation was observed, but as not all natural isolates form rosettes, this observation may or may not be related to the asplenic status of the patient. Parasite and host factors appear to be important in determining the effect of splenectomy on cytoadherence and rosette formation in human falciparum malaria.

Of the four human malaria parasites, *Plasmodium falciparum* is the only species which is sequestered in deep vascular beds as the intraerythrocytic parasite matures to the trophozoite and schizont stages. By so doing, it escapes the clearance mechanisms of the spleen and is thus able to proceed to schizogony. Sequestration results from the cytoadherence of erythrocytes infected with trophozoites and schizonts of *P. falciparum* to vascular endothelium; this cytoadherence requires an interaction between specific parasite ligands and endothelial cell receptors. At the points of contact are electron-dense protrusions on the surfaces of the erythrocytes, known as knobs. In *P. knowlesi* (3), *P. falciparum* (4, 7), and *P. fragile* (8) infections of monkeys, the spleen has been shown to be essential for maintaining the expression of parasite antigens on the surfaces of infected erythrocytes (IRBC), including the cytoadherent ligands. Parasite antigens are not detected on the surfaces of schizont-infected erythrocytes from splenectomized animals by immune sera from intact animals (3). Infected cells from splenectomized animals are not sequestered in vivo and do not bind to endothelial or melanoma cells in vitro (4, 7). However, the ability to express surface antigens and to cytoadhere is restored with the passage of parasitized cells from splenectomized animals through intact hosts (7, 8). The spleen also appears to be necessary for rosette formation, the agglutination of noninfected erythrocytes around infected ones, as rosette formation is markedly reduced in *P. fragile*-infected erythrocytes from splenectomized animals (6).

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It is not known how the spleen affects the surface antigen expression of plasmodium-infected erythrocytes. Studies with cloned *P. knowlesi* and *P. fragile* parasites suggest a modulating influence of the splenic environment which permits parasite antigen expression rather than the selective expansion of a phenotypically distinct variant within a mixed population (2, 8).

In the present report, we examined the cytoadherence and rosette formation properties of *P. falciparum*-parasitized erythrocytes from a splenectomized patient. Ultrastructural studies were also performed to determine whether knobs were present on the surfaces of these cells.

MATERIALS AND METHODS

IRBC were obtained from a patient who developed *P. falciparum* malaria 1 year after splenectomy following abdominal trauma. He had had previous attacks of malaria prior to the surgery. The complete absence of functioning splenic tissue was confirmed by computerized tomography of the abdomen and technetium scanning. The cells were washed three times with RPMI 1640 medium (Flow Laboratories, Ayreshire, Scotland) and cultured in vitro (18) in RPMI 1640 medium supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–24 mM $NaHCO_3$–penicillin (100 U/ml)–streptomycin (100 μg/ml)–10% heat-inactivated human AB serum (Flow Laboratories). Parasites were examined by light microscopy every 8 to 10 h, and when they had matured to the late trophozoite stage during the first parasite cycle in vitro, they were prepared for cytoadherence and rosetting assays.

The cytoadherence of IRBC to C32 melanoma cells (American Type Culture Collection, Rockville, Md.), which express both the putative receptor molecule CD36 (16) and

intercellular adhesion molecule 1 (ICAM-1) (5), and to DS-1 cells, a subclone of C32 melanoma cells which expresses only ICAM-1 (a kind gift from David Simmons, Imperial Cancer Research Fund, Oxford, United Kingdom), was studied as previously described (11). In brief, monolayers of C32 and DS-1 cells were prepared at a density of 10^5 cells per coverslip (measuring 22 by 22 mm). The monolayers were fixed in 1% formalin for 1.5 h and stored at 4°C. A 2% suspension of IRBC in supplemented RPMI 1640 medium was added to duplicate monolayers, and these were incubated at 37°C in 5% CO_2 for 90 min with gentle rocking every 15 min. At the end of the incubation, the coverslips were gently rinsed four times in RPMI 1640 medium and air dried. The monolayers were fixed in methanol, stained with 10% Giemsa stain, and examined microscopically. The number of IRBC adherent to 1,000 melanoma cells was counted along two perpendicular axes per coverslip. Results were expressed as the number of IRBC which adhered to 100 C32 or DS-1 cells.

Rosette formation was assessed by the method of David et al. (6) at the same time as cytoadherence. Ten microliters of a 10% suspension of IRBC in supplemented RPMI 1640 medium was mixed with 10 μl of heat-inactivated fetal bovine serum (Flow Laboratories). Ten microliters of the mixture was placed on a glass slide and covered with a coverslip (22 by 22 mm). One hundred IRBC were examined under oil immersion by phase-contrast microscopy. IRBC were identified by the presence of a golden brown refractile malaria pigment. The percentage of IRBC with two or more uninfected erythrocytes attached was scored.

For ultrastructural studies, IRBC containing trophozoites and schizonts were also fixed for 2 to 4 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Fixed cells were washed three times in cacodylate buffer by centrifugation in a Microfuge and finally spun into small pellets, which were then postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 1 h. The pellets were dehydrated in a series of acetone solutions of increasing concentrations and, after 100% acetone, were processed separately for scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For SEM, pellets were critical-point dried and then crumbled gently onto SEM stubs coated with double-sided adhesive tape. The cells were sputter coated with gold (5 to 10 nm thick). For TEM, pellets were passed through a 1:1 mixture of acetone-TAAB epoxy resin and finally embedded in TAAB resin. Sections were stained with uranyl acetate and lead citrate.

RESULTS

Cytoadherence and rosette formation. IRBC were seen to cytoadhere to C32 but not DS-1 cells. The mean number of IRBC per 100 melanoma cells at 0.4% parasitemia was 19 ± 0.2. No noninfected erythrocytes adhered to the monolayers. In contrast, no rosette formation was observed in triplicate slide preparations.

Ultrastructure. TEM revealed that all IRBC containing maturing trophozoites and schizonts possessed typical knobs on their surfaces (Fig. 1A and B). Uninfected cells, rings, and early trophozoites did not show knoblike features. Knobs were also detected clearly by SEM (Fig. 2).

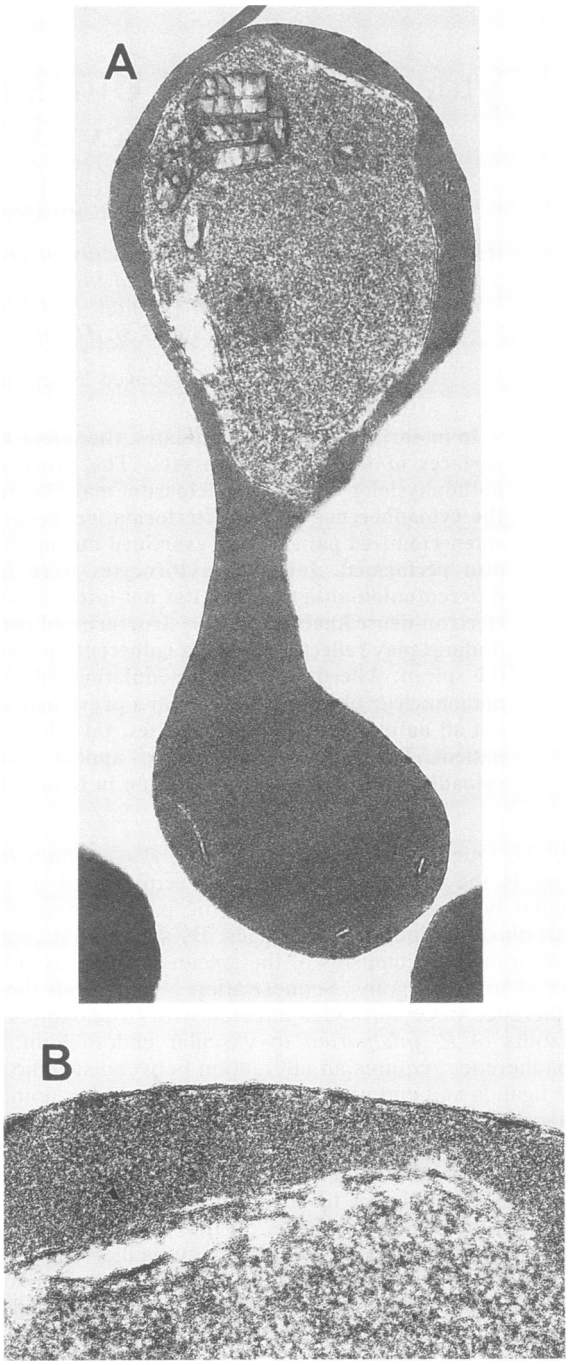

FIG. 1. (A) TEM of a section through an IRBC cultured from a splenectomized patient, showing a trophozoite. Knobs are visible on the surface. Magnification, ×18,000. (B) Detail of panel A showing knobs at a higher magnification (×92,000).

DISCUSSION

Malaria due to *P. falciparum* infection was previously reported for three splenectomized patients (1, 12, 14). Parasites of all intraerythrocytic developmental stages, i.e., rings, trophozoites, and schizonts, were found in the peripheral blood smears for two of them (1, 12). On the basis of this limited information, it has been suggested that parasitized erythrocytes are not sequestered in vivo in asplenic individuals, presumably as a result of the adverse effect of the splenectomy on surface antigen expression and cytoadherence.

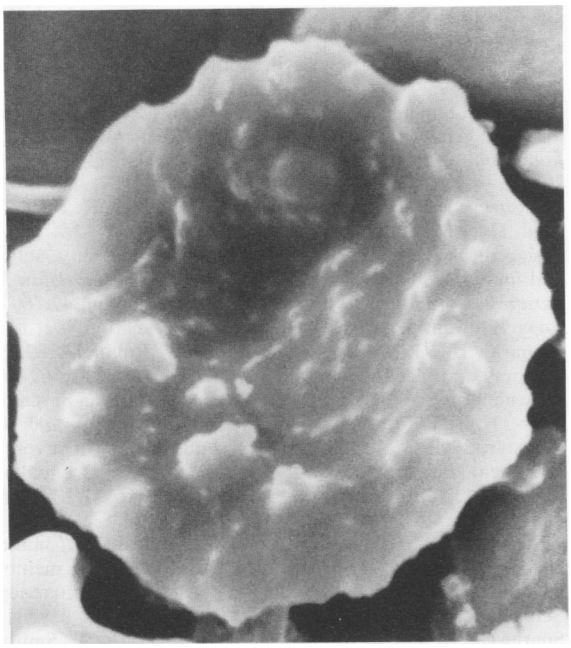

FIG. 2. SEM of an erythrocyte from the same culture as that shown in Fig. 1A and B, with an appearance typical of an IRBC. Magnification, ×12,500.

Evidence from primate studies indicates a more complex interaction between parasites and the spleen. The effect of splenectomy depends on the number of cycles and passages of the asexual blood-stage parasites in splenectomized animals as well as the intrinsic properties of the parasites (2–4, 7, 8). During the initial passage in splenectomized aotus monkeys of a laboratory-adapted *P. falciparum* isolate, knobs were retained (K^+), although there was a 4- to 16-fold reduction in the agglutination titer with immune sera, with a corresponding reduction in the degree of in vivo sequestration and in vitro binding (B^+) to cultured endothelial or melanoma cells (3). By the second passage, there was a complete loss of the ability to cytoadhere. As the parasites were further passaged, the inability to cytoadhere was accompanied by a progressive reduction in the proportion of K^+ cells. When a mixture of K^+ and K^- cells was passaged through an intact animal, the parasites regained the ability to cytoadhere and to be sequestered during the first passage. Similarly, in a *P. falciparum* infection of saimiri monkeys, the switch from K^+ B^- to K^+ B^+ occurred after 5 to 10 cycles of development during the first passage (7), whereas in *P. fragile*-infected toque monkeys, phenotypic changes have been documented as early as after 2 cycles of development during the first passage (8). In contrast, the Malayan-Camp CH/Q strain of *P. falciparum* remains K^+ B^+ even after multiple serial passages in splenectomized animals, and *P. coatneyi* does not become nonsequestering when passaged in splenectomized monkeys (4). A stable K^- clone of *P. falciparum* has also been reported (13).

The present study is the first report of the in vitro cytoadherence and rosette formation properties as well as the ultrastructure of *P. falciparum*-infected erythrocytes from a splenectomized patient. The presence of any residual functioning splenic tissue was excluded by ultrasonographic studies of the abdomen and technetium scanning. As with all natural parasite isolates from Thailand so far tested (11), these IRBC adhered to C32 melanoma cells. The degree of binding was relatively low compared with that of laboratory-adapted parasite lines and clones, but this result was likely to be related to the low parasitemia at which the cytoadherence assay was performed and the lower binding generally observed for natural parasite isolates tested during the first cycle in vitro (17). Erythrocytes containing trophozoites and schizonts were not seen in peripherhal blood smears for this patient, suggesting that cytoadherence may also have occurred in vivo. The IRBC did not form rosettes. However, as not all natural *P. falciparum* isolates form rosettes (9, 10) and as IRBC from this patient were agglutinated by a panel of hyperimmune sera (1a), it is unlikely that the absence of rosette formation was a result of the lack of surface antigen expression.

The molecular basis of the cytoadherence of IRBC from this splenectomized patient appeared to be similar to that of the cytoadherence of IRBC from intact hosts. Knobs were seen on the surfaces of the IRBC in ultrastructural studies. The adherence to C32 and not DS-1 cells indicates that, as with IRBC from intact hosts, the binding of IRBC from the splenectomized patient to melanoma cells occurred mainly via CD36 and not ICAM-1 (15).

How may the present observations be interpreted in light of the findings in primates? The situation in splenectomized patients is obviously different from experimental infections, as the former acquire their infections through the injection of sporozoites. Parasites are said to retain their cytoadherent phenotype after passage through mosquitoes (4). If that is the case, the parasites in our patient would have been K^+ B^+ at the start of the infection, as it is more than likely that the mosquito would have acquired the infection from an intact host. However, for parasitemia to become patent, the parasites would have had to have undergone several developmental cycles in the splenectomized host without the modulating effects of a spleen. The fact that the parasites remained K^+ B^+ suggests that either the isolate had a stable phenotype, as in the case of the Malayan-Camp CH/Q strain, or the modulating role of the spleen might have been assumed by other organs of the mononuclear phagocytic system, such as the liver. The observation that parasite clearance, another function attributed to the spleen in acute malaria, was normal in this patient (13a) supports the latter contention. Comparative studies of the behavior of parasites from naive and previously infected splenectomized individuals would clarify whether the capacity of the mononuclear phagocytic system to assume a modulating function in surface antigen expression is related to previous exposure to *P. falciparum*. The findings of this report underscore the importance of both parasite and host factors in determining the effect of splenectomy on the cytoadherence and rosette formation of IRBC in human falciparum malaria.

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