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Clinical Correlates of In Vitro *Plasmodium falciparum* Cytoadherence

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To determine whether isolates of *Plasmodium falciparum* have intrinsically different cytoadherent properties and whether these differences contribute to the clinical severity of human falciparum malaria, we studied the cytoadherence to C32 melanoma cells in vitro of 59 parasite isolates from patients with naturally acquired infections in Thailand. Parasitized erythrocytes adhere to these melanoma cells principally via the glycoprotein CD36, which is also expressed on most vascular endothelium. In vitro cytoadherence was significantly greater for isolates from patients with biochemical evidence of severe malaria. The cytoadherent properties of *P. falciparum* parasites may thus be a virulence factor in human falciparum malaria. However, there was no correlation between the degree of in vitro cytoadherence and cerebral symptoms, which suggests that other receptors and/or host factors may be important in the adherence of malaria parasites to cerebral vascular endothelium. The cytokines tumor necrosis factor, interleukin-1, and gamma interferon, which have been implicated in the pathogenesis of cerebral malaria and are known to promote intercellular adhesion in other systems, did not enhance the cytoadherence of *P. falciparum* isolates to C32 melanoma cells.

The central pathological event in human falciparum malaria is the cytoadherence of erythrocytes containing mature stages of *Plasmodium falciparum* to capillary and postcapillary venular endothelia in the deep vascular beds of vital organs (8). This process, termed sequestration, results in alterations in microcirculatory blood flow, metabolic dysfunction, and local toxicity, and, as a consequence, many of the complications seen in severe falciparum malaria (22). Sequestration occurs in all vital organs, although the degree of microvascular packing of infected cells varies considerably. The process is greatest in the brain (8) and is thought to cause coma in cerebral malaria. In human *P. falciparum* infection, parasitized erythrocytes either sequester or are removed from the circulation (principally by the spleen). The balance between sequestration, which allows parasite survival to schizogony, and splenic clearance is a major determinant of the rate of increase and magnitude of the infecting parasite burden. Within this paradigm, increased pathogenicity is associated with either greater cytoadherence or impaired clearance and possibly with a particular pattern of vital organ sequestration.

To determine whether isolates of *P. falciparum* have intrinsically different cytoadherent properties and whether these differences may contribute to the clinical severity of human falciparum malaria, we have studied the cytoadherence of fresh isolates of *P. falciparum* to C32 melanoma cells in vitro. These neoplastic cells share common surface determinants with cultured human endothelial cells for attachment of parasitized erythrocytes (19) and are much easier to propagate in vitro. The effects of the cytokines tumor necrosis factor (TNF), interleukin-1 (IL-1), and gamma interferon (IFN-γ) on cytoadherence were also examined. Circulating TNF levels have been shown to correlate positively with mortality in *P. falciparum* malaria (6), and all three cytokines are involved in intercellular adhesion in other systems (5, 18).

MATERIALS AND METHODS

Patients. We studied 59 consecutive patients with untreated acute *P. falciparum* malaria whose parasites matured in culture in vitro. These were adult Thai patients who either had had a few falciparum malaria attacks in the past or were experiencing their first infection. On admission, a complete physical examination and routine hematological and biochemical tests were performed. *P. falciparum* malaria was confirmed by the demonstration of asexual parasites in peripheral blood smears. Parasite counts (number of parasites per microliter) were estimated by quantitating the number of infected erythrocytes per 1,000 erythrocytes examined on thin smears. Five milliliters of blood was taken for parasite culture before antimalarial therapy was begun. Washed erythrocytes were either put into culture immediately or were resuspended in RPMI 1640 medium and kept overnight at 4°C.

Clinical assessment of severity. Patients were categorized into three clinical groups according to World Health Organization criteria (24). (i) Cerebral malaria: patients with unrousable coma in falciparum malaria (nonpurposive response to painful stimuli or worse) (21). (ii) Severe falciparum malaria: conscious patients who required admission to a hospital and parenteral antimalarial treatment and who had elevated bilirubin (>50 μmol/liter) and aspartate aminotransferase (>50 Reitman-Frankel units/liter) or elevated serum creatinine (>200 μmol/liter). Anemia was not used in

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this classification because of its multifactorial etiology and longer time course. Patients with preexisting renal or liver disease were excluded. (iii) Uncomplicated falciparum malaria: patients with none of the above.

Reagents. The tissue culture medium used for the culture of malaria parasites was RPMI 1640 medium (Flow Laboratories, Ayreshire, Scotland) supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Flow Laboratories), 25 mM $NaHCO_3$ (Flow Laboratories), gentamicin sulfate (Sigma Chemical Co., St. Louis, Mo.) at 0.15 mg/ml, and 10% (vol/vol) human AB serum (Merseyside Blood Transfusion Service, Liverpool, United Kingdom). For the culture of melanoma cells, heat-inactivated fetal bovine serum (Flow Laboratories, North Ryde, Australia) was used instead of human serum. Recombinant TNF, IL-1, and IFN-γ (Genzyme Co., Boston, Mass.) were aliquoted, stored at −70°C, and used within 6 months of purchase.

Melanoma cell monolayers. Human amelanotic melanoma cells (ATCC CRL 1585, designation C32r; American Type Culture Collection, Rockville, Md.) were cultured and plated onto 22-mm square glass coverslips at 10^5 cells per coverslip as described previously (18). The monolayers were fixed in 1% Formalin (37% [wt/vol] formaldehyde; Fisher Scientific) and stored at 4°C. A new batch was prepared every 4 to 6 weeks. Fresh unfixed melanoma cell monolayers were used in each study of the effect of TNF, IL-1, and IFN-γ on cytoadherence.

Melanoma cell binding assay. The melanoma cell binding assay used was described previously (20). Briefly, infected blood obtained from patients was cultured in vitro for 24 to 48 h until the majority of the parasites were judged morphologically to be at the late trophozoite/early schizont stage. One milliliter of a 2% (vol/vol) suspension in tissue culture medium supplemented with a single batch of AB serum was added to each petri dish containing a fixed melanoma cell monolayer. Parasite isolates which showed above 5% parasitemia were diluted with uninfected group O erythrocytes and tested at at least two dilutions below 5%. The petri dishes were incubated at 37°C in 5% CO_2 for 1.5 h with gentle rocking by hand every 15 min. Following incubation, the coverslips were washed four times with RPMI 1640 medium and then allowed to dry in air, fixed in methanol, and stained with 10% Giemsa for 20 min. Dried coverslips were mounted on glass slides with DPX mountant. Each coverslip was examined by light microscopy under oil immersion by two independent investigators, and the number of parasitized erythrocytes adherent to at least 1,000 melanoma cells was counted along a horizontal and a vertical axis. The mean count for duplicate slides was calculated and expressed as the number of infected erythrocytes per 100 melanoma cells. The coefficient of variation was less than 5% in 80% of the counts and ranged between 5 and 10% in the remainder.

Cytokines. Fresh monolayers were incubated for 4 to 12 h at 37°C with TNF (200 to 2,000 U; 10 to 100 ng/ml), IL-1 (5 to 10 U/ml), and IFN-γ (100 to 1,000 U; 40 to 400 ng/ml). At the end of the incubation, the monolayers were rinsed in RPMI 1640 medium, after which 1 ml of a 2% infected cell suspension was added to each dish. The binding assay was carried out as before.

Statistical analysis. Cytoadherence of parasites from the three patient groups was compared by using the Kruskal-Wallis one-way analysis of variance with multiple comparisons between groups. Correlations were assessed by Spearman's rank correlation coefficient.

RESULTS

P. falciparum parasites obtained from the peripheral blood of patients varied in the stage of ring form development. To ensure that all isolates were tested at a similar stage of maturation, parasites were cultured in vitro for variable periods of time (24 to 40 h) and examined microscopically every 6 to 8 h until they reached the pigmented trophozoite/early schizont stage. Once this stage of development was reached, there was a period of approximately 8 to 12 h during which cytoadherence remained stable.

Cytoadherence and parasitemia. The degree of cytoadherence of different parasite isolates varied considerably and correlated significantly with the admission parasitemia of the patient ($r = 0.755$, $P < 0.001$, $n = 59$). In preliminary experiments, serial dilutions of six isolates with normal group O erythrocytes were tested (Fig. 1). The tight linear relationship ($r = 0.99$ to 1.00) demonstrated between cytoadherence and parasitemia for all six isolates, which showed initial parasitemias of 2.4 to 10.6%, validated the process of testing parasite isolates either at their original parasitemia level or diluted to a parasitemia level within this range. Cytoadherence was then normalized (corrected binding) by dividing the "raw" binding by the percent parasitemia at the time of the binding assay so that all isolates were compared at an extrapolated value at 1% parasitemia. When corrected binding was plotted against admission parasitemia (Fig. 2), a much weaker association ($r = 0.286$) was demonstrated which appeared to be weighted heavily by three outlying points.

Clinical correlates. The cytoadherence of *P. falciparum* isolates to C32 melanoma cell monolayers from different patient groups is shown in Fig. 3. The clinical and laboratory features of the three categories of patients are summarized in Table 1. The preponderance of male patients reflects the epidemiology of malaria in Thailand in that transmission is mainly of the focal, "forest-fringe" type and men are exposed for occupational reasons. The majority of the patients ($n = 33$) had uncomplicated malaria, while 18 had evidence of organ dysfunction other than cerebral malaria. Eight met the criteria for cerebral malaria, of whom seven also had evidence of other vital organ dysfunction. There was marked variation in the corrected binding, confirming that fresh parasite isolates from patients with naturally acquired infections have diverse cytoadherent properties. The median corrected binding of parasite isolates from patients with uncomplicated malaria (42 infected erythrocytes [IRBC] per 100 melanoma cells) was significantly lower than that of isolates from conscious patients with severe malaria (85 IRBC per 100 melanoma cells; $P < 0.001$). In contrast, the median corrected binding of isolates from patients with cerebral malaria (45 IRBC per 100 melanoma cells) was similar to that of isolates from patients with uncomplicated malaria ($P > 0.05$) and was significantly lower than that of isolates from patients with severe malaria but without cerebral involvement ($P < 0.05$). Pooling of data for both severe groups still gave a median corrected binding of 73 IRBC per 100 melanoma cells, which was significantly higher than that of isolates from patients with uncomplicated malaria ($P < 0.001$).

Effects of cytokines on cytoadherence. Pretreatment of melanoma cell monolayers with any of the cytokines singly or in combination for 4 to 12 h did not have any consistent enhancing effect on the cytoadherence of 10 *P. falciparum* isolates (data not shown).

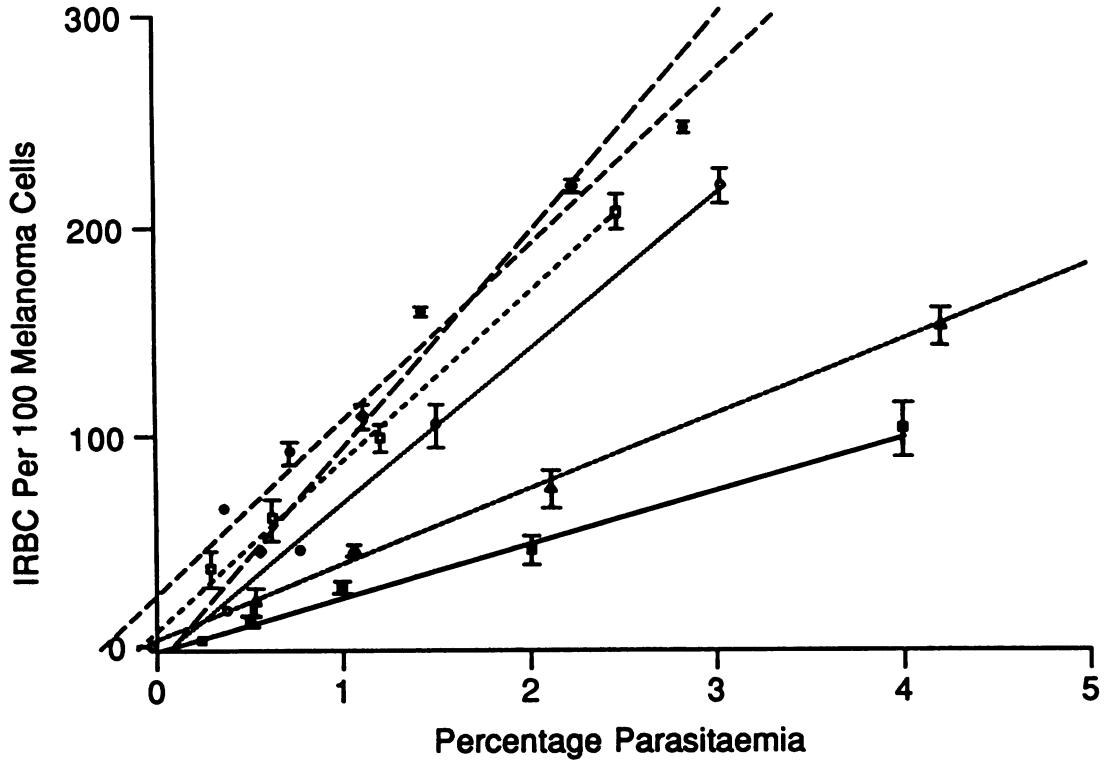

FIG. 1. Effect of trophozoite/schizont parasitemia on cytoadherence of *P. falciparum*. Plotted points are means of duplicate experiments for each of six isolates showing initial parasitemia from 2.4 to 10.6% (r = 0.99 to 1.00). Only data for observations obtained at less than 5% parasitemia are shown.

DISCUSSION

Falciparum malaria is a major cause of death in the tropics. Deep vascular sequestration of parasitized erythrocytes is considered the central pathological event in this infection and, as a result, considerable interest has been focused on the mechanisms underlying this process. The cytoadherence of parasitized erythrocytes to vascular endothelium and certain melanoma cell lines (including the C32 cell line) involves interaction between parasite antigens expressed on the surface of the infected erythrocyte and receptors on endothelial cells. To date, three molecules have been implicated as receptors for parasite ligands: the adhesive glycoprotein thrombospondin (16), the leukocyte differentiation antigen CD36 (12), and intercellular adhesion molecule 1 (ICAM-1) (4). Although both CD36 and ICAM-1 are expressed on C32 cells, cytoadherence to these cells occurs largely through CD36, as shown by inhibition experiments with monoclonal antibodies to the two receptors (2, 4, 12). Furthermore, parasitized erythrocytes can adhere directly to purified CD36 immobilized on plastic (12) or expressed on transfected COS cells (13).

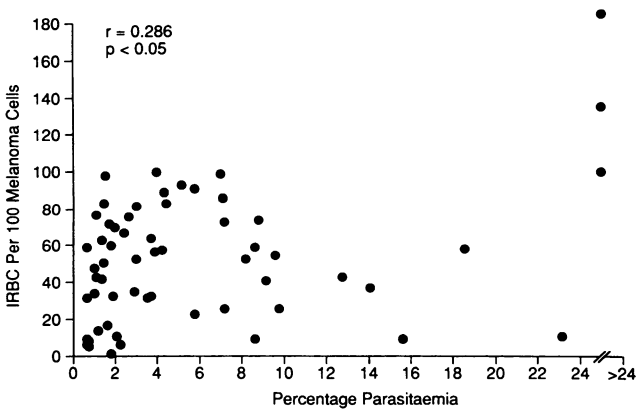

FIG. 2. Corrected binding versus admission parasitemia.

In the present study, cytoadherence of parasitized erythrocytes was found to correlate significantly with parasitemia in vivo. It stands to reason that with more parasitized cells, more would adhere. However, the aim of the current study was to determine if there are intrinsic parasite properties which affect cytoadherence independently of parasitemia and host factors. We eliminated the variation in degree of cytoadherence due to parasitemia per se by comparing the binding of all isolates at the same level of parasitemia (1%). The use of this corrected binding and the fact that in vitro culture largely removes host factors means that we examined only the ability of isolates to cytoadhere.

By so doing, we demonstrated that malaria parasite isolates from natural infections of humans have diverse intrinsic capacities for cytoadherence. The weak association between corrected binding and parasitemia in vivo suggests that higher levels of parasitemia may have resulted from parasite isolates which had bound to a greater extent, allowing a higher multiplication rate. However, the finding should be interpreted with caution as the correlation coefficient was relatively low.

The factors which determine these cytoadherent properties are not fully characterized but may be related to the expression of a family of parasite proteins with different

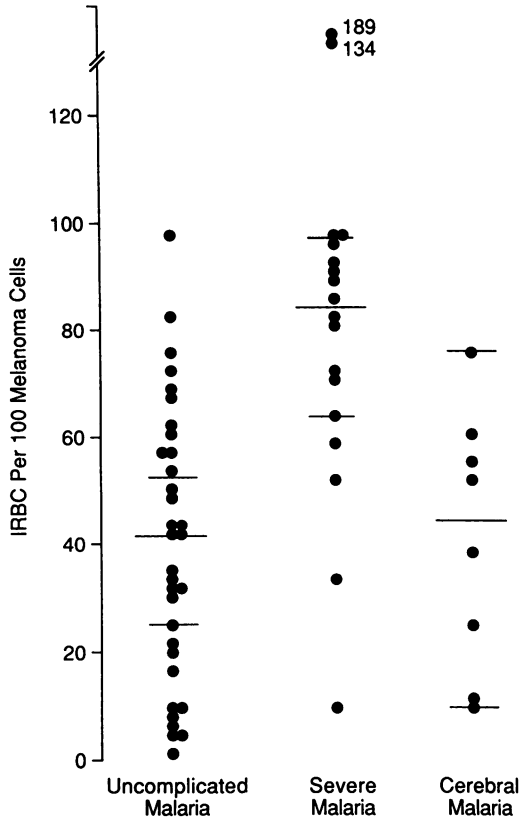

FIG. 3. Comparison of corrected binding for isolates from patients with uncomplicated, severe, and cerebral malaria. The bars indicate the median ± 95% confidence intervals.

molecular weights on the surface of the infected erythrocytes (9). Cytoadherence is associated with the expression of a larger form of the protein (molecular weight, 2.6×10^5) which may contain the adhesive epitopes. Parasites isolated from patients with natural infections may consist of multiple populations expressing different forms of the surface protein, or individual parasites may be capable of producing variant forms of the protein. The degree to which a given isolate cytoadheres in vivo will then depend on various parasite properties and also factors related to the host. These include immune status (20), blood pH (10), and physical determinants such as the shear forces of blood flow within the microvasculature (17).

For *P. falciparum* parasites isolated from conscious patients with severe malaria, there was increased cytoadherence of infected erythrocytes to melanoma cells in vitro compared with isolates from patients with uncomplicated disease. Patients with severe malaria were divided into two groups: cerebral malaria (unrousable coma) or severe disease with raised serum creatinine or bilirubin and aspartate aminotransferase, reflecting impaired renal function and a combination of muscle, liver, and erythrocyte damage. This suggests that the cytoadherent properties of parasites contribute to virulence and supports the hypothesis that cytoadherence to CD36 on vascular endothelial cells is an important pathological mechanism in vivo. Parasites which sequester more readily than others (either earlier in the developmental cycle or to a greater extent) are less likely to be removed by the spleen. This will result in a greater multiplication rate and a more rapid expansion of the parasite burden (23).

The distribution of organ pathology secondary to sequestration in severe malaria is related presumably to the expression and density of receptor molecules on vascular endothelial cells in different tissues, and this in turn determines the distribution of microvascular obstruction, local toxicity, and interference with metabolic function. The pattern of organ dysfunction in severe falciparum malaria varies between patients. Some may become comatose with little evidence of other vital organ dysfunction (21), whereas others may die with acute renal failure, pulmonary edema, hypoglycemia, and lactic acidosis without prior loss of consciousness. In African children, renal dysfunction and pulmonary edema are very unusual in severe malaria (11), whereas these manifestations develop in over half the Thai adults with lethal infections.

By use of monoclonal antibody OKM5, the CD36 antigen has been demonstrated on the vascular endothelium of the

TABLE 1. Clinical and laboratory features of patients with uncomplicated, severe, and cerebral malaria

Parameter	Value for patients with malaria type:		
	Uncomplicated (n = 33)	Severe (n = 18)	Cerebral (n = 8)
Age (yr)	22.7 ± 7.0[a]	25.4 ± 10.1	33.3 ± 13.4
Sex (M:F)	27:6	13:5	6:2
Parasite count (geometric mean/μl)	99,519	279,209	116,396
Haematocrit (%)	35.8 ± 8.5	36.1 ± 8.0	32.0 ± 8.4
Blood urea nitrogen			
mg/dl	17.8 ± 6.7	40.5 ± 35.3	61.9 ± 42.4
mmol/liter	6.3 ± 2.4	14.4 ± 12.6	22.0 ± 15.1
Serum creatinine			
mg/dl	1.2 ± 0.4	2.6 ± 2.7	2.9 ± 1.5
μmol/liter	106.2 ± 35.4	230.2 ± 239.0	256.7 ± 132.8
Total bilirubin			
mg/dl	1.6 ± 1.1	6.4 ± 8.1	11.7 ± 18.9
μmol/liter	27.2 ± 18.7	108.8 ± 137.7	198.9 ± 321.3
Aspartate aminotransferase			
Reitman-Frankel units[b]	33.6 ± 7.8	86.9 ± 64.3	69.4 ± 32.1
μmol/liter	0.56 ± 0.13	1.45 ± 1.07	1.16 ± 0.54

[a] Mean ± standard deviation.
[b] Normal range, 0 to 40 U.

liver, kidney, and lung (7), which may explain the association between binding to melanoma cells in this study and clinical complications involving these organs. However, the demonstration of CD36 on cerebral endothelium from either routine autopsy materials or patients who died of cerebral malaria required the use of a different monoclonal antibody, MAb 8A6 (2), while other investigators have consistently failed to detect the CD36 antigen on cerebral vascular endothelium (3). This suggests that the CD36 molecule is expressed differently on cerebral endothelium or is absent. The variation in molecular expression of CD36 or its absence may explain the lack of association between cytoadherence to melanoma cells in vitro and the clinical syndrome of cerebral malaria found in this study and raises the possibility that CD36 may not be the principal cerebral endothelial receptor for *P. falciparum*-infected erythrocytes. Our results agree with those of the only previous attempt to correlate in vitro measures of cytoadherence and clinical findings, in which no differences in melanoma cell binding were found between *P. falciparum* isolates from Gambian children with cerebral and uncomplicated malaria (10).

Using a line of *P. falciparum* parasites selected for cytoadherence to human umbilical vein endothelial cells, Berendt et al. (4) have demonstrated that some parasitized erythrocytes cytoadhere via (ICAM-1). Whether this receptor molecule has any relevance to the cytoadherence of *P. falciparum* in vivo remains to be determined, but our findings do suggest that receptors other than CD36 may be involved in the cytoadherence of parasitized erythrocytes in human falciparum malaria.

The cytokines TNF, IL-1, and IFN-γ, either singly or in combination, did not enhance the cytoadherence of parasitized erythrocytes to C32 melanoma cells. Since these cytokines act mainly through the induction of receptor molecules on the cell surface, the lack of effect is probably due to the fact that CD36 is already maximally expressed on C32 melanoma cells (1). There is now limited evidence that TNF and IL-1, which upregulate ICAM-1 expression on human umbilical vein endothelial cells (15), may actually enhance the cytoadherence of certain laboratory-adapted parasites to these cells (4). Further studies with isolates obtained from natural infections are needed in order to explain the role of cytokines in the pathogenesis of severe falciparum malaria.

The results of this prospective study have shown that parasitized erythrocytes from patients with severe falciparum malaria have intrinsically greater cytoadherence to melanoma cells in vitro than do those from patients with uncomplicated infections. Thus, the cytoadherent properties of *P. falciparum* may be an important virulence factor. The potential for inhibiting or reversing cytoadherence with antibodies or receptor analogs should be actively explored.

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