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Plasmodium falciparum Gametocyte Adhesion to C32 Cells via CD36 Is Inhibited by Antibodies to Modified Band 3

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Plasmodium falciparum **gametocyte-infected erythrocytes are characterized by their ability to sequester in the microvasculature of various organs, primarily the spleen and bone marrow. This phenomenon is thought to play a critical role in the development and survival of the sexual stages. Little is known, however, about ligands on the gametocyte-infected erythrocyte. Infection of erythrocytes with mature asexual stages of *P. falciparum* (trophozoites and schizonts) has been shown to induce modification of the erythrocyte anion transporter, band 3, and this has been linked to the acquisition of an adherent phenotype. Here, we demonstrate for the first time that immature gametocyte-infected erythrocytes also express modified band 3. In vitro binding assays demonstrate that gametocyte-infected erythrocytes of the 3D7 strain utilize this surface receptor for adhesion to C32 amelanotic melanoma cells via the host cell receptor CD36 (platelet glycoprotein IIIb). Adhesion of gametocyte-infected erythrocytes to CD36-transfected CHO cells is also dependent on modified band 3. However, modified band 3 does not mediate adhesion of gametocyte-infected erythrocytes to intercellular adhesion molecule 1, a second host receptor for gametocytes expressed on C32 cells.**

The absence of mature asexual-stage parasites (trophozoites/schizonts) from the peripheral circulation of an infected host is a characteristic feature of *Plasmodium falciparum* infection (24, 49). This phenomenon is due to parasite sequestration—the attachment of erythrocytes infected with mature asexual stages to the microvascular endothelia of various organs (32, 45) by a process known as cytoadhesion (2). Infected erythrocytes are found in almost all organs, but the lungs, kidneys, and gut are the primary sites and in severe cases the brain (24, 30, 40). The presence of aggregated erythrocytes in the microvasculature leads to occlusion of small blood vessels (45) and appears to be a major contributory factor to the pathology of the disease (25).

Sequestration is not confined to the asexual stages; developing *P. falciparum* sexual stages also sequester. The only stages found circulating in the peripheral blood are the immature asexual stages (rings) and the mature sexual stages (stage V gametocytes). Immature gametocytes, like asexual-stage parasites, preferentially inhabit specific organs, but the organs involved are quite distinct from those in which asexual stages are found. Within 24 h of erythrocyte invasion, gametocyte-infected erythrocytes become sequestered in the microvasculature of the bone marrow and spleen (39) and remain sequestered for 8 to 10 days before reentering the circulation as mature gametocyte-infected erythrocytes which are competent for transmission via the mosquito. While sequestration of the asexual stages is thought to be important in avoiding immune clearance by the spleen, the importance of gametocyte sequestration in the parasite life cycle is unclear. However, *P. falciparum* sexual-stage maturation is a slow and complex process, accompanied by profound morphological changes, and cytoadhesion at a specific site may provide nutritional or environmental factors required for efficient development and survival of the sexual stages.

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In vitro binding assays using both endothelial and C32 melanoma cells have revealed specific interactions between host receptors and ligands on asexual-stage-infected erythrocytes. Known receptors include CD36 (platelet glycoprotein IIIb [1, 3, 9]), ICAM-1 (intercellular adhesion molecule 1 [6, 7, 9]), VCAM-1 (vascular cell adhesion molecule 1 [29]), ELAM-1 (endothelial leukocyte adhesion molecule 1 [9, 29]), and chondroitin sulfate A (34). The C32 amelanotic melanoma cell line is the most commonly used model for adhesion of the asexual-stage parasites. This cell line expresses both CD36 and ICAM-1 (33). We have recently shown that CD36 and ICAM-1 can also mediate adhesion of stage I to IV gametocyte-infected erythrocytes to C32 cells (33). Ligands on infected erythrocytes are less well defined, but both host- and parasite-derived proteins have been implicated. Over the first 24 h of infection with asexual-stage *P. falciparum*, distinct morphological changes occur in the erythrocyte membrane. These alterations may include both modification of preexisting host receptors and the insertion of parasite-derived antigens into the erythrocyte membrane. The major asexual-stage parasite receptors are a polymorphic group of high-molecular-weight trypsin-sensitive proteins, termed PfEMP1 (*P. falciparum* erythrocyte membrane protein 1), encoded by the *var* gene family (5, 41, 42). The expression of specific *var* family genes is associated with particular antigenic and adhesive phenotypes (5, 41, 42) and has been shown to mediate adhesion of asexual-stage-infected erythrocytes to CD36 and ICAM-1 (4, 18) and thrombospondin (4). In addition to surface expression of parasite-encoded proteins such as PfEMP1, infection with *P. falciparum* induces specific changes in host proteins. Modification of the major membrane protein of the erythrocyte, band 3, has been linked to the acquisition of cytoadherence (12–16, 19).

Band 3 is the anion transporter present in all erythrocytes, which, at a million copies per cell, is the most abundant integral membrane protein in the cell (14). Native band 3 exists in the form of monomers, dimers, and tetramers. The single polypeptide chain has a molecular mass of 95 kDa, comprising a 55-kDa membrane-spanning domain and a 43-kDa cytoplasmic domain. The complete amino acid sequence has been

determined (43), and predicted regions of hydrophobicity and hydrophilicity have been used to model the secondary structure (48). Extensive studies by Sherman and coworkers (10, 12–16, 19, 20, 23) have implicated forms of band 3 in the adhesion of erythrocytes infected with the asexual stages of the Gambian FCR3 strain of *P. falciparum*. Monoclonal antibodies (MAb) raised against infected erythrocytes recognize adherent asexual-stage-infected erythrocytes and inhibit their adhesion to C32 cells in a dose-dependent manner (14, 15). The surface antigen recognized has been shown to be a truncated 65-kDa form of band 3 (13). Infection is believed to lead to exposure of a cryptic epitope of band 3 (localized to amino acids 546 to 553) (12) which mediates adhesion. This hypothesis is supported by the specific inhibition of C32 cell binding by the synthetic peptide 3d (10), which contains the sequence recognized by the anti-band 3 antibodies 1C4 and 1F4, while 3ds, with a scrambled arrangement of the same residues, fails to block adhesion.

Here we demonstrate the presence of modified band 3 on gametocyte-infected erythrocytes of the 3D7 strain of *P. falciparum* and show specific inhibition of gametocyte-infected erythrocyte cytoadhesion to C32 melanoma cells by antibodies against modified band 3 and synthetic peptides based on the amino acid sequence of band 3. Furthermore, we suggest that CD36 acts as the major receptor for modified band 3.

MATERIALS AND METHODS

Parasite culture and purification. The 3D7 strain of *P. falciparum* was routinely cultured in vitro according to established protocols (44). Gametocytes were harvested following 12 days in semiautomatic continuous culture (31) and were enriched by centrifugation over a discontinuous Percoll gradient (70:45% interface) (31) and by subsequent sorbitol treatment (35). Mature asexual stages (trophozoites/schizonts) were enriched by Plasmagel (Laboratoire Roger Bellon, Neuilly, France) selection (26). Viability and subsequent development of both parasite stages were not impaired as a result of these enrichment procedures (data not shown).

Cell culture. The amelanotic melanoma C32 cell line (European Collection of Animal Cell Culture no. 870090201) was maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium containing 20% fetal calf serum, 1× nonessential amino acids, 100 μg of penicillin per ml, 100 U of streptomycin per ml, and 2 mM L-glutamine (all from Gibco, Paisley, United Kingdom). Medium was changed every second day. Once confluent, cells were detached for subculture by digestion with 0.5% trypsin–0.2% EDTA (Sigma Chemical Co., Poole, United Kingdom). Expression of CD36 and ICAM-1 was confirmed by fluorescence staining with MAb 8A6 and 15.2, respectively. C32 cells do not express lymphocyte function associated antigen 1 (LFA-1) by flow cytometry (data not shown). Chinese hamster ovary (CHO) cells transfected with human CD36 (CHO-C7) or mock transfected (vCHO), kindly donated by Irwin Sherman, were maintained in Hams F-12 nutrient mixture supplemented as above but excluding the NEAA (22). Expression of CD36 and absence of ICAM-1 were confirmed by flow cytometry with MAb 8A6 and 15.2, respectively (data not shown). CHO-C7 cells expressing high levels of CD36 were enriched by mini-MACS separation (Miltenyi Biotec, Inc.) according to the manufacturer's instructions. For adherence assays 2.5×10^3 cells/ml were seeded into 16-well Lab-Tek chamber slides (Nunc, Naperville, Illinois) and cultured for 48 h at 37°C and 5% CO_2. 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 was blocked with 1% bovine serum albumin in PBS for 2 h at room temperature.

Cell adhesion assays. Parasite binding assays were carried out as previously described (33) by using the C32 amelanotic melanoma cell line and the 3D7 strain of *P. falciparum*. Briefly, enriched parasites were resuspended to 10% hematocrit in binding medium (10.62 g of RPMI 1640 per liter without L-glutamine, 2 g of glucose per liter, and 1% human serum [pH 7.2]). Variable parasitemias were prepared using uninfected erythrocytes at 10% hematocrit. An aliquot of 50 μl of infected erythrocytes was added to each well, and slides were incubated at 37°C for 90 min on a rocking table. Chambers were removed from slides prior to four washes for 10 min each in washing medium (binding medium without serum). Parasites were visualized by methanol fixation and Giemsa staining, and the number of parasites bound to 500 cells was determined. The possible adherence of low levels of contaminating asexual-stage parasites in sexual-stage adhesion assays was excluded on the basis of distinct morphological characteristics. 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.

Antibodies and reagents. Unless otherwise stated, all reagents were purchased from Sigma Chemical Co. The following MAb were used for surface immunofluorescence detection of modified band 3: 1C4 (11) and 3H3 (15) murine MAb, which recognize amino acids 540 to 550 and 546 to 555, respectively, and were raised by intraperitoneal inoculation of asexual-stage-infected erythrocytes (16); and 1F4, an antipeptide MAb which recognizes amino acids 542 to 555 (20). All three antibodies recognize exofacial epitopes of the modified band 3 molecule and do not recognize the unmodified molecule (13, 15, 20). All were kindly donated by I. Sherman and I. Crandall. A mouse immunoglobulin G1 (IgG1) isotype was used as a control. All antibodies were used at 10 μg/ml. Second-layer antibody was biotin-conjugated rabbit anti-mouse IgG and fluorescein isothiocyanate-conjugated avidin (Serotec). Both were stored at 4°C and used at 1 μg/ml diluted in Tris-buffered saline. Murine anti-human MAb to CD36 (8A6) (3) and ICAM-1 (15.2) (17), provided by J. Barnwell and N. Hogg, respectively, were each used at a saturating concentration of 10 μg/ml. Peptides 3d and 3ds containing residues 546 to 555 (10) (donated by I. Sherman and I. Crandall) were reconstituted in sterile double-distilled water to 20 mg/ml and used at 1, 50, and 100 μg/ml diluted in binding medium.

Peptide and antibody inhibition assays. Fixed C32 cells or CHO cells were preincubated with 50 μl of 3d or 3ds peptide, MAb 8A6 (IgG1; anti-CD36), MAb 15.2 (IgG1; anti-ICAM-1), or the isotype control (IgG1; Sigma) 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 previously described. Antibodies to infected erythrocytes, IC4, IF4, and 3H3, were added to the binding assay mixture at the same time as the infected erythrocytes.

Immunofluorescence staining. Immunofluorescence staining was carried out as described by Crandall and Sherman (13) with minor modifications. Briefly, nonspecific binding was blocked with 50% heat-inactivated horse serum for 15 min at room temperature. After being washed three times with Tris-buffered saline (80 mM Tris [pH 7.4], 150 mM NaCl), the cells were stained with the relevant primary antibody for 60 min at room temperature. Cells were washed as described previously, and biotin conjugate was added for 30 min at room temperature in a humidifying chamber. Specific antibody staining was detected using 1/500 avidin-fluorescein isothiocyanate conjugate for 30 min at room temperature. After being washed, cells were mounted in PBS-glycerol. Staining was visualized using a Zeiss fluorescence microscope.

Antigen extraction and Western blotting. Antigen was prepared for Western blotting (immunoblotting) as described previously (13). Briefly, parasites were enriched by Plasmagel selection for asexual stages and Percoll for sexual stages. The parasitemias of the enriched populations were 50 and 95%, respectively. Asexual contamination of the gametocyte preparation, as determined on the basis of distinct morphological characteristics after Giemsa staining, was negligible (0.4%). Packed cells (100 to 300 μl) adjusted to equalize parasite numbers, were lysed with 50 mM Tris, pH 7.4–1% Triton X-100–1 mM phenylmethylsulfonyl fluoride (PMSF)–25 μg of leupeptin per ml–25 μg of aprotinin per ml. The lysate was centrifuged for 5 min at 14,000 × *g*, and the insoluble pellet was then washed three times in lysis buffer. Modified band 3 was extracted by incubation for 1 h at 4°C with 50 mM Tris, pH 7.4–1 M KCl–1 mM PMSF–25 μg each of leupeptin and aprotinin per ml. The extracted protein was centrifuged at 14,000 × *g* for 5 min and dialyzed overnight against distilled water. Extracted antigen was stored at −20°C. Antigen was separated on a sodium dodecyl sulfate–8% polyacrylamide gel and blotted onto nitrocellulose (13). Blots were blocked overnight with 5% milk in PBS-Tween 20 and then incubated with 1 μg of anti-band 3 MAb 3H3 or IgG1 (isotype control) per ml for 2 h on a rocking table at room temperature. Blots were washed three times with PBS-Tween 20, incubated for 1 h with peroxidase-labelled goat anti-mouse IgG (Bio-Rad), washed, and developed by enhanced chemiluminescence (Amersham).

RESULTS

Indirect immunofluorescence, using anti-modified band 3 antibodies. Immunofluorescence was first used to evaluate the expression of modified band 3 across all developmental stages of *P. falciparum*. MAb 1C4, 3H3, and 1F4 recognized the surface of stage I to IV gametocyte-infected erythrocytes, resulting in a stippled pattern of surface immunofluorescence (Fig. 1A). Staining intensity of 1C4 and 1F4 was unaffected by prior fixation in ice-cold methanol (data not shown). In contrast, intensity of 3H3 staining was increased following methanol fixation. Similar staining patterns were obtained with mature (trophozoite/schizont) asexual stages (Fig. 1B). In contrast, uninfected ring-stage and stage V gametocyte-infected erythrocytes did not react with the antibodies (Fig. 1C). This clearly demonstrates that MAb 1C4, 3H3, and 1F4 recognize surface epitopes exposed only on erythrocytes infected with mature asexual stages or immature gametocytes of the 3D7a strain of *P. falciparum*. Differences in staining intensity

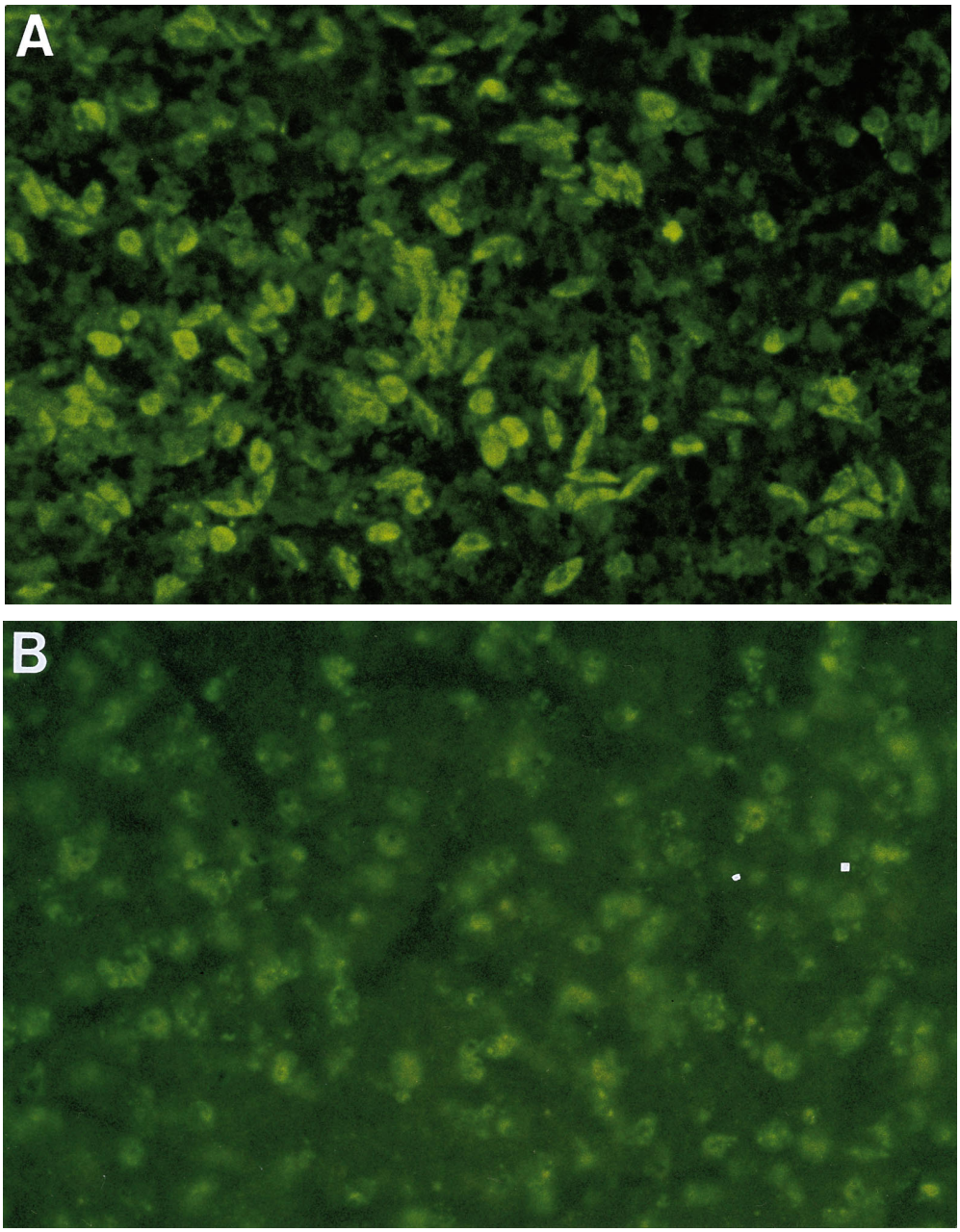

FIG. 1. Immunofluorescence staining of gametocyte-infected (A) and trophozoite-infected (B) erythrocytes with 1C4 at 10 μg/ml. 1C4 recognized the surface of infected erythrocytes displaying the characteristic stippled pattern.

with 3H3, 1F4, and 1C4 following methanol fixation may reflect subtle differences in the epitopes recognized, which overlap but are not identical.

Confirmation of modified band 3 in *P. falciparum* gametocytes by Western blot analysis. To confirm the expression of modified band 3 on gametocyte-infected erythrocytes, Western blot analysis was performed, using antigen extracts from both sexual- and asexual-stage-infected erythrocytes (Fig. 2). As previously reported for the FCR-3 strain (13), a number of proteins were detected in asexual-stage-infected cell preparations. The bands detected included native band 3 and the truncated 65-kDa modified band 3, as well as others of intermediate molecular mass. Similar results were obtained with MAb 1C4 (data not shown). The pattern of bands in the gametocyte extract was similar, but the level of band 3 detected was slightly lower than in the asexual stages at comparable protein levels (lanes 5 to 8). No bands were detected with the isotype control (lanes 1 to 4). Western blot analysis of uninfected erythrocyte antigen preparations were also negative (lane 5).

Anti-modified band 3 antibodies and specific peptide significantly inhibit adhesion of *P. falciparum* gametocytes to C32 cells. Having confirmed the expression of modified band 3 on gametocyte-infected erythrocytes, its functional role was determined in C32 cell adhesion assays. As seen in Fig. 3A, adhesion of gametocytes was specifically inhibited by MAb 1F4 and 1C4

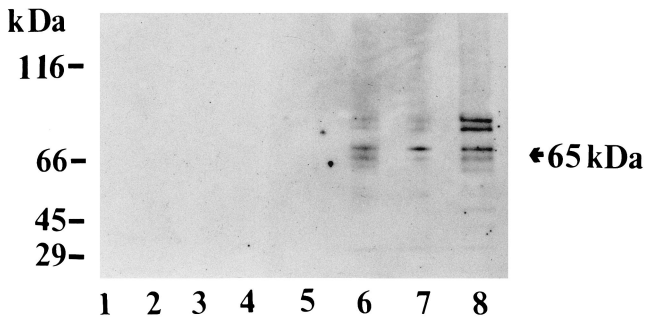

FIG. 2. Western blot analysis using MAb 3H3 at 1 μg/ml. Lanes 1 to 4, isotype control; lanes 5 to 8, MAb 3H3. Lanes 1 and 5, uninfected erythrocytes; lanes 2 and 6, gametocytes (10 μg/ml); lanes 3 and 7, trophozoites (5 μg/ml); lanes 4 and 8, trophozoites (10 μg/ml). Blots were produced by transferring proteins from an 8% gel. Molecular masses are indicated.

($P < 0.05\%$) but was not significantly affected by MAb 3H3 or by the IgG1 isotype matched control. Antibody inhibition curves were similar to those obtained in directly comparable studies using trophozoites (Fig. 3B). In both cases MAb 1F4 was more effective at inhibition than MAb 1C4, but neither was able to cause complete inhibition of adhesion. Crandall and Sherman (15), using the FCR strain of *P. falciparum*, obtained significant inhibition in the presence of MAb 3H3. The differences in these data may be due to strain-dependent differences or to the different assay systems used. The poor blocking effect of MAb 3H3, combined with the surface fluorescence, suggests that affinity of 3H3 for the native epitope might be lower than that of the other MAb.

Peptide 3d, which spans the epitope recognized by antibody 1F4, blocked adhesion of gametocytes (Fig. 4A) and asexual-stage-infected erythrocytes (Fig. 4B) in a dose-dependent manner. Maximum inhibition saturation was achieved at 100 μg/ml for trophozoites and 50 μg/ml for gametocytes. The percent reduction of binding observed in the presence of 3d was similar for both parasite stages. The specificity of this inhibition was confirmed by the inability of the scrambled control 3ds peptide to inhibit adhesion at concentrations of up to 100 μg/ml.

Modified band 3 mediates adhesion to host cells via the surface receptor CD36. As previously demonstrated, adhesion of sexual- and asexual-stage parasites to C32 cells can be inhibited by antibodies to the C32 cell surface receptors CD36 and ICAM-1 (7, 28, 33). Complete inhibition of binding can be achieved in the presence of both antibodies (Fig. 5A and B). Binding of infected erythrocytes is not significantly inhibited by antibodies to major histocompatibility complex class I, although this cell surface molecule is highly expressed (data not shown). Adhesion of *P. falciparum* to C32 cells is therefore mediated by the combination of CD36 and ICAM-1. As infected erythrocytes express neither ICAM-1 nor LFA-1 (data not shown), these data suggested that modified band 3 could serve as a ligand for one or both of these receptors. To test this, blocking experiments were performed using a combination of antibodies to receptor-ligand pairs (Fig. 5C and D). Saturating concentrations of anti-CD36 or anti-ICAM-1 inhibited gametocyte binding by 67 and 35.6%, respectively. Coaddition of anti-band 3 MAb (MAb 1C4) and anti-CD36 had no detectable effect above that of anti-CD36 antibody alone. However, when MAb 1C4 was included in addition to anti-ICAM-1, inhibition was enhanced, to almost 100%. Control antibody directed at Wrb, another surface receptor on erythrocytes, had no detectable effect on binding of parasitized erythrocytes alone or in combination with anti-CD36 or anti-ICAM-1 (Fig. 5C), showing that the inhibition was unlikely to be due to steric hindrance. Again the characteristics of inhibition were similar in directly comparable studies with trophozoites/schizonts (Fig. 5D). These data strongly suggest that the CD36-dependent adhesion of parasitized erythrocytes to C32 cells is mediated via modified band 3. In contrast, ICAM-1-dependent adhesion is mediated by a second distinct ligand expressed on infected cells.

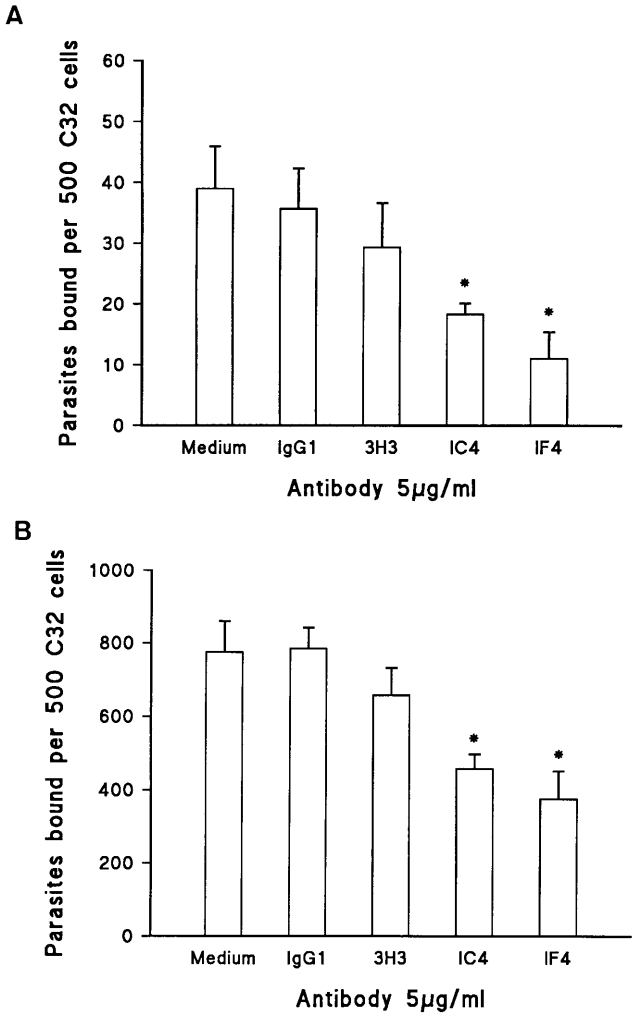

FIG. 3. Adhesion to C32 cells and inhibition by anti-modified band 3 antibodies. (A) Binding inhibition of sexual-stage-infected erythrocytes. Percoll-enriched gametocytes were incubated with C32 cells at 50% parasitemia and 10% hematocrit. (B) Binding inhibition of asexual-stage-infected erythrocytes. Trophozoite/schizont stage-infected erythrocytes were incubated with C32 cells at 10% parasitemia and 10% hematocrit. All assays were carried out in triplicate. Values represent means ± standard errors of the means. Asterisks denote significance at $P < 0.05$ by Student's *t* test analysis.

Modified band 3 mediates gametocyte adhesion to CD36-transfected CHO cells. To confirm the role for modified band 3 in mediating adhesion of gametocyte-infected erythrocytes to CD36, binding assays were carried out using CD36-transfected CHO cells which do not express ICAM-1 (data not shown) (Fig. 6). Binding of infected gametocytes was significantly inhibited by antibodies to modified band 3 and to CD36, while the isotype control had no effect (Fig. 6A). Adhesion was also significantly inhibited in the presence of 3d but not 3ds peptide (Fig. 6B). Inhibition curves were similar to those obtained in

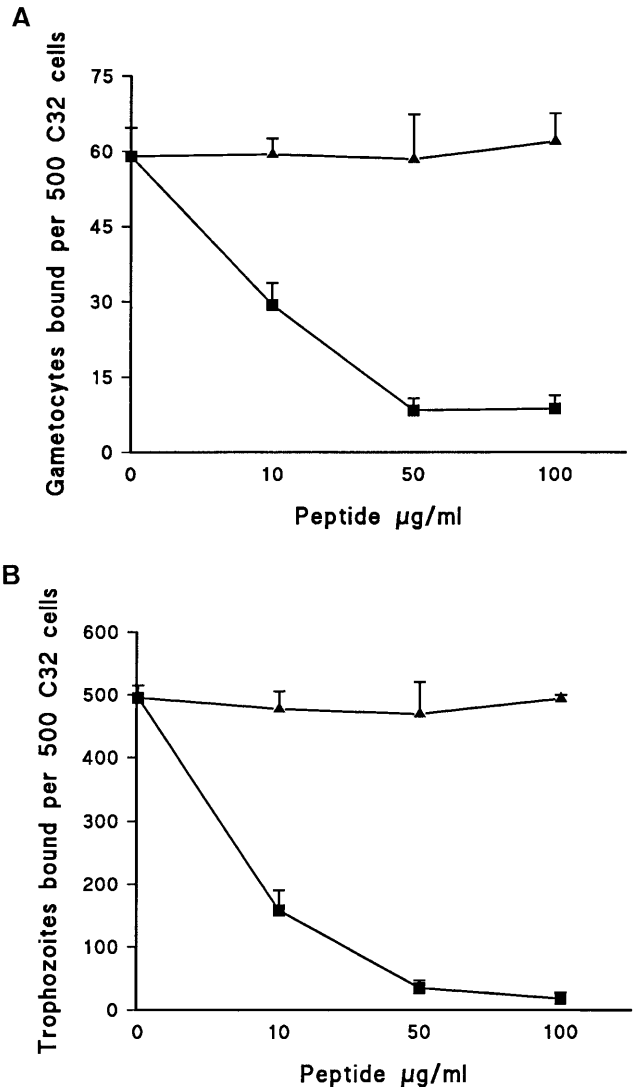

FIG. 4. Effect of peptides on adhesion of *P. falciparum*. Dose-dependent inhibition of sexual- and asexual-stage adhesion by the 3d peptide (■) and the scrambled version 3ds peptide (▲). (A) Percoll-enriched gametocytes were incubated with C32 cells at 50% parasitemia and 10% hematocrit. (B) Plasmagel-enriched trophozoites were incubated with C32 cells at 10% parasitemia and 10% hematocrit. Values represent means ± standard errors of the means for triplicate wells.

directly comparable studies using asexual-stage-infected erythrocytes (data not shown).

DISCUSSION

We have recently described binding of gametocyte-infected erythrocytes to C32 cells, albeit at lower levels than asexual-stage parasites (33). Extensive studies by Sherman and coworkers have implicated a modified form of band 3 in the adhesion of erythrocytes infected with mature asexual stages of the Gambian FCR-3 strain of *P. falciparum* (10, 12–16, 19, 20). The experiments reported here demonstrate, for the first time, the presence of modified band 3 on gametocytes and implicate modified band 3 in interactions with CD36 expressed by C32 cells, thereby mediating adhesion of gametocytes.

The pattern of inhibition of gametocyte binding by anti-band 3 antibodies and specific peptide (amino acid residues 542 to 555) was almost identical to that seen with asexual-stage-infected erythrocytes. We have confirmed the presence of modified band 3 in gametocytes by Western blotting and surface immunofluorescence. On gametocyte stages I to IV, levels of surface immunofluorescence are comparable to that on the asexual stages. The absence of detectable levels of modified band 3 on stage V gametocytes is consistent with the inability of mature gametocytes to cytoadhere and their presence in the peripheral circulation of infected hosts. The mechanism by which the protein is lost is not known. In the mixed-stage gametocyte preparations, band 3 levels were lower than those in asexual-stage-infected cells, and there may be progressive degradation or shedding throughout the period of gametocyte development, so that by the time maturity is reached no band 3 protein is left and the cells are released from the endothelium.

Inhibition of binding to C32 cells by specific antibody combinations directed at receptor-ligand pairs indicated that band 3 adhesion is mediated via the host cell receptor CD36, but not ICAM-1. This was confirmed using CD36-expressing CHO cells, which are ICAM-1 negative. These results are consistent with those Crandall et al. obtained using CHO cells transfected with CD36 (12). Clonal selection of parasites which bind to ICAM-1 has been reported (36), suggesting that adhesion to this receptor is likely to be dependent on parasite-encoded genes. ICAM-1 is therefore more likely to be a receptor for the PfEMP-1 family of parasite ligands, which have been linked to an adhesive phenotype (5, 41, 42). CD36, ICAM-1 (4, 18), and thrombospondin (4) have been shown to mediate adhesion of asexual-stage-infected erythrocytes via PfEMP-1. Work to investigate whether PfEMP-1 is expressed on gametocyte-infected erythrocytes and whether it mediates adhesion of the sexual stage is under way. In contrast, the ability of the 3d peptide, which is based on the amino acid sequence of a host-encoded protein, to inhibit adhesion suggests that binding of gametocyte-infected erythrocytes does not involve a parasite-derived protein. However, the possibility of molecular mimicry by the parasite cannot be excluded. The almost complete inhibition of binding by the 3d peptide compared to the partial blocking obtained using antibody is at odds with the interpretation that band 3 binds to CD36 alone, but it should be noted that this blocking was achieved only at high concentrations of peptide. Also, the kinetics of inhibition by the small peptide are likely to be very different from those of the antibodies, and the two types of inhibition are not directly comparable. The ability of a specific peptide derived from the band 3 sequence to block adhesion, whereas scrambled peptide had no effect, suggests that the inhibition was not due to the presence of peptide alone but also to interference with interactions between newly exposed band 3 surface epitopes and proteins on the surface of C32 cells.

Studies by Guthrie and colleagues (20) using two MAbs, 1C4 and B6, which both recognize the same amino acid residues (542 to 555) of band 3 but in different conformations, have revealed differing effects in cytoadhesion assays. Adhesion is significantly inhibited by MAb 1C4 but unaffected by MAb B6, thereby suggesting that the modification of band 3 leading to the adhesive phenotype of asexual stages may be a consequence of an alteration in the surface topography of the native molecule (20). Infection of erythrocytes with *P. falciparum* asexual stages induces a conformational change involving amino acid residues 542 to 555 of the native band 3 molecule, which then conveys the adhesive phenotype (10, 13–15). The conformational change of band 3 in asexual-stage-infected erythrocytes results in a protein which is protease sensitive (37). The precise mechanism of achieving this modification,

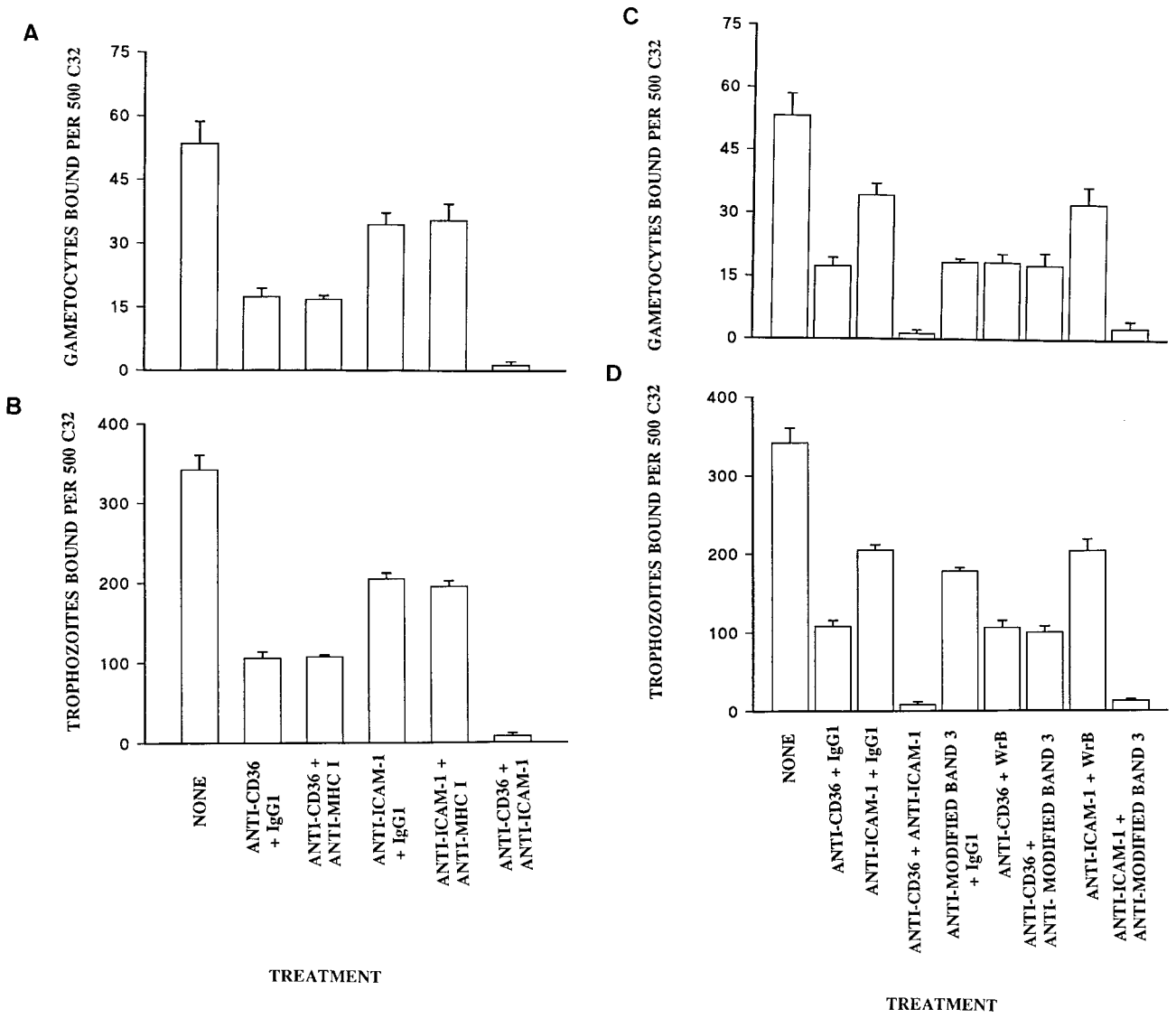

FIG. 5. Adhesion of infected erythrocytes to C32 cells is mediated via CD36 and modified band 3. Binding inhibition of gametocyte-infected erythrocytes (A) and asexual stages (B) by preincubation with saturating concentrations of anti-CD36 and anti-ICAM-1. Effect of double inhibition with antibodies directed against receptor-ligand pairs on the adhesion of gametocyte-infected erythrocytes (C) and asexual-stage-infected erythrocytes (D). Antibodies to host receptors (anti-CD36, anti-ICAM-1, and IgG1 isotype control) were preincubated with each other (bars 2 to 4, left to right) or with antibodies to the ligand on the infected erythrocyte (anti-modified band 3 MAb 1C4 or isotype-matched control Wrb) (bars 5 to 9, left to right). Asexual- and sexual-stage parasites added, per well, were 5 and 50%, respectively. Values are means ± standard errors of the means for triplicate wells.

however, is as yet unidentified. It is very likely that the same modification is occurring in erythrocytes containing sexual stages of the parasite, as our data reveal comparable inhibition properties for both stages. Intensive studies are under way to further characterize the modification.

Having demonstrated the role for modified band 3 in mediating adhesion of gametocyte-infected erythrocytes, it is clear that other factors must also be important in adhesion, since the slightly lower levels of modified band 3 detected in gametocytes are not sufficient to explain the order of magnitude difference in adhesion between asexual- and sexual-stage-infected erythrocytes (33). Expression of modified band 3 in asexual-stage-infected cells does not correlate with the adhesive phenotype. Direct association between modified band 3 and CD36 has been reported for some strains but not for others, suggesting that other molecules may be required to stabilize the interaction (4). The distribution of band 3 may also be important. The intraerythrocytic development of *P. falciparum* leads to distinct morphological changes in the erythrocyte. One such change linked to asexual cytoadhesion is the expression of electron-dense protrusions, known as knobs, on the erythrocyte surface (20). The close contact between knobs and endothelium is visible by both scanning and transmission electron microscopy (46). Expression of modified band 3 has been colocalized to knobs on asexual-stage-infected erythrocytes (47); however, knob-independent, band 3-dependent adhesion of asexual stage-infected erythrocytes has been observed (8, 21, 47). The parasite-encoded KAHRP (knob-associated histidine-rich protein) expressed in the submembrane region of knobs may interact with the cytoskeleton, enabling stabilization and clustering of band 3, thereby increasing the binding capacity of the infected erythrocyte for the endo-

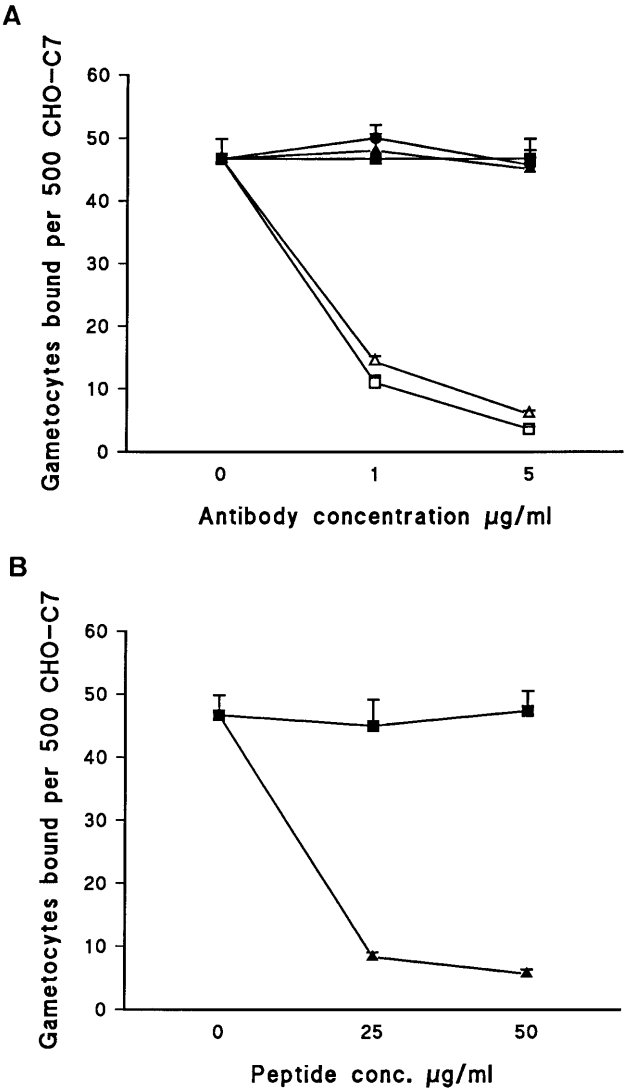

FIG. 6. Adhesion of gametocyte-infected erythrocytes to CD36-transfected CHO cells (CHO-C7). (A) Dose-dependent inhibition of gametocyte-infected erythrocyte adhesion after incubation with antibodies directed against CD36 (□) and modified band 3 (△) or with IgG1 (▲) and Wrb (●) controls or medium alone ■. (B) Dose-dependent inhibition of adhesion by 3d peptide (■) and the scrambled version 3ds (▲). Values are means ± standard errors of the means for triplicate wells.

thelial cell (20). This concentration of band 3 in asexual stage parasite-associated knobs may partly explain the more efficient binding of the asexual stages compared to that of gametocytes, which lack knobs (27, 38). We intend to determine the distribution of modified band 3 in gametocytes by immunoelectron microscopy.

In conclusion, using cellular substrates, we have determined CD36-modified band 3-dependent adhesion for both asexual and sexual stages of *P. falciparum* of the 3D7 strain. In contrast, ICAM-1-dependent adhesion of parasite-infected erythrocytes to C32 cells is not mediated via modified band 3. However, it is clear that there are also major differences between asexual stages and gametocytes both in terms of in vivo sequestration patterns and in vitro adhesive properties. Studies are now under way to characterize gametocyte-specific receptors and the cell types which express them.

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