INFECTION AND IMMUNITY, June 2000, p. 3455–3462 Vol. 68, No. 6
0019-9567/00/$04.00+0
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A Model for Sequestration of the Transmission Stages of *Plasmodium falciparum*: Adhesion of Gametocyte-Infected Erythrocytes to Human Bone Marrow Cells

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Received 28 December 1999/Returned for modification 23 February 2000/Accepted 16 March 2000

With the aim of developing an appropriate in vitro model of the sequestration of developing *Plasmodium falciparum* sexual-stage parasites, we have investigated the cytoadherence of gametocytes to human bone marrow cells of stromal and endothelial origin. Developing stage III and IV gametocytes, but not mature stage V gametocytes, adhere to bone marrow cells in significantly higher densities than do asexual-stage parasites, although these adhesion densities are severalfold lower than those encountered in classical CD36-dependent assays of *P. falciparum* cytoadherence. This implies that developing gametocytes undergo a transition from high-avidity, CD36-mediated adhesion during stages I and II to a lower-avidity adhesion during stages III and IV. We show that this adhesion is CD36 independent, fixation sensitive, stimulated by tumor necrosis factor alpha, and dependent on divalent cations and serum components. These data suggest that gametocytes and asexual parasites utilize distinct sets of receptors for adhesion during development in their respective sequestered niches. To identify receptors for gametocyte-specific adhesion of infected erythrocytes to bone marrow cells, we tested a large panel of antibodies for the ability to inhibit cytoadherence. Our results implicate ICAM-1, CD49c, CD166, and CD164 as candidate bone marrow cell receptors for gametocyte adhesion.

Human malaria is caused by several species of protozoan parasites within the genus *Plasmodium*. The majority of malaria-related mortality is due to infection with the species *Plasmodium falciparum*. Microscopic examination of the peripheral blood of people infected with this species commonly reveals only two developmental forms: early trophozoite stages, termed "rings," and mature (stage V) gametocytes. Trophozoites are asexual forms that rapidly multiply, often causing disease, whereas gametocytes have no known association with disease but are the only form of the parasite that is infective to mosquitoes. All other stages of the parasite's development are sequestered in the deep vasculature among various host organs.

Early stage V gametocytes of *P. falciparum* appear during in vitro culture 8 to 10 days after emergence from the parent schizont (14, 22), suggesting that, in vivo, stage I to IV gametocytes spend a minimum of 7 days sequestered in host tissues. Recent observations of gametocyte emergence following antimalarial treatment of clinical malaria cases are consistent with this estimate of the duration of gametocyte sequestration (G. A. T. Targett and C. J. Drakeley, unpublished data). This is in marked contrast to the sequestration of asexual parasites, which is assumed to be in the order of 22 to 26 h in duration (9, 21). Furthermore, existing evidence from limited in vivo studies indicates that the distribution of sequestered gametocytes is measurably different from that of sequestered asexual parasites within the host. In a study of 22 Gambian children, Smalley et al. (23) found that immature gametocytes (stages II, III, and IV) had a 5-fold-higher prevalence and 10-fold-higher average density in bone marrow biopsies than in peripheral blood. Mature asexual parasites (late trophozoites and schizonts) were equally scarce in both compartments. It is of great interest to elucidate the mechanisms by which maturing gametocytes sequester during this lengthy period, as strategies aimed at reversal of the process are likely to be effective transmission-blocking interventions.

The primary mechanism mediating sequestration of the asexual-stage *P. falciparum*-infected erythrocytes (IRBC) is cytoadherence. The variant antigen PfEMP1 and other parasite-encoded ligands on the surface of the infected erythrocyte bind to receptors expressed by host endothelium in the fine vasculature of a number of organs. In the case of gametocytes, recent in vitro work from Day, Hayward, Piper, and colleagues (6, 10, 15) has demonstrated that cytoadherence of very early sexual-stage parasites to C32 melanoma cells is indistinguishable from that of their asexual counterparts by several criteria. First, the host endothelial receptor CD36 is a crucial component of the binding interaction. Second, asexual parasites and early-stage gametocytes from the same laboratory line bind to C32 cells at similar densities. Third, analysis of the expression of members of the *var* gene family and agglutination profiles with immune sera strongly suggests that the repertoire of PfEMP1 expressed by these very young gametocytes is similar or identical to the repertoire of asexual parasites from the same genotype or isolate. Fourth, electron microscopic observation of early gametocytes shows that they have knobs on the infected erythrocyte surface. These are macromolecular com-

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plexes that are strongly implicated in stabilizing and enhancing CD36-dependent cytoadherence, especially under shear flow conditions (5).

An important finding of these studies is that the binding interaction between early gametocytes and CD36 does not continue through to maturity. From stage IIb on, knobs are not visible in electronmicrographs, the pattern of PfEMP1 expression changes, and the numbers of gametocytes binding to CD36, as measured by in vitro depletion assays, plummets (6, 10). There is thus an apparent cessation of asexual-type cytoadherence quite early in gametocyte development. What, then, are the processes that mediate sequestration of stage IIb, III, and IV gametocytes over the several days that must elapse prior to their release into the peripheral circulation? Previous studies in our laboratory clearly indicate that stage III and IV gametocytes do bind to both CD36 and the adhesion molecule ICAM-1 expressed on the surfaces of cultured C32 melanoma cells in vitro (18, 19). Modified band 3 was implicated as a parasite-induced ligand in the binding of gametocytes to CD36 but not to ICAM-1 (19). These results appear to be at odds with those of Day et al., which may be because the results produced by the two assay methods are not comparable (6). Taken together, these studies show that (i) adhesion of very early stage gametocytes (I and IIa) is virtually indistinguishable from that of asexual parasites and (ii) adhesion of later immature gametocytes (IIb, III, and IV) is quantitatively and qualitatively distinct from the cytoadherence of erythrocytes infected with asexual-stage parasites. This would imply that the C32 melanoma cell is a poor model system for investigating the complete sequestered development of gametocytes and that more appropriate models of sexual-stage adhesion are needed.

The observations of shared usage of receptors such as CD36 and ICAM-1 cannot explain reported differences in sequestration sites between asexual- and sexual-stage parasites in vivo (23). In view of the reported role of bone marrow as a site of gametocyte sequestration in vivo, we have been investigating the binding of later immature gametocytes (stages III and IV) to cultured cells derived from human bone marrow. Using the stromal-cell line L88/5 (24), we have recently found CD36-independent cytoadherence of *P. falciparum* gametocytes at a level measurably higher than that of asexual parasites (unpublished data). In this report, we significantly extend these studies using transformed human bone marrow endothelial cells (trHBMEC). The results demonstrate that *P. falciparum* gametocytes adhere to human bone marrow cells in a manner that is fixation sensitive, CD36 and modified band 3 independent, stimulated by tumor necrosis factor alpha (TNF-α), and inhibited by four novel monoclonal antibodies (MAbs) and by anti-ICAM-1 antibodies. We identify four host receptors implicated in gametocyte cytoadherence and consider the parasite ligands that may be binding to them.

MATERIALS AND METHODS

Parasite culture and purification. The 3D7 strain of *P. falciparum* was routinely cultured in vitro according to established protocols (26). Gametocytes from both semiautomatic continuous cultures (16) and standard flask cultures were enriched by one of two methods. For the majority of experiments, sexual-stage parasites were obtained from the 70%–45% interface of a discontinuous Percoll gradient formed as described by Riley et al. (16). For those experiments in which depletion assays were used to enumerate gametocyte binding, sexual-stage parasites were obtained from the 30%–45% and 45%–54% interfaces of a Percoll gradient formed as described by Day et al. (6). Mature asexual-stage parasites (trophozoites and schizonts) were enriched by flotation on Plasmagel (Laboratoire Roger Bellon, Neuilly, France) (13).

Cell culture. The simian virus 40-transformed human bone marrow-derived L88/5 stromal cell line was maintained in RPMI 1640 medium–2 mM L-glutamine–10% fetal calf serum (FCS) (Life Technologies, Inc., Paisley, United Kingdom) under 5% CO_2. For experiments requiring fixed cells, fixation was performed in 1% formaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature prior to binding.

trHBMEC (20) were the kind gift of Babette Weksler. The cells were maintained on surfaces coated with gelatin (1%) as previously described (8) in a culture medium consisting of Dulbecco's modified Eagle's medium–5% FCS–10 mM HEPES–100 U of penicillin ml^{-1}–100 μg of streptomycin ml^{-1}–3 mM glutamine plus vitamins (all components were from Life Technologies Inc.) under 5% CO_2.

Cytoadherence assays. Adhesion assays were performed in either 5-cm-diameter plastic petri dishes or 16-well Lab-Tek chamber slides (Nunc, Naperville, Illinois) using previously described protocols (18). Bound parasites were stained with Giemsa stain and counted in triplicate wells for each experiment; all data shown are mean values estimated from these counts. Each experiment was repeated at least three times.

Depletion assays with L88/5 cells were performed in 5-cm-diameter petri dishes essentially as described by Day et al. (6), and all volumes used in the assay were adjusted accordingly. Washing was performed at a lower stringency than described (6): the plates were rinsed three times for several minutes each time in binding medium without serum (i.e., RPMI 1640 plus 0.2% glucose) at 37°C with gentle agitation.

Antibodies and reagents. All antibodies employed in this study have been previously described by us (18, 19) except MAbs obtained from the Leucocyte Differentiation Antibody (LDA) Workshop, kindly donated by David Katz. The identities of this series were not known to the investigator; 52 MAbs with designations between A1 and A130 were used for the experimental work. LDA hybridoma clone names for those MAbs that bound to trHBMEC and/or L88/5 cells, and their specificities where known, are given (see Table 2) using information available from the LDA website (Human Leukocyte Differentiation Antigens Database [http://www.mh-hannover.de/projekte/hlda7/hldabase/select.htm]). All other reagents were obtained from the Sigma Chemical Co. unless otherwise stated.

Antibody inhibition studies. Fixed or unfixed trHBMEC or L88/5 cells were preincubated at 37°C for 60 min with various antibodies in a 50-μl volume, as previously described (18). Optimal antibody concentrations were independently determined for each reagent (data not shown).

Cytokine stimulation of cells prior to binding assays. Cells were stimulated in vitro with human TNF-α or interleukin-1β (IL-1β) at a concentration of 10 to 1,000 U ml^{-1}. Cytokines were added to the culture medium, and the cells were cultured for 4, 24, or 48 h.

Surface fluorescence by flow cytometry. Cells (2.5×10^5) were added to 6-ml Falcon tubes (Becton Dickinson), and nonspecific binding sites were blocked with 10% heat-inactivated FCS in fluorescence-activated cell sorter (FACS) buffer (PBS–1% FCS–1% sodium azide). The cells were blocked for 30 min at 4°C. After being washed with 2 ml of FACS buffer at $300 \times g$ for 10 min, the cells were stained with the relevant primary antibody for 45 min on ice and then washed as described previously. Specific-antibody binding was then detected with fluorescein-conjugated goat $F(ab)_2$ fragment to mouse immunoglobulin G (IgG) (Cappel). The cells were washed as described above and fixed for 10 min with 1% paraformaldehyde–PBS. Detection of receptor expression was done with a FACScan (Becton Dickinson) with LYSIS II software. Five thousand cells were run per sample. The data shown are representative of at least three experiments with similar results.

Modulation of cell surface receptor function by divalent cations. To test for the effects of divalent-ion concentrations, 5 mM EDTA and/or 5 mM EGTA was added to the binding medium and was present throughout the course of the adhesion assay. The cells were preblocked and washed as previously described.

RESULTS

Cytoadherence of gametocytes and asexual parasites to trHBMEC. *P. falciparum* gametocytes (stages III and IV) exhibit a significant level of binding to trHBMEC by our cytoadherence protocol (Fig. 1). Giemsa-stained preparations clearly show stage III and IV gametocytes bound to trHBMEC (Fig. 2). Stage V gametocytes are not seen to bind. The observed gametocyte adhesion is at a higher level than that mediated by either C32 melanoma cells or L88/5 stromal cells and is highly sensitive to prior formaldehyde fixation (Fig. 1). Erythrocytes infected with asexual parasites also bind to trHBMEC, but the level of this adhesion is two- to threefold lower than that we have observed for gametocytes and is not fixation sensitive (Fig. 1), strongly suggesting that distinct receptor-ligand interactions participate in the binding of parasites at these two stages of development. Having shown that gametocyte binding to trHBMEC is fixation sensitive, we used unfixed trHBMEC for all subsequent experiments.

Depletion assays were performed in an attempt to quantify

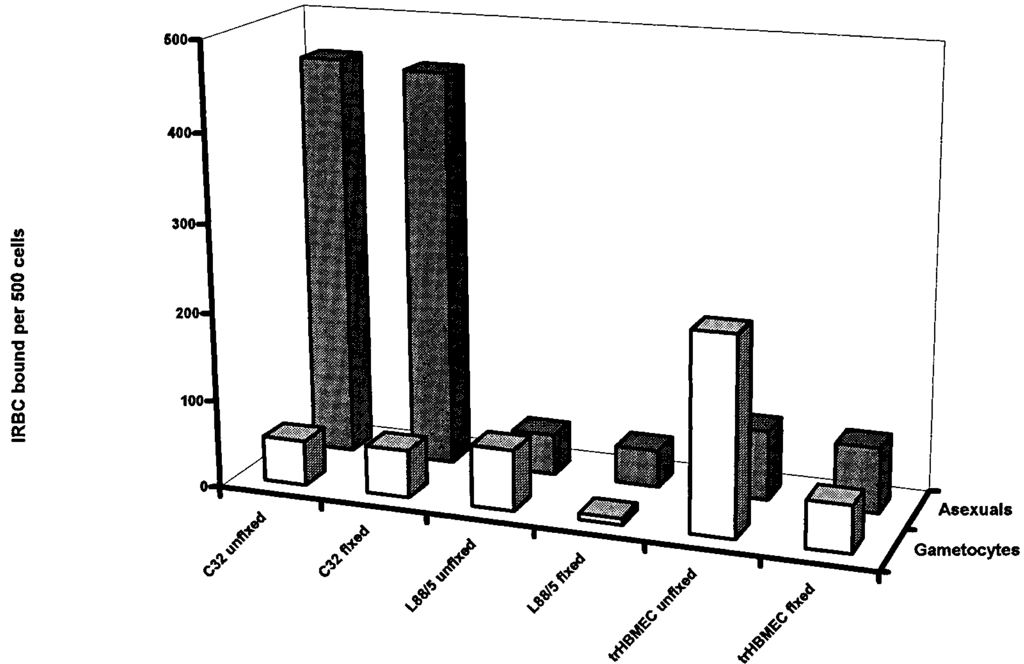

FIG. 1. Fixation sensitivity of adhesion by IRBC harboring either stage III and IV gametocytes or asexual-stage parasites to three different cell lines. The results of a representative set of adhesion assays performed on the same day with a single preparation of gametocyte-infected erythrocytes and a single preparation of asexual-stage-parasite-infected erythrocytes are shown. The IRBC numbers represent the means of triplicate wells in a single experiment. In each case, the standard error was less than 10% of the calculated mean value (not shown).

gametocyte binding to L88/5 bone marrow cells in a way that would permit direct comparison with the data of Day et al. (6). Gametocyte binding was observed at the densities observed for L88/5 cells (unpublished data). Duplicate, blinded, and stage-specific gametocyte counts by two different microscopists before and after binding to immobilized L88/5 cells failed to demonstrate significant depletion of any gametocyte stage (data not shown). To explain these results, we calculated the percentage of sexual-stage parasites binding to both L88/5 and trHBMEC at the gametocytemia used in our assays, and the

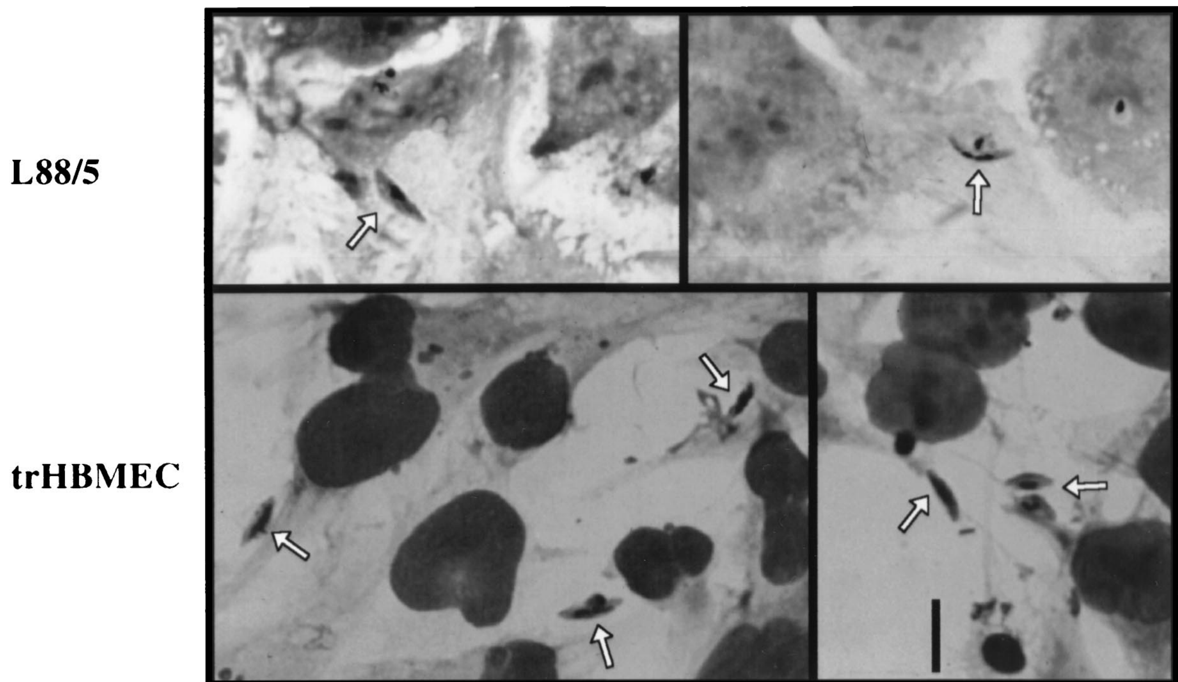

FIG. 2. Adhesion of developing gametocytes to cultured human bone marrow cells. Developmental stage III and IV gametocytes (arrows) bound to L88/5 stromal cells (upper) and trHBMEC (lower) are shown. Bar (lower right), 9 μm.

TABLE 1. Expected depletion of gametocytes caused by adhesion to immobilized bone marrow cells

Cell line	No. of gametocytes binding per 500 cells[a]	No. of gametocytes added	Expected gametocyte depletion (%)	Source
L88/5	79	1.7×10^6	0.0004	Unpublished data
trHBMEC	243	1.7×10^6	0.0014	This study
C32	56	1.7×10^6	0.003	18

[a] The average number of bone marrow cells per well is about 5,000.

estimated depletion expected (Table 1). These calculations show that the frequency of binding to either of these bone marrow cell lines is not sufficient to produce an observable depletion of stage III and IV gametocytes.

Physiological requirements of gametocyte cytoadherence to trHBMEC. The binding of gametocytes to bone marrow endothelial cells was further characterized in cytoadherence assays performed with trHBMEC and *P. falciparum* gametocytes at 50% parasitemia and 10% hematocrit under different conditions. These experiments demonstrated that binding requires human serum and is partly ablated if heat-inactivated serum is used. Replacing human serum with fetal bovine serum drastically reduced binding, and no measurable binding occurred under serum-free conditions (Fig. 3a). Furthermore, the addition of either 5 mM EDTA or 5 mM EGTA significantly inhibited binding—inhibition was enhanced when both were added together, each at 5 mM (Fig. 3b), indicating that under these assay conditions the chelators are not saturating at 5 mM. The data suggest that other divalent cations in addition to calcium contribute to the binding interaction, as EGTA alone only partially ablates adhesion. Gametocyte adhesion to trHBMEC is therefore dependent on human serum components and requires the presence of divalent cations.

Characterization of receptors on the surfaces of trHBMEC: TNF-α stimulation. Pretreatment of trHBMEC cells with the cytokine TNF-α significantly increased the density of gametocyte binding, whereas pretreatment with IL-1β had no effect (Fig. 4). The effect of TNF-α was maximal at a concentration of 1,000 U/ml and increased with the duration of pretreatment, peaking at 48 h (data not shown). Previous studies have shown that ICAM-1 plays a role in gametocyte adhesion (18), and TNF-α is a known regulator of ICAM-1 expression. We therefore investigated the possibility that ICAM-1 might be one receptor for gametocytes on trHBMEC.

Coincubation with an anti-ICAM-1 MAb partially ablated TNF-α-stimulatable cytoadherence (Fig. 4), whereas an anti-major histocompatibility complex (MHC) class 1 MAb and IgG1 and IgG2a isotype controls had no effect on binding (data not shown). This suggests that TNF-α stimulates adhesion by up-regulation of surface ICAM-1 expression and that ICAM-1 on trHBMEC is a receptor for gametocytes. This is also the case for L88/5 stromal cells (unpublished data). Nevertheless, saturating levels of anti-ICAM-1 MAb are unable to ablate adhesion completely with either model, so ICAM-1 is probably only one of a number of receptors which participate in the interaction between gametocytes and the surfaces of bone marrow cells. Furthermore, ICAM-1-mediated cytoadherence is not fixation sensitive (18) and therefore is unlikely to be the primary mechanism of gametocyte-specific adhesion to bone marrow cells in vitro. Adhesion to CD36 via modified band 3 can also be ruled out as a major mechanism because this, too, is insensitive to fixation (19) and because an anti-CD36 MAb is unable to inhibit gametocyte binding (see below) (Fig. 5).

Characterization of receptors on the surfaces of trHBMEC: inhibition with MAbs. Our results suggest that previously identified receptors for IRBC cannot fully account for the binding of gametocytes to trHBMEC. We therefore screened for novel receptors using a panel of 47 MAbs from the LDA Workshop. Exhaustive gametocyte adhesion inhibition assays with all 47 antibodies identified a number that inhibited gametocyte binding to bone marrow cells (Fig. 5). Subsequent flow cytometric analysis with each antibody provided an extensive profile of receptor expression on the surfaces of trHBMEC and L88/5 cells (Table 2). Of the four antibodies that inhibited gameto-

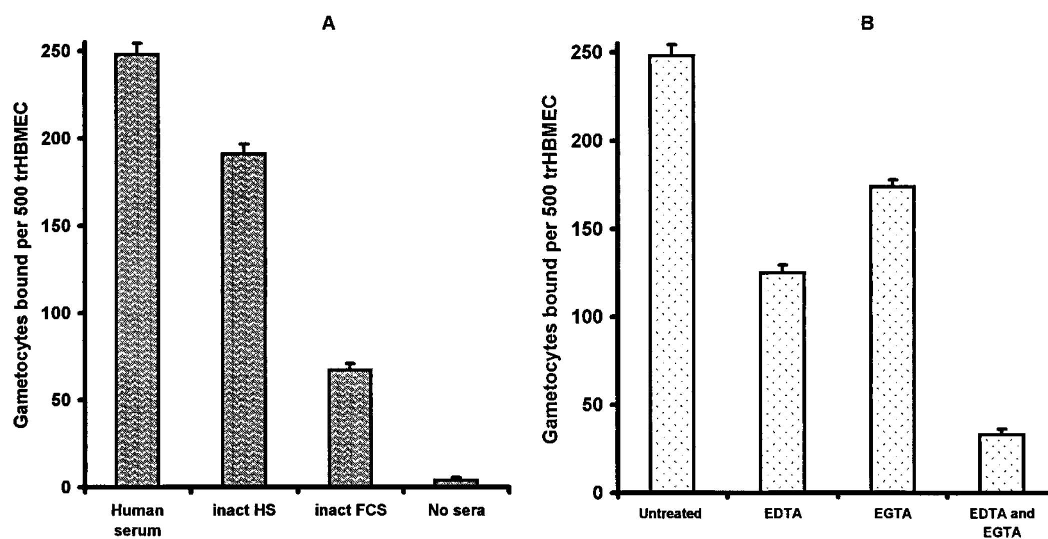

FIG. 3. Adhesion of *P. falciparum* gametocytes to trHBMEC is dependent on serum and divalent cations. (A) Gametocytes were incubated at 50% parasitemia and 10% hematocrit with trHBMEC in the presence of different sera at a concentration of 1% (vol/vol) in binding medium. HS, human serum; inact., heat inactivation of serum at 56°C for 30 min. (B) Gametocytes were incubated at 50% parasitemia and 10% hematocrit with trHBMEC in the presence of 5 mM EDTA, 5 mM EGTA, 5 mM EDTA plus 5 mM EGTA, or medium alone. The gametocyte numbers are the means of triplicate wells in a single experiment, and the error bars represent standard errors of the mean.

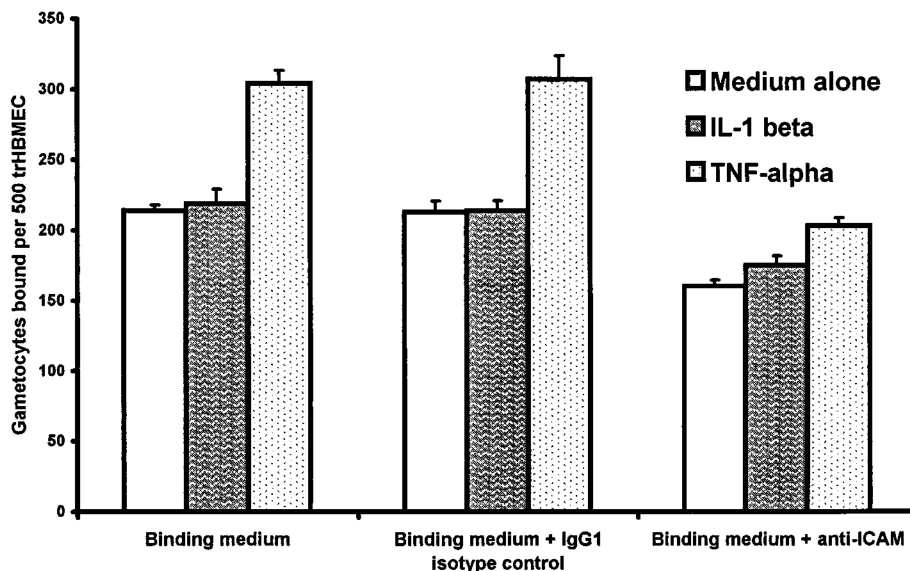

FIG. 4. Adhesion of *P. falciparum* gametocytes to trHBMEC stimulated by IL-1β and TNF-α. Adhesion of gametocytes following pretreatment of trHBMEC with IL-1β (1,000 U/ml) or TNF-α (1,000 U/ml) compared to medium alone in the presence and absence of an anti-ICAM-1 MAb is shown. The gametocyte numbers are the means of triplicate wells in a single experiment, and the error bars represent standard errors of the mean.

cyte adhesion, three of them (A002, A030, and A124) detected expression of the target antigen on one or both of the bone marrow cell lines (Table 2). The fourth, A115, is specific for the same surface molecule as A124.

Three of the LDA Workshop MAbs, A002, A030, and A115, significantly inhibited adhesion of gametocytes to both L88/5 cells and trHBMEC compared to binding medium alone, anti-MHC class I, anti-ICAM-1, anti-CD36, and anti-IgG controls (Fig. 5). A fourth MAb, A124, inhibited gametocyte adhesion to trHBMEC but not to L88/5 cells. Dose-response experiments with MAbs A002 and A030 clearly demonstrated that inhibition of binding to both cell types was dose dependent (data not shown). A002 and A030, but not A124, also recognize the surfaces of L88/5 stromal cells. None of these MAbs inhibited binding of erythrocytes infected with asexual-stage parasites to either cell line. In contrast, an anti-ICAM1 MAb

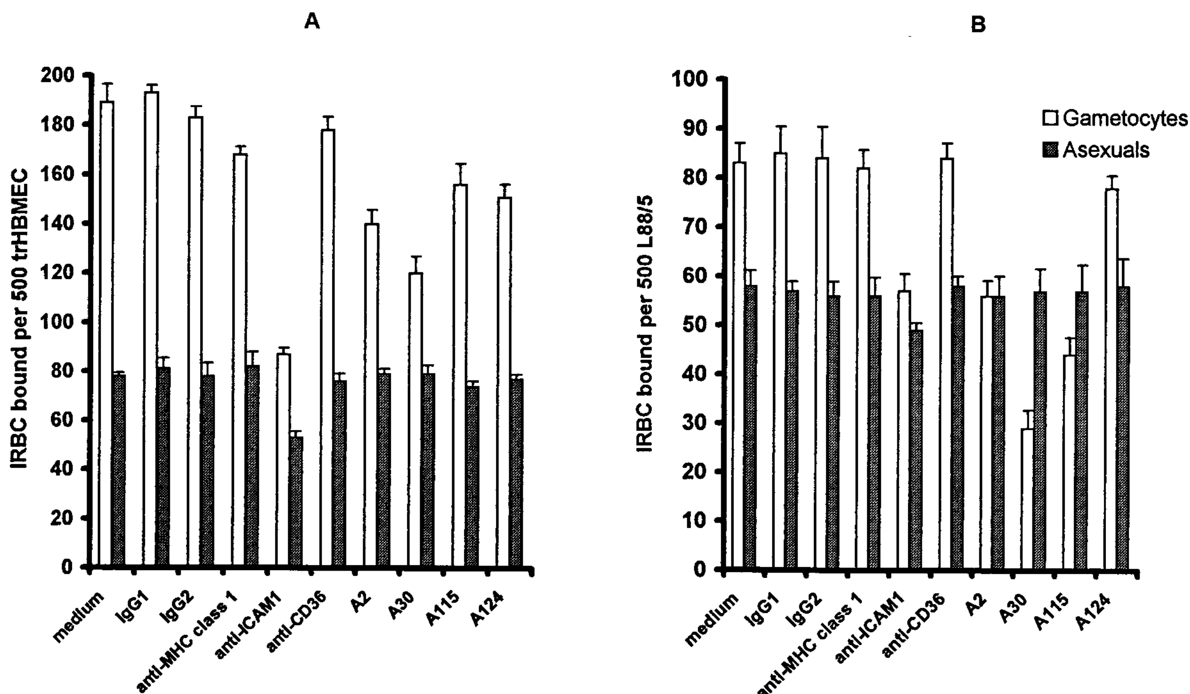

FIG. 5. Inhibition of adhesion of *P. falciparum* gametocytes and asexual-stage parasites to bone marrow cells by pretreatment with MAbs from the LDA panel, anti-MHC class I, anti-CD36, anti-ICAM-1, and isotype controls. (A) Adhesion of gametocytes and asexual-stage parasites following pretreatment of trHBMEC with the antibodies shown. (B) Adhesion of gametocytes and asexual-stage parasites following pretreatment of L88/5 cells with the antibodies shown. The gametocyte numbers are the means of triplicate wells in a single experiment, and the error bars represent standard errors of the mean.

TABLE 2. MAbs obtained from the LDA Workshop: reactivity with the surface of bone marrow cells and effect on adhesion of gametocyte-infected erythrocytes[a]

Antibody	Clone	Type	Specificity[b]	Characteristics[c]			
				L88/5		trHBMEC	
				Effect on adhesion	Detection on surface	Effect on adhesion	Detection on surface
A002	ASC-1	Mouse IgG1	CD49c	++	+	+	+
A004	ASC-2	Mouse IgG1	NK	−	−	−	+
A006	ASC-5	Mouse IgG1	CD49c	−	+	−	+
A007	ASC-6	Mouse IgG1	CD49c	−	+	−	+
A008	ASC-8	Mouse IgG1	CD104	−	−	−	+
A009	ASC-9	Mouse IgG2a	CD104	−	−	−	+
A010	ASC-10	Mouse IgG1	CD49c	−	+	−	+
A013	Lia1/2	Mouse IgG1	CD29	−	+	−	+
A025	F430C-5	Mouse IgG2a	CD105	−	+	−	+
A029	J3-119	Mouse IgG2a	CD166	−	+	−	+
A030	J4-81	Mouse IgG2a	CD166	+++	+	++	+
A047	MEM63	Mouse IgG1	CD58	−	+	−	+
A091	VC5	Mouse IgG1	CD49e	−	+	−	+
A097	B-D12	Mouse IgG1	CD71	−	+	−	+
A101	4A11	Mouse IgM	NK	−	−	−	+
A102	ASC-11	Mouse IgG1	NK	−	−	−	+
A105	CJ1250	Mouse IgM	NK	−	+	−	+
A107	CNA42	Mouse IgM	NK	−	−	−	+
A108	AD2	Mouse IgG1	CD165	−	+	−	+
A109	3A6	Mouse IgG1	CD166	−	+	−	+
A111	F4-35H7	Mouse IgG1	CD146	−	+	−	+
A112	F432G3	Mouse IgG1	CD146	−	+	−	+
A113	F439E	Mouse IgG1	CD146	−	+	−	+
A115	105A5	Mouse IgM	CD164	++	−	+	−
A117	J1-G3B	Mouse IgG1	NK	−	+	−	+
A119	J3-E1B	Mouse IgG1	CD98	−	+	−	+
A120	H8E3	Mouse IgG3	NK	−	+	−	+
A124	9E10	Mouse IgG3	CD164	−	−	+	+
A126	RMcB	Mouse IgG2b	NK	−	−	−	+

[a] All antibody data are from the LDA website (http://www.mh-hannover.de/projekte/hlda7/hldabase/select.htm).
[b] NK, not known.
[c] +, 15 to 30% inhibition of adhesion/positive reaction with cell surface; ++, 30 to 50% inhibition of adhesion; +++, >50% inhibition of adhesion; −, no effect on adhesion/not detected on cell surface.

inhibited adhesion of both asexual-stage-parasite- and gametocyte-infected erythrocytes to trHBMEC and L88/5 cells (Fig. 5), whereas an anti-CD36 MAb had no effect upon the adhesion of parasites at either stage. This is consistent with our failure to detect CD36 on the cell surface of either bone marrow cell line by flow cytometry (data not shown).

DISCUSSION

The mechanism of sequestration of erythrocytes infected with asexual-stage parasites of *P. falciparum* has been extensively studied, and a number of in vitro models of cytoadherence have been developed using these stages. Much less is known of the cytoadherent properties of developing sexual-stage parasites, although it is known that the spleen and bone marrow are both sites of sequestration in vivo (7, 25). Developing asexual trophozoites and schizonts are also found sequestered in the microvasculature of the spleen, but bone marrow appears to be a site favored only by developing gametocytes (23). Ours is the first study of gametocyte adhesion to cells derived from this tissue and the first to indicate the existence of gametocyte-specific receptors on host cells.

Our results demonstrate that erythrocytes harboring developing *P. falciparum* gametocytes selectively bind to human bone marrow cell lines of both stromal and endothelial origin. This adhesion is fixation sensitive and independent of both CD36 and modified band 3. Adhesion is stimulated by prior treatment of cells with TNF-α, is partially inhibited by anti-ICAM-1 antibodies, and is also partially inhibited by three novel MAbs obtained from the LDA Workshop. This adhesion is therefore qualitatively distinct from that observed between C32 melanoma cells and erythrocytes harboring either asexual parasites or gametocytes (6, 18, 19). In addition, there is a marked quantitative difference between these two in vitro models of cytoadherence. The density with which gametocytes bind to bone marrow cells in our assay system (0.1 to 0.5 per cell) is 2- to 10-fold lower than the density with which asexual parasites bind to formaldehyde-fixed C32 cells in our hands (0.8 to 1 per cell) at the same starting parasitemia. Furthermore, it is up to 30-fold lower than the binding densities reported by Day et al. (6) using unfixed C32 cells (1.2 to 3.2 per cell). According to our calculations, the frequency of binding of gametocytes to our bone marrow cell lines is not sufficient to produce an observable depletion effect (Table 1). Therefore, adhesion of stage III and IV gametocytes to two different human bone marrow cell lines, as measured in our assay system, is of a markedly lower avidity than the CD36-mediated knob-dependent adhesion of early-developing gametocytes to C32 melanoma cells (6). Furthermore, we conclude from our data that gametocyte adhesion to bone marrow is mediated by a set of receptors distinct from those involved in CD36-mediated adhesion of gametocytes to C32 melanoma cells.

ICAM-1 is a major receptor on C32 cells for the adhesion of erythrocytes infected with both asexual parasites and gameto-

cytes. Inhibition studies with an anti-ICAM-1 MAb both with and without TNF-α stimulation demonstrate that ICAM-1 has a role in the adhesion we have observed. However, ICAM-1 is unlikely to be the receptor mediating gametocyte-specific adhesion to either L88/5 or trHBMEC, as this molecule appears to be a receptor for the observed low-level asexual-parasite adhesion to both cell lines (Fig. 5). Furthermore, adhesion of gametocyte-infected erythrocytes to ICAM-1 is not fixation sensitive (18), whereas prior fixation of either L88/5 cells or trHBMEC greatly reduces the level of gametocyte adhesion but not asexual adhesion, as illustrated in Fig. 1. The avidity of asexual-parasite adhesion to ICAM-1 differs from adhesion to CD36 in that it is a rolling rather than a stationary interaction (3). The low avidity of gametocyte binding to bone marrow cells that we have observed is therefore consistent with both the involvement of ICAM-1 and the demonstrated absence of CD36.

Inhibition studies with a blind panel of MAbs from the LDA Workshop identified four MAbs which significantly inhibited gametocyte adhesion to trHBMEC (Table 2). Three of these also inhibited gametocyte binding to L88/5 cells. These reagents and their specificities are as follows: A002 (ASC-1), which recognizes CD49c, or VLA-3 ($\alpha_3\beta_1$), a member of the β-1 integrin receptor family; A030 (J4-81), which recognizes CD166, or activated-leukocyte cell adhesion molecule, a member of the immunoglobulin superfamily; A115 (105A5) and A124 (103B2/9E10), both of which recognize CD164, or MGC-24, a heavily glycosylated sialomucin (Human Leukocyte Differentiation Antigens Database [http://www.mh-hannover.de/projekte/hlda7/hldabase/select.htm]). Our results suggest that these three molecules are candidate receptors for *P. falciparum* gametocyte adhesion but not for adhesion of asexual parasites, and each warrants further investigation.

CD49c is heavily implicated in cell-cell and cell-matrix adhesion interactions in a variety of tissues (1) and is thought to bind laminin, fibronectin, and collagen; CD49c expression is stimulated if cells are grown on fibronectin (11). As this and other β-1 integrins are widely distributed among tissues and cell lineages, it cannot directly contribute to the bone marrow specificity of gametocyte adhesion. Nevertheless, in this study the A002 antibody inhibited adhesion of gametocytes, but not asexual parasites, to L88/5 and also blocked gametocyte adhesion to trHBMEC. Integrins in general are known to be functionally modulated by divalent cations and are upregulated in the presence of a fibronectin substratum (11). We have shown that divalent cations are required for maximal gametocyte adhesion to both L88/5 cells and trHBMEC. Gametocyte adhesion experiments with trHBMEC grown with and without a fibronectin substrate may provide further evidence of integrin involvement in gametocyte binding. Interestingly, a total of four MAbs that bind CD49c recognized the surfaces of both L88/5 cells and trHBMEC, but only one of these inhibited gametocyte adhesion (Table 2). This is suggestive of a specific receptor-ligand interaction that is only inhibited by those antibodies that bind to the receptor in such a way that this interaction is hampered.

CD166 is a ligand for the lymphocyte antigen CD6, a molecule thought to have an important role in regulating the immune response, and T-cell development in particular (2). Although expression of CD166 (referred to in some studies as HCA) has been demonstrated in a number of human and other vertebrate cell lineages, Cortes et al. (4) have investigated its expression in human bone marrow, particularly the stroma. These authors report expression of this molecule on a number of marrow-derived cells, including the L88/5 stromal line. Its major function is thought to be in adhesion of $CD6^+$ hemopoietic progenitor cells (2, 4). As seen for CD49c, only one of the three anti-CD166 MAbs that recognize the bone marrow cell surface is able to disrupt gametocyte adhesion, suggesting a specific receptor-ligand interaction.

Finally, CD164 is an adhesive glycoprotein expressed by hematopoietic progenitors and bone marrow stroma (27, 28). It includes 16 potential sites for O-linked glycan attachment, 9 possible N-linked glycosylation sites, and a putative glycosaminoglycan attachment site (28). In view of the high level of glycosylation of CD164, it is intriguing that neuraminidase treatment of L88/5 cells prior to addition of *P. falciparum* gametocytes causes a measurable inhibition of adhesion (17). Both of the MAbs recognizing this protein, A115 and A124, inhibited gametocyte adhesion to both L88/5 cells and trHBMEC. However, in flow cytometry experiments, the only positive identification of this protein on the cell surface was with A124 on trHBMEC (Table 2). Therefore, CD164 is probably scarce on the cell surface. In view of the inhibition of gametocyte binding caused by pretreatment of trHBMEC with A124 and A115, and the presence of CD164 on the surfaces of these cells as indicated by flow cytometry with A124, we plan to study the effect of neuraminidase on gametocyte adhesion to trHBMEC.

This work demonstrates that adhesion of *P. falciparum* gametocytes, like that of erythrocytes infected with asexual parasites, involves the simultaneous use of multiple host receptors. The identity of the gametocyte-expressed IRBC ligand(s) that binds to these receptors remains uncertain. At present, there is little compelling evidence that variable antigens are expressed on the surfaces of later-developing gametocytes (10, 15). We have recently demonstrated transcription of a member of the *clag* multigene family in gametocytes isolated from the peripheral blood of Gambian children (C. J. Sutherland, unpublished data), and at least one member of this gene family is strongly implicated in cytoadherence to CD36 (12). We are also investigating expression of members of the *rif/stevor* multigene family in developing cultured gametocytes and in mature gametocytes isolated from the peripheral blood of Gambian children (Sutherland, unpublished). Our inability to completely block gametocyte adhesion to bone marrow cells with any single MAb suggests that a number of different ligand-receptor pairs are involved in gametocyte sequestration in the bone marrow. A combinatorial approach to antibody-mediated blocking studies may be needed for complete inhibition of binding, as we have previously shown in the case of gametocyte adhesion to C32 melanoma cells (19).

What is the relevance of our findings to in vivo sequestration of *P. falciparum* sexual stages and the transmission of malaria from human to mosquito? The adhesion we have observed is of a much lower avidity than the CD36-mediated adhesion implicated in the sequestration of erythrocytes harboring asexual parasites. This suggests that it would not be binding at sufficient strength to anchor or immobilize gametocytes under the shear flow conditions characteristic of the microvasculature. Furthermore, the absence of knobs on gametocyte-infected erythrocytes after stage II (6) is further evidence that they are unable to participate in the high-avidity adhesive interactions of either asexual- or early-sexual-stage parasites. The bone marrow, however, is permeable to erythrocytes, and therefore gametocytes are likely to encounter the surface receptors of marrow endothelial and stromal cells in the absence of significant shear flow forces. This may also be the case in the splenic pulp, another site of gametocyte sequestration (7). We postulate that low- to moderate-avidity interactions between unidentified ligands on the surface of the gametocyte-infected erythrocyte and a variety of receptors on bone marrow cells mediate

sequestration of *P. falciparum* sexual-stage parasites. Among these receptors are ICAM-1 and probably one or more among CD49c, CD166, and CD164. Upon maturation of a gametocyte, its period of sequestration ends and it is released into the periphery, where it becomes accessible to biting *Anopheles*. Interventions which interrupt this period of sequestered maturation may compromise the infectivity of the gametocyte and therefore contribute to a reduction in malaria transmission.

ACKNOWLEDGMENTS

This work was supported by a Wellcome Trust studentship to N.J.R. and by a Wellcome Trust program grant to G.A.T.T.

We thank Babette Weksler for kindly providing trHBMEC and for useful discussions and David Katz for providing MAbs from the LDA Workshop.

REFERENCES

1. **Bosman, F. T.** 1993. Integrins: cell adhesives and modulators of cell function. Histochem. J. **25:**469–477.
2. **Bowen, M. A., J. Bajorath, M. D'Egidio, G. S. Whitney, D. Palmer, J. Kobarg, G. C. Starling, A. W. Siadak, and A. Aruffo.** 1997. Characterisation of mouse ALCAM (CD166): the CD6-binding domain is conserved in different homologs and mediates cross-species binding. Eur. J. Immunol. **27:** 1469–1478.
3. **Cooke, B. M., and R. L. Coppel.** 1995. Cytoadhesion and falciparum malaria: going with the flow. Parasitol. Today **11:**282–287.
4. **Cortes, F., F. Deschaseaux, N. Uchida, M.-C. Labastie, A. M. Friera, D. He, P. Charbord, and B. Peault.** 1999. HCA, an immunoglobulin-like adhesion molecule present on the earliest human hematopoietic precursor cells, is also expressed by stromal cells in blood-forming tissues. Blood **93:**826–837.
5. **Crabb, B. S., B. M. Cooke, J. C. Reeder, R. F. Waller, S. R. Caruana, K. M. Davern, M. E. Wickham, G. V. Brown, R. L. Coppel, and A. F. Cowman.** 1997. Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. Cell **89:**287–296.
6. **Day, K. P., R. E. Hayward, D. Smith, and J. G. Culvenor.** 1998. CD36-dependent adhesion and knob expression of the transmission stages of *Plasmodium falciparum* is stage-specific. Mol. Biochem. Parasitol. **93:**167–177.
7. **Garnham, P. C. C.** 1931. Observations on *Plasmodium falciparum* with special reference to the production of crescents. Kenya E. Africa Med. J. **8:**2–21.
8. **Gaugler, M.-H., C. Squiban, M. Claraz, K. Schweitzer, B. Weksler, P. Gourmelon, and A. van der Meeren.** 1998. Characterisation of the response of human bone marrow endothelial cells to *in vitro* irradiation. Br. J. Haematol. **103:**980–989.
9. **Gravenor, M. B., M. B. van Hensbroek, and D. Kwiatkowski.** 1998. Estimating sequestered parasite population dynamics in cerebral malaria. Proc. Natl. Acad. Sci. USA **95:**7620–7624.
10. **Hayward, R. E., B. Tiwari, K. P. Piper, D. I. Baruch, and K. P. Day.** 1999. Virulence and transmission success of the malarial parasite *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA **96:**4563–4568.
11. **Hemler, M. E.** 1990. VLA proteins in the integrin family: structures, functions, and their role on leukocytes. Annu. Rev. Immunol. **8:**365–400.
12. **Holt, D. C., D. L. Gardiner, E. A. Thomas, M. Mayo, P. F. Bourke, C. J. Sutherland, R. Carter, G. Myers, D. J. Kemp, and K. R. Trenholme.** 1999. The cytoadherence-linked asexual gene family of *Plasmodium falciparum*: are there roles other than cytoadherence? Int. J. Parasitol. **29:**939–944.
13. **Jensen, J. B.** Concentration from continuous culture of erythrocytes infected with trophozoites and schizonts of *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. **27:**1274–1276.
14. **Lensen, A., A. Bril, M. van de Vegte, J. van Gemert, W. Eling, and R. Sauerwein.** 1999. *Plasmodium falciparum*: infectivity of cultured, synchronised gametocytes to mosquitoes. Exp. Parasitol. **91:**101–103.
15. **Piper, K. P., R. E. Hayward, M. J. Cox, and K. P. Day.** 1999. Malaria transmission and naturally acquired immunity to PfEMP-1. Infect. Immun. **67:**6369–6374.
16. **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.
17. **Rogers, N. J.** 1996. Ph.D. thesis. University of London, London, United Kingdom.
18. **Rogers, N. J., O. Daramola, G. A. T. Targett, and B. S. Hall.** 1996. CD36 and intercellular adhesion molecule 1 mediate adhesion of developing *Plasmodium falciparum* gametocytes. Infect. Immun. **64:**1480–1483.
19. **Rogers, N. J., G. A. T. Targett, and B. S. Hall.** 1996. *Plasmodium falciparum* gametocyte adhesion to C32 cells via CD36 is inhibited by antibodies to modified band 3. Infect. Immun. **64:**4261–4268.
20. **Schweitzer, K. M., P. Vicart, C. Delouis, D. Paulin, A. M. Drager, M. M. Langenhuijsen, and B. B. Weksler.** 1997. Characterization of a newly established human bone marrow endothelial cell line: distinct adhesive properties for hematopoietic progenitors compared with human umbilical vein endothelial cells. Lab. Investig. **76:**25–36.
21. **Silamut, K., and N. J. White.** 1993. Relation of the stage of parasite development in the peripheral blood to prognosis in severe falciparum malaria. Trans. R. Soc. Trop. Med. Hyg. **87:**436–444.
22. **Sinden, R. E., and M. E. Smalley.** 1979. Gametocytogenesis of *Plasmodium falciparum in vitro*: the cell-cycle. Parasitology **79:**277–296.
23. **Smalley, M. E., S. Abdalla, and J. Brown.** 1980. The distribution of *Plasmodium falciparum* in the peripheral blood and bone marrow of Gambian children. Trans. R. Soc. Trop. Med. Hyg. **75:**103–105.
24. **Thalmeier, K., P. Meisser, G. Reisenbach, M. Falk, A. Brechtel, and P. Dormer.** 1994. Establishment of two permanent human bone marrow stromal cell lines with long-term post-irradiation feeder capacity. Blood **83:**1799–1807.
25. **Thomson, D.** 1914. The origin and development of gametes (crescents) in malignant tertian malaria: some observations on flagellation etc. Ann. Trop Med. Parasitol. **8:**85–104.
26. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. Science **193:**673–675.
27. **Verfaillie, C. M.** 1998. Adhesion receptors as regulators of the hematopoietic process. Blood **92:**2609–2612.
28. **Zannettino, A. C., H. J. Buhring, S. Niutta, S. M. Watt, M. A. Benton, and P. J. Simmons.** 1998. The sialomucin CD164 (MGC-24v) is an adhesive glycoprotein expressed by human hematopoietic progenitors and bone marrow stromal cells that serves as a potent negative regulator of hematopoiesis. Blood **92:**2613–2628.

Editor: W. A. Petri, Jr.