Serum Lipoproteins Promote Efficient Presentation of the Malaria Virulence Protein PfEMP1 at the Erythrocyte Surface

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Plasmodium falciparum causes the most virulent form of human malaria. This apicomplexan parasite undergoes cyclical asexual development inside the mature red blood cells (RBCs) of its host. RBCs are highly differentiated and have a limited metabolic repertoire and no protein synthesis or protein-trafficking capability. The virulence of P. falciparum is due in large part to the export of proteins to the host cell compartment. These exported proteins modify the properties of the host cell membrane, thereby facilitating access to nutrients and providing a mechanism for evading the immune system. Of particular importance is a parasite-encoded family of proteins known as P. falciparum erythrocyte membrane protein 1 (PfEMP1). Members of this large variant family mediate adhesion of infected RBCs to a number of host molecules, including ICAM-1, CD36, complement receptor-1, and chondroitin sulfate A (CSA) (6, 37, 51, 54).

The adhesion process sequesters infected RBCs away from the peripheral circulation and prevents phagocytic clearance in the spleen. The accumulation of infected RBCs within the microvasculature in vital organs such as the brain and the placenta leads to complications such as cerebral malaria, which is often fatal, and placental malaria, which predisposes to fetal and maternal death (34, 53). In areas of intense transmission, the main burden of disease is borne by children in the age group 0 to 5 years. Repeated exposure to multiple strains of P. falciparum leads to the development of partial immunity based on immune recognition of PfEMP1 and other antigens. As a consequence, severe disease is seen less commonly in adults; during pregnancy, however, women are again susceptible to disease due to the adhesion of infected RBCs to a novel population of binding sites in the placenta (24, 48).

Members of the PfEMP1 family are large (200- to 350-kDa) integral membrane proteins that are presented at the host cell surface. The highly diverse N-terminal adhesive domain is comprised largely of a self-associating protein called the knobs-associated histidine-rich protein (KAHRP). The knobs provide a raised platform that enhances presentation of the adhesive domain of PfEMP1. Individual P. falciparum clones have a repertoire of 50 to 60 different var genes, but only one PfEMP1 variant is expressed at the surface of a mature-stage-infected RBC in any one cycle (13). Switching expression between different var genes allows the parasite to evade the host’s immune response (17, 64).

Transcription of var gene(s) is initiated in early-ring-stage parasites, and the level of transcript peaks approximately 12 h after RBC invasion (37). However, transfer of the newly synthesized protein to the RBC surface requires about 9 h, first appearing at the RBC membrane about 16 h after invasion. Moreover, a substantial proportion of the PfEMP1 population appears to remain in an intracellular pool associated with the Maurer’s clefts (25, 35).

PfEMP1 is a major target of acquired antibodies (39, 63). However, despite its importance both as a virulence factor and...
as an immune target, relatively little is known of the mechanisms and machinery for delivering it to the RBC membrane. It was initially assumed that like other integral membrane proteins, PFEMP1 is cotranslationally inserted into the parasite endoplasmic reticulum membrane and subsequently delivered to the RBC membrane via a series of vesicle-mediated trafficking events (15). However, given that RBCs lack endogenous machinery for vesicle-mediated transport and that recent studies argue against the export of parasite-encoded classical trafficking components to the RBC cytoplasm (1), the pathway for trafficking across the host cell cytoplasm is clearly highly unusual.

We have recently shown that depletion of cholesterol from the RBC membrane inhibits the delivery or presentation of PFEMP1 at the RBC membrane (23). In this work, we show that efficient presentation of the PFEMP1 variants expressed by the A4 and CS2 parasite strains is dependent on the presence of serum components.

MATERIALS AND METHODS

Parasite culture. *P. falciparum* parasites of the A4, CS2, and FAF-EA8 lines were cultured in medium containing 8% human serum in RPMI 1640 supplemented with hypoxanthine and glibatamate as described previously (25) using blood donated by the Australian Red Cross Blood Service, Melbourne. A transfected *P. falciparum* line expressing a PFEMP1 fragment-green fluorescent protein (GFP) chimera (K1.119.TMATS-GFP) (33) was kindly provided by Ellen Kneuefer and Alan Cowman, the Walter and Eliza Hall Institute of Medical Research. The A4 clone was derived from *P. falciparum* IT4/25/5 by micromanipulation (50) and was kindly donated by Chris Newbold, Oxford, United Kingdom. A4-infected RBCs were frequently selected for binding to immobilized BC6 monoclonal antibody (MAb), which recognizes the A4-specific PFEMP1 molecule (25). Flow cytometric analysis of freshly selected A4 parasite-infected RBCs with BC6 MAb showed positive staining on the surfaces of 70 to 80% of A4-infected RBCs. The CS2 line was derived from isolate FAF-EA8 (which is genetically the same as IT4/25/5) by panning for adhesion to Chinese hamster ovary cells and to immobilized CSA (Sigma). A4 and CS2 parasites were cultured in medium containing 8% human serum in RPMI-HEPES. Bound cells were fixed by incubation in 2% glutaraldehyde in 0.1 M HEPES, pH 7.5, and examined using fluorescence microscopy, thin smears of *P. falciparum*-infected RBCs were prepared on slides and fixed in methanol-acetone (1:1 [vol/vol]) at 0°C for 10 min. Binding of primary antibodies was detected with AlexaFluor 568-conjugated anti-mouse IgG secondary antibodies. Samples were viewed with an inverted Leica TCS-SP2 confocal microscope using a 100× oil immersion objective (numerical aperture, 1.4). The fluorescence from GFP was observed with an argon ion laser (488 nm) and AlexaFluor 568 with a helium-neon laser (543-nm line) matched with the appropriate dichroic mirrors (55). Semiquantitative image analysis of 8-bit confocal images was performed using ImageJ software.

Western analysis. Percoll-purified trophozoite cultures (~5% parasitemia) were washed in PBS, and the pellet was disrupted with 1% Triton X-100 in PBS. The sample was recentrifuged and the supernatant added to reducing SDS sample buffer. Proteins were separated on a 6% acrylamide gel. Proteins were transferred to nitrocellulose and processed for antigen detection with a chemiluminescence system (ECL Amersham). Rabbit anti-PFEMP1 AT5 antisera (1:1,000) was used for antigen detection, and the secondary antibody was horseshadish peroxidase-coupled sheep anti-rabbit IgG (1:2,000; Chemicon). Cytoadherence assays. Adhesion assays were performed as described previously (8). Recepients used were CSA (from bovine trachea [Sigma]; 0.4 to 50 µg/ml), CD36 purified from platelets (gift of M. Berndt, Baker Institute, Australia), and recombinant human ICAM-1 (Bender Med Systems) diluted in PBS and coated in triplicate spots on the bases of 25-ml plastic petri dishes by overnight incubation at 4°C. After blocking for 30 min with PBS-1% bovine serum albumin (BSA) or casein blocker (Pierce), plates were washed with RPMI-HEPES, and mature pigmented trophozoites (3 to 10% parasitemia) were re-suspended at 5 to 7% hematocrit in adhesion medium (RPMI supplemented with 25 mM HEPES, 50 µg/ml bovine serum albumin, 2.5 µg/ml gentamycin, and 5% pooled human plasma); in some cases, parasites were resuspended in RPMI-HEPES with 1% BSA. Thirty-five microliters of this suspension was added per receptor spot and incubated at 37°C for 45 min. Unbound cells were removed by washing with RPMI-HEPES. Bound cells were fixed by incubation in 2% glutaraldehyde in PBS and then stained with Giemsa stain and counted. Infected erythrocytes bound in eight fields on each receptor spot were counted by light microscopy.
RESULTS

Surface exposure of PfEMP1 is decreased in A4 strain-infected RBCs matured in lipid-enriched serum albumin. The amount of surface PfEMP1 on the infected RBCs can be analyzed in a semiquantitative fashion by flow cytometric analysis if a suitable reagent specifically recognizing the surface-exposed domain of PfEMP1 is available (68). As each parasite strain can express up to 60 different var genes, methods for selecting parasites expressing a particular variant of PfEMP1 are needed for these analyses. The A4 parasite can be selected for binding to the BC6 MAb, which specifically recognizes an epitope within the external domain of the A4 PfEMP1 (25, 35). Trophozoite-stage A4 parasites developing in RBCs maintained in 8% serum are about 70 to 80% BC6 reactive for a period of a few weeks after selection and then slowly lose reactivity as they switch away from the A4 genotype (29). A three-layer antibody labeling protocol enables the detection of surface binding of the BC6 MAb to intact infected RBCs as a FITC signal, while ethidium bromide staining can be used to distinguish uninfected RBCs from infected RBCs (23). Flow cytometric analysis of the labeled cells provides estimates of the percentage of BC6-positive cells and the MFI of the infected RBCs (Fig. 1A).

AlbuMAX is a commercially available serum substitute that is often used to maintain parasite cultures (18, 22, 42, 49). To investigate the importance of serum components in promoting PfEMP1 delivery or presentation at the infected RBC surface, we have used our flow cytometric assay to examine the effects of culturing cells in this serum substitute. We synchronized the infected RBCs at ring stage (2 to 12 h) and allowed the parasites to mature for a period of 20 h in either 8% serum (Alb) or maintained in AlbuMAX for 20 h and then in serum for 48 h (A → S). To facilitate comparison between samples, the MFI for ethidium bromide-positive cells in the 20 h serum sample was taken as 100%. The data represent the average ± SD for triplicate determinations. (C) A4 strain parasites were collected at time points ranging from 1 to 72 h prior to analysis at the mid-trophozoite stage and either transferred to medium containing 0.5% AlbuMAX I or maintained in medium containing 8% serum and prepared for flow cytometry. The MFI for the last serum control was taken as 100%. The data represent the average ± SD for triplicate determinations on two separate days. The fluorescence values at the 15- to 72-h AlbuMAX time points are significantly different (P < 0.005, Student’s t test) from the average value for the serum control samples, as indicated by the asterisks. The P value for the 1-h AlbuMAX sample compared with the average value for the serum samples is 0.05.

![Graph A](image1.png)

**FIG. 1.** Comparison of surface presentation of PfEMP1 in A4 strain-infected RBCs maintained in serum or AlbuMAX. (A) Early-ring-stage A4 strain parasites (~2 to 12 h) were incubated for 20 h in medium containing 8% serum or 0.5% AlbuMAX I or maintained in AlbuMAX for 20 h and then in serum for 48 h (A → S). Samples were incubated with BC6 MAb followed by rabbit anti-mouse IgG and then FITC-labeled pig anti-rabbit IgG plus ethidium bromide (EtBr). The cell preparations were analyzed by flow cytometry, with the data representing the ethidium bromide and fluorescein (FITC-BC6) fluorescence signals. The cross lines indicate the cell populations selected for analysis; the upper quadrants represent infected RBCs, with the right upper quadrant representing BC6-positive infected RBCs. The MFI values for BC6 staining of ethidium bromide-positive infected RBCs for this experiment are 296, 118, and 295, and the BC6-positive cell fractions are 79%, 22%, and 83%, respectively. (B) The BC6 reactivity of infected RBCs maintained in serum (Ser) was compared with that of cells incubated in AlbuMAX for 20 h (Alb) or maintained in AlbuMAX for 20 h and then in serum for 48 h (A → S). To facilitate comparison between samples, the MFI for ethidium bromide-positive cells in the 20 h serum sample was taken as 100%. The data represent the average ± SD for triplicate determinations. (C) A4 strain parasites were collected at time points ranging from 1 to 72 h prior to analysis at the mid-trophozoite stage and either transferred to medium containing 0.5% AlbuMAX I or maintained in medium containing 8% serum and prepared for flow cytometry. The MFI for the last serum control was taken as 100%. The data represent the average ± SD for triplicate determinations on two separate days. The fluorescence values at the 15- to 72-h AlbuMAX time points are significantly different (P < 0.005, Student’s t test) from the average value for the serum control samples, as indicated by the asterisks.
tained in serum was taken as 100% (Fig. 1B). Surprisingly, the MFI of the sample maintained in AlbuMAX was only about 40% of the control level (Fig. 1A and B). Analysis of data from several experiments showed that the MFI of the surface-accessible PIEMP1 in AlbuMAX-incubated infected RBCs varied from 10% to 40% of the serum control, depending on the degree of synchronicity of the ring-stage parasites and the time since selection of the A4 parasites (data not shown). These data show that components of the serum are needed during the maturation of the intracellular parasite for efficient delivery or presentation of PIEMP1 at the RBC surface. Medium supplemented with a lower level of serum (4%) also allowed efficient surface presentation of PIEMP1 (data not shown).

Switching of surface-expressed PIEMP1 variants occurs at a rate of about 2% per generation (29, 50). To determine whether the loss of surface accessibility of the A4 PIEMP1 variant during incubation in AlbuMAX might be due to a switching event, we incubated ring-stage parasites for 20 h in the presence of AlbuMAX I and then transferred them back to serum for 48 h and analyzed the surface presentation of PIEMP1 at the mid-trophozoite stage of the next cycle (Fig. 1A and B). These infected RBCs have an MFI (98% ± 4%) similar to that of the controls. This suggests that presentation of PIEMP1 at the infected RBC surface can be very rapidly converted from high to low levels and back again and is unlikely to involve gene switching.

To examine the effect of the time of incubation of the infected RBCs in AlbuMAX I on the efficiency of PIEMP1 presentation in A4 parasites, we exchanged the serum-containing medium for AlbuMAX-based medium at different times prior to the point (mid-trophozoite stage) at which they were harvested for analysis. As shown in Fig. 1C, samples maintained in AlbuMAX I medium for 15 to 72 h prior to analysis showed markedly decreased PIEMP1 surface presentation. By contrast, a sample of trophozoite-stage parasites exchanged into AlbuMAX-containing medium only 1 hour prior to analysis showed a number of BC6-positive cells (98% ± 6%) similar to that seen for the serum control (data not shown) and only a relatively small decrease (78% ± 4%) in the MFI (Fig. 1C). The data show that when infected RBCs are deprived of serum during maturation, there is a loss of efficiency of PIEMP1 transfer to or presentation at the infected RBC surface. However, exchange into AlbuMAX medium has little effect on PIEMP1 that has already been presented at the infected RBC surface. Thus, it is unlikely that AlbuMAX prevents binding of the BC6 MAb or directly interferes with the flow cytometric analysis.

Lipid-enriched serum albumin and purified serum lipoproteins support parasite maturation and reinvasion at similar levels. PIEMP1 is first expressed on the infected RBC surface at about 16 h postinvasion and peaks at about 24 h postinvasion (35). To ensure that the effect of AlbuMAX on PIEMP1 presentation was not due to a gross effect on the maturation of A4 parasites to the trophozoite stage, we estimated the trophozoite-stage parasitemia (relative to the initial ring-stage parasitemia) in cultures maintained for 20 h in different media (Table 1). After 20 h, the entire population of the parasites in the 8% serum and 0.5% AlbuMAX samples had reached the trophozoite stage (i.e., the 20- to 38-h stage), as assessed by examining Giemsa-stained smears under light microscopy (60).

Similar results were obtained when smears were assessed at 28 h posttreatment (data not shown). Moreover, the parasites were able to complete the life cycle within 48 h and to reinvoke with a similar efficiency (Table 1). By contrast, medium with a higher concentration of AlbuMAX I (1%) did appear to inhibit maturation to the trophozoite stage and reinvasion (Table 1).

### TABLE 1. Characteristics of *P. falciparum* cultures maintained in different media

| Supplement          | Maturation ratio (20 h) | Invasion ratio (48 h) | RBC chol level (%) |
|---------------------|-------------------------|-----------------------|-------------------|
| 8% serum            | 1.0 ± 0.1               | 1.7 ± 0.2             | 100               |
| 0.5% AlbuMAX        | 0.9 ± 0.1               | 1.4 ± 0.1             | 82 ± 6            |
| 1% AlbuMAX          | 0.6 ± 0.2               | 0.4 ± 0.1             | 90 ± 5            |
| 2× PCi              | 0.9 ± 0.1               | 1.2 ± 0.3             | 84 ± 19           |
| 4× PCi              | 0.9 ± 0.1               | 0.9 ± 0.3             | 90 ± 19           |
| 8× PCi + chol*      | 1.1 ± 0.1               | 0.2 ± 0.1             | 92 ± 17           |
| LDL                 | 1.2 ± 0.2               | 1.2 ± 0.4             | 75 ± 14           |
| HDL                 | 0.8 ± 0.1               | 0.6 ± 0.1             | 86 ± 6            |

* Smears of duplicate samples were prepared and stained with Giemsa stain, and 1,000 cells per slide were counted. PC vesicles (4% PC) were added in 0.5% AlbuMAX. Data represent the mean ± SD for duplicate experiments on three separate days.

* Smear-positive ring-stage parasites (2 to 12 h, 5% parasitemia) were incubated for 20 h, and the maturation ratio was then determined as the number of cells infected with trophozoite (>20-h)-stage parasites relative to the initial parasitemia.

* Smears of duplicate samples were prepared and stained with Giemsa stain, and 1,000 cells per slide were counted. PC vesicles (4% PC) were added in 0.5% AlbuMAX. Data represent the mean ± standard error of the mean for duplicate experiments on five separate days.

* Relative to PC concentration in serum.

### Maintenance of *P. falciparum*-infected RBCs in lipid-enriched serum albumin does not affect total levels of PIEMP1 expression or trafficking of PIEMP1 to the Maurer’s clefts.

To determine whether there was any change in the overall level of PIEMP1 expression or a change in the PIEMP1 variants that are expressed, we undertook Western blotting analysis of RBCs infected with A4 parasites that had been cultured for 24 h in either 8% serum or 0.5% AlbuMAX (Fig. 2A). Full-length A4 PIEMP1 migrates as a band with a molecular mass of ~240 kDa. Similar levels of this band were observed in A4-infected RBCs maintained for 24 h in either serum or AlbuMAX. The data represent a typical gel from an experi-

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ment performed three times. Similar results were obtained using 3D7 strain parasites.

Maurer’s clefts are parasite-derived structures in the host cell cytoplasm that are thought to represent an intermediate depot for PfEMP1 en route to the RBC membrane (15, 38). The appearance of PfEMP1 at the Maurer’s clefts was examined by immunofluorescence microscopy (Fig. 2B). As reported previously, most of the PfEMP1 band (B and C) Smears of A4 strain trophozoite-stage cultures maintained for 20 h in either 8% serum or 0.5% AlbuMAX were fixed and probed with rabbit anti-PfEMP1 ATS (B) or mouse anti-KAHRP (C) serum followed by AlexaFluor 568-labeled anti-rabbit or -mouse IgG and examined by confocal fluorescence microscopy. Bar, 5 μm.

FIG. 2. Effect of incubation of A4 strain-infected RBCs in serum or AlbuMAX on the total level of PfEMP1 in trophozoite-stage parasites. (A) Early-ring-stage A4 strain parasites (~2 to 12 h) were incubated for 20 h in medium containing 8% serum (Ser) or 0.5% AlbuMAX (Alb). A Triton X-100-insoluble pellet was extracted with 2% SDS and subjected to SDS-PAGE (5% acrylamide) and Western blot analysis. The arrowhead indicates the main PfEMP1 band. (B and C) Smears of A4 strain trophozoite-stage cultures maintained for 20 h in either 8% serum or 0.5% AlbuMAX were fixed and probed with rabbit anti-PfEMP1 ATS (B) or mouse anti-KAHRP (C) serum followed by AlexaFluor 568-labeled anti-rabbit or -mouse IgG and examined by confocal fluorescence microscopy. Bar, 5 μm.

In a semiquantitative analysis of total pixel values for 25 cells, the integrated fluorescence intensity of AlbuMAX-cultured cells was 95% of that for the serum-cultured cells. The average numbers of Maurer’s clefts were 20 ± 6 (standard deviation [SD]) and 17 ± 6 (SD) in AlbuMAX- and serum-cultured cells, respectively. Similarly, there were no obvious differences in the levels or patterns of expression of KAHRP (Fig. 2C). Culturing for 20 h in AlbuMAX also had no obvious effect on the distribution of a GFP-PfEMP1 fragment chimera in K1-TmATS-GFP transfectants (33) (data not shown).

These data suggest that the defect in the surface accessibility of PfEMP1 is not due to a decrease in the total level of expression of PfEMP1 or in the trafficking of the protein to the Maurer’s clefts but presumably lies at the level of transfer of PfEMP1 from the Maurer’s clefts to the RBC membrane or in the presentation of PfEMP1 at the RBC surface.

Serum contains more phospholipid and cholesterol and less fatty acid than AlbuMAX. AlbuMAX is a preparation of lipid-loaded BSA which has been used extensively as a serum substitute in the culture of malaria parasites and mammalian cell lines. To compare the lipid composition of AlbuMAX with that of serum, we extracted the lipid from aliquots of 8% serum and 0.5% AlbuMAX and subjected the samples to TLC employing sequential separation steps with two solvent systems (32) (Fig. 3). This system allows the effective separation of amphipathic and neutral lipid species. Semiquantitative analysis of the TLC plates indicates that 8% serum contains more total lipid than 0.5% AlbuMAX I. In particular, the levels of phospholipid, cholesterol, and triacylglycerol are higher. AlbuMAX II has a lipid composition very similar to that of AlbuMAX I (data not shown). When the AlbuMAX I concentration is increased to 4%, the levels of cholesterol and phospholipid are similar to those in 8% serum, but much higher levels of fatty acid are observed. As shown in Table 1, the higher fatty acid level appears to be toxic to parasite growth, and P. falciparum cannot be maintained in 1 to 4% AlbuMAX I (Table 1, data not shown, and reference 36). This precluded an analysis of the effects of higher levels of AlbuMAX I on the surface presentation of PfEMP1.

LDL and HDL promote surface presentation of PfEMP1 in P. falciparum-infected RBCs. We were interested to identify the serum component(s) responsible for promoting efficient surface presentation of PfEMP1. The serum lipoprotein HDL.

FIG. 3. Analysis of the lipid compositions of serum and AlbuMAX. TLC analysis of lipid classes in aliquots of 8% serum (S) and 0.5% and 4% AlbuMAX I (Alb). The samples were extracted in organic solvent and separated using a combination of two mobile phases as described in Materials and Methods. The lipid standards (3 μg each) are SM, PS, PC, phosphatidylethanolamine (PE), monosaccharide (MAG), diacylglycerol (DAG), cholesterol (Chol), fatty acid (FA), triacylglycerol (TAG), and CE. Std, standards. The fatty acid, cholesterol, and phospholipid spots are arrowed. Note that the CE spot comigrates with colored serum components.
has previously been shown to be sufficient to supply essential lipids to the intracellular parasite and thereby maintain the growth of *P. falciparum* in vitro (26, 31). We confirmed that HDL and LDL support parasite growth over the 20-h maturation period (Table 1) and examined the ability of purified LDL and HDL to promote PfEMP1 presentation at the infected RBC surface. Serum lipoproteins were isolated by density centrifugation (14). LDL and HDL were observed as opalescent bands near the top and middle of the KBr gradient. A lipoprotein-depleted fraction of serum was collected from the bottom of the gradient and subjected to a further centrifugation step before use. All samples were dialyzed extensively against PBS and analyzed by SDS-PAGE and TLC. The purified fractions were added back to RPMI (with or without 0.5% AlbuMAX) in volumes designed to give levels equivalent to those in 8% serum.

The lipoprotein-depleted fraction was not able to support efficient PfEMP1 presentation at the infected RBC surface (Fig. 4), indicating that it is the lipoprotein fractions rather than low-molecular-weight species that are the important components of serum. Indeed, we found that purified LDL substantially enhanced PfEMP1 presentation. The percentage of BC6-positive cells in LDL-supplemented samples was 102% ± 8% of the control (data not shown), while the MFI of the infected RBCs was 74% ± 6% (Fig. 4). HDL was consistently less effective than LDL but also provided a substantial enhancement of PfEMP1 surface presentation compared with AlbuMAX (Fig. 4). These data indicate that lipoproteins represent a major component of serum needed for PfEMP1 surface presentation. It is important to note that mixtures of LDL or HDL with AlbuMAX allowed levels of PfEMP1 surface presentation similar to those seen for the lipoproteins alone (Fig. 4), although these combinations were more effective at supporting the long-term growth of the parasites (data not shown). These data indicate that the 0.5% AlbuMAX does not contain a species that inhibits PfEMP1 presentation but rather lacks a component (the serum lipoproteins) needed for effective surface presentation of PfEMP1.

Lipoproteins are thought to promote parasite growth by acting as a lipid source (26). We therefore examined the ability of synthetic phospholipid vesicles to support PfEMP1 presentation. Small unilamellar vesicles comprised of PC with or without an equimolar concentration of cholesterol were generated by sonicating the lipids in RPMI before supplementing the medium with 0.5% AlbuMAX. These vesicles were not able to support PfEMP1 presentation even when added at up to eight times the equivalent concentration of PC present in 8% serum (Fig. 4), although higher concentrations of the vesicles did cause some cell lysis. Phosphatidylserine and SM vesicles also had little effect (data not shown). The data indicate that lipid components must be supplied in the context of lipoprotein particles to effect efficient PfEMP1 presentation.

**Incubation in AlbuMAX slightly modulates host cell cholesterol levels, but this does not correlate with PfEMP1 exposure.** We considered the possibility that incubation in AlbuMAX might alter the level of cholesterol in RBC membranes, which could in turn affect the trafficking or presentation of PfEMP1. We have previously shown that depletion of host cell cholesterol by ~50% using methyl-β-cyclodextrin decreases PfEMP1 surface presentation by ~50% (23). Other studies have also shown that the availability of antigenic epitopes on *P. falciparum*-infected RBCs is affected by cholesterol levels (4, 5, 12). Some authors have reported that altered cholesterol levels also affect the accessibility of endogenous RBC proteins to chemical reagents and antibodies (11, 58), although we showed that...
CD59 accessibility was not affected (23). We have therefore measured relative cholesterol levels in cultures of *P. falciparum* (A4 strain, ~10% parasitemia) maintained for 24 h in either AlbuMAX- or serum-containing medium and compared these data with data for cultures maintained in AlbuMAX supplemented with lipoproteins or lipid vesicles (Table 1). In all cases where the infected RBCs are incubated in AlbuMAX-containing medium, there was a small (~10 to 20%) decrease in cholesterol levels in the membranes of the infected erythrocyte samples. However, the effect on cholesterol levels did not correlate with the observed effect on PIEMP1 surface presentation. For example, the level of cholesterol was decreased in samples incubated with HDL in 0.5% AlbuMAX, which showed increased levels of PIEMP1 surface presentation compared to the AlbuMAX-incubated cells.

**CS2 strain parasite-infected RBCs also show decreased surface presentation of PIEMP1 when cultured in lipid-enriched serum albumin.** We were interested to determine whether the effect of AlbuMAX was observed for strains of *P. falciparum* other than the A4 strain. The CS2 parasite has been selected for binding to CSA (51). A PIEMP1 variant encoded by the *var2csa* gene is the main CSA-binding ligand expressed by this parasite line and is thought to be the main PIEMP1 variant responsible for placental sequestration (19, 56). We have developed a rabbit polyclonal antiserum that specifically recognizes RBCs infected with CS2 strain parasites (but not uninfected RBCs). It is likely that this polyclonal antiserum mainly recognizes the external domain of VAR2CSA PIEMP1 (20, 48). Preimmune serum from the same rabbit gives only background reactivity (Fig. 5A). Similarly, pooled human hyperimmune serum from Malawian pregnant women recognizes surface epitopes on RBCs infected with CS2 strain parasites, while control nonimmune serum from Australian volunteers does not recognize these cells (Fig. 5B). The availability of these reagents offers the possibility of examining the surface presentation of PIEMP1 in a strain of *P. falciparum* expressing another PIEMP1 variant. For this work, we compared two different lipid-rich albumin preparations, AlbuMAX I and II.

CS2 parasites were maintained in serum for a period of several weeks, and early-ring-stage parasites were then incubated for 48 h in either 8% serum or 0.5% AlbuMAX II prior to analysis of the level of surface-accessible PIEMP1. The rabbit polyclonal antiserum recognized surface epitopes in AlbuMAX-incubated cells at less than 20% of the level for the serum-cultured infected RBCs (Fig. 5A), while the immune human serum reactivity was decreased to about 60% (Fig. 5B). These data indicate that surface presentation of VAR2CSA PIEMP1 also falls substantially when infected RBCs are maintained in serum-free medium. The fact that the immune human serum reactivity remains higher suggests that the immune serum is also recognizing other surface-exposed epitopes in infected RBCs that are not altered during incubation in AlbuMAX. The CS2 parasite has a very stable adhesion phenotype (J. Beeson and M. Duffy, unpublished data), suggesting that it undergoes switching at a rate much lower than that seen for other parasite lines. We were therefore interested in the fact that the surface accessibility of CS2 PIEMP1 recovered substantially when the parasites were returned to serum-containing medium for an additional cycle (Fig. 5C), again arguing against a switching event. Similar experiments were performed using AlbuMAX I rather than AlbuMAX II, with very similar results (Fig. 5C). This indicates that neither of the commercially available AlbuMAX preparations is able to efficiently support PIEMP1 presentation.

**Infected RBCs show decreased cytoadherence to some receptors when maintained in the absence of serum.** We examined the effect of culturing parasites (CS2 and its parent line, FAF-EA8) in medium supplemented with AlbuMAX II or serum on the cytoadherence of infected RBCs to CD36, CSA, and ICAM-1. We initially evaluated whether serum influences the adhesion of infected RBCs when included in the adhesion medium. For FAF-EA8-infected RBCs, adhesion to CD36 was lower when the adhesion medium contained 1% BSA rather than 10% serum (Fig. 6A and B). This effect was observed for infected RBCs maintained in either serum or AlbuMAX but was more dramatic for AlbuMAX-grown parasites. This suggests that serum factors facilitate binding to CD36. By contrast, the adhesion of FAF-EA8 to ICAM-1 and of CS2 to CSA was not substantially influenced by the presence or absence of serum in adhesion medium (data not shown). Subsequently, adhesion assays were generally performed in medium containing 10% serum.
When FAF-EA8 parasites were cultured for one cycle in AlbuMAX II, there was reduced adhesion of infected RBCs to CD36 compared to what was seen for a serum-maintained culture of the parasites (Fig. 6C; \( P < 0.01 \), Wilcoxon rank sum test). These data indicate that factors in serum may contribute both to the level of surface-expressed PfEMP1 epitopes and to the ability of PfEMP1 to adhere to CD36. The adhesion of infected RBCs to ICAM-1 was also lower when parasites were cultured in AlbuMAX compared to what was seen when parasites were cultured with serum (Fig. 6D). We also examined the effect of culture in AlbuMAX on the cytoadherence of CS2 parasites to CSA. We found similar levels of adhesion of serum- and AlbuMAX-matured infected RBCs to CSA (three experiments; data not shown). The data suggest that the alteration in the surface presentation of PfEMP1 that occurs when infected RBCs are cultured in AlbuMAX affects adhesion to some receptors but not others.

**DISCUSSION**

An increased understanding of the factors that control the trafficking and surface presentation of PfEMP1 could lead to novel ways of interrupting this process. However, the ability of parasites to switch between up to 60 different var genes has complicated efforts to assess the factors controlling PfEMP1 delivery to and presentation at the host cell surface. In this work, we have employed different *P. falciparum* lines that have been selected to express particular PfEMP1 variants whose N-terminal domains are recognized by specific reagents—a MAb in the case of A4 parasites and rabbit or human immune sera in the case of CS2 strain parasites.

A4 strain parasites selected for binding to immobilized BC6 MAb are initially 70 to 80% positive for surface expression of the A4 PfEMP1 variant (30). We found that when freshly selected ring-stage A4 parasites were cultured for 20 h in medium containing the serum substitute AlbuMAX, the surface reactivity with the BC6 MAb declined to 10 to 40% of the level of a control sample maintained in serum. By contrast, transferring trophozoite-stage parasites to AlbuMAX medium for 1 h did not greatly affect BC6 reactivity. The data indicate that a component of normal human serum needs to be present in the medium when PfEMP1 is being presented at the host cell surface in the late-ring/early-trophozoite stage of infection. This effect is not restricted to A4 parasites, as CS2 strain parasites, which express a different PfEMP1 variant termed VAR2CSA, also lost surface reactivity with immune sera when cultured in lipid-loaded albumin. The decrease in antibody surface reactivity could result from a decrease in the delivery of PfEMP1 to the surface or an altered surface conformation that inhibits the binding of reagents at the external surface.

The effects on PfEMP1 surface presentation translated into an effect on the level of adhesion of FAF-EA8-infected RBCs to immobilized CD36 and ICAM-1 but did not affect the binding of CS2-infected RBCs to CSA. This differential effect may relate to the nature of the adhesion receptor (10, 16) or to the level of surface presentation of PfEMP1. The differences may depend on the strength of adhesion to different receptors, such that tight interactions are less affected by a decrease in PfEMP1 surface density or presentation than weaker interactions. Alternatively, it is possible that the effect may be due to the interaction of the antisera with exofacial regions of PfEMP1 different from those involved in cytoadhesion or to a difference in apparent avidity, dependent upon the Duffy-like binding domain substructure of CSA binders or the involvement of non-PfEMP1 molecules in CSA binding. A prior study reported that AlbuMAX-cultured parasites adhered to a number of different receptors under conditions of physiologically relevant flow. However, no direct comparison was reported for the adhesion of AlbuMAX-cultured parasites versus that of serum-cultured parasites (18).

Our data argue against a switching event, because the total level of PfEMP1 expression is not substantially altered, as judged by semiquantitative immunofluorescence and Western blot analysis. Moreover, high levels of surface presentation of A4 PfEMP1 were reprimed when AlbuMAX-incubated infected RBCs were returned to serum-containing medium for another cycle. This suggests that the defect must be in PfEMP1 trafficking or surface presentation. Indeed, the defect appears to
lie beyond the point of trafficking of PiEMP1 to the Maurer’s clefts, as there were no obvious differences in the levels of Maurer’s cleft labeling.

Human serum contains multiple proteins involved in the transport of nutrients and waste products, immune defense, and homeostasis. Two major classes of proteins, serum albumin and the serum lipoproteins, are involved in the delivery of lipid components. During intraerythrocytic development, *P. falciparum* increases its phospholipid content sixfold as the parasite generates the membranes needed for growth and division (7, 43, 62, 66). The parasite has little de novo lipid biosynthetic capability. Therefore, the bulk of the fatty acid and polar head groups needed for lipid synthesis are scavenged from the host serum as either intact phospholipids, lysophospholipids, or free fatty acids (2, 27, 43, 61, 62, 67).

HDL is thought to represent a major lipid source for parasites grown in serum-containing medium (26); however, AlbuMAX is routinely used in many malaria laboratories as a more readily available and consistent supplement for maintaining malaria parasites (18). Once adapted to growth in AlbuMAX, most parasite strains appear to have similar rates of multiplication, indicating that the requirements for in vitro growth are readily met. Indeed, we found no major differences in maturation or reinvasion when parasites were maintained in either serum or AlbuMAX over the periods examined in this study. This indicates that the observed dramatic decrease in the surface accessibility of PiEMP1 is probably not due to the lack of an essential nutrient but must be due to a direct effect on the PiEMP1 delivery or presentation processes.

We analyzed the lipid components of serum and AlbuMAX and found that serum contains higher levels of phospholipid, cholesterol, and neutral lipids, presumably carried by serum lipoproteins, and lower levels of free fatty acid. Increasing the level of AlbuMAX used to supplement the medium increased the levels of phospholipid and cholesterol but also increased the fatty acid component and did not support parasite growth. We found that a lipoprotein-depleted serum fraction supported only low levels of PiEMP1 surface presentation, indicating that lipid-containing components are important. Lipoproteins are particles with diameters of 10 to 500 nm and have a hydrophobic core of triacylglycerols and CEs protected by a monolayer of phospholipids, cholesterol, and specialized proteins (57). We found that LDL and HDL, when added at levels similar to those present in serum, enhanced PiEMP1 surface presentation. LDL was consistently more effective than HDL in promoting PiEMP1 accessibility, while HDL has previously been shown to be more effective in supporting parasite growth (26). Synthetic phospholipid or phospholipid/cholesterol vesicles were not effective, indicating that the lipid component(s) needs to be supplied as lipoproteins.

We considered the possibility that AlbuMAX incubation might lead to a net loss of cholesterol from the host membrane, which might adversely affect the properties of the host cell membrane. Indeed, we observed a small decrease in the level of cholesterol in the membranes of cultures of infected RBCs incubated in AlbuMAX-containing medium compared with cells incubated in serum-containing medium. This decrease is presumably due to the transfer of cholesterol from the RBCs to albumin. However, this effect did not correlate with the observed effect on PiEMP1 surface presentation. Therefore, while decreased cholesterol levels in the host cell membrane may contribute to the decreased efficiency of the surface presentation of PiEMP1, additional factors must be involved. It is possible that the serum lipoproteins bind directly to the RBC surface and promote PiEMP1 delivery or presentation.

These studies add to our understanding of the pathway for trafficking of PiEMP1 to the RBC surface. Recent studies show that PiEMP1 is trafficked through the endomembrane system of the parasite to the parasitophorous vacuole membrane, where a conserved protein export element/vacuolar transport signal (PEXEL/VTS) motif is recognized; this motif directs the transfer of the protein to the host cell cytoplasm (28, 40, 41). It has been suggested that PiEMP1 is transported through the parasite’s secretory apparatus and into the host cell cytoplasm as a chaperoned complex and is inserted into a bilayer environment only at nascent or mature Maurer’s clefts (33, 46). The final event in the transfer of PiEMP1 to the RBC surface may involve insertion into cholesterol-rich microdomains in the RBC membrane. Alternatively, the cholesterol-rich regions may promote presentation of PiEMP1 epitopes at the RBC surface (23). The work presented here suggests that the presence of lipoproteins at the time of transfer of PiEMP1 to the RBC surface facilitates these processes.

A number of studies have reported an altered lipid composition in lipoproteins from patients with *P. falciparum* infections (3, 21, 44, 45, 59). However, the extent and nature of the changes differ between different studies, and it is difficult to predict the effects of these changes on the efficiency of presentation of PiEMP1 in infected RBCs in vivo. It remains possible nonetheless that lower levels of serum lipoproteins could reduce the severity of *P. falciparum* infections. An increased understanding of the mechanism involved could lead to new mechanisms for interfering with the surface expression of this major virulence factor and immune target.

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