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Parasite Virulence Factors during Falciparum Malaria: Rosetting, Cytoadherence, and Modulation of Cytoadherence by Cytokines

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To determine virulence factors of isolates of *Plasmodium falciparum* and the potential role of cytokines in cerebral malaria, 46 Malagasy patients presenting with cerebral ($n = 10$), severe ($n = 10$), and uncomplicated ($n = 26$) malaria were enrolled in a study. The capacity of 21 of 46 *P. falciparum* isolates to form rosettes in vitro and to adhere to human umbilical vein endothelial cells (HUVECs) that express intercellular adhesion molecule-1 receptors and to C32 amelanotic melanoma cells that express mainly CD36 receptors was investigated together with the effects of tumor necrosis factor alpha (TNF-α), granulocyte macrophage–colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and IL-6 alone and in two-by-two combinations on the cytoadherence of infected erythrocytes to HUVECs. Plasma levels of these cytokines were also measured in the patients at admission. The percentage of rosette formation was higher for the isolates from patients with cerebral ($n = 6$; 19.5%) and severe ($n = 6$; 30.5%) malaria than for those from patients with uncomplicated malaria ($n = 9$; 5%) ($P < 0.002$). The cytoadherence properties of the isolates did not differ among the three groups whatever the target cell used, but adherence to melanoma cells was systematically higher than that to HUVECs. Adhesion to HUVECs was increased more after TNF-α stimulation than after GM-CSF, IL-3, or IL-6 stimulation ($P < 0.01$). Only the combination of TNF-α and IL-3 enhanced cytoadherence more than TNF-α used alone ($P < 0.02$). No difference in the modulation of cytoadherence by cytokines was found in relation to the severity of the disease. TNF-α and IL-6 levels in peripheral blood were higher in the patients with cerebral and severe malaria than in the patients with uncomplicated malaria ($P < 0.005$). Most of the patients' sera contained little or no IL-3 or GM-CSF. Our results challenge the role of intercellular adhesion molecule-1 as the principal receptor mediating the cytoadherence of *P. falciparum*-infected erythrocytes and contrast with data obtained in the murine model.

Cerebral malaria (CM) is the most severe complication of *Plasmodium falciparum* infection and one of the main causes of infant mortality in Africa. The pathophysiology of CM is starting to be elucidated. The main hypothesis involves sequestration of erythrocytes infected with *P. falciparum* trophozoites and schizonts in cerebral capillaries and postcapillary veinules (19). This phenomenon has been observed in necropsy specimens; it occurs in all organs but is more marked in the brain during CM. The main events leading to sequestration and altered microcirculation are cytoadherence of parasitized erythrocytes to endothelial cells and/or rosette formation, i.e., agglutination of noninfected erythrocytes around erythrocytes parasitized with trophozoites and schizonts (40). At least five endothelial cell receptors able to bind infected erythrocytes have been identified: thrombospondin, CD36 (platelet glycoprotein IIIb or IV), and intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1, and endothelial leukocyte adhesion molecule-1 (1, 2, 23, 28).

The involvement of cytokines in the pathophysiology of CM has also been suggested. In vitro, tumor necrosis factor alpha (TNF-α) can modulate the expression of ICAM-1 on endothelial cells (25). In humans, peripheral blood concentrations of TNF-α and interleukin-6 (IL-6) are markedly raised in severe malaria (SM) and CM (9, 16, 17), but their direct pathogenic role is much debated. TNF-α, IL-3, and granulocyte macrophage–colony-stimulating factor (GM-CSF) are essential for the onset of neurological symptoms in mice infected with *P. berghei* (6, 8). To our knowledge GM-CSF and IL-3 have never been measured in CM patients.

It is not clear why only 1 to 2% of patients infected with *P. falciparum* develop SM or CM, although several genetic, nutritional, and immunological host factors have been implicated, together with a behavioral factor such as delayed or inappropriate treatment. Strain virulence and particularly the capacities to adhere to endothelial cells, to form rosettes, and to multiply are also probably involved (10). To determine whether parasite virulence factors are associated with clinical gravity markers, we studied the capacity of *P. falciparum* isolates to form rosettes and to adhere to human umbilical vein endothelial cells (HUVECs) and human C32 amelanotic melanoma cells in two in vitro models of cytoadherence, together with the sensitivity of the isolates to

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chloroquine and to quinine in vitro. We also studied the effects of TNF-α, IL-3, IL-6, and GM-CSF on the cytoadherence of parasitized erythrocytes to cultured endothelial cells. Finally, we measured plasma levels of TNF-α, IL-3, IL-6, and GM-CSF in the corresponding patients at admission to hospital.

MATERIALS AND METHODS

Study area. The study was carried out between January 1991 and March 1992 in the high plateau area of Madagascar in Antananarivo, the capital city, and in Ankazobe, a village situated 100 km north of Antananarivo. The high plateau area is an area where malaria is endemic and is characterized by unstable seasonal transmission between January and June.

Patients. All adults and children admitted to Befelatanana Hospital or the Military Hospital at Antananarivo, or to Ankazobe Hospital, and presenting with *P. falciparum* malaria and parasitemia above 1% were enrolled in the study. The diagnosis was confirmed by examination of Giemsa-stained thin blood smears, and parasite density was determined by counting the number of parasitized erythrocytes per 10,000 erythrocytes. On admission, full clinical histories were recorded, and a complete physical examination and routine hematological (hemoglobin, erythrocyte, leukocyte, and platelet counts) and biochemical (serum bilirubin and creatinine) tests were performed. Patients were classified into three clinical groups according to World Health Organization criteria (41), as follows: group 1, patients with CM; group 2, patients with SM; group 3, patients with acute uncomplicated malaria (UM). Informed consent to participate in the study was given by the patient or by the family.

Parasites. After hematological and biochemical tests, 10 ml (5 ml in the case of children) of venous blood was drawn onto EDTA on admission before treatment. Plasma was aliquoted, frozen, and stored at −70°C until cytokine assay. The erythrocytes were washed three times with RPMI 1640 medium (GIBCO, Paisley, Scotland); 1 ml of the pellet was immediately used for in vitro sensitivity testing, and the remainder was mixed with an equal volume of 30 g of mannitol per liter, 28% glycerol, and 6.5 g of NaCl per liter and cryopreserved in liquid nitrogen. For the cytoadherence and rosette formation assays, infected erythrocytes were thawed and cultured until the parasites matured to late trophozoite and schizont stages in candle jars in standard conditions (36). For isotopic counting in the cytoadherence test, infected erythrocytes were labelled for at least 24 h in hypoxanthine-free culture medium containing 3 μCi of ^{3}H-hypoxanthine (Amersham, Buckinghamshire, United Kingdom) per ml.

Cell culture. HUVECs were obtained from four to nine human umbilical cords as described previously (15). They were cultured in candle jars in 25-cm^2 Primaria culture flasks (Becton-Dickinson, Lincoln Park, N.J.) with E199 medium (Flow Laboratories, Irvine, Scotland) supplemented with 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Sigma Chemical Co., St. Louis, Mo.), 15 mM $NaHCO_3$, 2 mM L-glutamine (Flow Laboratories), penicillin (100 U/ml), streptomycin (100 μg/ml), and 20% inactivated fetal calf serum. For the binding assay, confluent cells were subcultured for 2 to 3 days to confluence on 15-mm round Thermanox tissue culture coverslips (Nunc, Naperville, Ill.) precoated with fibronectin (2 μg/cm^2; Sigma) in 24-well flat-bottomed plates (Becton-Dickinson). C32 human amelanotic melanoma cells (ATCC CRL 1585, designation C32r; American Type Culture Collection, Rockville, Md.) were cultured under the same conditions with RPMI 1640 medium supplemented with 10% inactivated fetal calf serum and plated on 15-mm round coverslips at 2×10^4 cells per cm^2. After 48 h of culture, the monolayer was fixed with 1% formalin and stored at 4°C until the binding assay (38).

In vitro sensitivity testing. Sensitivity to chloroquine and quinine was determined with the isotopic semimicro in vitro drug sensitivity test (18). The 50% inhibitory concentration (IC_{50}) was calculated by log dose/probit analysis. The threshold of resistance to chloroquine and quinine was defined as an IC_{50} of >100 and >500 nmol/liter, respectively.

Rosette formation assay. Ten microliters of the parasite culture was mixed with a small amount of 0.001% acridine orange and distributed under a coverslip (22 by 22 mm); 100 consecutive parasitized erythrocytes were examined with a ×40 lens under incident UV light (4). Tests were performed in duplicate at the thawing parasite density (1 to 3%). The percentage of parasitized erythrocytes to which two or more uninfected erythrocytes were attached was noted.

Binding assay. Immediately prior to use, confluent endothelial cells, fixed melanoma cells, and cultured parasitized erythrocytes were washed three times with RPMI 1640 medium. RPMI 1640 medium (0.5 ml) supplemented with 10% pooled human serum (pH 6.9) containing erythrocytes (4% hematocrit) was added to each well with adherent endothelial cells or melanoma cells. All tests were performed in duplicate at 1% of parasitized erythrocytes. The plates were incubated at 37.5°C in candle jars for 1.5 h with gentle rocking every 30 min. The coverslips were then washed three times in RPMI 1640 medium, fixed with 2% glutaraldehyde, stained with 10% Giemsa, mounted on glass slides, and examined under a light microscope. The number of parasitized erythrocytes attached per 500 target cells was then determined. The mean of duplicate counts was calculated and expressed as the number of infected erythrocytes per 100 target cells. An alternative assay that used an isotopic counting method adapted previously to the melanoma cell binding assay (42) was used to study the effects of cytokines on cytoadherence (see below). To compare the isotopic and optical assay results, we tested the binding of three isolates at five different parasite densities. Results of the two methods were strongly related ($r' = 1$ in each case).

Effects of cytokines on cytoadherence. Recombinant TNF-α, GM-CSF, IL-3, and IL-6 (Genzyme Co., Boston, Mass.) were diluted in phosphate-buffered saline (pH 7.2) supplemented with 1% bovine serum albumin to obtain working concentrations and then aliquoted and stored at −70°C until cell stimulation. Confluent HUVECs were incubated for at least 24 h with high concentrations of TNF-α (5, 10, and 20 ng/ml) to obtain maximal ICAM-1 expression (25, 26). GM-CSF, IL-3, and IL-6 were also used at three high concentrations each (5, 10, and 20, 5, 10, and 20, and 10, 50, and 100 ng/ml, respectively). The effects on cytoadherence were also investigated with two-by-two combinations of the four cytokines at the highest concentrations. Unstimulated HUVECs were used as controls. Before the binding assay, unincorporated ^{3}H-hypoxanthine was removed from the culture medium by washing the erythrocytes three times with RPMI 1640 medium containing 50 μg of hypoxanthine per ml. The erythrocytes were then suspended, and the cytoadherence assay was performed as described above. Following incubation, the coverslips were washed three times with RPMI 1640 medium and transferred directly to scintillation vials. The results were expressed as the percentage of modulation for each cytokine as follows: percentage

TABLE 1. Clinical and laboratory features of patients with CM, SM, and UM

Parameter	Value for given patient group (mean ± SD)		
	CM (n = 10)	SM (n = 10)	UM (n = 26)
Age (yr)	25.4 ± 9.7	19.9 ± 10	15.3 ± 11.9
Sex ratio (male/female)	1	2.3	1.4
Duration of symptoms (days)	3.2 ± 2.1	3.3 ± 2.7	1.3 ± 1.9
Parasite density (geometric mean per μl)	99,761	115,570	80,574
Hemoglobin (g/dl)	8.6 ± 3.8	7.6 ± 3.9	11.2 ± 2.4
Erythrocyte count (μl, 10^6)	3.1 ± 1.4	2.7 ± 1.4	4.1 ± 0.8
Leukocyte count (per μl)	6,750 ± 3,865	4,000 ± 2,582	7,569 ± 3,598
Platelet count (μl, 10^3)	68.1 ± 54.4	65.9 ± 54.8	134.7 ± 78
Serum bilirubin (μmol/liter)	31.8 ± 24.7	25.4 ± 15.5	12 ± 7.1
Serum creatinine (μmol/liter)	227.4 ± 245.3	118 ± 61.8	78.1 ± 31

of modulation = [(cpm after cytokine stimulation − cpm of control) × 100]/cpm of control. Modulation of cytoadherence was considered significant when binding was enhanced by 50% or more (31).

Cytokine assay. Plasma cytokine concentrations were assayed with commercial kits according to the manufacturers' instructions. Enzyme-linked immunosorbent assays were used to measure the concentrations of IL-3 (Amersham), IL-6 (Amersham), and GM-CSF (Genzyme). Plasma TNF-α was assayed with a competitive inhibition radioimmunoassay (IRE-Medgenix, Fleurus, Belgium). The detection limits were 7.4 pg/ml for IL-3, 3 pg/ml for IL-6, 4 pg/ml for GM-CSF, and 6 pg/ml for TNF-α. For statistical purposes, samples in which cytokines were undetectable were arbitrarily assigned values of one-half the detection limits. Plasma samples from six healthy Malagasy volunteers with negative thick-blood smears were used as controls.

Statistical analysis. Unpaired comparisons between groups were performed with the Mann-Whitney U test and the Kruskal-Wallis test. Paired comparisons within groups were made with the Wilcoxon signed-rank test and Friedman's test. Correlations were identified by using the Spearman rank correlation coefficient. Differences in proportions were tested for with the Fisher exact test. P values of 0.05 or less were considered significant.

RESULTS

Patients. Forty-six patients were enrolled in the study (10 with CM, 10 with SM, and 26 with UM). The clinical and the laboratory features of the three groups of patients are summarized in Table 1. In the CM group, coma was usually associated with other organ dysfunctions: renal failure (n = 4), pulmonary or urinary tract infection (n = 2), massive bleeding (n = 2), hepatic dysfunction (n = 2), severe anemia (n = 2), and circulatory collapse (n = 1). In the SM group, patients presented with one (n = 6) or several of the following organ dysfunctions: obnubilation (n = 5), renal failure (n = 2), hepatic dysfunction (n = 3), severe anemia (n = 5), and spontaneous bleeding (n = 1). On admission, 38 patients (81%) had an axillary temperature of >37.5°C, and the remaining patients reported a history of fever in the past 24 h. Patients were given standard chloroquine or quinine treatment. One patient in whom chloroquine therapy failed was successfully treated with amodiaquine (25 mg/kg for 3 days). The outcome was favorable in all but the three patients (all with CM) who died 1, 2, and 3 days after initiation of treatment.

In vitro sensitivity. Forty-six in vitro tests were performed, with a success rate of 83%. Two isolates were resistant in vitro to chloroquine (IC_{50} = 218 and 359 nmol/liter); the remaining isolates had a median IC_{50} of 29.5 nmol/liter (range, 12.5 to 87 nmol/liter), and no isolates were resistant in vitro to quinine (median IC_{50} = 143 nmol/liter; range, 25 to 418 nmol/liter).

In vitro cytoadherence and rosette formation. After thawing, 21 isolates grew to the trophozoite and schizont stages at a parasitemia above 1% and could thus be included in the study of cytoadherence and rosette formation; 6, 6, and 9 isolates, respectively, were from patients with CM, SM, and UM. The isolates were cultured for 25 to 40 h. In each case, trophozoites and schizonts represented more than 80% of the parasite stages.

(i) Rosette formation. Rosette formation was observed in 17 (81%) of the 21 isolates, with considerable differences in the percentage of rosette formation (range, 5 to 43%). All isolates obtained from patients with CM and SM formed rosettes in vitro compared with only five of the nine isolates from patients with UM ($P < 0.03$). The median number of rosettes per 100 parasitized erythrocytes was significantly different among the three groups ($P < 0.002$); the median value was higher in the CM (19.5%) and SM (30.5%) groups than in the UM group (5%) ($P < 0.05$ and $P < 0.002$, respectively) (Fig. 1).

(ii) Binding. Whatever the target cell used, cytoadherence properties varied widely among the isolates. No difference was found in the pattern of binding to HUVECs and to melanoma cells between isolates from the three groups of patients (Fig. 2). Cytoadherence was significantly higher on melanoma cells (median, 50 parasitized erythrocytes per 100 melanoma cells; range, 14 to 350) than on HUVECs (median, 18 parasitized erythrocytes per 100 HUVECs; range, 7 to 91) (P = 0.001). There was no correlation between binding to HUVECs and binding to melanoma cells.

(iii) Effects of cytokines on cytoadherence. TNF-α stimulation induced a morphological modification of endothelial cells into an elongated fibroblastic shape, whereas IL-3, IL-6, and GM-CSF did not. The combination of TNF-α with each of the other three cytokines induced the same modification. For each cytokine, no difference in the pattern of cytoadherence modulation was observed with the three concentrations used. Cytoadherence varied markedly after cytokine stimulation. TNF-α enhanced cytoadherence significantly (modulation, >50%) in 5 of 21 isolates (4 isolates from patients with SM and 1 isolate from a patient with UM). Adhesion to HUVECs increased more after TNF-α stimulation (median percentage of modulation, 24%; range, −12 to 126%) than after GM-CSF (median, −3%; range, −34% to 44%), IL-3 (median, 11%; range, −20 to 52%) and IL-6

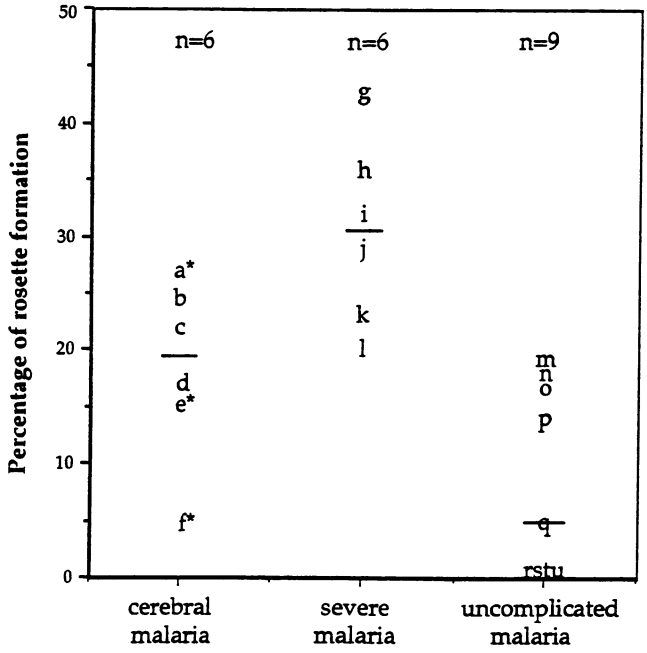

FIG. 1. Comparison of rosette formation with 21 isolates of *P. falciparum* from patients with CM, SM, and UM. The bars indicate the median values, and the asterisks indicate fatal outcome.

(median, 8%; range, −22 to 50%) stimulation (all $P < 0.01$) (Fig. 3). In contrast to the other cytokines, GM-CSF seems to decrease cytoadherence. Only the combination of TNF-α and IL-3 enhanced adherence more than TNF-α alone ($P < 0.02$). No difference was observed in HUVEC cytoadherence modulation by the cytokines relative to the severity of the disease (Fig. 4). For a given isolate, the capacity of TNF-α to modulate cytoadherence correlated with the capacity of this isolate to bind to unstimulated HUVECs ($r' = 0.47$; $P < 0.05$).

Plasma cytokine concentrations. In contrast to GM-CSF, plasma concentrations of TNF-α and IL-6 were higher in the patients than in the controls (all $P < 0.003$) (Table 2). TNF-α and IL-6 levels correlated positively with the parasitemia,

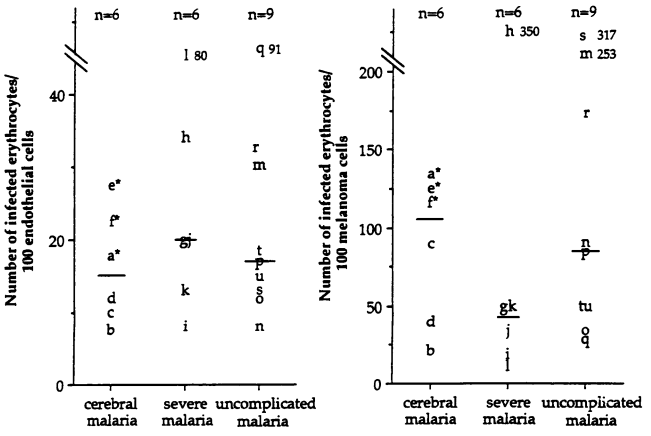

FIG. 2. Comparison of cytoadherence to HUVECs and to C32 amelanotic melanoma cells of erythrocytes infected with 21 isolates of *P. falciparum* in relation to the severity of the disease. The bars indicate the median values, and the asterisks indicate fatal outcome.

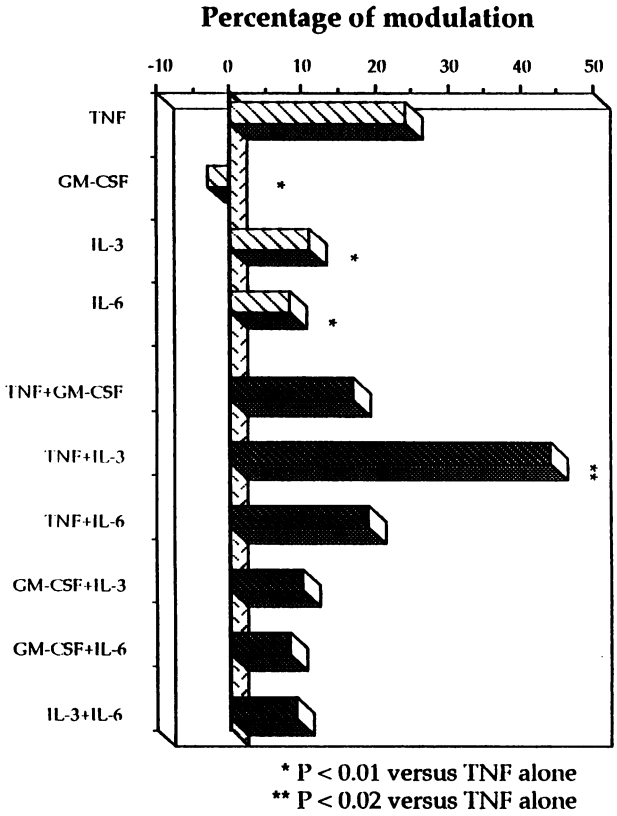

FIG. 3. Modulation of cytoadherence after HUVEC stimulation with TNF-α (20 ng/ml), GM-CSF (20 ng/ml), IL-3 (20 ng/ml), or IL-6 (100 ng/ml) and with two-by-two combinations of the cytokines. The binding assay was performed with 21 isolates of *P. falciparum*. Results are expressed as median percentages of modulation relative to controls (without cytokine stimulation).

serum bilirubin, and temperature (all $r' \geq 0.35$; all $P < 0.03$) and correlated negatively with the platelet count (all $r' \leq -0.5$; all $P < 0.002$). TNF-α levels correlated positively with IL-6 levels ($r' = 0.76$; $P = 0.0001$) and with the percentage of rosette formation observed with the corresponding isolate ($r' = 0.57$; $P < 0.02$). TNF-α levels in peripheral blood differed among the three groups of patients ($P < 0.005$). The geometric mean TNF-α concentration was higher in CM and SM than in UM (all $P < 0.03$), and patients with complicated malaria (i.e., CM and SM) had higher geometric mean IL-6 levels (116 pg/ml) than patients with UM (32.7 pg/ml) ($P < 0.03$). In the three patients who died, TNF-α and IL-6 concentrations on admission were 186, 96, and 313 pg/ml and 240, 17, and 471 pg/ml, respectively. IL-3 was detectable in one patient with CM, two patients with SM, and one patient with UM (7.4 to 12.5 pg/ml).

DISCUSSION

Of the virulence factors studied in vitro (rosette formation and cytoadherence), only rosette formation was related to the severity of the disease. Our results confirm previous studies performed in The Gambia (4, 37) in which all isolates from CM patients led to rosette formation. Nevertheless, the pathophysiologic significance of rosettes in CM remains to be established. Rosettes have rarely been described in the brain postmortem (27), and rosette formation is negatively

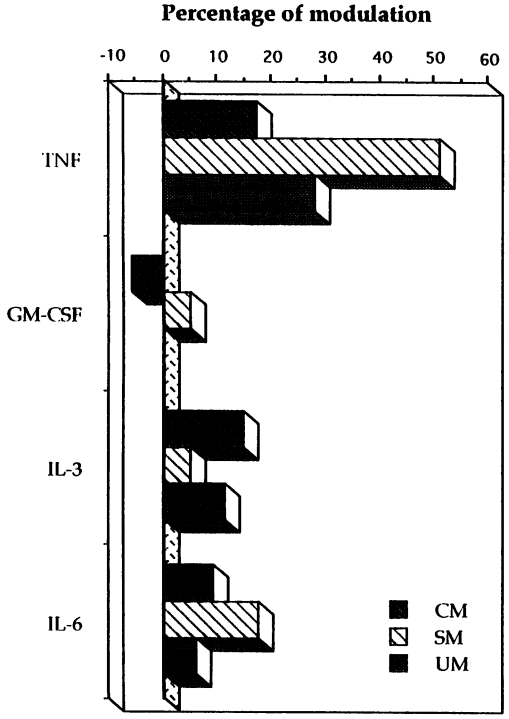

FIG. 4. Comparison of cytoadherence modulation after HUVEC stimulation with TNF-α (20 ng/ml), GM-CSF (20 ng/ml), IL-3 (20 ng/ml), and IL-6 (100 ng/ml) with 21 isolates of *P. falciparum* from patients with CM (n = 6), SM (n = 6), or UM (n = 9). Results are expressed as median percentages of modulation relative to controls (without cytokine stimulation).

correlated with adherence, suggesting that isolates of *P. falciparum* have a propensity for either rosette formation or cytoadherence but not both (13). Alternatively, infected erythrocytes that have formed rosettes may not be able to adhere unless the rosettes are disrupted first (11). Nevertheless, we and other workers (39) observed formed rosettes binding to HUVECs and melanoma cells, although they had not been disrupted. Furthermore, disruption of rosettes prior to incubation with melanoma cells did not affect the cytoadherence capacity of wild isolates (37). The role of cytokines in rosette formation has not been studied, and why TNF-α levels in blood should be positively correlated with in vitro rosette formation is not clear and requires further investigations.

Although resistance to antimalarial drugs cannot be considered a true virulence factor, we studied it because resistance, and therefore ineffective treatment, can lead to the development of CM (10). The chloroquine and quinine IC_{50} values did not correlate with the severity of the disease in this study, in which 35% of the patients stated that they had taken chloroquine before admission to hospital.

TABLE 2. Comparison of levels of TNF-α, IL-6, and GM-CSF in plasma in patients with CM, SM, and UM

Patient group	Cytokine concn (pg/ml)[a]		
	TNF-α	IL-6	GM-CSF
Controls (n = 6)	<6[b]	<3[b]	4.9 (4.3–5.6)
CM (n = 10)	111[c] (44–280)	101 (38–266)	4.1 (3.4–4.8)
SM (n = 10)	149[c] (71–312)	133 (69–253)	4.5 (3.8–5.3)
UM (n = 26)	45[c] (29–71)	33 (15–71)	5.2 (4.4–6.3)

[a] Geometric mean; 95% confidence interval is given in parentheses.
[b] $P < 0.003$; Mann-Whitney U test versus patients.
[c] $P < 0.005$; Kruskall-Wallis test.

Cytoadherence is thought to be the essential factor at the origin of CM. In vitro models of cytoadherence are numerous, but their receptor expression differs. HUVECs show low-level basal ICAM-1 expression and lack CD36 receptors (34). Therefore, HUVECs are not representative of endothelial cells in general because endothelial cells from other tissues, including human dermal microvascular endothelial cells and human brain endothelial cells, express receptors such as CD36 (32, 34). As there are large variations in ICAM-1 expression on HUVECs (12), we pooled cells from at least four cords. C32 melanoma cells express both types of receptor, with a predominance of CD36, which is mainly responsible for cytoadherence in this model. In the present study, we used both HUVECs and melanoma cells because the respective pathophysiological roles of ICAM-1 and CD36 are still controversial. The modulation of cytoadherence by cytokines was only studied with HUVECs as cytoadherence of parasitized erythrocytes to melanoma cells is unaffected by cytokines (14). A relationship between in vitro cytoadherence to CD36 and ICAM-1 and disease severity has not been formally demonstrated. Isolates from SM patients adhere more to melanoma cells and plastic-absorbed CD36 than do isolates from UM patients, but binding does not differ between isolates from CM and UM patients (14, 20, 22). Our results are consistent with these studies, as no difference in adherence to either cell type was observed among the isolates from patients with CM, SM, and UM. Isolates did, however, consistently adhere more to melanoma cells than to HUVECs. The absence of any relationship between the results of in vitro models of cytoadherence and clinical features raises a further hypothesis. Such cell systems may not be accurate models of sequestration or CM and should thus perhaps be considered rather as binding models, reflecting the ability of isolates to adhere to specific receptors. On the other hand, it has not been formally demonstrated that clinical manifestations stem from sequestration and cytoadherence. Sequestration of parasitized erythrocytes is observed more often in patients dying of CM but also occurs in patients with no neurological symptoms (19). CM with no evidence of cerebral sequestration has also been reported (3, 35). Conversely, rosette formation is related to the severity of the disease, but the presence of rosette formation during CM is still controversial.

High TNF-α levels have been considered a prognostic factor in children with CM (9). TNF-α increases the in vitro adherence of erythrocytes infected with *P. falciparum* ITO4 to endothelial cells by causing ICAM-1 overexpression (2). It has therefore been suggested that a TNF-α-induced increase in surface expression of ICAM-1 on endothelial cells in brain capillaries could lead to sequestration and, thus, CM. Isolates from patients with CM would therefore be expected to show higher binding to HUVECs stimulated with TNF-α. In our study, TNF-α enhanced the binding (modulation, >50%) of only 5 of 21 isolates. TNF-α stimulation of HUVECs did not enhance the binding of any isolates from CM patients but enhanced the binding of isolates that spontaneously show high binding to unstimulated HUVECs.

Although we did not formally demonstrate the exclusive role of the ICAM-1 receptor in the enhancement of cytoadherence after TNF-α stimulation, ICAM-1 may participate in 75% at least (23). Therefore, the involvement of other

receptors, including receptors that remain to be identified in cytoadherence phenomena, is probable but would occur to a lower extent. TNF-α induces thrombospondin secretion by cultured endothelial cells (5), and although thrombospondin could alone mediate the binding of infected erythrocytes, CD36 is most probably the receptor for thrombospondin (30). CD36 is not, however, expressed on HUVECs (34). Vascular cell adhesion molecule-1 and endothelial leukocyte adhesion molecule-1 are absent from unstimulated endothelial cells, but their expression can also be increased by TNF-α. However, the kinetics and the concentrations required to obtain maximum expression are different from those of ICAM-1 (24, 33).

The TNF-α concentrations used for stimulation were more than 2.5 times higher than the concentration in the peripheral blood of children dying of CM (9). The IL-6 concentrations used were also more than 20 times higher than the highest concentration found in the patients. These high levels were chosen to obtain a maximal effect, and it should be noted that levels of cytokines in peripheral blood do not necessarily reflect local secretion in the brain. In our results, GM-CSF, IL-3, and IL-6 did not modify cytoadherence, but their effects on ICAM-1 and CD36 expression on HUVECs have not yet been studied. The increased adherence observed with the combination of TNF-α and IL-3 suggests an additive effect and probably a different mode of action of IL-3 and TNF-α.

These results suggest that ICAM-1 is not the principal cytoadherence receptor, particularly in CM, but they do not rule out its involvement. Indeed, high ICAM-1 expression on HUVECs induced by TNF-α stimulation enhanced cytoadherence of 5 of 21 isolates only and showed no relation to disease severity. Moreover, more isolates seem to express the ligand for CD36 (a receptor expressed mainly by melanoma cells) than that for ICAM-1 (a receptor expressed by HUVECs), as confirmed by our in vitro results and by a previous study with plastic plates coated with purified CD36 and ICAM-1 receptors (22).

To complete the in vitro results, we also measured cytokine levels in the patients. Our results confirmed previous studies in which TNF-α and IL-6 levels were related to clinical and biological markers of severity (9, 16, 17, 21, 29), but high levels in peripheral blood were not specifically associated with CM or death. Most of the patients' sera contained little or no IL-3 or GM-CSF. Only TNF-α is elevated during the course of malaria and also enhances cytoadherence to stimulated HUVECs. But the combination of high levels of TNF-α in the peripheral blood and increased binding is not specific for CM, as was observed in two cases of SM and one case of UM. Our results also underline the contrast between human disease and the murine model. The mechanisms involved in humans and those in mice are quite different, particularly with regard to cell sequestration in brain capillaries. In the murine model, GM-CSF and IL-3 are essential mediators of CM, whereas IL-6 is involved in hypergammaglobulinemia (7). In humans, IL-6 is a marker of severe malaria, whereas GM-CSF and IL-3 are increased little if at all during malarial attack. Furthermore, these cytokines did not modify in vitro cytoadherence to HUVECs.

In summary, only rosette formation in vitro correlated with the severity of the disease in this study. TNF-α enhanced in vitro cytoadherence of *P. falciparum*-infected erythrocytes to HUVECs but only with isolates with a high capacity for binding to unstimulated HUVECs. Further studies are required to elucidate the role of the receptors potentially involved in cytoadherence and the role of cytokines during CM.

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