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Inflammatory reactions in placental blood of *Plasmodium falciparum*-infected women and high concentrations of soluble E-selectin and a circulating *P. falciparum* protein in the cord sera

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SUMMARY

To better understand reasons for increased susceptibility to malaria in pregnancy; and the interrelationships between maternal malaria, local immune reactions and the development of the fetus, concentrations of soluble interleukin-10 (IL-10), cytokine receptors, adhesion molecules, a *Plasmodium falciparum* protein, glutamate-rich protein (GLURP) and antibodies to *P. falciparum* rhoptry-associated protein-1 were measured among 105 Gambian women and their neonates. Peripheral blood concentrations of IL-10, soluble cytokine receptors and soluble adhesion molecules were found to be different from those concentrations measured in the placenta. Markers of inflammatory reactions: IL-10, sIL-2R, sIL-4R, and soluble tumour necrosis factor receptor I (sTNF-RI) were found in high concentrations in the placenta, indicating that inflammatory reactions take place in the placenta which has been regarded as an immunoprivileged site. Concentrations of soluble vascular cell adhesion molecule-1 (sVCAM-1) and soluble intracellular adhesion molecule-1 (sICAM-1), potential adhesion receptors for malaria parasites, were associated with an active *P. falciparum* infection in the placenta although the associations did not reach significance. *P. falciparum* exoantigen, GLURP, was detected in cord blood indicating transplacental passage of malarial antigens. Concentrations of E-selectin were higher in cord blood samples compared with peripheral blood samples. This appeared to be associated with development of cord endothelial cells and not with *P. falciparum* infection.

INTRODUCTION

The placenta provides nutrition for the fetus and protects the fetus from rejection by maternal immune responses. Suppression of cellular immune responses may make pregnant women more susceptible to a number of infectious diseases, including malaria.[1]

When malaria infection occurs during pregnancy the placenta is frequently infected with malaria parasites and much higher parasitaemias may be found within the placenta than in the peripheral blood.[2] Infection of the placenta results in pathological changes including thickening of the trophoblast basement membrane.[3] These changes may be the reason why malaria in pregnancy is associated with a reduction in birth weight.[4]

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Placental *Plasmodium falciparum* infections and low birthweight are more prevalent among primigravidae than among multigravidae. A number of hypotheses have been forwarded to explain these phenomena including impaired immunity in pregnancy and unique immuno/physiological regulation of the first pregnancy.[2,4,5] Several explanations have been suggested to explain the large numbers of malaria parasites within placentas compared to peripheral blood. *P. falciparum* parasites may use different receptor–ligand combinations to sequester in the placenta compared with those used for sequestration in other parts of the body.[6,7] The expression of new and different surface epitopes in the placenta may be the reason for the impaired immune recognition of these epitopes in primigravidae.

Suppression of inflammatory and specific cellular immune reactions may occur in the placenta in order to protect the fetus from immune reactions directed against paternal antigens expressed by the fetus. Cortisol, an immunosuppressive hormone is found in increased concentrations in the placenta among primigravidae.[8] Furthermore, immune responses in the placenta may be biased towards a T helper type-2 (Th2) type

of response.[9] Studies in rodent models[10] have suggested that a Th2 type of response may impair efficacy in controlling new malaria infections.

Because immune responses in the placenta may differ from those seen at other sites, we have studied markers of inflammatory reactions in the placenta to determine whether inflammatory reactions are suppressed or stimulated in the placenta in response to *P. falciparum* infections.

MATERIALS AND METHODS

Study site, donors and blood collection

Patient recruitment, sample collection and the methods by which malaria was diagnosed have been reported in detail previously.[8,11]

In brief, 105 women were studied at Basse Health Centre, The Gambia, West Africa. Upon delivery and with informed consent of the mother, a maternal venous and cord blood sample were collected and a biopsy was taken from an off-centre position of the decidual face of the placenta. Blood films were prepared from maternal peripheral venous, placental and cord blood and stained with Giemsa to detect malaria parasites. Placental sections were fixed in 10% formalin, routinely processed in paraffin wax, sectioned at 3 μm and stained with haematoxylin and Giemsa to detect parasites and pigment For the purposes of the analyses reported in this paper, women were placed into three groups: 1, infected: parasites in placental blood films and/or placental sections; 2, previously infected: malaria pigment in fibrin or in cells within fibrin, in the absence of parasites; 3, not infected: absence of parasites or pigment. Details of this classification have been documented elsewhere.[12] The frequency of parasitized placentas (group 1) was 26·4%; and an additional 29·8% of placentas showed pigment in fibrin only (group 2).[3]

Determination of interleukin-10 (IL-10), soluble cytokine receptors and adhesion molecules

Enzyme-linked immunosorbent assay (ELISA) kits were used as specified by the manufacturers to measure plasma IL-10 (Endogen, Cambridge, MA), sIL-2R (T Cell Diagnostics, Woburn, MA), sIL-4R (RD systems, Minneapolis, MN), soluble tumour necrosis factor receptor I (sTNF-RI) (RD systems), soluble intracellular adhesion molecule-1 (sICAM-1) (T Cell Diagnostics), soluble vascular cell adhesion molecule-1 (sVCAM-1) (RD systems) and E-selectin (RD systems). Assay sensitivities were 3 pg/ml, 24 U/ml, 5 pg/ml, 30 pg/ml, 0·3 ng/ml, 2 ng/ml and 2 ng/ml, respectively.

Antibody reactivity with RAP-1

Recombinant RAP-1 was produced by subcloning a fragment of the *RAP-1* gene[13] encoding amino acids 23–294 into a pDS expression vector containing six C-terminal histidine residues to aid purification.[14] Antibody reactivities in plasma to RAP-1 were measured by an indirect ELISA method as described previously.[15] To account for day-to-day variation, levels of reactivities were calculated as ELISA units (EU): $(OD_{sample} - OD_{background})/(OD_{positive\,control} - OD_{background}) \times 100$.

Reactivity was considered to be positive when $EU > EU_{mean} + 3 \times SD$, where EU_{mean} was the mean of 23 Danish controls.

Determination of soluble P. falciparum *glutamate rich protein (GLURP)*

Levels of GLURP of *P. falciparum*[16] were measured by a competition ELISA. Nunc (Roskilde, Denmark) maxisorp plates were used. Rabbit antiserum against the 3′repetitive region, RII, of GLURP, diluted 1:1000 in 150 μl carbonate buffer, pH 9·6, was added to each well and left overnight at 4°. Plasma (5 μl) were preincubated with 245 μl of biotinylated recombinant GLURP RII (1·2 μg/ml) at 37° for 90 min. The mixture of GLURP and test sera (150 μl) were then added to the ELISA wells and the residual GLURP binding capacity was estimated by incubation for 1 hr at 37°. After washing, 150 μl peroxidase conjugated streptavidin (Dako, Denmark) diluted 1:5000 in dilution buffer was added and incubation performed for 1 hr at 37°. After a final wash, 100 μl of substrate were added and the colour reaction stopped after 30 min. with H_2SO_4. Absorbance was read at 492 nm. Serum mixed with buffer served as a control.

Percent inhibition of GLURP binding was calculated from the formula $[100 - (\text{ELISA OD}_{sample}/\text{ELISA OD}_{control})] \times 100$. All tests were performed in duplicate. Cut off levels of inhibition were calculated using values obtained in plasma samples from 23 adult Danes.

Statistics

The paired *t*-test was used for paired intergroup comparisons after testing for normal data distributions (Kolmogorov–Smirnov test). Ten markers were investigated and *P*-values $< 0{\cdot}005$ were therefore considered significant.

The Spearman rank order correlation coefficient (*r*) was used for evaluation of parameter associations. *P*-values less than 0·005 and *r*-values higher than 0·5 were considered significant.

All calculations were performed using SigmaStat (Jandel Scientific, San Rafael, CA) software.

RESULTS

Concentrations of IL-10 and soluble cytokine receptors

Concentrations of IL-10, sIL-2R, sIL-4R and sTNF-RI were all higher in placental blood than in maternal peripheral blood or cord blood samples (Table 1 and Fig. 1a and b).

Levels of sIL-4R were higher in the peripheral blood than in cord blood but concentrations of IL-10, sIL-2R and sTNF-RI were similar in peripheral blood and cord blood samples.

Levels of IL-10 were higher in placentas with active infection (mean: 45 μg/ml, SD 36; $n = 9$) compared with placentas with no infection (mean 23 μg/ml, SD 46; $n = 22$), although the difference was not significant ($P = 0{\cdot}06$). No associations were found between malaria infection and the concentrations of sIL-2R, sIL-4R or sTNF-RI. No associations between levels of IL-10 or soluble cytokine receptors and number of pregnancies were detected.

Concentrations of soluble adhesion molecules

Concentrations of sVCAM-1 and of sICAM-1 (Table 1 and Fig. 1c and d) were significantly higher in placental blood than in cord blood samples. There were no significant differences in sVCAM-1 or sICAM-1 concentrations between peri-

Table 1. Levels of IL-10, soluble cytokine receptors and soluble adhesion molecules in placental blood, peripheral venous blood and cord blood. Means and SDs in parentheses are shown

	Placental blood a	Venous blood b	Cord blood c	Statistical analysis		
				a vs b	a vs c	b vs c
IL-10 (pg/ml) ($n=54$)	35 (47)	14 (57)	5 (14)	$P=0.003$	$P=0.0001$	$P=0.12$
sIL-2R (U/ml) ($n=54$)	648 (491)	335 (485)	302 (171)	$P=0.0001$	$P=0.0001$	$P=0.32$
sIL-4R (pg/ml) ($n=53$)	94 (65)	64 (55)	35 (33)	$P=0.0003$	$P=0.0001$	$P=0.0002$
sTNF-RI (ng/ml) ($n=54$)	6.7 (2.3)	1.6 (1.1)	1.9 (1.2)	$P=0.0001$	$P=0.0001$	$P=0.10$
sVCAM-1 (ng/ml) ($n=52$)	930 (472)	819 (621)	607 (338)	$P=0.09$	$P=0.0001$	$P=0.008$
sICAM-1 (ng/ml) ($n=54$)	162 (76)	171 (75)	61 (41)	$P=0.14$	$P=0.0001$	$P=0.0001$
sE-selectin (ng/ml) ($n=54$)	78 (48)	36 (27)	96 (56)	$P=0.0001$	$P=0.007$	$P=0.0001$

pheral blood and placental blood. Levels of placental sVCAM-1 were correlated with sICAM-1 ($r=0{\cdot}60$, $P<0{\cdot}005$).

Concentrations of sE-selectin (Table 1 and Fig. 1e) were higher in cord and in placental blood samples than in peripheral blood samples.

Levels of sICAM-1 were higher in placentas with active infection (mean: 204 ng/ml, SD 113; $n=11$) compared with placentas with no infection (mean: 140 ng/ml, SD 80; $n=24$) although the difference was not significant ($P=0{\cdot}06$).

Levels of sVCAM-1 were higher in placentas with previous infection (mean: 1107 ng/ml, SD 699; $n=19$) and in placentas with active infection (mean: 1159 ng/ml, SD 722; $n=11$) than levels in placentas with no infection (mean: 734 ng/ml, SD 455; $n=24$) although the difference was not significant ($P=0{\cdot}04$ in both cases).

No associations were found between malaria infection and the concentrations of sE-selectin.

No significant associations were detected between levels of sICAM-1, sE-selectin or sVCAM-1 and number of pregnancies.

Malaria antibody reactivities to RAP-1

Immunoglobulin G (IgG) reactivities to RAP-1 ($n=69$) were significantly higher in the peripheral blood (mean relative OD 0·75 (SD 0·77)) than in cord blood (mean relative OD 0·68 (SD 0·71)) although the difference only approached significance ($P=0{\cdot}006$). IgG reactivities in placental samples (mean relative OD 0·84 (SD 0·76)) were also higher than in cord blood ($P=0{\cdot}001$). There were no significant differences between peripheral blood and placental IgG reactivities ($P=0{\cdot}76$).

IgM reactivities to RAP-1 ($n=69$) were significantly higher in placental samples (mean relative OD 3·94 (SD 5·80)) than in cord blood (mean relative OD 2·06 (SD 2·27)) ($P=0{\cdot}002$). IgM reactivities to RAP-1 in peripheral blood (mean relative OD 3·82 (SD 6·07)) were also higher than in cord blood ($P=0{\cdot}002$).

Only 1 out of 72 cord sera were IgM positive when compared to the cut off level defined from IgM reactivities of the Danish controls.

Cord blood factors

Levels of sICAM-1 and levels of sE-selectin were associated ($r=0{\cdot}68$; $P<0{\cdot}005$).

Malaria antigen (GLURP) levels

Levels of circulating GLURP ($n=42$) in placenta (mean 29%, SD 21), in cord blood (mean 31%, SD 30) and in the peripheral blood (mean 24%, SD 18) were not significantly different. Twelve of 42 cord sera (28·6%) were positive for GLURP.

Levels of GLURP were negatively associated with levels of sE-selectin, although the association was not significant ($r=-0{\cdot}43$, $P=0{\cdot}006$). Levels of GLURP were higher in cord sera if the corresponding placenta was infected (mean 56·8%, SD 36·6, $n=6$) than in cord sera where the corresponding placenta was not infected (mean 25·6%, SD 28·9 $n=18$) or had a previous infection (mean 27·4%, SD 25·2, $n=15$) but the difference was not significant ($P=0{\cdot}02$ and $P=0{\cdot}03$, respectively).

DISCUSSION

The placenta grows and differentiates from extraembryonic fetally derived tissue. It separates the circulatory systems of the fetus and the mother. All exchanges of material take place in the placenta. The trophoblast layer protects the placenta and the fetus from immunological rejection by maternal leucocytes. Trophoblast which covers the placenta lacks classic major histocompatiblity complex (MHC) class I and II antigens. The lack of MHC expression and general immunosuppression in the placenta, mediated partly by steroid hormones, are likely to be reasons why placentas are not rejected by maternal leucocytes. The price to be paid for avoiding rejection may be an increased susceptibility of the placenta to some infections including malaria.

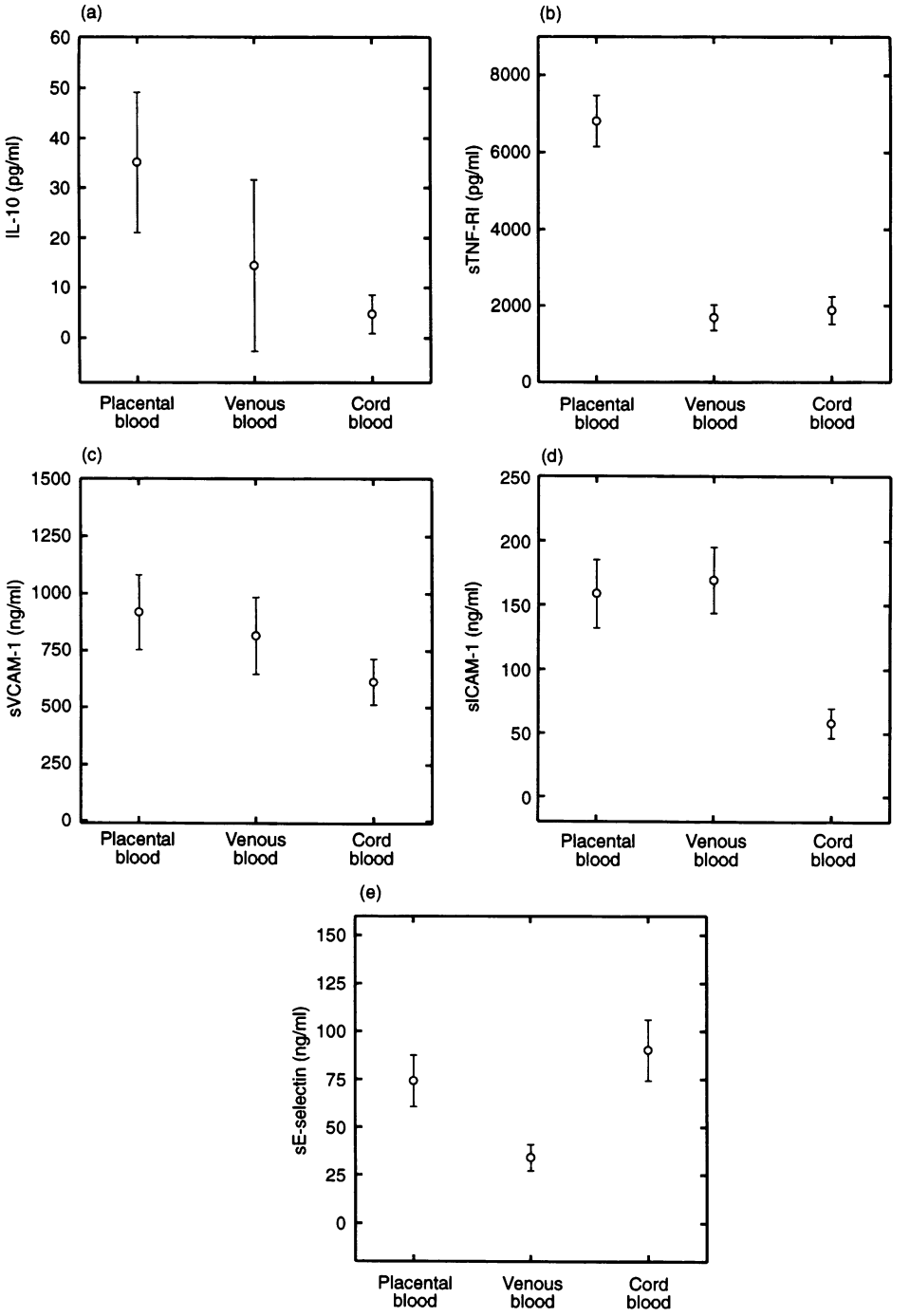

Figure 1. Levels of proteins in placental, venous and cord blood samples collected from Gambian women. (a) IL-10 ($n=54$); (b) sTNF-RI ($n=54$); (c) sVCAM-1 ($n=52$); (d) sICAM-1 ($n=54$); (e) sE-selectin ($n=54$).

Pregnant women are susceptible to malaria and placentas may carry a high parasite burden, but parasites are seldom detected in cord blood in endemic regions. Placental infections are associated with a decrease in mean placental weight and birth weight.[17]

The placenta is normally regarded as a site with no or minor immune activation. However, we found evidence of local inflammatory reactions in most placentas examined as levels of sTNR-I, sIL-2R and sIL-4R were increased in placental blood compared with venous blood. These inflammatory reactions may help to control parasite replication but they may also contribute to placental pathology and harm the fetus. TNF-RI is produced by the syncytiotrophoblast and the soluble receptor may bind and neutralize TNF-alpha in the placenta.[18]

Concentrations of cytokines and soluble cytokine receptors in the peripheral blood are often measured in connection with studies of organ-specific disease manifestations like cerebral malaria. Levels of IL-10, sTNF-RI, sIL-2R and sIL-4R were much higher in placental blood compared with peripheral

blood showing that concentrations of cytokines and cytokine receptors in the peripheral blood do not necessarily reflect concentrations in the blood of a particular organ like the placenta. It is uncertain what was the cause of increased concentrations of IL-10 and soluble cytokine receptors in placentas that showed no evidence of malaria infection. Human placental cytotrophoblasts produce IL-10[19] which is induced by inflammatory reactions but which may also suppress cellular immune reactions in the placenta as elsewhere.

Levels of sICAM-1 and sVCAM-1 were associated with an active infection of placenta. Both adhesion molecules have been reported to bind infected erythrocytes[20,21] and ICAM-1 mediate parasite sequestration in placenta.[7] Levels of these adhesion molecules in Gambian children were associated with severity of malaria.[22] Some of the molecules are also involved in placental development. VCAM-1 play a role in the development of placenta and in the formation of the umbilical cord.[23,24]

The physiological fetal environment engages neonatal cells preferentially in a Th2 response.[25] No increase in markers of inflammatory reactions were detected in the cord blood. Whilst malaria parasites are occasionally detected in cord blood soluble malarial antigens can cross the placenta[26] and we detected transplacental passage of GLURP in 28·6% of the tested cord sera. Late gestational exposure may prime immune responses prior to birth.[27]

Decreased levels of IgG to RAP-1 were detectable in cord blood indicating a dilution of antibodies from placenta to cord. Only one cord serum was IgM reactive to RAP-1, indicating that this assay is not a sensitive marker of fetal exposure to *P. falciparum* proteins.

Interestingly the profiles of soluble adhesion molecules in cord blood samples were strikingly different from that seen in placental and in peripheral blood. Levels of soluble ICAM-1 were low in cord blood, the reduced expression of ICAM-1, a receptor of infected erythrocytes, could contribute to the low transplacental transfer of parasites. Levels of E-selectin were increased in cord blood. Soluble E-selectin is normally regarded as a marker of endothelial inflammatory reactions but this appears not to be the case in cord blood. E-selectin has also been reported to be a marker of proliferating endothelium[28] and E-selectin may play a role in the cord development.

In conclusion, we found that concentrations of IL-10 and soluble cytokine receptors in the the peripheral blood do not reflect concentrations of these molecules in placental blood. The high concentrations of these molecules in placenta indicates that inflammatory reactions take place in the placenta. The association between active malaria infection of placenta and concentrations of sICAM-1 and sVCAM-1 indicate a role of these molecules in placental malaria. High concentrations of soluble E-selectin in cord blood may be associated with cord blood development while the presence of a *P. falciparum* exoantigen in cord blood indicate potential priming of the fetal immune system.

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