INFECTION AND IMMUNITY, Apr. 1997, p. 1251–1257 Vol. 65, No. 4
0019-9567/97/$04.00+0

Cytoadherence of *Plasmodium falciparum* to Intercellular Adhesion Molecule 1 and Chondroitin-4-Sulfate Expressed by the Syncytiotrophoblast in the Human Placenta

BERTRAND MAUBERT,[1] LARRY J. GUILBERT,[2] AND PHILIPPE DELORON[1]*

Institut de Médecine et d'Epidémiologie Africaine, Institut National de la Santé et de la Recherche Médicale U13, Hôpital Bichat, 75018 Paris, France,[1] and Department of Medical Microbiology and Immunology, University of Alberta, Calgary, T6G 2H7 Alberta, Canada[2]

Received 7 October 1996/Returned for modification 15 November 1996/Accepted 14 January 1997

Late stages of *Plasmodium falciparum*-infected erythrocytes (IRBCs) frequently sequester in the placentas of pregnant women, a phenomenon associated with low birth weight of the offspring. To investigate the physiological mechanism of this sequestration, we developed an in vitro assay for studying the cytoadherence of IRBCs to cultured term human trophoblasts. The capacity for binding to the syncytiotrophoblast varied greatly among *P. falciparum* isolates and was mediated by intercellular adhesion molecule 1 (ICAM-1), as binding was totally inhibited by 84H10, a monoclonal antibody specific for ICAM-1. Binding of the *P. falciparum* line RP5 to the syncytiotrophoblast involves chondroitin-4-sulfate (CSA), as this binding was dramatically impaired by addition of free CSA to the binding medium or by preincubation of the syncytiotrophoblast with chondroitinase ABC. ICAM-1 and CSA were visualized on the syncytiotrophoblast by immunofluorescence, while CD36, E-selectin, and vascular cell adhesion molecule 1 were not expressed even on tumor necrosis factor alpha (TNF-α)-stimulated syncytiotrophoblast tissue, and monoclonal antibodies against these cell adhesion molecules did not inhibit cytoadherence. ICAM-1 expression and cytoadherence of wild isolates was upregulated by TNF-α, a cytokine that can be secreted by the numerous mononuclear phagocytes present in malaria-infected placentas. These results suggest that cytoadherence may be involved in the placental sequestration and broaden the understanding of the physiopathology of the malaria-infected placenta.

In areas in which *Plasmodium falciparum* is endemic, pregnant women, even if clinically protected before pregnancy, are more likely to develop the malaria disease than their nonpregnant counterparts, and this increased susceptibility to malaria is more pronounced during the first pregnancy (for reviews, see references 6 and 26). One of the most striking features of malaria during pregnancy is that at delivery, women frequently present with parasitized placentas (4). Placental parasite load is associated with maternal anemia and low birth weight of the offspring (26), one of the leading risk factors for neonatal mortality and morbidity in areas in which malaria is endemic (24), thus posing a major public health challenge.

On the maternal face of a normal, term placenta, about 50 lobules can be observed. Each lobule consists in an intervillous space, into which maternal erythrocytes circulate, and a villus that contains the fetal blood and bathes in the maternal intervillous spaces. Maternal erythrocytes of the intervillous spaces are in contact with the epithelial lining of the villi, termed the trophoblast. The villous trophoblast comprises two layers, the superficial one being syncytial (the syncytiotrophoblast) and the inner one being mononuclear (the cytotrophoblast) (32). The syncytiotrophoblast derives from fusion and differentiation of the underlying cytotrophoblast and is in direct contact with maternal erythrocytes. In *P. falciparum*-parasitized placentas, the placental barrier efficiently inhibits fetal infection and *P. falciparum*-infected erythrocytes (IRBCs) are encountered only in intervillous spaces. At delivery, several features related to the *P. falciparum* infectious process, past or active, have been described, associated in different combinations: the macrophage concentration in the intervillous spaces, syncytiotrophoblastic damage, an excess of perivillous fibrinoid deposits, trophoblastic basal lamina thickening, and villous fibrosis (43). All these features are indicative of a local inflammatory process (8).

The mechanisms of placental colonization by IRBCs are still poorly understood. Three observations suggest that IRBCs are sequestered in the intravillous spaces of the placenta: at delivery IRBCs are more frequently found in the placenta than in the peripheral blood (25); parasite densities are often much higher in the placenta than in the peripheral blood (4, 16); and lastly, most of the *P. falciparum* parasites in placental blood are at mature stages, while these stages are absent from the peripheral blood (43). All these features suggest that placental sequestration involves a cytoadherence phenomenon. The cytoadherence of IRBCs to endothelial cells has been extensively studied because it is thought to be one of the leading causes of cerebral malaria (for a review, see reference 39). Various cell adhesion molecules (CAMs) expressed by endothelial cells are able to specifically bind IRBCs: CD36 (1), intercellular adhesion molecule 1 (ICAM-1; CD54) (2), thrombospondin (36), E-selectin (also called ELAM-1 or CD62-E), vascular cell adhesion molecule 1 (VCAM-1; CD106) (31), and chondroitin-4-sulfate (also called chondroitin sulfate A [CSA]) (35, 37). A recent report demonstrated that parasites isolated from the placenta (but not those from the peripheral blood of a nonpregnant host) were able to bind placental sections through CSA (15), suggesting that a parasite subpopulation was responsible for maternal malaria. The availability of a pure population of human villous cytotrophoblast cells that differentiate in culture into mature syncytiotrophoblasts provided us with the opportunity to further investigate the mechanisms underlying the binding of IRBCs to the human syncytiotrophoblast.

* Corresponding author. Mailing address: INSERM U13/IMEA, CHU Bichat, 46 rue Henri Huchard, 75018 Paris, France. Phone: 33 1 40256355. Fax: 33 1 40256351. E-mail: deloron@bichat.inserm.fr.

In this paper, we describe an in vitro model of IRBC cytoadherence to the human syncytiotrophoblast and characterize molecules mediating this adherence.

MATERIALS AND METHODS

Monoclonal antibodies, biological reagents, and chemicals. Several mouse anti-human monoclonal antibodies (MAbs) were used: OKM5 against CD36 (Ortho Diagnostic Systems, Roissy, France), 84H10 against ICAM-1 (Serotec, Oxford, United Kingdom), 12B6 against E-selectin (Immunotech, Marseille, France), 1G11 against VCAM-1 (Immunotech), MAB2030 against CSA (Chemicon International-Euromedex, Souffelweyersheim, France), 50H.19 against CD9 (22, 28), ZK-31 against desmosomes (21) (Sigma, Saint Quentin Fallavier, France), and TM18, which binds to the trophoblast membrane (a gift of D. Bellet, Institut Gustave Roussy, Villejuif, France). A mouse immunoglobulin G1 (IgG1) isotypic control MAb (clone 679.1Mc7; Immunotech) was used at the same concentrations as its controlled partners. Recombinant tumor necrosis factor alpha (TNF-α) expressed in the yeast *Saccharomyces cerevisiae* and human recombinant epidermal growth factor (EGF) (from *S. cerevisiae*) were purchased from Sigma; chondroitinase ABC (from *Proteus vulgaris*) and CSA were obtained from Fluka (Saint Quentin Fallavier, France). Type AB human serum was from the Transfusion Center of Pontoise (France), fetal calf serum (FCS) was obtained from Seromed (Berlin, Germany), and horse serum and glutaraldehyde were purchased from Sigma.

Parasites. *P. falciparum* isolates were obtained from patients of the Bichat Hospital in Paris and cryopreserved in liquid nitrogen. Before assaying for cytoadherence, samples were thawed and cultured overnight to pigmented stages (late trophozoite to young schizont) according to the standard method (41). The *P. falciparum* isolates were then washed twice and suspended at a 30% hematocrit in binding medium (BM), consisting of RPMI 1640, 25 mM HEPES, 10% human serum, and no bicarbonate. Its pH value was adjusted to 6.4 by addition of HCl (1 N). Most experiments were conducted during the first cycle of in vitro growth. The *P. falciparum* line RP5 (given by J. Gysin, Institut Pasteur, Lyon, France) has been derived from the Palo Alto strain by five selection steps on Sc1707, a *Saimiri* brain vasculature endothelial cell clone expressing CSA and no other receptor able to bind IRBCs (17).

Isolation and culture of human placental trophoblast cells. The complex structure of the syncytia makes their isolation in pure form difficult. This problem was overcome by the isolation of purified populations of villous cytotrophoblast cells that give rise to syncytia in vitro (for a review, see reference 11). Cytotrophoblast cells were purified by negative selection from human placentas obtained from vaginal delivery, as previously reported (44). Briefly, chorionic villous tissue from term placentas was dissected and subjected to repeated digestions with trypsin and DNase. Erythrocytes were removed by hemolysis. The remaining cells were incubated with 50H.19, a MAb specific for CD9, and after being washed were eluted through glass bead columns coated with goat anti-mouse polyclonal antibodies (Biotex, Edmonton, Alberta, Canada). Because CD9 is expressed by virtually all mesenchymal cells in the placenta but not by the trophoblast, cytotrophoblast cells do not bind to the columns and are eluted. Purified cells were cryopreserved in liquid nitrogen until testing was performed.

We used the Parafilm method to create microwells on culture-treated petri dishes (Corning, Bagneaux-sur-Loin, France), as described by Hasler et al. (18). Briefly, 5-mm-diameter holes were punched in a piece of Parafilm, the paper backing was removed, and the Parafilm was pressed very firmly onto the surface of the petri dish. Each hole in the Parafilm delimited a shallow microwell in which 20 to 40 μl of cell suspension could be deposited. Plates were sterilized by UV irradiation for 30 min. Cytotrophoblast cells were thawed rapidly in a 37°C water bath, washed twice in cold IMDM (Gibco-BRL, Cergy-Pontoise, France), and suspended at 1.6 million/ml in IMDM supplemented with 10% FCS and 50 μg of gentamicin per ml. Twenty microliters of this suspension was added to each well. After a 4-h incubation at 37°C in a 5% CO_2 atmosphere, nonadherent cells were removed by gentle washings with warm IMDM. Petri dishes were then filled with 5 ml of IMDM supplemented with 10% FCS, 50 μg of gentamicin per ml, and 5 ng of EGF per ml, which favors the differentiation of the cytotrophoblast cells (27). Syncytium formation is observed with a phase-contrast microscope after 48 h of culture, but full cell differentiation requires 7 days. The culture medium was changed every week. Cytoadherence assays were performed between days 7 and 14, with no evidence of variation.

Assay of IRBC binding to the syncytiotrophoblast. Syncytiotrophoblast cultures were rinsed twice with warm phosphate-buffered saline (PBS), and the Parafilm was taken off. Cells were washed with PBS and then with BM, and 20 μl of a suspension of IRBCs in BM was deposited into each well. Plates were incubated for 40 min with continuous gentle shaking at room temperature. For removal of nonadherent cells, petri dishes were immersed in washing medium (WM), consisting of BM without human serum, and turned upside down to allow nonadherent cells to fall from the trophoblast culture by gravity alone. Twenty minutes later, the cells were fixed for 60 min with a 2% glutaraldehyde solution in WM, rinsed with PBS, stained for 15 min with 3% Unna blue (RAL Réactifs, Paris, France), and air dried. In some experiments, cells were incubated with 30 μl of a 200-U/ml solution of TNF-α for 16 h prior to addition of IRBCs. Binding was quantified by light microscopy, with at least 50 fields being examined through a 50× objective under oil immersion. Results are expressed as the number of IRBCs adhering to 1 mm^2 of the cell monolayer and are given as means ± standard errors of the means (SEMs) for triplicate samples. The effect of pH on binding was investigated by using BM and WM with pH values ranging from 6.4 to 7.6, adjusted by addition of 1 N HCl or 1 N NaOH. Washes were performed at the same pH values as incubations.

Cytoadherence to CHO-CD36 and rosetting assays. Transfected Chinese hamster ovary (CHO) cells expressing CD36 (CHO-CD36; a gift of R. Howard) were cultured as described previously (18). Binding plates were prepared by adding 40 μl of a suspension containing 75×10^3 cells/ml to each well. After a 24-h culture period, cells were fixed with formaldehyde and then kept at 4°C for less than 2 months. Cytoadherence was tested as described for the syncytiotrophoblast except results were reported as the number of IRBCs per 100 cells. The rosetting assay was performed by the standard procedure (9). Briefly, 50 μl of IRBC suspension in BM was mixed with 50 μl of 0.01% acridine orange in BM. Ten microliters of the suspension was placed under a coverslip (22 by 22 mm), and 100 IRBCs were examined under a fluorescence microscope. The number of IRBCs that bound at least two uninfected erythrocytes was determined.

Immunostaining of CAMs on the syncytiotrophoblast surface. Cytoadherence receptors were assessed by indirect immunofluorescence on TNF-α-stimulated (200 U/ml) syncytiotrophoblast cultures for 6, 16, or 40 h or unstimulated syncytiotrophoblast cultures. Cells were rinsed in PBS, saturated with horse and human sera for 30 min, and incubated for 1 h with primary MAbs (concentrations, 2 μg/ml for anti-ICAM-1 and anti-CD36 MAbs and 1 μg/ml for anti-VCAM-1 and anti-E-selectin MAbs; controls included an isotypic MAb and absence of MAb). Cells were then incubated for 30 min with a biotinylated horse anti-mouse IgG (heavy plus-light chains) (Vector Laboratories Inc., Burlingame, Calif.) at a dilution of 1/50 prior to incubation with fluorescein avidin D (Vector) at a dilution of 1/100. All steps were performed at 4°C, and all dilutions were in PBS containing 5% human serum and 5% horse serum. Slides were mounted in VECTASHIELD mounting medium (Vector) and examined by fluorescence microscopy. For assessment of CSA, unfixed cells were incubated for 45 min with 0.5 U of chondroitinase ABC per ml in RPMI-HEPES buffer and rinsed with PBS, and the following steps were performed as described above (anti-CSA MAb was used at 1/400).

Modulation of cytoadherence capacity by TNF-α and CAM-specific reagents. Syncytiotrophoblast cultures were stimulated with TNF-α (200 U/ml) for 16 h prior to addition of IRBCs. Control wells received no TNF-α. The binding assay was then performed as described above. Alternatively, syncytiotrophoblast cultures (stimulated by TNF-α or unstimulated) were incubated for 30 min with 10% human serum in PBS and then for 1 h with MAbs specific for CD36 (5 to 20 μg/ml), ICAM-1 (0.2 to 20 μg/ml), E-selectin (20 μg/ml), or VCAM-1 (20 μg/ml). Controls included medium alone, an isotypic control (which does not bind to the trophoblast), and TM18, a MAb that binds to the syncytiotrophoblast membrane (1/500 dilution). After a 40-min incubation, cells were washed before the binding assay was performed. Because of the lack of availability of a CSA-specific MAb capable of inhibiting the binding, three experiments were performed to assess the involvement of CSA as an IRBC receptor. First, the binding capacity of the CSA-adherent line RP5 was assessed. Second, a competition test was performed with RP5 suspended in BM containing 1 mg of free CSA per ml. Third, syncytiotrophoblast cultures were incubated for 45 min with a solution consisting of 0.5 U of chondroitinase ABC per ml in RPMI-HEPES (37) and then washed extensively with PBS before the cytoadherence capacity of RP5 was assessed.

RESULTS AND DISCUSSION

In order to obtain syncytiotrophoblasts, we cultured purified cytotrophoblast cells that after 7 days in the presence of EGF fuse together to form syncytial units (44) and that can be maintained in vitro for at least 2 weeks. One-week cultures contained fewer than one vimentin-positive (mesenchymal) cell per square millimeter, and 2-week cultures remained free of fibroblasts and macrophages. After stimulation with EGF, the cytotrophoblast differentiated into a syncytiotrophoblast that secreted human chorionic gonadotropin (27, 44). Syncytialization was monitored by phase-contrast microscopy and confirmed by assessing for the presence of desmosomes, which form intercellular junctions in most epithelia (14). By using indirect immunofluorescence with ZK-31, a MAb specific for desmosomes, and ethidium bromide to stain nuclei, we verified that 7-day-culture cells were a patchwork: 70% of the nuclei were in multinucleated syncytia, while the remaining cells were mononuclear.

Immunofluorescence detection of ICAM-1 and CSA on the syncytiotrophoblast. The expression of CAMs by some tissues is regulated by the cytokine microenvironment. For example,

TABLE 1. Identification of CAMs by immunofluorescence on the syncytiotrophoblast cultures[a]

Duration of TNF-α stimulation (h)	CAM expression level				
	ICAM-1	CD36	ELAM-1	VCAM-1	CSA
0	+	−	−	−	++
4	++	−	−	−	++
16	+++	−	−	−	++
40	+++	−	−	−	++

[a] Results are presented as relative fluorescence achieved with anti-ICAM-1 (84H10), anti-CD36 (OKM5), anti-E-selectin (12B6), anti-ICAM-1 (1G11), or anti-CSA (MAB2030). −, negative; +, weak positive; ++, medium-strength positive; +++, strong positive.

TNF-α-induced expression of ICAM-1 has been reported for human umbilical vein endothelial cells (HUVECs) (2), human lung endothelium cells (29), *Saimiri* brain endothelial cells (17), and astrocytes (40). Thus, trophoblast cells were stimulated by TNF-α for 4 h (the optimum length of time for E-selectin expression on HUVECs), 16 h (the optimum length of time for ICAM-1 and VCAM-1 expression on HUVECs), and 40 h before immunostaining was performed on unfixed cells. Table 1 shows that only ICAM-1 and CSA were found to be present on the syncytiotrophoblast. In the absence of cytokine stimulation, staining with MAb against ICAM-1 was distinct but weak, present only on some syncytiotrophoblasts, and more intense on the edge of the syncytial units; staining intensity was much higher on TNF-α-stimulated cells. The amount of CSA varied between syncytial units but was homogeneous over the whole surface of each unit and did not vary after TNF-α stimulation (Fig. 1). There was no staining with IgG1 control MAb or with MAbs against CD36, E-selectin, or VCAM-1, even after TNF-α stimulation.

CSA is expressed on many cell types, including human syncytiotrophoblasts (15). Salafia et al. (38) reported the absence of ICAM-1 on the human trophoblast, but to our knowledge its presence has never been assessed in *P. falciparum*-infected placentas. ICAM-1 is present on brain capillary endothelial cells from people who died from cerebral malaria but not on those from people who died from other causes (42). The expression of ICAM-1 by the syncytiotrophoblast in vitro and in the placentas of some human immunodeficiency virus-infected women (17a) demonstrates that ICAM-1 is expressed on human placentas in vivo, at least under certain circumstances. This expression involves immune responses accompanied by systemic release of inflammatory cytokines since, in addition to TNF-α, gamma interferon and interleukin-1α also strongly upregulate syncytiotrophoblast expression of ICAM-1 (17a). In *P. falciparum*-infected placentas, TNFα, gamma interferon, and interleukin-1α are likely to be released locally by the heavy monocytic infiltration (43) and by the trophoblast itself (10). These mediators also induce placental alterations (45) that modify maternal-fetal exchanges and contribute to intrauterine growth retardation, which is likely to be involved in the low birth weight of offspring from mothers with *P. falciparum*-infected placentas (6).

IRBCs bind to the syncytiotrophoblast. The binding of IRBCs to the syncytiotrophoblast (Fig. 2) was specific to IRBCs, since more than 90% of cells adhering to the cell culture were parasitized while the initial percentage of parasitized cells was much lower (0.2 to 4%). Adherence was maximal when parasites were at the late trophozoite-young schizont stage. When parasites were tested at the ring or late schizont stage, the former did not adhere and binding of the latter was weaker than that of young schizonts. Since cultures contained both multinucleated syncytiotrophoblasts and mononucleated cytotrophoblasts, we next investigated the cellular distribution of IRBC adherence. Ten-day-culture cells were incubated with parasites, washed as described above, and then fixed with acetone-ethanol (1:1). Desmosomes were then visualized by immunofluorescence, and IRBCs were observed by light microscopy on the same slide. More than 90% of IRBCs bound to multinucleated cells, although some adherence to cytotrophoblasts was observed.

The Parafilm method was adapted to our goal, given the low number of cells required, the ease of removal of nonadherent cells, and the ability to use any type of plate. IRBC binding to the syncytiotrophoblast was profoundly affected by test conditions. In initial experiments, conducted without shaking, at a 3% hematocrit and a pH value of 6.8, as advised by Marsh et al. for C32 cells (23), cytoadherence did not exceed 30 IRBCs/mm^2. Continuous shaking, high hematocrit (30%), and gentle removal of nonadherent cells were found to be important parameters for optimal binding of IRBCs to the syncytiotrophoblast. We observed no difference in adhesion at room temperature and at 37°C (data not shown). Conversely, IRBC adherence to the syncytiotrophoblast was profoundly influenced by the BM pH value, being maximal at low pH values (6.4) (Fig. 3). The pH value in vivo in the vicinity of the syncytiotrophoblast membrane is unknown. Although a pH of 6.4 is not physiologically observed in the blood flow of other organs, it is within the range expected at inflammatory sites. Malaria parasites produce large amount of lactic acid (20), and the microenvironment in intervillous spaces could favor the stagnation of acids. Given the high density of parasites usually observed in infected placentas, the blood pH in these placentas is likely to be rather low.

TNF-α increases the capacity of the syncytiotrophoblast to bind IRBCs. The number of IRBCs that bind HUVECs increases when HUVECs are incubated with TNF-α. This increase is related to upregulation of the CAMs expressed by HUVECs (2, 31). Similarly, preincubation of the syncytiotrophoblast with TNF-α increased by 3.4-fold ± 0.4-fold (mean ± SEM) the number of bound IRBCs (Table 2). This increase was observed with different *P. falciparum* isolates and with syncytiotrophoblast cultures prepared from all four placentas tested. In pregnant women, *P. falciparum*-infected placentas are colonized by numerous macrophages (43) likely to release TNF-α, which is also secreted in vivo by the trophoblast itself (10). Thus, further experiments were performed on 16-h TNF-α-stimulated cultures.

Natural isolates of *P. falciparum* vary in their capacity to bind to the syncytiotrophoblast. Table 3 reports the cytoadherence of nine *P. falciparum* isolates to the syncytiotrophoblast. Parasites were tested (on 16-h TNF-α-stimulated syncytiotrophoblast cultures) at the patients' blood densities, ranging from 0.2 to 4%. Since in our model, as in models using C32 cells or transfected CHO cells (18, 19), adherence is a linear function of the parasite density (data not shown), results were adjusted to a 1% parasite density by dividing the number of adherent IRBCs per square millimeter by the parasite density of the inoculum (19). For the nine isolates tested, the adjusted number of bound IRBCs was highly variable, ranging from 14 to more than 200 per mm^2. Binding was not impaired by rosette formation (Table 3) as, except for isolate DEV, less than 10% of IRBCs were engaged in rosetting even though the rosetting test was performed in BM, whose low pH favors rosetting (9).

To further identify the CAM(s) involved in adherence, two approaches were used. The possible involvement of CD36,

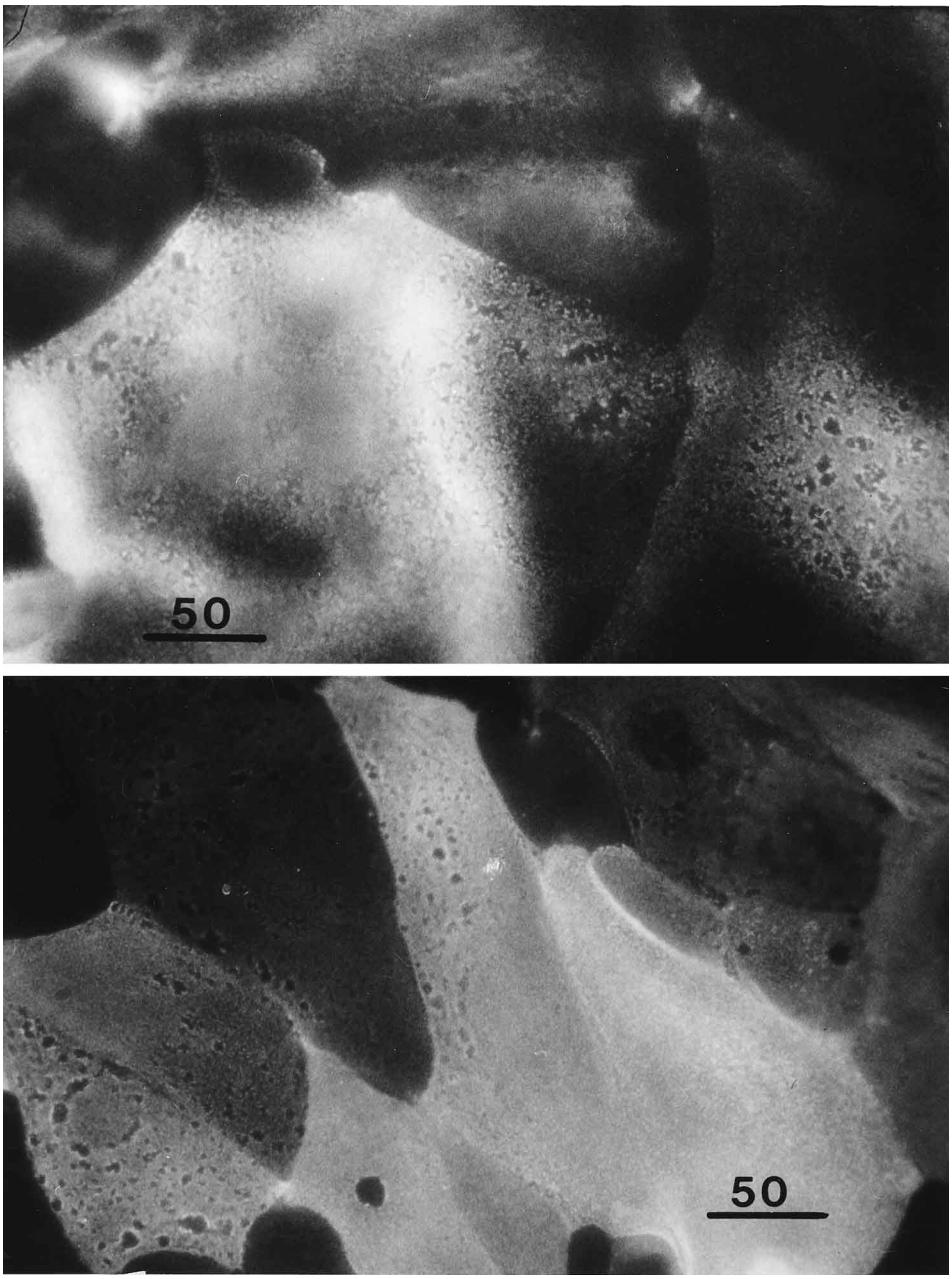

FIG. 1. Immunofluorescence staining of ICAM-1 and CSA on the surface of in vitro-cultured human syncytiotrophoblast cells. (Upper panel) Syncytiotrophoblast cultures were incubated with TNF-α for 16 h and then with 84H10, a MAb against ICAM-1. (Lower panel) Syncytiotrophoblast cultures were treated with chondroitinase ABC and incubated with MAB2030, a MAb against CSA. In both cases, staining was revealed by using biotinylated anti-mouse IgG and fluorescein avidin D. Bars = 50 μm.

ICAM-1, E-selectin, and/or VCAM-1 was investigated by incubating syncytiotrophoblast cultures with CAM-specific antibodies before performing the cytoadherence assay. The involvement of CSA was investigated following two approaches: in the first, free CSA was added to the binding medium, and in the second, syncytiotrophoblast CSA was hydrolyzed by chondroitinase ABC.

Binding of *P. falciparum* to the syncytiotrophoblast is mediated by ICAM-1 and CSA. Figure 4 shows a typical test of the inhibition of adherence of a *P. falciparum* isolate, using TNF-α-stimulated syncytiotrophoblast cultures. Similar results were obtained with unstimulated cells and with two other isolates. The binding of IRBCs from all three isolates tested was almost completely inhibited (98% ± 2%) by 84H10, the MAb against ICAM-1, at 2 μg/ml. MAbs against CD36, E-selectin, and VCAM-1, as well as the addition of free CSA or the hydrolysis of CSA (a constituent of the extracellular matrix and the glycocalyx surrounding the cell membrane), had no effect. The three *P. falciparum* isolates tested had the ability to bind CHO-CD36 (Table 3), and OKM5, the MAb specific for CD36, blocks the binding of IRBCs to C32 melanoma cells (1). Thus, the lack of effect of OKM5 in our model is probably related to the lack of CD36 expression by the syncytiotrophoblast, as suggested by immunofluorescence studies. The frequency of

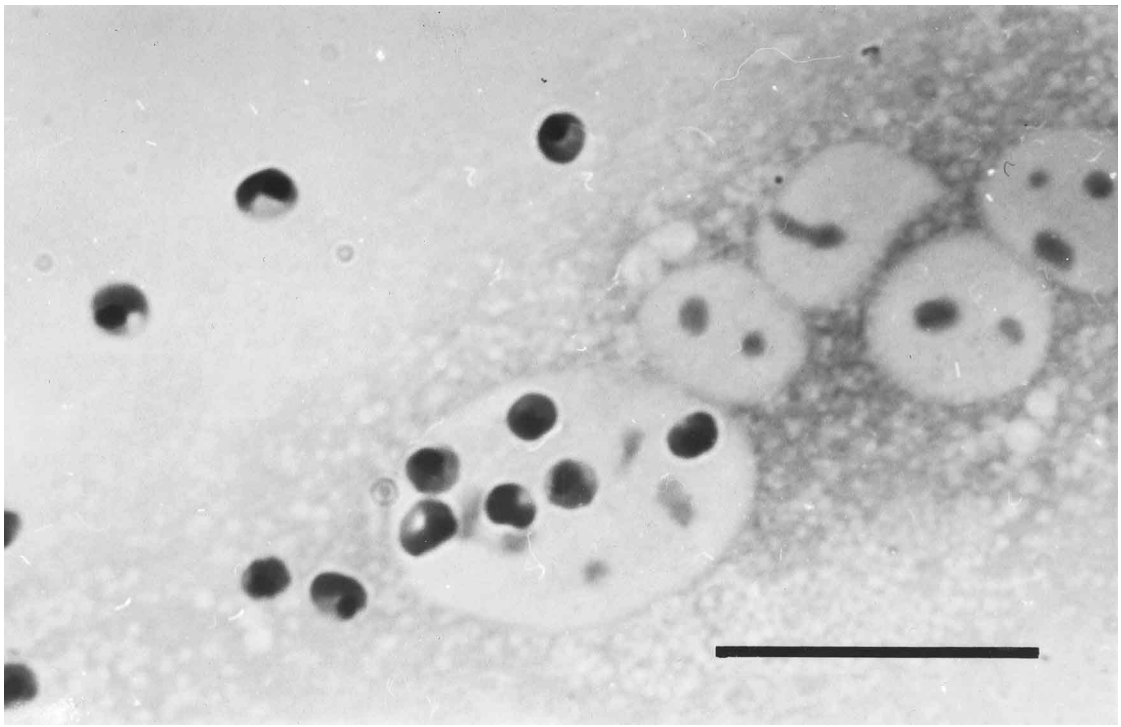

FIG. 2. Binding of *P. falciparum*-infected erythrocytes to the syncytiotrophoblast. Syncytiotrophoblast cultures were incubated with a *P. falciparum*-infected erythrocyte suspension (parasite density, 1%) as described in Materials and Methods. After gentle washing to remove nonadherent erythrocytes and fixation, cells were stained with Unna blue. Bar = 50 μm.

natural isolates binding E-selectin and VCAM-1 is low, and one cannot rule out the possibility that all isolates tested lacked the ability to bind these CAMs. Furthermore, the efficiencies of 12B6 and 1G11, the MAbs against E-selectin and VCAM-1, at blocking the binding of IRBCs to cells expressing E-selectin and VCAM-1 have not been reported yet. Nevertheless, the absence of immunostaining of E-selectin and VCAM-1 on the surface of the syncytiotrophoblast strongly suggests that these CAMs are not involved in mediating cytoadherence in our model. Figure 5 shows that the CSA-selected line RP5 also bound to the syncytiotrophoblast (152 ± 47 IRBCs/mm^2). However, in contrast to our findings with *P. falciparum* isolates, this binding was significantly impaired by free CSA at 1 mg/ml (inhibition, 98.9% ± 1.3%; $P < 0.05$) or by hydrolysis of cell surface proteoglycans by chondroitinase ABC (inhibition, 98.6% ± 1.0%; $P < 0.05$), demonstrating the involvement of CSA in our model.

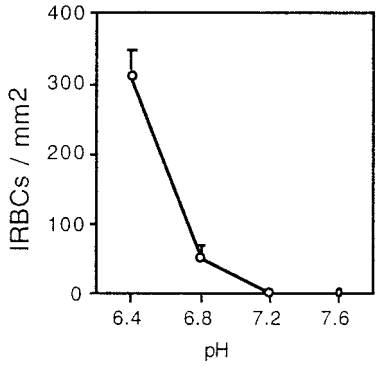

FIG. 3. Effect of pH on IRBC binding to the syncytiotrophoblast. Data are means of values for nine wells ± SEMs. The experiment was repeated twice, with similar results, using two isolates. See text for details.

Overall, our data demonstrate that ICAM-1 and CSA are expressed by the human syncytiotrophoblast. *P. falciparum* isolates from the peripheral blood bind to the syncytiotrophoblast via ICAM-1, but we obtained no evidence for widespread use of CSA. Indeed, we did not observe binding to CSA with any of the patient isolates, and thus the relevance of CSA in binding IRBCs remains unclear. However, recent data show that unlike isolates from peripheral blood, *P. falciparum* isolates obtained from the placenta are able to bind to CSA (15). The involvement of ICAM-1 in the adherence of patient isolates to the syncytiotrophoblast is in agreement with the considerable variation in binding capacity of the different parasite isolates tested. Indeed, studies with parasites from Papua New Guinea (33) and various African countries (13, 34) showed that most isolates bound ICAM-1, but the adherence level varied greatly among isolates. In addition, the flow-controlled study of Cooke

TABLE 2. Binding of *P. falciparum* isolates to different lines of human syncytiotrophoblast previously incubated for 16 h with or without 200 U of TNF-α per ml

Isolate	SCTB line[a]	No. of IRBCs bound/mm^3 with[b]:		Stimulated/ unstimulated ratio
		No stimulation	TNF-α stimulation	
LEL	5	36.0 ± 1.2	90.0 ± 10.0	2.5
LEL	3	56.3 ± 5.3	125.9 ± 14.8	2.2
GUY	3	70.0 ± 25.8	352.0 ± 42.5	5.0
GUY	4	122.1 ± 22.0	411.0 ± 28.0	3.4
GAY	2	7.2 ± 2.1	26.6 ± 4.0	3.7

[a] SCTB, syncytiotrophoblast.
[b] Data are means of values for triplicate samples ± SEMs.

TABLE 3. Binding of *P. falciparum* isolates to the human syncytiotrophoblast (SCTB), to uninfected erythrocytes (uRBCs), and to CD36-transfected CHO cells (CHO-CD36)

Isolate	Binding capacity with[a]:		
	SCTB[b]	uRBCs[c]	CHO-CD36[d]
JAN	14 ± 1	5.4	ND[e]
LEL	166 ± 33	7.8	425 ± 84
SAN	71 ± 28	0.8	179 ± 40
DEV	41 ± 3	26.0	ND
DUC	10 ± 1	8.0	ND
PER	189 ± 127	0.0	ND
MBA	15 ± 9	7.2	ND
SOU	147 ± 130	1.0	ND
GUY	228 ± 80	5.0	195 ± 80

[a] Experiments involving binding to SCTB and CHO-CD36 were done in triplicate, and values are means ± SEMs.
[b] Number of IRBCs per square millimeter.
[c] Percentage of IRBCs binding two or more uRBCs.
[d] Number of IRBCs per 100 CHO cells.
[e] ND, not done.

et al. (12) suggested that most IRBCs were not stopped by ICAM-1 but rolled on ICAM-1-coated surfaces. Shear flow must be taken into account when studying adherence in cerebral malaria, as capillaries have a tubelike structure, for which the flow rate is constant on the whole surface. Conversely, placenta intervillous spaces possess a wider structure. The maternal flow rate is high at the point where blood springs into intervillous spaces but is lower near the edges (30). Local low flow rates would facilitate the binding of IRBCs to ICAM-1 and justify, in our assay, washing with minimal shear flow. The weakness of the ICAM-1–IRBC binding could also explain why the binding of IRBCs to the placenta is reported in histological studies as a rare event (7, 43).

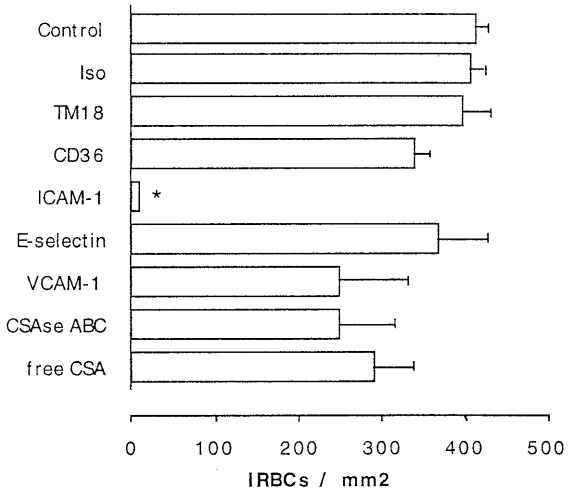

FIG. 4. Modulation of cytoadherence of *P. falciparum* to the syncytiotrophoblast by anti-CAM MAbs, free CSA, and hydrolysis of CSA. Ten-day syncytiotrophoblast cultures were stimulated for 16 h with TNF-α (200 U/ml). Cells were incubated for 1 h with various MAbs specific for CAMs (CD36, ICAM-1, E-selectin, and VCAM-1). Controls included an IgG1 isotypic control (Iso) and a MAb directed against the trophoblast surface (TM18). In the case of CSAse ABC, syncytiotrophoblast cultures were pretreated with this enzyme. After the various treatments of the syncytiotrophoblast cultures, IRBCs were added and the cytoadherence assay was performed as described in Materials and Methods. In the case of CSA, syncytiotrophoblast cultures were left untreated but free CSA (1 mg/ml) was added to the binding assay medium. Data represent the means of values from triplicate samples ± SEMs of a single experiment with a patient isolate (GUY in Table 3). Similar experiments were conducted with two other isolates (LEL and SAN) with similar results. Similar experiments conducted without TNF-α stimulation revealed a similar inhibition effect.

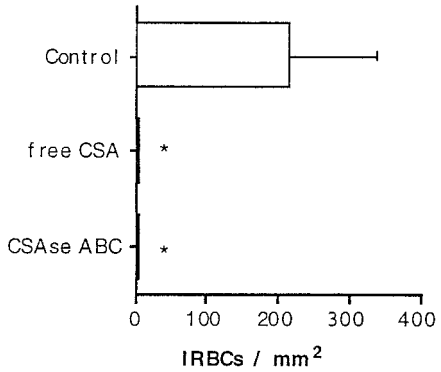

FIG. 5. Inhibition of the binding of the *P. falciparum* line RP5 to the syncytiotrophoblast by CSA or by pretreatment with chondroitinase ABC. For the control, syncytiotrophoblast cultures were incubated with a suspension of erythrocytes infected by *P. falciparum* line RP5 (parasite density, 1%) and treated as described in Materials and Methods. In the case of CSA, syncytiotrophoblast cultures were left untreated but free CSA (1 mg/ml) was added to the binding assay medium. In the case of CSAse ABC, syncytiotrophoblast cultures were incubated with the enzyme at 0.5 U/ml for 45 min and then washed before addition of parasites. The experiment was repeated twice with similar results. Data are means ± SEMs of values from triplicate samples.

PfEMP-1 (*P. falciparum*-infected erythrocyte membrane protein 1), the best-characterized ligand implicated in cytoadherence (5), belongs to a polymorphic family of antigenic molecules, explaining why selection by cytoadherence leads to the selection of antigenic variants (3). Cytoadherence in the placenta could select minor parasite subpopulations (15) expressing particular surface antigens that may be rarely selected outside of pregnancy. The novelty of the surface antigens expressed by placenta-bound parasites could account for the lack of strain-specific immunity, for the persistence of the infection for a longer period of time at the placental level than at the peripheral level, and for the higher susceptibility of pregnant women to *P. falciparum*. In addition, during their first pregnancies, women with parasitized placentas will acquire a relative immunity to placental IRBCs, which explains why multigravid women are less susceptible to placental infection that primigravid women. The binding of IRBCs to the human syncytiotrophoblast provides a new field of investigation, including the study of (i) selection of *P. falciparum* subpopulations that adhere to the syncytiotrophoblast, (ii) the consequences of such a binding for the syncytiotrophoblast physiology, (iii) the screening of maternal antibodies able to prevent such ICAM-1- or CSA-dependent adherence, and (iv) the identification of trophoblastic factors that modulate cytoadherence.

ACKNOWLEDGMENTS

We are indebted to Gérard Chaouat and Geneviève Milon for their advice and valuable discussion. We also thank Leonardo Basco for *P. falciparum* patient isolates, Jürg Gysin and Bruno Pouvelle for the RP5-selected *P. falciparum* line, Russell Howard for CHO cells transfected by CD36 cDNA, and D. Bellet for TM18 monoclonal antibody.

This work was supported by Institut National de la Santé et de la Recherche Médicale (INSERM). B.M. is a recipient of a fellowship from the French Ministry of High Education and Research.

REFERENCES

1. **Barnwell, J. W., C. F. Ockenhouse, and D. M. Knowles II.** 1985. Monoclonal antibody OKM5 inhibits the in vitro binding of *Plasmodium falciparum*-infected erythrocytes to monocytes, endothelial, and C32 melanoma cells. J. Immunol. **135:**3494–3497.

2. **Berendt, A. R., D. L. Simmons, J. Tansey, C. I. Newbold, and K. Marsh.** 1989. Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for *Plasmodium falciparum*. Nature **341:**57–59.
3. **Biggs, B. A., R. F. Anders, H. E. Dillon, K. M. Davern, M. Martin, C. Petersen, and G. V. Brown.** 1992. Adherence of infected erythrocytes to venular endothelium selects for antigenic variants of *Plasmodium falciparum*. J. Immunol. **149:**2047–2054.
4. **Blacklock, D. B., and R. M. Gordon.** 1925. Malaria parasite in the placental blood. Ann. Trop. Med. Parasitol. **19:**37–45.
5. **Borst, P., W. Bitter, R. McCulloch, F. Van Leeuwen, and G. Rudenko.** 1995. Antigenic variation in malaria. Cell **82:**1–4.
6. **Brabin, B. J.** 1991. The risks and severity of malaria in pregnant women. Applied Field Research in Malaria Report 1. Special Programme for Research and Training in Tropical Diseases (TDR)/FIELDMAL/1. World Health Organization, Geneva, Switzerland.
7. **Bray, R. S., and R. E. Sinden.** 1979. The sequestration of *Plasmodium falciparum* infected erythrocytes in the placenta. Trans. R. Soc. Trop. Med. Hyg. **73:**716–719.
8. **Bulmer, J. N., F. N. Rasheed, N. Francis, L. Morrison, and B. M. Greenwood.** 1993. Placental malaria. I. Pathological classification. Histopathology **22:**211–218.
9. **Carlson, J., G. Holmquist, D. W. Taylor, P. Perlmann, and M. Wahlgren.** 1990. Antibodies to a histidine-rich protein (PfHRP1) disrupt spontaneously formed *Plasmodium falciparum* erythrocyte rosettes. Proc. Natl. Acad. Sci. USA **87:**2511–2515.
10. **Chen, H. L., Y. Yang, X. L. Hu, K. K. Yelavarthi, J. L. Fishback, and J. S. Hunt.** 1991. Tumor necrosis factor alpha mRNA and protein are present in human placental and uterine cells at early and late stages of gestation. Am. J. Pathol. **139:**327–335.
11. **Contractor, S. F., and S. R. Sooranna.** 1993. Trophoblast cell culture as a model for studying placental function, p. 504–526. *In* C. W. G. Redman, I. L. Sargent, and P. M. Starkey (ed.), The human placenta. A guide for clinicians and scientists. Blackwell Scientific Publications, Oxford, United Kingdom.
12. **Cooke, B. M., A. R. Berendt, A. G. Craig, and J. McGregor.** 1994. Rolling and stationary cytoadhesion of red blood cells parasitized by *Plasmodium falciparum*: separate roles for ICAM-1, CD36 and thrombospondin. Br. J. Haematol. **87:**162–170.
13. **Cooke, B. M., S. Morris-Jones, B. M. Greenwood, and G. B. Nash.** 1995. Mechanisms of cytoadhesion of flowing, parasitized red blood cells from Gambian children with *Plasmodium falciparum* malaria. Am. J. Trop. Med. Hyg. **53:**29–35.
14. **Douglas, G. C., and B. F. King.** 1990. Differentiation of human trophoblast cells in vitro as revealed by immunocytochemical staining of desmoplakin and nuclei. J. Cell Sci. **96:**131–141.
15. **Fried, M., and P. E. Duffy.** 1996. Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. Science **272:**1502–1504.
16. **Garnham, P. C. C.** 1938. The placenta in malaria with special reference to reticulo-endothelial immunity. Trans. R. Soc. Trop. Med. Hyg. **32:**13–48.
17. **Gay, F., C. Robert, B. Pouvelle, S. Peyrol, A. Scherf, and J. Gysin.** 1995. Isolation and characterization of brain microvascular endothelial cells from *Saimiri* monkeys. An in vitro model for sequestration of *Plasmodium falciparum*-infected erythrocytes. J. Immunol. Methods **184:**15–28.

17a. **Guilbert, L. J.** Unpublished data.

18. **Hasler, T., G. R. Albrecht, M. R. Vanschravendijk, J. C. Aguiar, K. E. Morehead, B. L. Pasloske, C. Ma, J. W. Barnwell, B. Greenwood, and R. J. Howard.** 1993. An improved microassay for *Plasmodium falciparum* cytoadherence using stable transformants of Chinese hamster ovary cells expressing CD36 or intercellular adhesion molecule-1. Am. J. Trop. Med. Hyg. **48:**332–347.
19. **Ho, M., B. Singh, S. Looareesuwan, T. M. E. Davis, D. Bunnag, and N. J. White.** 1991. Clinical correlates of in vitro *Plasmodium falciparum* cytoadherence. Infect. Immun. **59:**873–878.
20. **Jensen, M. D., M. Conley, and L. D. Helstowski.** 1983. Culture of *Plasmodium falciparum*: the role of pH, glucose and lactate. J. Parasitol. **69:**1060–1067.
21. **Lang, A. B., B. F. Odermatt, and J. R. Ruettner.** 1986. Monoclonal antibodies to human cytokeratins: application to various epithelial and mesothelial cells. Exp. Cell Biol. **54:**61–72.
22. **MacLean, G. D., J. Seehafer, A. R. E. Shaw, M. W. Kieran, and B. M. Longenecker.** 1982. Antigenic heterogeneity of human colorectal cancer cell lines analyzed by a panel of monoclonal antibodies. I. Heterogeneous expression of Ia-like and HLA-like antigenic determinants. J. Natl. Cancer Inst. **69:**357–364.
23. **Marsh, K., V. M. Marsh, J. Brown, H. C. Whittle, and B. M. Greenwood.** 1988. *Plasmodium falciparum*: the behavior of clinical isolates in an in vitro model of infected red blood cell sequestration. Exp. Parasitol. **65:**202–208.
24. **McCormick, M. C.** 1985. The contribution of low birth weight to infant mortality and childhood morbidity. N. Engl. J. Med. **312:**82–90.
25. **McGregor, I. A.** 1984. Epidemiology, malaria and pregnancy. Am. J. Trop. Med. Hyg. **33:**517–525.
26. **Menendez, C.** 1995. Malaria during pregnancy: a priority area of malaria research and control. Parasitol. Today **11:**178–193.
27. **Morrish, D. W., D. Bhardwaj, L. K. Dabbagh, H. Marusyk, and O. Siy.** 1987. Epidermal growth factor induces differentiation and secretion of human chorionic gonadotropin and placental lactogen in normal human placenta. J. Clin. Endocrinol. Metab. **65:**1282–1287.
28. **Morrish, D. W., A. R. E. Shaw, J. Seehafer, D. Bhardwaj, and M. T. Paras.** 1991. Preparation of fibroblast-free cytotrophoblast cultures utilizing differential expression of the CD9 antigen. In Vitro Cell. Dev. Biol. **27A:**303–306.
29. **Muanza, K., F. Gay, C. Behr, and A. Scherf.** 1996. Primary culture of human lung microvessel cells: a useful in vitro model for studying *Plasmodium falciparum*-infected erythrocyte cytoadherence. Res. Immunol. **147:**149–163.
30. **Muller, L. M. M.** 1993. Ultrasound assessment of the placenta, and clinical assessment of placental growth, p. 155–189. *In* C. W. G. Redman, I. L. Sargent, and P. M. Starkey (ed.), The human placenta. A guide for clinicians and scientists. Blackwell Scientific Publications, Oxford, United Kingdom.
31. **Ockenhouse, C. F., T. Tegoshi, Y. Maeno, C. Benjamin, M. Ho, K. E. Kan, Y. Thway, K. Win, M. Aikawa, and R. R. Lobb.** 1992. Human vascular endothelial cell adhesion receptors for *Plasmodium falciparum*-infected erythrocytes: roles of endothelial leukocyte adhesion molecule-1 and vascular cell adhesion molecule-1. J. Exp. Med. **176:**1183–1189.
32. **Panigel, M.** 1993. The origin and structure of the extraembryonic tissues, p. 3–32. *In* C. W. G. Redman, I. L. Sargent, and P. M. Starkey (ed.), The human placenta. A guide for clinicians and scientists. Blackwell Scientific Publications, Oxford, United Kingdom.
33. **Reeder, J. C., S. J. Rogerson, F. Al-Yaman, R. F. Anders, R. L. Coppel, S. Novakovic, M. P. Alpers, and G. V. Brown.** 1994. Diversity of agglutinating phenotype, cytoadherence and rosette-forming characteristics of *Plasmodium falciparum* isolates from Papua New Guinean children. Am. J. Trop. Med. Hyg. **51:**45–55.
34. **Ringwald, P., F. Peyron, J. P. Lepers, P. Rabarison, C. Rakotomalala, M. Razanamparany, M. Rabodonirina, J. Roux, and J. Le Bras.** 1993. Parasite virulence factors during falciparum malaria: rosetting, cytoadherence, and modulation of cytoadherence by cytokines. Infect. Immun. **61:**5198–5204.
35. **Robert, C., B. Pouvelle, P. Meyer, K. Muanza, H. Fujtoka, M. Aikawa, A. Scherf, and J. Gysin.** 1995. Chondroitin-4-sulphate (proteoglycan), a receptor for *Plasmodium falciparum*-infected erythrocyte adherence on brain microvascular endothelial cells. Res. Immunol. **146:**383–393.
36. **Roberts, D. D., J. A. Sherwood, S. K. Spitalnik, L. J. Panton, R. J. Howard, V. M. Dixit, W. A. Frazier, L. H. Miller, and V. Ginsburg.** 1985. Thrombospondin binds falciparum malaria parasitized erythrocytes and may mediate cytoadherence. Nature **318:**64–66.
37. **Rogerson, S. J., S. C. Chaiyaroj, K. Ng, J. C. Reeder, and G. V. Brown.** 1995. Chondroitin sulfate A is a cell surface receptor for *Plasmodium falciparum*-infected erythrocytes. J. Exp. Med. **182:**15–20.
38. **Salafia, C. M., N. Haynes, V. J. Merluzzi, and R. Rothlein.** 1991. Distribution of ICAM-1 within decidua and placenta and its gestational age-associated changes. Pediatr. Pathol. **11:**381–388.
39. **Sherman, I. W., I. E. Crandall, N. Guthrie, and K. M. Land.** 1995. The sticky secrets of sequestration. Parasitol. Today **11:**378–384.
40. **Shrikant, P., I. Y. Chung, M. E. Ballestas, and E. N. Benveniste.** 1994. Regulation of intercellular adhesion molecule-1 gene expression by tumor necrosis factor-alpha, interleukin-1 beta, and interferon-gamma in astrocytes. J. Neuroimmunol. **51:**209–220.
41. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. Science **193:**673–675.
42. **Turner, G. D. H., H. Morrison, M. Jones, T. M. E. Davis, S. Looareesuwan, I. D. Buley, K. C. Gatter, C. I. Newbold, S. Pukritayakamee, B. Nagachinta, N. J. White, and A. R. Berendt.** 1994. An immunohistochemical study of the pathology of fatal malaria. Evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule 1 in cerebral sequestration. Am. J. Pathol. **145:**1057–1069.
43. **Walter, P. R., Y. Garin, and P. Blot.** 1982. Placental pathologic changes in malaria. Am. J. Pathol. **109:**330–342.
44. **Yui, J., M. Garcia-Lloret, A. J. Brown, R. C. Berdan, D. W. Morrish, T. G. Wegmann, and L. J. Guilbert.** 1994. Functional, long term cultures of human term trophoblasts purified by column-elimination of CD9 expressing cells. Placenta **15:**231–246.
45. **Yui, J., M. Garcia-Lloret, T. G. Wegmann, and L. J. Guilbert.** 1994. Cytotoxicity of tumor necrosis factor-alpha and gamma-interferon against primary human placental trophoblasts. Placenta **15:**819–835.

Editor: S. H. E. Kaufmann