Ex Vivo Desequestration of *Plasmodium falciparum*-Infected Erythrocytes from Human Placenta by Chondroitin Sulfate A

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We performed ex vivo experiments with *Plasmodium falciparum*-infected human placentas from primi- and multigravida women from Cameroon. All women, independent of their gravida status, had anti-chondroitin sulfate A (CSA) adhesion antibodies which cross-reacted with heterologous strains, such as FCR3 and Palo-Alto (FUP1), which were selected for CSA binding. These antibodies, directed against the surface of infected erythrocytes obtained by flushing with CSA (IRBC\textsuperscript{CSA}), were restricted to the immunoglobulin G3 isotypes. Massive desequestration of parasites was achieved with soluble CSA but not with anti-ICAM-1 and anti-CD36 monoclonal antibodies. All of the CSA-flushed parasites were analyzed immediately by using in vitro assays of binding to *Saimiri* brain endothelial cells (SBEC) expressing various adhesion receptors. Parasites derived from all six placentas displayed the CSA adhesion phenotype. However, only partial inhibition of adhesion was observed in the presence of soluble CSA or when Sc1D SBEC were treated with chondroitinase ABC. These results suggest that an additional adhesive molecule of IRBC\textsuperscript{CSA} which binds to an unidentified receptor is present in the placenta. This new phenotype was lost once the parasites adapted to in vitro culture. We observed additional differences in the CSA adhesion phenotype between placental parasites and in vitro-cultured parasites panned on endothelial cells carrying CSA. The minimum size of fractionated CSA required for a significant inhibition of placental IRBC\textsuperscript{CSA} adhesion to Sc1D cells was 1 to 2 kDa, which contrasts with the 4-kDa size necessary to reach equivalent levels of inhibition with panned IRBC\textsuperscript{CSA} of this phenotype. All placental IRBC\textsuperscript{CSA} cytoadhered to Sc1D SBEC, which express only the CSA receptor. Panning of IRBC\textsuperscript{CSA} on these cells resulted in a significant quantitative increase of IRBC cytoadhering to the CSA of Sc1D cells but did not change their capacity for adhesion to CSA on normal placenta cryosections. Our results indicate that the CSA binding phenotype is heterogeneous and that several distinct genes may encode *P. falciparum*-CSA ligands with distinct binding properties.

In high-risk regions of the world, pregnancy is frequently complicated by *Plasmodium falciparum* malaria infection. If the fetus remains protected from parasitemia during pregnancy, the placentas are often directly involved in protection, and more severe levels of infection appear to correlate with low birth weight and neonatal morbidity and mortality (6). The infection is characterized by sequestration of infected erythrocytes (IRBC) within the placenta, predominantly within the intervillous space where the maternal erythrocytes are in contact with the trophoblastic layer and the syncytiotrophoblast (5). In cases of low peripheral parasitemia, women can be asymptomatic, even if there is a large accumulation of parasites in the placenta. In the chronic stage an inflammatory reaction occurs, with accumulation of leukocytes and necrosis of the neighboring placental tissue (2).

Recently it has been demonstrated that a distinct subpopulation of parasites which bind chondroitin sulfate A (CSA) is responsible for *P. falciparum* adhesion in the placenta. This supports the view that parasite sequestration in the placenta could be the leading cause of maternal malaria (5). Another study suggested an involvement of ICAM-1 (13). Identifying the cytoadhesion phenotypes involved in maternal malaria is of major importance because CSA is the only receptor for which it has been possible to specifically reverse IRBC adhesion in vivo, by injecting a 50-kDa CSA in a *P. falciparum*-infected *Saimiri sciureus* monkeys (15). Therefore, if cytoadhesion to CSA is the main adhesion phenotype involved in maternal malaria, it is important to investigate the possibility of desequestrating bound parasites from the placenta by the injection of soluble CSA. The objective of the present study was to elucidate the feasibility of reversing IRBC sequestration in human placentas by flushing pieces of placenta with CSA immediately after child delivery. We further analyzed the adhesion phenotypes of the desequestered IRBC by inhibition assays with placenta cryosections and *Saimiri* brain microvascular endothelial cells (SBEC) in the presence of different potential inhibitors. Our results show that the predominant adhesion phenotype of all placenta-derived IRBC is adhesion to CSA.

MATERIALS AND METHODS

Placentas. Five of seven malaria-infected placentas collected in Yaoundé, Cameroon, were from primigravida women, one was from a secundigravida woman, and one was from a multigravida (4-gravida) woman (Table 1). Several biopsies of approximately 0.5 cm\textsuperscript{3} were removed from the maternal-facing surface of each placenta between the midportion and the border and were immediately frozen by immersion in liquid nitrogen. This technique eliminated the possibility of artifacts caused by fixative agents such as formalin. Serial 7-μm cryosections of each biopsy were fixed with methanol for 2 min and stained with Giemsa stain and also with hematoxylin-eosin. The presence of cytoadherent...
IRBC (with apparent direct contact with the syncytiotrophoblastic layer) was measured as the mean number of IRBC ± standard error (SE) per 20 high-power microscopic fields (Leitz Diplan microscope; magnification, ×1,000).

Placenta 938 was classified as showing an active-chronic infection, and the other three were classified as showing a more active or moderately active chronic infection, according to previously described criteria (5). The placenta was collected at the beginning of the malaria transmission season.

Collection of IRBC from placentas. A transverse piece approximately 12 by 12 cm was removed from the area between the center and the edge of each placenta immediately after delivery and injected with 40 ml of 0.9% NaCl containing 5 IU of heparin (Choay, Gentilly, France) per ml. The tissue was then immersed in the same saline solution. After thorough external rinsing of the piece of placenta with fresh heparinized saline, it was injected repeatedly at several sites in the maternal compartment with a total of 500 ml of heparinized RPMI 1640 by using 20-ml syringes with a 0.8- by 50-mm needle (Beckton Dickinson). The number of released RBC dropped to fewer than 1,000/ml after the last flushing. The same procedure (Table 1) was used for flushing of the placenta with RPMI 1640 containing 2.4 mM Na-bicarbonate, 2 mM glutamine, 50 mM hypoxanthine, 10 g of a 50-kDa soluble CSA obtained from bovine trachea (Fluka, Saint Quentin Fallavier, France) per ml. After flushing with CSA, a placental biopsy was taken and immediately frozen in liquid nitrogen for the estimation of residual IRBC on serial 7-μm cryosections. The recovered IRBC were assessed for their cytoadhesion property and composition and cryopreserved in liquid nitrogen. The number of IRBC required to perform cytoadhesion inhibition assays is 4 × 10^6. We therefore performed assays only when the number of collected IRBC was greater than 10^7 (Table 1). Otherwise, all of the collected IRBC were kept for cell cultures.

Culture of parasites. The IRBC subpopulation PA^CSA^ with a CSA adhesion phenotype, was selected from the PA^CSA^ strain by panning on Sc17 cells (16). Cryopreserved IRBC obtained by flushing with heparin (IRBC^HS^) or with CSA (IRBC^CSA^) were thawed and grown on human O^2^ RBC in RPMI 1640 containing 2.4 mM Na-bicarbonate, 2 mM glutamine, 50 mM hypoxanthine, 0.2% glucose, 0.5% Albumax (Gibco, Cergy Pontoise, France), and 10 μg of gentamicin per ml at 37°C in a humid atmosphere of 5% O_2, 5% CO_2 and 90% N_2. Nonimmune, non-C5a, non-Fcγ receptor IgG was added and expanded by culture. Cytoadhesion inhibition assays on SBEC and cryosections of human placentas. Cytoadhesion inhibition assays were performed with PA^CSA^ (16), FCR3^CSA^ (10), freshly flushed CSA^HS^, and IRBC^CSA^ immediately after flushing of the placentas and with non-C5a, non-Fcγ receptor IgG in IRBC^CSA^ and IRBC^HS^ obtained from cultures by using a previously described method (14, 16). Briefly, for cytoadhesion assays, 40 μl of a solution of 5 × 10^6 IRBC/ml diluted in cytoadhesion medium was spotted on confluent Sc1D (which express CD36, ICAM-1, and CSA), CHO, CHO-CD36, and CHO-ICAM-1 cells (10) grown on 12-dot immunofluorescence assay slides (Institut Pasteur, Paris, France). For inhibition assays, the IRBC were either spotted alone after pretreatment of the cells with 0.5 U of chondroitinase ABC per ml or spotted with either 100 μg of a 50-kDa CSA (Fluka) per ml, an equimolar concentration of different sizes of CSA polymers (1, 1.5, 2, 2.5, 3, 3.5, 5, and 7 kDa), 25 μg of 84H10 anti-ICAM-1 monoclonal antibody (MAb) (Immunotech, Marseille, France) per ml, 5 μg of FAB152 anti-CD36 MAb (a gift from L. Edelman) (14, 16) per ml, or normal immune anti-P. falciparum plasma at dilutions of 1/5, 1/10, 1/20, 1/40, and 1/80. An adhesion inhibition assay was also performed with culture-adapted IRBC^HS^ and IRBC^CSA^ by using unfixed 7-μm cryosections of normal human placenta, according to a procedure described elsewhere (9). All assays were performed in duplicates or triplicates, and the inhibitions are expressed as a percentage control value.

Preparation of CSA molecules of different sizes. A 10-mg/ml solution of a 50-kDa CS (Sigma, St. Louis, Mo.) was adjusted to pH 7.5 with 0.5 U of chondroitinase ABC per ml for 30 min at 20°C. The sample was boiled for 10 min to stop the reaction. Control CSA was prepared in the same way but without the addition of chondroitinase ABC. The different-sized molecules present in the digested sample were separated by exclusion chromatography with Bio-Gel P30 (150-4154; Bio-Rad, Ivry sur Seine, France), and their sizes were determined by comparison with the eluted profile of standards (a gift from H. Lortat-Jacob). The elution medium was 1 M NaCl. The collected fractions were dialyzed against water and lyophilized. Cytoadhesion inhibition activity was tested by diluting the fraction in 0.15 M NaCl at a concentration of 4 mM. The fractions were mixed with equal volumes of suspensions of 1 × 10^7 IRBC/ml of cytoadhesion medium to give final concentrations of 2 mM CSA and 5 × 10^6 IRBC/ml. Control CSA was treated in the same way, with the final concentration of 2 mM corresponding to 0.1 mg/ml. The molecules that purified and tested in the inhibition assays were 1, 1.5, 2.5, 3, 3.5, 5, and 7 kDa in size.

Determination of the IgG isotypes directed against IRBC^CSA^, Plasma samples were diluted 1:10 with cytoadhesion medium, and 100 μl was incubated with 10 μg of a PA^CSA^ pellet for 30 min at 4°C. After washing with cytoadhesion medium, goat anti-human IgG (chain-specific)-fluorescein isothiocyanate conjugate (F-6380, Sigma, St. Louis, Mo.); anti-IgG1 (0280), IgG3 (0282), or IgG4 (0283) MAb (Immunotech); or IgG2 MAb (1-9513; Sigma, Saint Quentin Fallavier, France) was added to the recommended working dilution and incubated for 30 min at 4°C. After washing, anti-IgG isotype MAb were visualized by incubating the pellet for an additional 30 min with an anti-mouse IgG (Fc-specific) fluorescent isothiocyanate conjugate (F-8864; Sigma). The fluorescence intensity was examined by exhaustive photon reassignment microscopy (CELLScan; Scanalytics, Billerica, Mass.) (4).

Statistical analysis. Results of IRBC cytoadhesion inhibition assays are expressed as the mean value ± SE. The Mann-Whitney test was employed to evaluate the statistical significance of data obtained from the assays and for comparison of cytoadhesion levels.

RESULTS

Density of placental parasites before and after flushing. To evaluate the parasite density in the placentas, three serial 7-μm cryosections of frozen placental biopsies were stained with Giemsa and hematoxylin-eosin stains and examined for parasite and pigment deposits. The number of sequestered IRBC (Table 1) ranged between 0.86 ± 0.23 and 110 ± 34.70 IRBC/20 high-power fields, reflecting rather low adhesion rates compared to those of cultured CSA binding parasites to placental sections (Table 2) (except for placenta 193). Histopathological analysis revealed that placenta 193 could be classified as having an active-chronic infection based on the presence of IRBC in the intervillous space, pigment and phagocytosed erythrocytes in monocytes, and the deposit of

<table>
<thead>
<tr>
<th>TABLE 1. Characteristics of IRBC collected before and after flushing of pieces of placentas with heparin and CSA</th>
<th>Placenta no.</th>
<th>No. of gravida</th>
<th>Successive treatments of placentas</th>
<th>No. of cytoadherent IRBC/20 fields (mean ± SE)</th>
<th>No. of IRBC collected by each flushing</th>
</tr>
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<tbody>
<tr>
<td>42</td>
<td>1</td>
<td>Heparin</td>
<td>15.8 ± 1.1</td>
<td>8.9 × 10^8</td>
<td></td>
</tr>
<tr>
<td>940</td>
<td>1</td>
<td>Heparin</td>
<td>0.3 ± 0.3</td>
<td>7.3 × 10^8</td>
<td></td>
</tr>
<tr>
<td>939</td>
<td>1</td>
<td>CSA</td>
<td>0.2</td>
<td>7.0 × 10^7</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>1</td>
<td>Heparin</td>
<td>2.7 ± 2.5</td>
<td>1.6 × 10^7</td>
<td></td>
</tr>
<tr>
<td>193</td>
<td>1</td>
<td>Heparin</td>
<td>11.0 ± 35</td>
<td>7.4 × 10^8</td>
<td></td>
</tr>
<tr>
<td>42DJ</td>
<td>2</td>
<td>Heparin</td>
<td>3.6 ± 1.4</td>
<td>3 × 10^9</td>
<td></td>
</tr>
<tr>
<td>938</td>
<td>4</td>
<td>Heparin</td>
<td>14.2 ± 3.3</td>
<td>6.3 × 10^7</td>
<td></td>
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pigment and cells within the fibrin (3). The other placentas presented an aspect of active infection, with parasitized erythrocytes, some monocytes, and moderate cell, pigment, or fibrin deposit.

**Recovery of placental IRBC.** Placentas were first flushed with heparin to prevent coagulation of the maternal blood. We assumed that the majority of cytoadherent IRBC would remain bound to syncytiotrophoblasts in the placenta. In a second step, we flushed the placentas with CSA and estimated the efficiency of the flushing procedure by comparing the initial counts of IRBC with those of residual IRBC (Table 1). After CSA flushing, there were no more IRBC present on Giemsa-stained cryosections of placentas 42DJ, 938, 940, 24, and 193. For placentas 42 and 939, the number of IRBC dropped from an initial 15.8 ± 1.1 and 19 ± 2.7 to 0.3 ± 0.3 and 0.2 ± 0.3 IRBC/20 high-power fields, respectively.

The total number of IRBC (Table 1) recovered by flushing with heparin varied between 1.6 × 10^5 and 3.1 × 10^5 IRBC/mg, and that obtained by flushing with CSA varied between <5 × 10^5 and 7.4 × 10^5 IRBC/mg. There was no correlation between recovered IRBC and initial parasite count. The most obvious reason for this was variation in thickness between the pieces of placenta. There was no correlation either between the ratio of CSA- to heparin-flushed IRBC and the repartition of the parasitic stages or between the number of recovered infected and uninfected RBC (data not shown).

**Receptor preference of placental parasites.** To assess the receptor preference of fresh and culture-adapted placental IRBC, we performed direct cytoadhesion assays with Sc1D cells, which express CSA, CD36, and ICAM-1. Freshly flushed IRBC were largely composed of IRBC of the CSA adhesion phenotype (Fig. 1A). Anti-CD36 and anti-ICAM-1 MAbs did not significantly affect the adhesion of freshly flushed IRBC. IRBC parasites, adapted to in vitro culture, conserve the CSA adhesion phenotype (Fig. 1B). The presence in some samples of the CD36 and ICAM-1 adhesion phenotypes probably resulted from the presence of rings in the flushed IRBC or from the switching of some parasites to different receptor phenotypes during laboratory culture.

Inhibition assays with freshly flushed IRBC from four placentas (Fig. 2) were significantly (P = 0.0286) but only partially inhibited by CSA (41% ± 19% to 85% ± 9%) and by chondroitinase ABC treatment of Sc1D cells (33% ± 1% to 66% ± 9%). Anti-ICAM-1 and anti-CD36 MAbs did not significantly inhibit cytoadhesion, suggesting that a subpopulation of adherent IRBC interacts with an unidentified receptor expressed by Sc1D cells. CSA-flushed parasites were, in most cases, significantly less inhibited by CSA and chondroitinase ABC than cultured parasites selected by two rounds of panning on CSA (data not shown). However, this distinct CSA phenotype of flushed parasites was transformed into a CSA phenotype highly sensitive to CSA inhibition after the IRBC were adapted to culture. It was impossible to count the cytoadhesion of freshly flushed IRBC and IRBC samples for samples 938 and 42DJ, because of their tendency to form large IRBC aggregates. This tendency to autoagglutinate disappeared after IRBC were maintained in culture.

IRBC from four placentas as well as within each placental population, different CSA binding phenotypes exist. The ones with higher affinity for Sc17-derived CSA are prob-
Inhibition (mean and SE) is expressed as a percentage of the corresponding control value obtained in the absence of inhibitor. Appropriately selected by the panning process, leading to the loss of ligands with low affinity. Furthermore, the panned parasites were significantly more sensitive to CSA inhibition and chondroitinase ABC treatment than CSA-flushed parasites, indicating that the panning selected parasites that had lost the capacity to bind to the unidentified receptor on Sc1D cells (data not shown).

Cytoadhesion of culture-maintained IRBC<sup>CSA</sup> to intact CHO cells which naturally express CSA was between 3,072 ± 222 and 10,273 ± 1,358 IRBC/mm<sup>2</sup> (not shown), whereas adhesion to chondroitinase ABC-treated normal CHO cells, CHO-CD36 cells, CHO-ICAM-1 transfecteds, ScC2 (CD36) cells, or Sc3A4 (ICAM-1) cells was as low as 1 ± 1 to 17 ± 14 IRBC/mm<sup>2</sup>. The lack of significant levels of cytoadhesion in these cell types revealed their cytoadhesion specificity for CSA (<i>P</i> < 0.0001). We investigated whether Sc17-panned placenta IRBC<sup>CSA</sup> conserved their ability to adhere to syncytiotrophoblasts after panning. We performed an inhibition assay on normal human placenta cryosections in the presence of CSA or after treatment with chondroitinase ABC. IRBC<sup>CSA</sup>, after two rounds of panning on Sc17 cells, maintained their full capacity to adhere to normal placenta cryosections (Table 2).

**Table 3. Cytoadhesion of culture-maintained IRBC<sup>CSA</sup> to Sc1D cells before and after two rounds of panning on Sc17 cells**

<table>
<thead>
<tr>
<th>Sample</th>
<th>IRBC/mm&lt;sup&gt;2&lt;/sup&gt; (mean ± SE)</th>
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<tr>
<td></td>
<td>Initially</td>
</tr>
<tr>
<td>42</td>
<td>104 ± 23</td>
</tr>
<tr>
<td>939</td>
<td>272 ± 15</td>
</tr>
<tr>
<td>24</td>
<td>126 ± 17</td>
</tr>
<tr>
<td>42DJ</td>
<td>2,008 ± 102</td>
</tr>
<tr>
<td>940</td>
<td>196 ± 15</td>
</tr>
<tr>
<td>193</td>
<td>330 ± 19</td>
</tr>
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</table>

Size-dependent CSA activity. While the inhibitory effect of CSA fragments smaller than 4 kDa on cytoadhesion of IRBC from peripheral isolates is not significant (14), in the present study cytoadhesion of three culture-adapted placental isolates (24<sup>CSA</sup>, 193<sup>CSA</sup>, and 939<sup>CSA</sup>) was significantly inhibited by 1-kDa CSA (the two extreme patterns of inhibition are presented in Fig. 3). CSA fragments of 1.5 kDa (42DJ<sup>CSA</sup> and 940<sup>CSA</sup>) or 2 kDa (42<sup>CSA</sup>) were required to significantly inhibit these three other culture-adapted placental isolates. The 5-kDa CSA fragments inhibited more than 90% of the cytoadhesion (no significant difference compared with the 50-kDa CSA inhibitory activity) for three of six placental isolates (193<sup>CSA</sup>, 939<sup>CSA</sup>, and 940<sup>CSA</sup>), while fragments of at least 9 kDa were necessary to reach similar levels of inhibition with peripheral IRBC (15). For the three other placental isolates, 74 to 81% of cytoadhesion was inhibited by the 5-kDa CSA fragments, and a 7-kDa CSA polymer inhibited >95% (Fig. 3).

**Inhibition of IRBC<sup>CSA</sup> cytoadhesion to Sc 1D by anti-<i>P. falciparum</i> immune plasma.** To assess the presence of cross-reactive anti-CSA adhesion antibodies in sera from women who had experienced <i>P. falciparum</i> infection during pregnancy (in Cameroon), we performed inhibition assays measuring adhesion of two heterologous laboratory strains, FCR3<sup>CSA</sup> and PA<sup>CSA</sup>, to Sc1D cells in the presence of plasma from the placenta donors. Cytoadhesion was significantly inhibited by plasma from all primi- and multigravida woman. This level varied between 62% ± 10% and 95% ± 3% for an initial plasma dilution of 1/5. The level of inhibition was dilution dependent for each plasma, with inhibition ranging between 0 and 33% ± 13% for a final dilution of 1/80 (the two extreme patterns of inhibition are presented in Fig. 4). Importantly, the same plasma dilution had comparable antibody inhibition titers against PA<sup>CSA</sup> (data not shown). Normal human sera from Caucasians showed no inhibition at serum dilutions of 1/5. There was no correlation between the gravida status of the donor and the level of inhibition.

CELLScan analysis of the six plasma samples from the placenta donors revealed the CSA adhesion phenotype PA<sup>CSA</sup> on the surface of infected erythrocytes at serum dilutions of 1/10 (Fig. 5). Surprisingly, only antibodies of the IgG3 isotype (but not IgG1, -2, and -4) reacted with the surface of intact IRBC.

**Discussion**

In this work we isolated cytoadherent parasites from different placenta immediately after child delivery from primigravida and multigravida women. Pieces of placenta were successively flushed with heparin and CSA, and the recovered IRBC populations were analyzed for their cytoadhesion receptor preferences. This approach allowed us to collect IRBC which cytoadhered exclusively to CSA. However, the cytoadhesion of IRBCs, collected immediately after flushing, to Sc1D cells (expressing CSA, CD36, and ICAM-1) was only partially inhibited by CSA or chondroitinase ABC treatment and was not inhibited by anti-CD36 and anti-ICAM-1 antibodies. This may imply the existence of another, yet-unidentified cytoadherent IRBC population. However, this particular adhesion phenotype was not stable once the parasites had been adapted to in vitro culture. We know from recent data that the CSA ligand of the FCR3 strain does not interact with CD36, ICAM-1, or any other known adhesion receptor (1a, 18), which suggests that another adhesion molecule was coexpressed in the placenta-derived parasites. It remains to be shown whether the new receptor observed on Sc 1D cells is involved in IRBC adhesion to placental tissue. Because the cytoadhesion inhibition assays were performed on SBEC and not on sections of placenta, it cannot be excluded that some IRBC express a phenotype allowing them to cytoadhere to an unknown receptor present on the syncytiotrophoblasts and not expressed by the SBEC. However, these IRBC would be indistinguishable from those cytoadhering to the unknown receptor expressed by SBEC (Sc
1D) before identification of at least one of the two potential receptors. As already shown by the study of Fried and Duffy (5), CD36 is not involved in placenta sequestration despite the nearly ubiquitous characteristics of *P. falciparum* strains for cytoadhesion to this receptor. Flushing of placentas with an anti-CD36 MAb did not result in release of IRBC (data not shown). Furthermore, the use of this antibody did not result in a significant inhibition of IRBC<sup>CSA</sup> or IRBC<sup>CSA</sup> cytoadhesion to Sc1D cells immediately after flushing. We detected only CD36 binding to cultured IRBC<sup>Hep</sup>. This was probably due to the initial presence of ring stage IRBC<sup>CD36</sup> in the flushed populations or to a phenotype switch during parasite culture. We were not able to confirm an involvement of ICAM-1 (13) in IRBC sequestration in the placentas studied here. The presence of ICAM-1 binding IRBC in cultures of IRBCHep samples is probably the result of the same phenomenon as previously described for the CD36 phenotype. Again, flushing the same placental pieces with an anti-ICAM-1 MAb did not result in a specific release of parasites with the ICAM-1 adhesion phenotype, and parasites of this phenotype did not significantly bind to placenta cryosections (data not shown). Maubert et al. (13) described the adhesion of peripheral blood IRBC from pregnant women to cultured term human trophoblasts and the possibility of abolishing this cytoadhesion with anti-ICAM-1 MAb 84H10. Furthermore, this cytoadhesion was not abolished when a 50-kDa soluble CSA from bovine trachea was used. In the same study, cytoadhesion of PA<sup>CSA</sup> (designated Rp5 by our laboratory at the time) was inhibited by soluble CSA but not by MAb 84H10. In an immunohistochemical
study (19) it was not possible to visualize ICAM-1 on normal term trophoblasts in the placenta. However, it was shown that expression of ICAM-1 on trophoblasts may occur as a consequence of an immunoinflammatory disorder at the intervillous villous level that triggers the production of tumor necrosis factor alpha, which then induces the expression of ICAM-1. Furthermore, sections of umbilical cord do not support the adhesion of placental parasites (5) despite the fact that umbilical cord endothelial cells (HUVEC) express predominantly ICAM-1. In the placenta, a relatively low-affinity interaction may be sufficient for adhesion of IRBC to syncytiotrophoblasts because of the low mechanical pressure exerted by the blood flow, estimated to be 600 ml/min at a hydrostatic pressure of 30 to 50 mm Hg in the maternal compartment of the placenta. In comparison, IRBC sequestered in the microvasculature can be exposed to blood pressures of 80 to 130 mm Hg (17). This might explain the abundance of the CSA adhesion phenotype in parasites flushed with heparin. Interestingly, when we compared the in vitro cytoadhesion characteristics of SBEC, syncytiotrophoblasts of placenta IRBC\textsuperscript{CSA} isolates, and laboratory strains such as Palo-Alto(FUP)\textsuperscript{CSA}, IPL/BRE\textsuperscript{CSA}, and FCR\textsuperscript{CSA} (9, 14, 18), a striking difference was observed in the size of CSA needed for inhibition of adhesion. In placental isolates, significant inhibition was obtained with CSA fragments as small as 1 to 2 kDa, while 4-kDa fragments are required for peripheral isolates. In addition, inhibition of greater than 90% was obtained with 5- to 7-kDa polymers of CSA for placental isolates, while 9-kDa polymers were necessary to achieve the same level of inhibition for peripheral isolates. The fact that adhesion of placental IRBC\textsuperscript{CSA} can be impaired by small CSA polymers suggests that these populations might be able to bind to small CSA chains. Again, these differences are probably due to variations in blood pressure between the microvasculature and placenta. One can assume that a low blood pressure facilitates weaker interactions, which require smaller CSA polymers. These variations in the sensitivity of IRBC\textsuperscript{CSA} cytoadhesion to the different CSA polymers, between placental and peripheral blood isolates or between different placental isolates, might be the first manifestation of a heterogeneity of this phenotype. The \textit{var} gene family is multigenic and might encode different \textit{P. falciparum}-CSA ligands with distinct binding properties, with the environmental pressure selecting for the development of some of the corresponding phenotypes.

We obtained evidence of another manifestation of the probable heterogeneity of the IRBC\textsuperscript{CSA} populations. When we panned IRBC\textsuperscript{CSA} on Sc17 cells, we observed an increase in the cytoadhesion ability of each placental isolate. This was not due to the presence of knobless IRBC in the cultures, as we used only gelatin-enriched suspensions. This was also not due to the presence of other adhesion phenotypes in the initial cultured IRBC\textsuperscript{CSA} placental isolates, as the level of cytoadhesion inhibition induced by soluble CSA or chondroitinase ABC pretreatment of the target cells was always greater than 95%. We assumed that panning selected for a subpopulation of IRBC\textsuperscript{CSA} having a strong interaction with Sc17 cells.

Other evidence for a role of CSA as a cytoadhesion receptor in the placenta comes from immunofluorescence studies showing an abundance in the placenta of thrombomodulin (9), a proteoglycan which has a CSA and is expressed by syncytiotrophoblasts in human and \textit{Saimiri} placenta. However, thrombomodulin is also found in abundance in microvascular endothelial cells in general and particularly in those in the microvasculature of the cerebellum (1); the lungs, heart, and kidneys (12); and other targets of sequestration.

The question as to whether IRBC\textsuperscript{CSA} derived from placenta interact only with syncytiotrophoblast CSA, as suggested by Fried and Duffy (5), remains. Given that the proteoglycan thrombomodulin is also expressed in the microvasculature of target organs such as the brain, kidney, heart, and lung, we would expect that IRBC\textsuperscript{CSA} cytoadhere in these tissues. The observed release of cells with the CSA adhesion phenotype in male \textit{Saimiri} monkeys following the injection of CSA (15) clearly demonstrates that, at least in this experimental model, sequestration is not restricted to the placenta. In addition, the development of antiadhesion antibodies directed against IRBC\textsuperscript{CSA} is an acquired humoral immune response which, in our small sample, is not restricted to multigravida women, as was previously suggested (7). Inhibition of the PA\textsuperscript{CSA} adhesion phenotype by IgG indicates that the primigravida women had developed anti-CSA cytoadhesion antibodies prior to contracting the placenta infection. Thus, we propose that the CSA binding phenotype is not restricted to primigravida women but that it might always be found in \textit{P. falciparum} parasites during pregnancy (5). Important questions that remain are (i) how effective such antibodies are at inhibiting cytoadhesion in vivo and (ii) at what stage anti-CSA adhesion immunity is developed.

In conclusion, our data clearly demonstrate that all placenta-derived parasites bind predominantly to CSA. Sequestration of cells with this phenotype can be specifically reversed ex vivo by a soluble bovine trachea CSA. Furthermore, the data clearly show that the CSA phenotype is heterogenous. All women had anti-CSA adhesion antibodies which cross-reacted with the surface of IRBC from heterologous strains selected for CSA binding. Interestingly, these antibodies directed against the surface of IRBC\textsuperscript{CSA} were restricted to the IgG3 isotypes. The corresponding \textit{P. falciparum} CSA ligand has been identified and cloned (1a), and work evaluating the potential of the CSA ligand for the development of an antidiisease vaccine that could protect pregnant women from a fetus-maternal pathology is in progress.

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