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Plasma Antibodies from Malaria-Exposed Pregnant Women Recognize Variant Surface Antigens on *Plasmodium falciparum*-Infected Erythrocytes in a Parity-Dependent Manner and Block Parasite Adhesion to Chondroitin Sulfate A[1]

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In areas of intense *Plasmodium falciparum* transmission, clinical immunity is acquired during childhood, and adults enjoy substantial protection against malaria. An exception to this rule is pregnant women, in whom malaria is both more prevalent and severe than in nonpregnant women. Pregnancy-associated malaria (PAM) in endemic areas is concentrated in the first few pregnancies, indicating that protective immunity to PAM is a function of parity. The placenta is often heavily infected in PAM, and placental parasites show a striking preference for chondroitin sulfate A (CSA) as an adhesion receptor. Plasma Abs from malaria-exposed multiparous women are able to interfere with binding of *P. falciparum* parasites to CSA in vitro, and acquisition of Abs interfering with CSA-specific parasite sequestration thus appears to be a critical element in acquired protection against PAM. Here we show that adults from an area of hyperendemic *P. falciparum* transmission generally possessed low levels of Abs specifically recognizing surface Ags expressed by a CSA-adhering parasite isolate, while unselected isolates were well recognized. In marked contrast, most third-trimester pregnant women from that area had very high plasma levels of such Abs. Plasma levels of Abs specifically recognizing the CSA-adhering isolate strongly depended on parity, whereas recognition of CSA-nonadhering isolates did not. Finally, we demonstrate a clear correlation between plasma levels of Abs recognizing the CSA-specific isolate and the ability to interfere with its sequestration to CSA in vitro. Our study supports the hypothesis that Abs inhibiting CSA-specific parasite sequestration are important in acquisition of protection against PAM. ***The Journal of Immunology*****, 2000, 165: 3309–3316.**

Plasmodium falciparum malaria remains as one of the leading health problems of the world. In malaria-endemic areas, substantial clinical protection is acquired during the first decade of life, and the majority of malaria-related morbidity and mortality is concentrated in young children (reviewed in Ref. 1). However, in contrast to the general absence of malaria in adults, pregnant women in endemic areas are highly susceptible to malaria, and both the prevalence and the severity of disease are higher in pregnant than in nonpregnant women (2). Possible quantitative or qualitative changes in the cellular immune system during pregnancy have been considered in explaining this phenomenon (3, 4). However, the fact that pregnancy-associated malaria (PAM),[3] which has adverse consequences for both mother and fetus, including maternal anemia, intrauterine growth retardation, and low birth weight (2), occurs mainly during the first couple of pregnancies in endemic areas is not easily explained by such a theory.

It is becoming increasingly apparent that acquired protective immunity to *P. falciparum* infection relies on Abs specifically recognizing variant parasite Ags expressed on the surface of late-stage-infected erythrocytes (LSIE) (5, 6). In this scenario, only parasites expressing variant Ags to which the host does not possess adequate specific Ab are likely to cause disease, and immunity is likely to depend on the accumulation of a large panel of Ab specificities recognizing different variants of such Ags.

PAM is often associated with sequestration of large quantities of parasites in the placenta, even when peripheral parasitemia is scant (2, 7). *P. falciparum* is unique among human malaria parasites in its ability to adhere to several host receptor molecules, including CD36, ICAM-1 (CD54), thrombospondin, vascular adhesion molecule 1, E-selectin, and chondroitin sulfate A (CSA) (8–16). Specifically, placental parasites have been shown to adhere preferentially to CSA, while parasites from nonpregnant malaria patients

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[3] Abbreviations used in this paper: PAM, pregnancy-associated malaria; CSA, chondroitin sulfate A; FCM, flow cytometry; LSIE, late-stage-infected erythrocytes; PfEMP1, *Plasmodium falciparum* erythrocyte membrane protein 1; sCSA, soluble CSA.

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rarely possess this phenotype (17). Furthermore, plasma from multigravid, but not primigravid, women from endemic areas can inhibit adhesion of placental parasites to CSA (18). Nevertheless, little detailed information about the parity dependency of inhibition of CSA-specific adhesion of *P. falciparum* is available. In the present study we have examined plasma from 163 third-trimester pregnant women with parities from 1 to 12 from an area of hyperendemic malaria transmission. Levels of Abs recognizing a CSA-adhering parasite isolate were compared with recognition of isolates without appreciable adhesion to CSA, and the relationship between Ab binding to LSIE, on the one hand, and with parity and inhibition of adhesion to CSA in vitro, on the other. We show that levels of Abs to the CSA-specific isolate are strongly associated with parity and with the ability to inhibit parasite adhesion to CSA. The data point to interference with CSA-dependent sequestration as the basis for parity-dependent acquisition of anti-PAM immunity, and suggest it as a target for vaccination against PAM.

Materials and Methods

Plasma donors

Plasma samples were collected from 163 third-trimester (gestational age, 34.8 ± 2.22 wk; mean ± 1 SD) pregnant women attending antenatal clinics in the village of Prampram, situated about 50 km east of Accra on the coast of Ghana. Additional samples were obtained from 40 healthy adults (20 men and 20 women) living in Gomoa Onyadze village about 50 km west of Accra. The pregnancy status of the latter group of women was not ascertained. Finally, a pool of plasma from adults living in Dodowa about 20 km outside Accra was used in some experiments (hyperimmune plasma pool).

Malaria transmission in southern Ghana is perennial, but peaks after the main wet season between July and September (19). *P. falciparum* is the dominant species, but *P. malariae* is also present (19). The entomological inoculation rate is approximately 8.5 infective bites/person/yr, and the dominant vector is *Anopheles gambiae* (20). Ethical clearance for the study was granted by the Ghanaian Ministry of Health (DMS-083).

Control samples were collected from healthy laboratory staff in Copenhagen. In addition, third-trimester plasma samples from 10 Danish women were included in the study. None of the control donors had any known exposure to malaria parasites, and most had never visited malaria-endemic areas.

Parasite isolates

Four *P. falciparum* isolates were used in the study. FCR3 is a long-term laboratory isolate (21), while isolates E2037 and E2039 were obtained from children with malaria, living in Dodowa village outside Accra. None of these isolates were subjected to in vitro selection for particular adhesion phenotypes. In addition to these unselected isolates, we used a CSA-adhering substrain (PA/CSA) of the Palo Alto strain (a gift from Jürg Gysin (Université de la Mediterranée, Marseilles, France)) selected for adhesion to CSA in vitro (15). All isolates were maintained in vitro by standard methods (22). High levels of binding to CSA in the PA/CSA isolate were secured by regular panning of infected erythrocytes on purified CSA (Sigma, St. Louis, MO) as previously described (16). All experiments on CSA-selected parasites were performed within 6 days of panning. All isolates were genotypically distinct according to PCR typing on merozoite surface protein 1, merozoite surface protein 2, and glutamate-rich protein, performed as described previously (23).

Isolation of LSIE

LSIE were separated from uninfected and ring-stage infected erythrocytes by magnet-activated cell sorting (Miltenyi BioTec, Bergisch Gladbach, Germany) or in some cases by gel flotation as described previously (24–27).

Parasite adhesion and agglutination

The cytoadhesion phenotype of all isolates was determined by adhesion assays to CD36, ICAM-1, and CSA as previously described (18, 28). The purified CD36 used in these assays was a gift from David Roberts and Arnab Pain (University of Oxford, Oxford, U.K.), whereas recombinant ICAM-1 and purified CSA were purchased from R&D Systems (Abingdon, U.K.) and Sigma, respectively.

The ability of isolates to agglutinate was determined by standard microagglutination assay as described previously (29).

Analysis of variant Ag-specific Abs by flow cytometry

Levels of plasma Abs recognizing variant LSIE surface Ags were measured by flow cytometry as described in detail previously (27). In brief, purified LSIE were labeled with ethidium bromide (0.1 mg/ml; 1 μl/1 $\times$ 10^5 LSIE) and sequentially exposed to test plasma (2.5 μl/1 $\times$ 10^5 LSIE), goat anti-human IgG (Dako, Glostrup, Denmark) diluted 1/250 in PBS, and FITC-conjugated rabbit anti-goat Ig (Dako) diluted 1/25. Samples were washed twice between each labeling step and once before analysis on a Coulter EPICS XL-MCL flow cytometer (Coulter Electronics, Luton, U.K.). For each sample, the mean fluorescence (expressed as channel number) was calculated using an ethidium bromide gate to identify LSIE. Labeling of uninfected erythrocytes was measured in a similar way, using uninfected erythrocytes rather than LSIE. All assays were performed using coded samples with no reference to donor parity, etc.

In some experiments with CSA-adhering LSIE, soluble CSA (sCSA; 10–500 μg/ml) was added either before or after labeling with plasma Ab.

Inhibition of parasite adhesion to CSA

Plasma-mediated inhibition of adhesion of parasitized erythrocytes to CSA was quantified essentially as described previously (18). In brief, 20 μl (10 μg/ml) of CSA (Sigma) was spotted onto Falcon petri dishes (Becton Dickinson, Brøndby, Denmark) and incubated for 3 h at room temperature. Subsequently, the drops were carefully removed, and the spots were blocked by 20 μl of BSA (20 mg/ml) for 30 min. After removal of the blocking buffer, 16 μl of LSIE (5% hemocrit, 5–20% parasitemia) preincubated for 60 min with 4 μl of test plasma was added to the CSA spots and incubated for 60 min at room temperature. In some assays LSIE were preincubated with sCSA (10–100 μg/ml) instead of plasma. Following incubation, unbound erythrocytes were removed by washing five times in PBS, and bound erythrocytes were counted (minimum of 15 fields) by microscopy ($\times$400) of the glutaraldehyde-fixed (0.5%, 10 min), Giemsa-stained spots. Again, all assays were performed using coded samples, with no reference to donor parity, etc. In assays of reversal of parasite adhesion, the preincubation step was omitted, and plasma was added only after parasites had been allowed to adhere to CSA for >30 min.

Statistical analysis and data presentation

Groupwise averages were compared by Kruskal-Wallis one-way ANOVA on ranks (H) followed by Dunn's test. Spearman's test (r_s) was used for analysis of parameter association. Analysis of possible confounding factors was performed using multiple linear regression analysis (t). $p < 0.05$ was considered significant. All experiments described in this paper were repeated a minimum of three times with similar results.

Results

Parasites selected on CSA have a distinct adhesion phenotype

Late-stage (amoeboid trophozoites and schizonts) *P. falciparum*-infected erythrocytes (LSIE) are able to adhere to a number of host receptor molecules (reviewed in Ref. 30). Most unselected parasite isolates investigated to date have shown affinity for CD36, whereas binding of LSIE to ICAM-1 is less common, and CSA binding is rare (31). We found that LSIE of all three unselected isolates adhered significantly to CD36, whereas none showed significant binding to either ICAM-1 or CSA (Table I). In contrast, the CSA-selected isolate PA/CSA showed strong affinity for CSA and did not bind to either CD36 or ICAM-1. The specificity of the binding to CSA was demonstrated by concentration-dependent inhibition of this adhesion by the addition of sCSA to the adhesion assay (data not shown). In conclusion, the parasite isolates all displayed the expected adhesion phenotype.

CSA-selected parasites are not well recognized by Abs in plasma from adults living in a malaria-endemic area

Plasma samples from individuals from malaria-endemic areas contain Abs that recognize variant LSIE surface Ags, and such Abs can be detected and quantified by flow cytometry (FCM) (27, 32). For all three unselected parasite isolates studied here, plasma from

Table I. *Parasite isolate-specific adhesion properties (LSIE bound/mm^2)*

Isolate	Ligand			
	BSA	CD36	ICAM-1	CSA
2037	73.2 ± 44.7	393.3* ± 147.8	89.3 ± 32.7	81.3 ± 48.7
2039	205.3 ± 140.0	1154.7* ± 230.6	137.3 ± 45.4	40.0 ± 27.8
FCR3	68.0 ± 43.5	1261.3* ± 228.4	52.0 ± 31.8	44.0 ± 30.4
PA/CSA	254.7 ± 85.7	272.0 ± 148.0	22.7 ± 20.2	6300.0* ± 559.5

*, Significantly (p(H) < 0.05) higher than background (BSA).

adults living in a hyperendemic malaria area (coastal Ghana) contained significantly higher levels (p(H) < 0.01 and $p < 0.05$, by Dunn's test) of Abs recognizing LSIE surface Ags compared with levels in plasma from nonexposed donors (Fig. 1*A* and data not shown). FCR3, which has been maintained in continuous in vitro culture for many years, was the unselected parasite isolate least recognized (not shown). In marked contrast to the plasma Ab-mediated pan-recognition of the unselected parasites, the CSA-selected parasite isolate PA/CSA was only poorly recognized by plasma from the same malaria-exposed Ghanaians (Fig. 1*B*). Even more strikingly, in not a single case did plasma samples from Ghanaian men contain Ab levels above levels in plasma from nonexposed control donors. The majority of plasma samples from Ghanaian women displayed a similar lack of Ab-mediated recognition of the CSA-selected parasite isolate (Fig. 1*B*). As a result, the recognition of CSA-selected parasites by the Ghanaian men or women did not differ significantly from that of plasma from donors without malaria exposure (p(H) = 0.09), and similarly, the recognition of CSA-selected parasites by a hyperimmune plasma pool was much less than the recognition of unselected parasites (Fig. 1). However, despite this general nonrecognition, a proportion of the plasma samples from the Ghanaian women did contain Abs specifically recognizing the CSA-selected parasites, and some at remarkably high levels (Fig. 1*B*). No reliable information regarding the pregnancy status of any of the Ghanaian women at the time of the collection of these blood samples was available, but with hindsight (see further below) it is tempting to speculate that these women either were pregnant or had recently been pregnant.

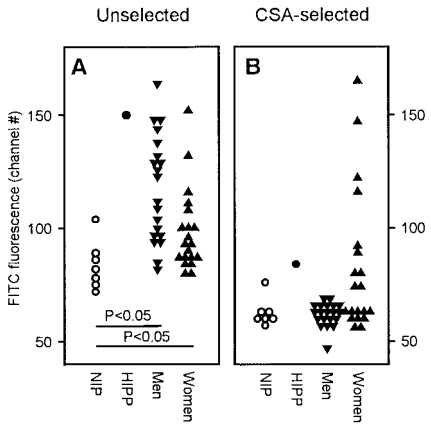

FIGURE 1. Recognition of variant surface Ags on erythrocytes infected with late developmental stages of *P. falciparum* malaria parasites by Abs in plasma from seven adults never exposed to malaria parasites (○, NIP), pooled plasma from Ghanaian adults selected for high titers of plasma Abs to variant surface Ags (●, HIPP), 20 men (▼), and 20 women (▲) living in an area of hyperendemic malaria transmission (Gomoa Onyadze, Ghana). Data obtained with one unselected parasite isolate (E2039, *A*) and one CSA-selected isolate (PA/CSA, *B*) are shown. Statistically significant differences are indicated (Dunn's test following Kruskal-Wallis one-way ANOVA on ranks).

Plasma Abs from malaria-exposed third-trimester pregnant women specifically recognize Ags on the surface of erythrocytes infected by CSA-selected parasites

To investigate the relationship between plasma Ab-mediated recognition of CSA-selected parasites and pregnancy in more detail we proceeded to analyze the levels of LSIE surface Ag-specific Abs in plasma from 163 malaria-exposed third-trimester pregnant women. In these samples the median level of Abs specific for the CSA-selected isolate was significantly higher (p(H)<0.001 and $p < 0.05$ for all pairwise differences (Dunn's test)) than for any of the unselected parasite isolates (Fig. 2*A*). This indicates that malaria-exposed third-trimester pregnant women have much higher

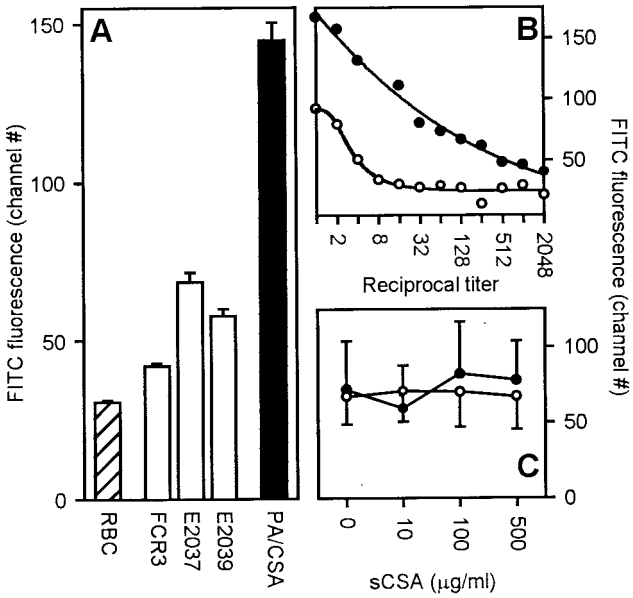

FIGURE 2. Recognition of variant erythrocyte surface Ags by Abs in plasma from third-trimester pregnant women from an area of hyperendemic malaria transmission (Prampram, Ghana). Medians and 95% confidence intervals of data from 163 women, obtained with uninfected erythrocytes (RBC) and erythrocytes infected with late developmental stages of unselected (FCR3, E2037, and E2039) and CSA-selected (PA/CSA) parasite isolates (*A*). All pairwise differences between groups were statistically significant ($p < 0.05$, by Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's test). Titration of a pool of plasma from pregnant women selected for high titers of plasma Abs to variant surface Ags against a CSA-selected (●, PA/CSA) and an unselected (○, E2039) parasite isolate (*B*) was performed. The effect of addition of sCSA before (●) or after (○) incubation of PA/CSA parasites with plasma from five plasma samples from malaria-exposed third-trimester pregnant women is shown (*C*).

titers of Abs recognizing LSIE infected by CSA-selected compared with unselected parasites. When CSA-selected and unselected LSIE were labeled by 2-fold dilutions of a pool of plasma from malaria-exposed pregnant women, significant labeling of CSA-selected LSIE remained at plasma dilutions where Abs recognizing any of the unselected parasite isolates were undetectable (Fig. 2*B* and data not shown). This supports the hypothesis of higher titers of Abs to CSA-adhering compared with unselected parasites in the pregnant women.

To test for a possible unspecific effect of pregnancy, we compared levels of Abs recognizing CSA-adhering LSIE in plasma from nonexposed third-trimester pregnant women, nonexposed nonpregnant adults, malaria-exposed men, malaria-exposed women, and malaria-exposed third-trimester pregnant women. The median Ab level in plasma from nonexposed pregnant women (median channel number, 41.8; $n = 10$) was not significantly different (by Kruskal-Wallis test, followed by Dunn's *post-hoc* test) from that of nonexposed, nonpregnant donors (35.2; $n = 14$). Levels in exposed males were low (42.3; $n = 20$), higher in plasma from malaria-exposed women (48.1; $n = 20$), and highest in plasma from malaria-exposed third-trimester pregnant women (68.8; $n = 5$). Binding of plasma Abs to CSA-selected LSIE could not be either inhibited or reversed (Fig. 2*C*) by sCSA at concentrations ranging from 10–500 μg/ml.

The finding of very high levels of Abs in plasma from malaria-exposed pregnant women, specifically recognizing variant Ags expressed by CSA-selected parasites prompted us to examine in more detail the apparently general inability of immune plasma to agglutinate CSA-selected LSIE (33) (our unpublished observations). When examining unselected parasite isolates, our FCM assay corresponds well to the conventional agglutination assay of Abs to variant-infected erythrocyte surface Ags (29). We thus speculated that the lack of agglutination of CSA-selected parasites might be due to an Ag-Ab ratio that is unfavorable for an agglutination reaction. However, agglutination assays using plasma from third-trimester pregnant women in 2-fold dilutions down to 1/2048 did not support this hypothesis, as the only (small) agglutinates observed when using CSA-selected LSIE were seen at low dilutions (1/1 to 1/4; data not shown). It thus appears that the general absence of agglutination CSA-selected LSIE is not simply due to the high plasma levels of potentially agglutinating Ab species.

Recognition of CSA-selected parasites by Abs in plasma from third-trimester pregnant women correlates with donor parity

The marked parity dependency of PAM in endemic areas has been proposed to reflect acquisition of protective Abs that specifically recognize placenta-adhering parasites (18). To investigate whether the high levels of third-trimester plasma Abs recognizing CSA-selected LSIE in our FCM assay were related to donor parity, we stratified the levels of Abs recognizing unselected and CSA-selected LSIE according to the parity of the plasma donor. The parity of the 163 plasma donors ranged from 1–12 (see Table II for details).

As shown in Fig. 3, levels of Abs recognizing CSA-selected parasites were significantly correlated to parity ($p(r_s) = 0.0008$), whereas this was not the case with respect to any of the three unselected parasite isolates ($0.09 < p(r_s) < 0.9$). Analysis of the Ab recognition of CSA-selected LSIE by multiple linear regression analysis considering parity and possible confounding factors, such as donor age, tribe, residence, gestational age, and malaria prophylaxis (Table II), identified parity as the only significant explanatory variable ($p(t) < 0.04$). Similar results were obtained if we reduced the number of independent variables included in the modeling. In contrast, parity did not contribute significantly to explaining sample variation when similar analyses were applied to the datasets obtained with unselected parasites ($p(t) > 0.5$ in all cases). Thus, the data clearly show that the level of plasma Abs recognizing Ags on the surface of CSA-selected, but not of unselected, parasite isolates depended on the parity of the plasma donors.

Plasma Abs recognizing CSA-selected parasites inhibit their adhesion to CSA

It has been reported previously that Abs capable of inhibiting CSA-specific adhesion of unselected *P. falciparum* parasites obtained from the placenta and peripheral blood of pregnant women can be found in the serum of multigravid women from malaria-endemic areas (18). As the level of FCM-detectable plasma Abs recognizing Ags on CSA-selected LSIE correlated with parity (Fig. 3), we investigated whether plasma samples with high Ab levels were more efficient in inhibiting adhesion of CSA-selected parasites to CSA than samples with low levels of such Abs. To this end we ranked all the third-trimester plasma samples according to their recognition of CSA-selected LSIE by FCM. The 10 plasma samples with the highest scores and the 10 samples with the lowest scores were then tested for their ability to inhibit the CSA-specific adhesion of the PA/CSA isolate (as before, these assays were always performed with coded samples). As shown in Fig. 4, there was a very clear relationship between the ability of a given plasma sample to score in the FCM assay and its ability to inhibit CSA-specific parasite adhesion in vitro.

Table II. *Plasma donor characteristics*

Parity	*n*	Age[a] (years)	Gestational Age[a] (wk)	Prophylaxis[b] 1st	2nd	3rd	Birth Weight[a] (kg)
1	30	19.0 ± 3.9	35.2 ± 2.0	46	46	52	2.84 ± 0.75
2	30	23.2 ± 3.5	34.7 ± 2.5	79	45	68	2.93 ± 0.40
3	30	25.6 ± 3.6	35.6 ± 2.6	67	44	50	2.82 ± 0.75
4	24	28.6 ± 4.4	34.5 ± 1.7	68	55	64	3.30 ± 0.46
5	19	30.6 ± 4.0	35.2 ± 1.7	70	53	65	3.22 ± 0.30
6	10	32.5 ± 6.5	35.0 ± 2.9	44	44	44	2.88 ± 0.39
7	7	32.7 ± 6.1	35.7 ± 1.8	57	50	50	3.50 ± 0.25
8	5	37.2 ± 5.5	32.8 ± 2.3	20	0	25	2.94 ± 0.28
≥9	8	35.0 ± 5.0	35.1 ± 1.6	63	50	86	2.84 ± 0.36

[a] Mean ± SD.
[b] Percentage of women reporting malaria prophylaxis in 1st, 2nd, and 3rd trimester.

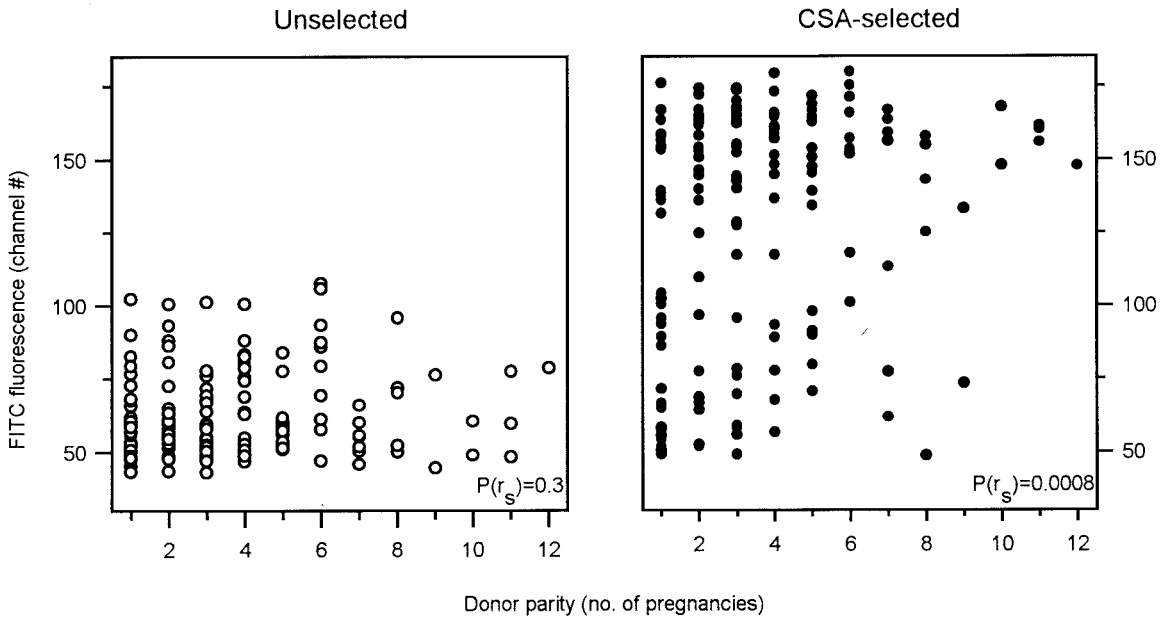

FIGURE 3. Parity dependence of variant erythrocyte surface Ag recognition by Abs in plasma from 163 third-trimester pregnant women from an area of hyperendemic malaria transmission (Prampram, Ghana). Data obtained with erythrocytes infected with late developmental stages of an unselected (○, E2039) and a CSA-selected (●, PA/CSA) parasite isolate and the corresponding statistical significance of the correlation between donor parity and median fluorescence are shown.

Ability of third-trimester plasma to inhibit CSA-specific adhesion correlates with Ab levels, not with parity

In a previous study third-trimester plasma from primigravid women were found to be uniformly unable to inhibit binding of placental LSIE to CSA in vitro (18). Nevertheless, we found that a considerable proportion of such samples contained high levels of Abs recognizing CSA-adhering LSIE (Fig. 3), and that high levels correlated with inhibition of adhesion to CSA in vitro (Fig. 4). We thus considered the possibility that the inhibitory capacity of Abs from primigravid and multigravid women differed independently

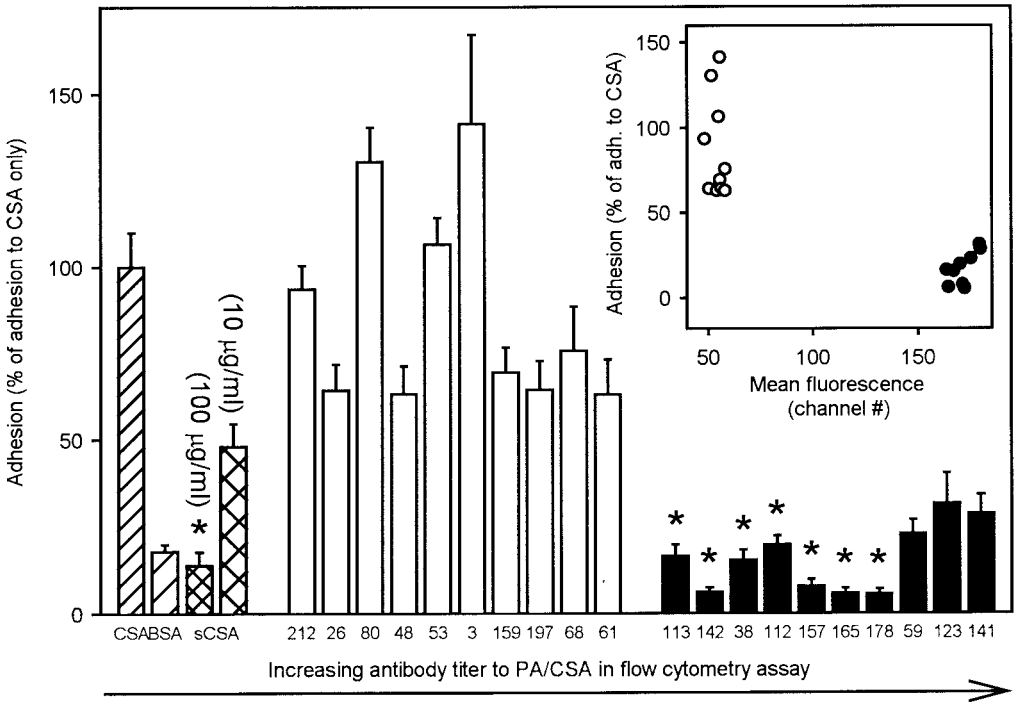

FIGURE 4. *Main panel*, Inhibition of CSA-specific adhesion of parasite isolate PA/CSA by sCSA and by plasma from malaria-exposed third-trimester pregnant women. Data (medians and 95% confidence intervals) on the adhesion to CSA in the presence of plasma selected for low (□) and high (■) titers of PA/CSA-specific Abs detected by FCM. Data are presented as the percent adhesion to CSA alone (positive control, ▨). Adhesion to BSA alone (background, ▨) and to CSA in the presence of soluble CSA (10 and 100 μg/ml, ▩) are shown for comparison. Asterisks indicate medians that were not significantly different from median adhesion to BSA alone (Kruskal-Wallis test followed by Dunn's test). Inset, Absolute relationship between plasma Ab levels (○, low levels; ●, high levels) detected by FCM and PA/CSA adhesion to CSA in the presence of the corresponding plasma sample.

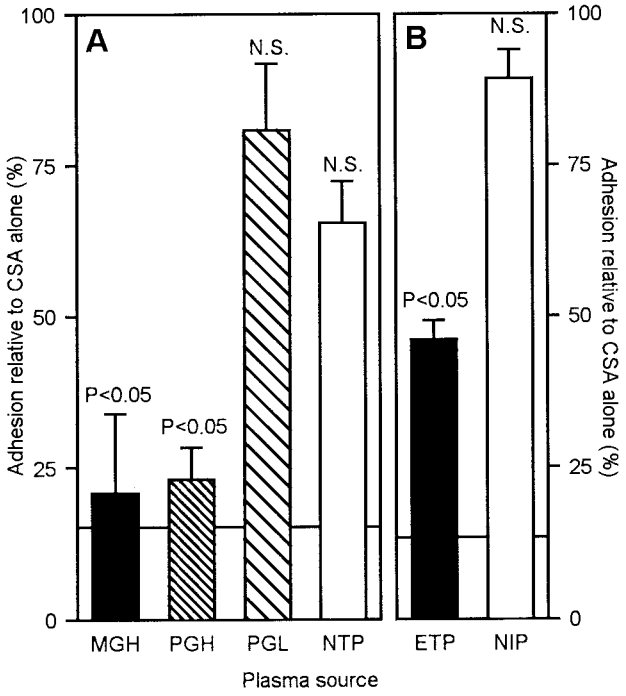

FIGURE 5. Plasma-mediated inhibition (*A*) and reversion (*B*) of adhesion of PA/CSA parasites to CSA in vitro by plasma (medians and 95% confidence intervals). Sources of plasma were multigravid malaria-exposed women with high Ab levels (MGH), primigravid malaria-exposed women with high (PGH) and low (PGL) Ab levels, nonexposed third-trimester pregnant women (NTP), malaria-exposed women (all parities, ETP), and nonexposed nonpregnant donors (NIP). All samples from pregnant women were from the third trimester. Horizontal lines indicate adhesion in plates not coated with CSA (background). The significance of the differences in median adhesion in the presence of plasma compared with adhesion to CSA alone is indicated (Kruskal-Wallis test followed by Dunn's test).

of the levels of such Abs. To test this hypothesis, we compared the ability of third-trimester samples from primigravid women with high and low plasma Ab levels and those from multigravid women with high plasma Ab levels to inhibit adhesion of LSIE to CSA. We found that the ability of plasma to inhibit adhesion depended on the levels of Abs recognizing CSA-adhering LSIE, but not on parity (Fig. 5*A*). Plasma from 10 third-trimester women without known exposure to malaria parasites and all containing low levels of Abs recognizing CSA-adhering LSIE were uniformly unable to affect LSIE binding to CSA (data not shown). Thus, our data support the hypothesis that the inhibitory capacity of plasma depends on the quantity, rather than the quality, of Abs binding to the surface of CSA-adhering LSIE, and that the parity dependency of protection against PAM is due to an increasing proportion of women having high Abs against CSA-adhering parasites with increasing parity.

Plasma Abs recognizing CSA-selected parasites can reverse established adhesion to CSA

We proceeded to examine whether third-trimester plasma Abs were capable of reversing already established binding of CSA-selected LSIE to CSA. Addition of third-trimester plasma from malaria-exposed, but not from nonexposed, women to CSA-selected parasites that had been allowed to adhere to CSA caused a partial reversion of this binding (Fig. 5*B*). In contrast, addition of nonimmune plasma had no significant effect on already established adhesion.

In conclusion, plasma Abs from Ghanaian women in their third trimester of pregnancy were shown to inhibit and even reverse binding of CSA-selected parasites to CSA. Furthermore, the levels of Abs to variant-specific surface Ags on CSA-selected LSIE detected by the FCM assay correlated strongly with this inhibitory capacity, suggesting this assay as a convenient tool to evaluate the degree of Ab-mediated protection against PAM.

Discussion

Parasites obtained from the placentas of *P. falciparum*-infected women adhere preferentially to CSA (17, 34), and plasma from multigravid, but not primigravid, women from endemic areas can inhibit adhesion of placental parasites to CSA (18). These findings suggest that acquisition of Abs specifically interfering with CSA-specific sequestration are of importance in the parity-dependent resistance to PAM that has repeatedly been observed in endemic areas (2). However, detailed analysis of the parity dependency of the prevalence and particularly the levels of such Abs has not previously been attempted and was consequently the aim of the present study.

Receptor-specific parasite adhesion appears to be mediated primarily through parasite-derived variant Ags in the membrane of LSIE. The best characterized such Ag is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is encoded by the multigene *var* family (35–37). Selection of *P. falciparum* for adhesion to CSA in vitro results in the expression of particular PfEMP1 variants capable of binding CSA, suggesting that PfEMP1 is involved in CSA-specific parasite adhesion (38, 39). We have recently developed a flow cytometric assay that is suitable for unbiased analysis of recognition of variant LSIE surface Ags by plasma Ab (27), with PfEMP1 as a main target Ag (27, 32). The assay correlates well with assays of Ab-dependent agglutination of LSIE (5, 29).

As expected from previous studies, most plasma samples from adults living in an area of hyperendemic malaria transmission (coastal Ghana) contained Abs labeling erythrocytes infected with each of three *P. falciparum* isolates that had not been subjected to in vitro selection pressure. In marked contrast, plasma levels of Abs recognizing the CSA-selected parasite isolate PA/CSA were not significantly different from levels in the plasma of unexposed donors. This result is in line with a previous study showing that sera from parasite-exposed males were unable to agglutinate placental (presumably CSA-specific) parasites (33). Another recent study showed lower agglutination rates of parasites from pregnant than from nonpregnant donors exposed to serum from nonpregnant individuals, although the sex of the serum donors was not indicated (40). The few among our plasma samples from Gomoa Onyadze that did label PA/CSA were exclusively from women. This marked gender difference seen only with the CSA-adherent isolate points to a relation to pregnancy, although we did not know the parity or pregnancy status of these female plasma donors.

Plasma from malaria-exposed, third-trimester pregnant women generally contained much higher levels of PA/CSA-specific Abs than of Abs recognizing unselected parasites. Furthermore, there was a highly significant association between levels of PA/CSA-specific Abs and parity, whereas this was not the case for any of the unselected isolates. Among the primigravid women, more than half had low PA/CSA-specific Ab levels, whereas the remaining women had levels as high as those in women of higher parity did. In fact, the main parity-dependent change was the decreasing proportion of women with low levels of PA/CSA-specific Abs with increasing parity, whereas there was no appreciable increase in the maximum Ab level with increasing parity. It thus appears that PAM induces a strong Ab response directed against CSA-adhering

parasites regardless of parity, and that the parity-dependent protection against PAM reflects an increasing likelihood of previous exposure to such parasites. In support of this idea, a very clear correlation between Ab levels in plasma samples and their capacity to inhibit CSA-specific adhesion was found between plasma levels of such Abs and the ability of plasma samples to inhibit CSA-specific adhesion. Furthermore, plasma samples from primigravid women with high plasma Ab levels were as efficient in this respect as samples from multigravid women. The fact that already established adhesion could be reversed by addition of third-trimester plasma, whereas Ab labeling of CSA-specific LSIE could not be reversed by sCSA suggests that the Ab-mediated mechanism of inhibition is steric hindrance. However, differences in the affinities of Abs and sCSA for the parasite CSA ligand may be contributory. Further studies are needed to clarify this issue.

In a study from an area of western Kenya it was reported that serum from multigravid women efficiently inhibited CSA-specific parasite adhesion, whereas serum samples from primigravidae were all completely inefficient in this respect (18). We found high levels of Abs recognizing CSA-selected parasites in a considerable proportion of plasma from primigravidae and a clear correlation between Ab levels and inhibition of adhesion regardless of parity. It thus seems that the samples from the primigravid women in the Kenyan study all had low levels of Abs specifically recognizing CSA-adherent parasites, although the endemicity there is much higher than that in our study area. This seeming paradox could be explained if Abs inhibiting CSA-specific parasite adhesion are acquired relatively late in the pregnancy, and if the samples assayed in the Kenyan study were obtained at an earlier gestational age than in our study. Parasite rates, anemia, and splenomegaly in primiparous women peak during the second trimester (2, 41). The subsequent reduction in these parameters may reflect the acquisition of inhibitory Abs, and indeed recent data showed lower agglutination rates in the sixth month of pregnancy than at delivery in first-time pregnant women from Cameroon (40). However, the fact that the serum samples used in the study from Kenya were actually obtained at term (M. Fried, personal communication) makes the above explanation of the apparent difference between the two studies less obvious. Detailed studies on the kinetics of the acquisition and decay of Abs interfering with CSA-specific parasite sequestration in areas of different endemicity are clearly needed.

In conclusion, we have shown that although levels of Abs specifically recognizing a CSA-adherent isolate of *P. falciparum* are generally low in an endemic population, levels are usually high in third-trimester pregnant women. Secondly, the proportion of such women with high Ab levels increases with increasing parity. Thirdly, levels of these Abs correlate with the ability of plasma to inhibit and reverse adhesion of the parasites to CSA. Taken together, our data strongly support the hypothesis that parity-dependent protection against PAM reflects acquisition of Abs interfering with CSA-specific placental sequestration of parasites. Furthermore, our study demonstrates a convenient method of measuring plasma levels of Abs recognizing the parasite Ags mediating this adhesion. These Ags constitute an obvious target for vaccination against PAM.

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