INFECTION AND IMMUNITY, Apr. 1996, p. 1480–1483 Vol. 64, No. 4
0019-9567/96/$04.00+0

CD36 and Intercellular Adhesion Molecule 1 Mediate Adhesion of Developing *Plasmodium falciparum* Gametocytes

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Received 3 October 1995/Returned for modification 13 December 1995/Accepted 12 January 1996

Plasmodium falciparum **trophozoite-infected erythrocytes adhere to the amelanotic melanoma C32 cell line in vitro. Here we demonstrate for the first time that immature gametocyte-infected erythrocytes also adhere to C32 cells, albeit at lower levels than trophozoites. However, anti-CD36 and anti-intercellular adhesion molecule 1 antibodies inhibit asexual and gametocyte adhesion by comparable percentages, suggesting a common dependency for binding to these cellular receptors.**

Plasmodium falciparum-infected erythrocytes are characterized by their ability to adhere to the endothelial cells lining the microvasculature of various organs, including the heart, gut, lung, and brain (7, 10, 15, 20, 22, 26, 32). Adhesion of the asexual (trophozoite/schizont)-stage-infected erythrocyte is implicated in malaria pathogenesis (16, 22), while sequestration of the erythrocytes infected with immature sexual stages is presumably vital to the development of mature gametocytes.

In vitro binding assays with erythrocytes infected with asexual-stage parasites (3, 8, 14, 30, 31, 33) have revealed specific interactions between one or more receptors on the host endothelium and ligands on such infected erythrocytes. Host cell receptors identified include CD36 (platelet glycoprotein IIIb) (2, 4, 9), intercellular adhesion molecule 1 (ICAM-1) (7–9), thrombospondin (1, 9, 18, 23), vascular cell adhesion molecule 1 (19), and endothelial leukocyte adhesion molecule 1 (19). CD36 and ICAM-1 are thought to be the major receptors in the adhesion of most *P. falciparum* isolates (6). Members of the PfEMP-1 family of variable surface-expressed parasite antigens have been proposed as parasite ligands mediating adhesion of asexual-stage-infected erythrocytes (5, 27, 28).

In contrast to the above-described studies, the adhesion of erythrocytes infected with sexual-stage parasites has been poorly described. Gametocytogenesis involves five stages, all of which remain intracellular. Within 24 h of erythrocyte invasion, immature male and female gametocytes (stage I) become sequestered in the microvasculature of the bone marrow and spleen (25). The gametocytes remain sequestered for 8 to 10 days, as judged by the absence of stage I to IV parasites from the peripheral circulation. Mature infectious gametocytes (stage V) reenter the circulation (6, 12). CD36 and thrombospondin have been suggested as receptors mediating binding of early (stage I and II) gametocytes (13) but not that of later developmental forms.

The C32 amelanotic melanoma cell line is the most commonly used model system for adhesion of asexual-stage parasites. This cell line expresses both CD36 and ICAM-1 (3). Conventional binding assays, however, preclude the study of gametocyte binding because of the requirement for large volumes of infected erythrocytes. We have developed and optimized an improved binding assay to enable gametocyte adhesion to be studied with the low yield of gametocytes obtainable in culture. Immature gametocytes of the 3D7 strain of *P. falciparum* were harvested following 12 days in semiautomatic continuous culture (21) and were enriched by centrifugation over a discontinuous Percoll gradient (70–45% interface) (21) and by subsequent sorbitol treatment (24). Asexual-stage parasites were cultured according to established protocols (29). Trophozoites and schizonts were enriched by Plasmagel (Laboratoire Roger Bellon, Neuilly, France) selection (17). The viability and subsequent development of parasites were not impaired as a result of these enrichment procedures (data not shown). Enriched parasites were resuspended to 10% hematocrit in binding medium, and parasitemia was varied by the addition of fresh uninfected erythrocytes. The percent gametocytemia was determined by using morphological criteria after Giemsa staining. For gametocyte adherence assays, 2.5×10^3 C32 cells were seeded into 16-well Lab-tek chamber slides (Nunc, Naperville, Ill.) and cultured for 48 h at 37°C and 5% CO_2 in 20% fetal calf serum in Dulbecco's minimum essential medium with nonessential amino acids. The cells were fixed with 1% formaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 20 min at room temperature. The fixative was aspirated, and nonspecific binding sites were blocked with 1% bovine serum albumin in PBS for 2 h at 4°C. Immediately prior to the addition of parasites, the blocking medium was removed and the cells were washed twice in binding medium (10.62 g of RPMI 1640 [without L-glutamine] per liter, 2 g of glucose per liter, and 1% human serum, pH 7.2). Infected erythrocytes (50 μl) were added to each well, and slides were incubated at 37°C for 90 min on a rocking table. Chambers were removed from the slides prior to four washes for 10 min each at 37°C in washing medium (binding medium without serum). Parasites were visualized after methanol fixation and Giemsa staining (Fig. 1), and the number of parasites bound to 500 C32 cells was determined (magnification, ×1,000). Under these conditions, there was no background binding of uninfected erythrocytes to C32 cells or of infected erythrocytes to slides. The possible adherence of low levels of contaminating asexual stages in sexual-stage assays was excluded on the basis of distinct morphological characteristics. Parasites in triplicate wells were counted, and data were analyzed by using Student's *t* test (significance level, $P < 0.05$). All experiments were repeated at least three times. Under these conditions, binding of gametocyte stages I to IV was clearly observed (Fig. 1). Stage V gametocytes were never observed, which is consistent with the presence of these parasites in the peripheral circulation and

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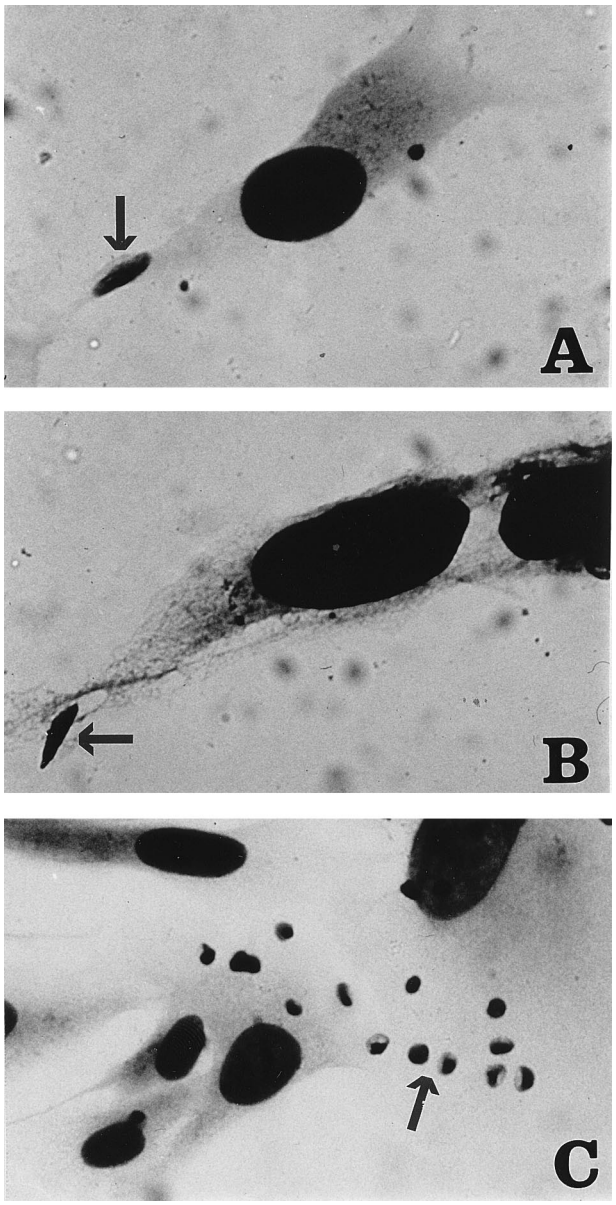

FIG. 1. Adherent parasites visualized by Giemsa staining. (A) Stage III gametocytes; (B) stage IV gametocytes; (C) trophozoites. Magnification, ×1,000 (oil immersion). Arrows indicate parasitized erythrocytes. Binding of trophozoites was not homogeneous; many may bind to one cell or a specific region of one cell, while other C32 cells fail to bind trophozoites. In contrast, gametocyte binding never exceeded two per cell.

their nonadhesive phenotype. In contrast to the binding of asexual-stage parasites under the same conditions, the number of adherent sexual-stage parasites was low (Fig. 2). At 5% infected erythrocytes, approximately 14 times more trophozoites than gametocytes adhere. Furthermore, whereas trophozoite binding reaches a plateau at above 7% parasitemia, binding of gametocytes continues to increase up to 50%, the maximum tested.

Given the role of CD36 and ICAM-1 in the adhesion of asexual-stage parasites, we investigated whether they mediate gametocyte adhesion. Fixed C32 cells were preincubated with 50 μl of monoclonal antibody (MAb) 8A6 (immunoglobulin G1 [IgG1]; anti-CD36) (4) or 15.2 (IgG1; anti-ICAM-1) (11) or an isotype control (IgG1; Sigma, Poole, United Kingdom) at various concentrations in binding medium at 37°C for 60 min. Unbound antibody was aspirated, and the cells were rinsed once with binding medium prior to the addition of parasites as described above. Both antireceptor antibodies inhibited adhesion of gametocytes in a dose-dependent fashion (Fig. 3A). MAb 8A6 resulted in significantly greater inhibition of gametocyte binding than did MAb 15.2 ($P < 0.05$) at all concentrations tested. The characteristics of antibody inhibition were similar in directly comparable studies using trophozoites (Fig. 3B). Furthermore, the percent reductions in specific binding observed for gametocytes and asexual-stage parasites were similar (Table 1). Binding of infected erythrocytes was not significantly inhibited by a MAb to major histocompatibility complex class I (W6/32), although this molecule is highly expressed, compared with CD36 and ICAM-1, on C32 cells (data not shown).

In summary, we have developed an in vitro binding assay which has enabled us to define receptors involved in adhesion of gametocyte-infected erythrocytes. While we have identified similarities between gametocytes and asexual stages in host-parasite adhesion interactions, differences between these

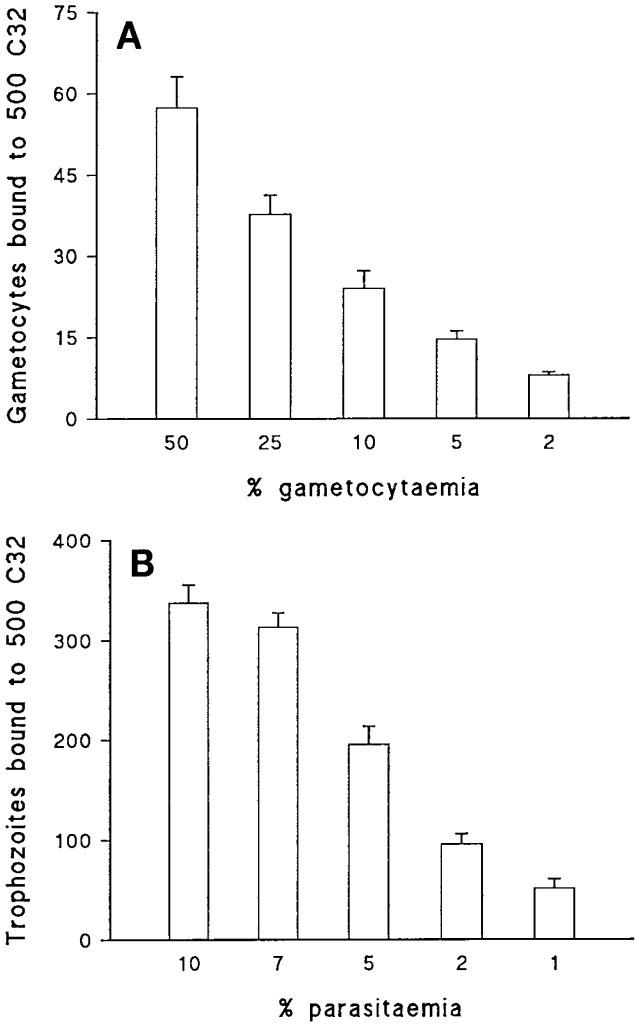

FIG. 2. Adhesion of infected erythrocytes over a range of parasitemia with gametocytes (A) and trophozoites (B). Values are means ± standard errors of the means for triplicate wells. Five hundred C32 cells were counted per well. Data are representative of three experiments.

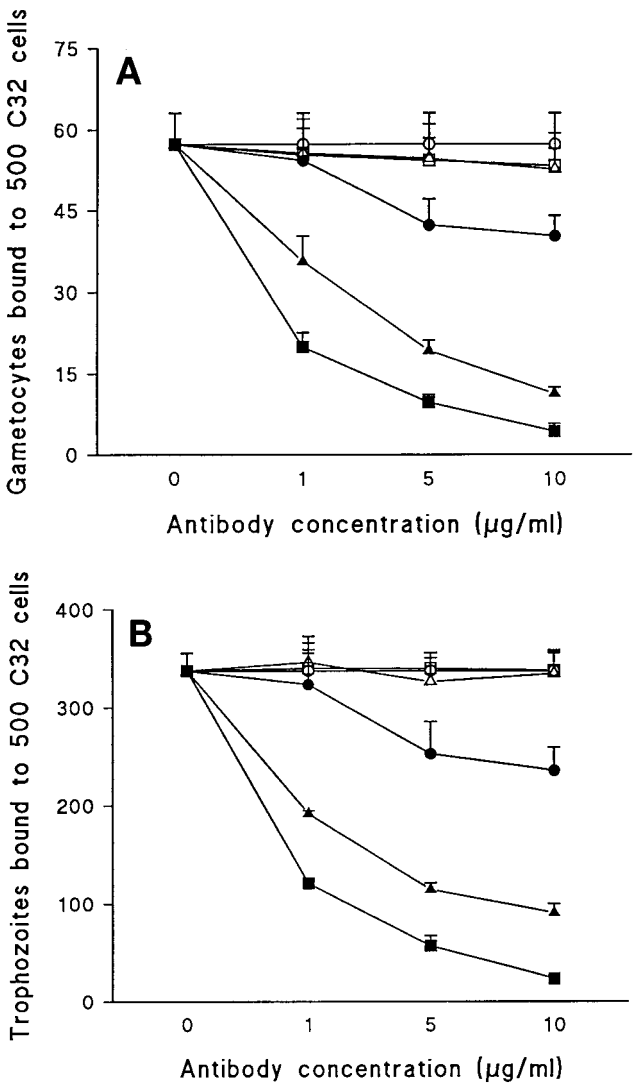

FIG. 3. Dose-dependent inhibition of gametocyte (A) and trophozoite (B) adhesion after incubation with MAb 8A6 (anti-CD36) (■), 15.2 (anti-ICAM-1) (▲), or W6/32 (anti-major histocompatibility complex class I) (●), with IgG1 (□) or IgG2a (△) isotype controls, or with medium alone (○). Asexual- and sexual-stage parasitemias added, per well, were 10 and 50%, respectively. Values are means ± standard errors of the means.

stages in adhesion properties and/or receptor expression clearly exist. Asexual parasites rupture from the host cell every 48 h, invading new erythrocytes, and they possess the ability to alter their surface receptors from generation to generation (5, 22, 27, 28). The recently cloned PfEMP-1 family of genes has been implicated in adhesion, and the study of these genes and their products may answer questions about expression and affinity of receptors. Once committed to gametocyte development, however, the parasite remains inside the erythrocyte, and no data on the ability of gametocytes to express or alter cell surface receptors are currently available. Work to investigate whether PfEMP-1 is expressed on gametocyte-infected cells and whether it mediates adhesion of the sexual stage is under way. The lower numbers of bound gametocytes compared with asexual stages may reflect the fact that only a subpopulation of gametocytes express the ligands for CD36 and ICAM-1 and are therefore capable of adhering to the C32 cell. Alternatively, gametocyte-infected erythrocytes may express ligands at a lower density or affinity for CD36 and ICAM-1 than asexual stages. Under the assay conditions we have described, it may not be possible to detect adhesion of parasites to low-affinity receptors. Less stringent washing, however, results in greater nonspecific binding of both infected and uninfected erythrocytes.

TABLE 1. CD36- and ICAM-1-dependent inhibition of parasite adhesion to C32 cells at 1 μg of antibody per ml

Antibody	% Gametocyte inhibition[a]	% Trophozoite inhibition[a]
8A6 (anti-CD36)	66.33 ± 0.62[b]	62.45 ± 1.15[b]
15.2 (anti-ICAM-1)	37.15 ± 0.96[b]	46.80 ± 2.12[b]
IgG1 (control)	0.58 ± 1.75	1.83 ± 0.74
W6/32 (anti-MHC I[c])	6.5 ± 0.64[d]	4.13 ± 0.48[d]
IgG2a (control)	3.37 ± 0.33	0.53 ± 0.27

[a] Values are means ± standard errors of the means for triplicate assays.
[b] $P < 0.05$ compared with IgG1 isotype control.
[c] MHC, major histocompatibility complex.
[d] Inhibition was not significant.

Our data do not preclude the possibility that receptors other than CD36 and ICAM-1 are involved in gametocyte adhesion. Indeed, the distinct sequestration sites of asexual and sexual stages suggest that other gametocyte-specific receptors and/or cellular targets may be involved. However, this work clearly demonstrates that later-stage gametocyte adherence shares at least some of the properties of adherence of asexual-stage parasites. The assay described will allow further characterization at the molecular level of the receptors on C32 cells and endothelial cells from various organs. Identification of the receptors vital to gametocyte adhesion may make it possible to interfere with sequestration of the immature gametocytes, thus interrupting the subsequent development and transmission of the mature sexual stages.

This work was supported by grants from the Wellcome Trust. N.J.R. is the recipient of a Wellcome Trust Studentship.

We thank Nancy Hogg and John Barnwell for providing MAbs 15.2 and 8A6, respectively, Chris Newbold for providing C32 amelanotic melanoma cells, and Martin Goodier and Paul Kaye for constructive discussion.

REFERENCES

1. **Asch, A. S., J. W. Barnwell, R. L. Silverstein, and R. L. Nachman.** 1987. Isolation of the thrombospondin membrane receptor. J. Clin. Invest. **79:** 1054–1061.
2. **Asch, A. S., I. Liu, F. M. Bricetti, J. W. Barnwell, F. Kwakye-Berka, A. Dokun, J. Goldberger, and M. Pernambuca.** 1993. Analysis of CD36 binding domains: ligand specificity controlled by dephosphorylation of an ectodomain. Science **262:**1436–1440.
3. **Barnwell, J. W.** 1989. Cytoadherence and sequestration in falciparum malaria. Exp. Parasitol. **69:**407–412.
4. **Barnwell, J. W., A. S. Asch, R. L. Nachman, M. Yamoya, M. Aikawa, and P. Ingravalla.** 1995. A human 88kd membrane glycoprotein (CD36) functions *in vitro* as a receptor for a cytoadherence ligand on *Plasmodium falciparum*-infected erythrocytes. J. Clin. Invest. **84:**765–772.
5. **Baruch, D. I., B. L. Pasloske, H. B. Singh, X. Bi, X. C. Ma, M. Feldman, T. F. Taraschi, and R. J. Howard.** 1995. Cloning the *P. falciparum* gene encoding PfEMP1, a malaria variant antigen and adherence receptor on the surface of parasitised human erythrocytes. Cell **82:**77–87.
6. **Berendt, A. R., D. J. P. Ferguson, and C. I. Newbold.** 1990. Sequestration in *Plasmodium falciparum* malaria: sticky cells and sticky problems. Parasitol. Today **6:**247–253.
7. **Berendt, A. R., A. McDowall, A. G. Craig, P. A. Bates, M. J. E. Sternberg, K. Marsh, C. I. Newbold, and N. Hogg.** 1992. The binding site on ICAM-1 for *P. falciparum*-infected erythrocytes overlaps, but is distinct from, the LFA-1 binding site. Cell **68:**71–81.
8. **Berendt, A. R., D. L. Simmons, J. Tansey, C. I. Newbold, and K. Marsh.** 1989. Intracellular adhesion molecule-1 is an endothelial cell adhesion receptor for *Plasmodium falciparum*. Nature (London) **341:**57–59.

9. **Chulay, J. D., and C. F. Ockenhouse.** 1990. Host receptors for malaria-infected erythrocytes. Am. J. Trop. Med. Hyg. **43:**6–14.
10. **Crandall, I., M. Kirkwood, and I. W. Sherman.** 1994. *P. falciparum*: Pfalhesin and CD36 form an adhesin/receptor pair that is responsible for the pH-dependent portion of cytoadherence/sequestration. Exp. Parasitol. **78:**203–209.
11. **Drasfield, I., C. Cabanas, J. Barrett, and N. Hogg.** 1992. Interaction of leucocyte integrins with ligand is necessary but not sufficient for function. J. Cell Biol. **116:**1527–1535.
12. **Garnham, P. C. C.** 1931. Observations on *Plasmodium falciparum* with special reference to the production of crescents. Kenya East Afr. Med. J. **8:**2–21.
13. **Gupta, S., and K. P. Day.** 1994. Strain theory of malaria transmission. Parasitol. Today **10:**476–480.
14. **Hasler, T., G. R. Albrecht, M. R. Van Schravendijk, 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 ICAM-1. Am. J. Trop. Med. Hyg. **48:**332–347.
15. **Hommel, M.** 1993. Amplification of cytoadherence in cerebral malaria: towards a more rational explanation of disease pathophysiology. Am. J. Trop. Med. Hyg. **87:**627–635.
16. **Iqbal, J., P. Perlmann, and K. Berzins.** 1993. Serological diversity of antigens expressed on the surface of erythrocytes infected with *Plasmodium falciparum*. Trans. R. Soc. Trop. Med. Hyg. **87:**583–588.
17. **Jensen, J. B.** 1978. Concentration from continuous culture of erythrocytes infected with schizonts and trophozoites of *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. **27:**1274–1276.
18. **Ockenhouse, C. F.** 1989. Identification of a platelet membrane glycoprotein as a falciparum malaria sequestration receptor. Science **243:**1469–1471.
19. **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 for endothelial leucocyte adhesion molecule-1. J. Exp. Med. **176:**1183–1189.
20. **Raventos-Saurez, C., D. K. Kaul, F. MaCaluso, and R. L. Nagel.** 1985. Membrane knobs are required for the microcirculatory obstruction induced by *Plasmodium falciparum*-infected erythrocytes. Proc. Natl. Acad. Sci. USA **82:**3829–3833.
21. **Riley, E. M., C. S. L. Ong, O. Olerup, S. Eida, S. J. Allen, S. Bennett, G. Andersson, and G. A. T. Targett.** 1990. Cellular and humoral immune responses to *Plasmodium falciparum* gametocyte antigens in malaria-immune individuals. J. Immunol. **144:**4810–4816.
22. **Roberts, D. J., A. G. Craig, A. R. Berendt, R. Pinches, G. Nash, K. Marsh, and C. I. Newbold.** 1992. Rapid switching to multiple antigenic phenotype in malaria. Nature (London) **357:**689–692.
23. **Roberts, D. J., J. A. Sherwood, S. L. Spitalnik, L. J. Panton, R. J. Howard, V. M. Dixit, W. A. Frazier, L. H. Miller, and V. Ginsburg.** 1985. Thrombospondin binds falciparum malaria parasitised erythrocytes and may mediate cytoadherence. Nature (London) **318:**64–66.
24. **Saul, A., P. Graves, and L. Edser.** 1990. Refractoriness of erythrocytes infected with *Plasmodium falciparum* gametocytes to lysis by sorbitol. Int. J. Parasitol. **20:**1095–1097.
25. **Smalley, M. E.** 1976. *Plasmodium falciparum* gametocytogenesis in vitro. Nature (London) **264:**271–272.
26. **Smith, H., J. A. Nelson, C. G. Gamberg, I. Crandall, and I. W. Sherman.** 1992. *Plasmodium falciparum*: cytoadherence of malaria-infected erythrocytes to human brain capillary and umbilical vein endothelial cells—a comparative study of adhesive ligands. Exp. Parasitol. **75:**269–280.
27. **Smith, J. D., C. E. Chitnis, A. G. Craig, D. J. Roberts, D. E. Hudson, D. S. Peterson, R. Pinches, C. I. Newbold, and L. H. Miller.** 1995. Switches in expression of *Plasmodium falciparum var* genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. Cell **82:** 101–110.
28. **Su, X.-Z., V. W. Heatwole, S. P. Wertheimer, F. Guinet, J. A. Herrfeldt, D. S. Peterson, J. A. Ravetch, and T. E. Wellems.** 1995. The large diverse gene family *var* encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. Cell **82:**89–100.
29. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. Science **193:**673–675.
30. **Udeinya, I. J., and C. O. Akagyeram.** 1993. Induction of adhesiveness in human endothelial cells by *Plasmodium falciparum*-infected erythrocytes. Am. J. Trop. Med. Hyg. **48:**488–495.
31. **Udeinya, I. J., P. A. Graves, R. Carter, M. Aikawa, of L. H. Miller.** 1983. *Plasmodium falciparum*: effect of time in continuous culture on binding to human endothelial cells and amelanotic melanoma cells. Exp. Parasitol. **56:** 207–214.
32. **Winograd, E., and I. W. Sherman.** 1989. Characterisation of a modified red cell membrane protein expressed on erythrocytes infected with the human malaria parasite *Plasmodium falciparum*. Possible role as a cytoadherence mediating protein. J. Cell Biol. **108:**23–30.
33. **Wright, P. S.** 1990. *Plasmodium falciparum*: a rapid assay for cytoadherence of [^{3}H]hypoxanthine labelled infected erythrocytes to human melanoma cells. Exp. Parasitol. **71:**346–349.

Editor: D. H. Howard