Depressed Natural Killer Cell Cytotoxicity against *Plasmodium falciparum*–Infected Erythrocytes during First Pregnancies

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We measured natural killer (NK) cell cytotoxicity and cortisol and prolactin concentrations in peripheral venous blood samples obtained from pregnant Gabonese women at the time of delivery. The NK cell–mediated cytotoxicity against *Plasmodium falciparum*–infected erythrocytes in vitro was lower in samples obtained from primiparous women than in samples obtained from multiparous women; cortisol concentrations were significantly higher in primiparous women than in multiparous women, and prolactin concentrations were significantly lower. The highest cortisol concentrations were found in the plasma of *P. falciparum*–infected primiparous women. A positive correlation was found between cortisol concentration and parasite load; an inverse correlation was found between the magnitude of the NK cell cytolytic effect and cortisol production. A positive correlation was found between this effect and prolactin production. Thus, depressed NK cell cytotoxicity against *P. falciparum*–infected erythrocytes is correlated with high cortisol concentrations and may contribute to increased susceptibility to malaria during pregnancy.

Malaria during pregnancy is a cause of maternal morbidity and mortality. People living in areas where malaria is highly endemic normally develop protective immunity against severe malaria and high-density parasitemia. However, this protection appears to be partially lost during pregnancy [1]. Women in their first and second pregnancies are most susceptible to *Plasmodium falciparum* infections [2]. The mechanisms responsible for the increased susceptibility to malaria during pregnancy—and particularly during the first pregnancy—have not been elucidated. Cell-mediated immune mechanisms are believed to be important in the development and maintenance of immunity to malaria; thus, impairment of this immunity may at least partly explain the susceptibility of pregnant women to malaria. There is convincing evidence suggesting that bilateral communication occurs between neuroendocrine and immune systems. Adrenal glucocorticoids and prolactin are hormones involved in immunoregulation, and cortisol suppresses the immune system; prolactin seems to stimulate the immune system [3, 4].

Natural killer (NK) cells are key effectors of the human innate immune system and are essential in maintaining pregnancy [5, 6]. Low NK cell cytotoxic activity is linked with increased sensitivity to human infections [7, 8]. This activity is partially inhibited by endogenous glucocorticoids [9, 10]. Furthermore, there is a relationship between serum cortisol concentrations and the suppression of malaria immunity during pregnancy.
[MATERIALS AND METHODS]

Study site and participants. This study took place in Libreville and Lambaréné, 2 cities in Gabon where P. falciparum malaria is hyperendemic, with a perennial mode of transmission and little seasonal fluctuation. The entomological inoculation rate measured in and around Lambaréné is ~50 infective bites per person per year [12], and the main vectors are Anopheles gambiae and Anopheles moucheti. One hundred one pregnant women (40 primiparous women and 61 multiparous women) were enrolled during 1–30 September 2002 during delivery in the Centre Hospitalier de Libreville and from the Albert Schweitzer Hospital in Lambaréné. All deliveries assessed for that study occurred between 6:00 p.m. and 12:00 p.m. As a control group, 30 healthy nonpregnant women in the same area and from the same age group were also recruited.

Informed consent was obtained from all participating women, and the study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital in Lambaréné and performed in accordance with the guidelines for human experimentation in clinical research of the Ministry of Public Health and Population of Gabon (Libreville).

Obtainment of plasma samples. Maternal venous blood samples were obtained at the time of delivery in sterile, heparinized tubes. Of the 12 mL of blood obtained, 2 mL was used for plasma collection and was immediately centrifuged for 10 min at 400 g with a Biofuge Pico centrifuge (Heraeus Instruments) to separate the pellet containing the packed erythrocytes from the plasma. All plasma samples were frozen at −80°C and were stored until they were assayed.

Malaria diagnosis. Thick and thin peripheral blood films were stained with Giemsa stain and examined by 2 microscopists following standard, quality-controlled procedures. Parasite load is expressed as the number of asexual forms of P. falciparum per microliter of blood. All infected women received quinine chemotherapy.

Endocrine assay. The concentrations of cortisol and prolactin in the plasma samples were estimated by use of the Mini-Vidas VB 02.96 system with standard Vidas cortisol and Vidas prolactin kits (bioMérieux).

Effector cell isolation. PBMCs were fractionated on a Ficoll-Paque gradient (Pharmacia) and separated by density gradient centrifugation (400 g) for 30 min. Mononuclear cells were washed twice with Ca2+ - and Mg2+-free PBS (Gibco-BRL) and frozen until assayed in Tübingen, Germany. At the time that the experiments were performed, cells were thawed and resuspended in RPMI 1640 (Sigma) containing penicillin (100 U/mL), streptomycin (100 µg/mL), and 10% fetal calf serum (Gibco-BRL). The viability was always >98%, as determined by the trypan blue exclusion test. NK cells were isolated by positive selection using MACS separation columns (Miltenyi Biotec) containing 20 µL of magnetic beads coated with anti-CD56 antibody (clone AF12-7H3.6.11 isotype: mouse IgG1; Miltenyi Biotec) according to the manufacturer’s recommendations. Cells obtained after positive selection were labeled with CD56 antibody (clone B159; Pharmingen) coupled to phycoerythrin and CD16 antibody (clone 3G8; Pharmingen), coupled to fluorescein (FITC), then analyzed in a flow cytometer to determine their purity. The isotype controls was the IgG1-FITC/IgG2a–phycoerythrin mixture of dual TAG (clone MOPC-21/UPC-10; Sigma). Facstar analysis of CD56+/CD16+ cells showed that the population of NK cells obtained was >90% pure.

Target cells. The NK cell cytotoxic activity was tested by using P. falciparum–parasitized heterologous RBCs (pRBCs). K562 cells, a myeloid tumor NK-sensitive cell line, were used as a positive control and uninfected erythrocytes (uRBCs) were used as a negative control. The cell line was maintained in continuous culture throughout the study period in complete medium containing RPMI 1640 (Sigma), 10% decomplemented fetal calf serum, 100 µg/mL streptomycin, 100 U/mL penicillin, and 5 µg/mL sodium pyruvate (Gibco-BRL) in culture flasks (Polylabo); the medium was changed every 2 days. A local strain of P. falciparum, referred to as “S007,” was used. Parasites were cultured in accordance with a modified version of the method described by Trager and Jensen [13], with freshly prepared blood-bank–derived O+ erythrocytes in culture medium (RPMI 1640 [Sigma], 25g/L gentamycin [Gibco-BRL], 5.94 g/L HEPES [Seromed], and 2.33 g/L NaHCO3) supplemented with 0.5% Albumax II (Gibco-BRL) and 0.006% hypoxanthine (Sigma) in an atmosphere containing 5% CO2, 5% O2, and 90% N2. The culture medium was endotoxin free and was changed every day. Schizonts were obtained by means of magnetic separation with SuperMacs columns (Miltenyi Biotec) and were used directly for the cytolytic assays. The P. falciparum culture was routinely tested for Mycoplasma contamination by PCR. Uninfected fresh erythrocytes were obtained from non-immune women.

Cytotoxicity assay. NK cell lytic activity was measured by a standard chromium-51 release cytotoxicity assay, as reported elsewhere [14]. In brief, after overnight preculture of cells at 37°C without any stimulation, 106 of NK cells were resuspended in 0.3 mL of complete medium. Target cells, K562, pRBCs, and uRBCs were incubated with 100 µCi of chromium (Na91[51Cr]-O4; Amersham) for 1 h at 37°C. Target cells were then washed 3 times, and ∼5 × 104 of cells were placed in each well of U-bottomed 96-well ELISA plates (Costar) containing increasing
numbers of NK cells. Effector:target ratios of between 50:1 and
1:1 were tested in triplicate. Effector and target cells were co-
incubated at 37°C for 16 h. The final volume was 200 μL per
well. The plates were then centrifuged, and the supernatant
(100 μL) of each well was collected and directly placed in a γ
counter (Cobra Auto-Gamma; Packard Camberra) to determine
the radioactivity. Maximal chromium release was determined
by adding 1% SDS (Sigma). Spontaneous release was deter-
mained by incubating target cells with culture alone. Sponta-
neous release never exceeded 20% of maximal release. The
percentage of specific cytotoxicity was calculated as fol-
low:s: specificity percentage = [(cpm Exp. – cpm Spont.)/(cpm
Max. – cpm Spont.)] × 100.

Statistical analysis. Correlations between continuous vari-
bles were assessed by the Spearman rank test, corrected for
ties, where a value of ρ of >0.25 (combined with P<.05) was
considered significant. The χ² test was used to compare pro-
portions. The nonparametric Wilcoxon sign rank test was used to
test the significance of differences between paired continuous
variables, and the Mann-Whitney U (2-group comparisons)
test or the Kruskal-Wallis (>2-group comparisons) test was used
to determine the significance of differences in unpaired con-
tinuous variables. A t test was used for age comparisons. Two-
tailed P values of <.05 were considered to be significant.

RESULTS

Study subjects. The mean age of women included in the
study was 22 years and was not significantly different between
pregnant and nonpregnant women. Primiparous women were
significantly younger than multiparous women (18 vs. 26 years;
P = .001). The mean number of previous pregnancies was 3
in the multiparous group. NK cell cytotoxic activity was as-
essed in 17 women (5 primiparous women, 9 multiparous
women at term, and 3 control subjects). There was no difference
in the distribution of the studied variables between this group
and the entire study population.

Infection status. P. falciparum was detected in the pe-
ripheral blood of 24 women (24%).Primiparous women were
significantly more likely to be infected than were multiparous
women (18 women [45%] vs. 6 women [10%], respectively;
P<.001). The median number of parasites per microliter of
blood was also significantly higher in primiparous women
(2320 parasites/μL) than multiparous women (300 parasites/
μL; P = .0107) and was not associated with age (data not shown).

Cortisol. Plasma cortisol concentrations were within the
expected range for full-term pregnancies (i.e., 3–7 times the
normal range) [15]. Cortisol concentrations were significantly
higher in multiparous and primiparous women than in control
subjects (P<.001) (figure 1A). Plasma cortisol concentrations
were also higher in primiparous women than multiparous
women (P = .001) (figure 1A). Among the primiparous
women, the plasma of P. falciparum–infected women contained
more cortisol than did the plasma of uninfected women
(P = .030) (figure 1B), and cortisol concentrations were also
significantly correlated with parasite load (ρ = 0.36; P = .027).

Prolactin. Plasma prolactin concentrations were signifi-
cantly lower in control subjects and primiparous women than
in multiparous women (P = .001) (figure 1C). The ratio of
prolactin to cortisol was higher in multiparous women than in
their primiparous counterparts (P = .001) (figure 1D). Prolact-
in concentrations were similar in P. falciparum–positive and
–negative primiparous women (data not shown).

NK cell cytolytic activity. We evaluated NK cell cytolytic
activity by using P. falciparum–pRBCs, uRBCs (negative con-
trols), and erythroleukemia cell line K562 (positive controls)
as targets (figure 2A). The cytolytic activity of NK cells on
pRBCs was specific and dependent on the effector cell density
(which was 50:1, 25:1, 10:1, 5:1, and 1:1). At 25:1, K562 and
pRBCs were specifically lysed by NK cells with similar efficiency
(mean ± SD, 63% ± 23% and 89% ± 29%, respectively). How-
ever, the lysis of uRBCs was nonspecific (mean ± SD, 21% ±
6%; figure 2A). We next compared the ex vivo activity of
NK cells from controls and from primiparous and multiparous
women on the lysis of pRBC (figure 2B). The NK cell cyto-
toxicity was lower in primiparous women than in either control
subjects (P = .002) or multiparous women (P = .004), but it
was similar in multiparous women and control subjects.

Correlation between hormone concentrations and NK cell
cytotoxicity. A significant inverse correlation was found be-
tween NK cell activity against P. falciparum–infected erythro-
cytes and cortisol concentrations (ρ = −0.86; P = .006). A
significant positive correlation was found between prolactin
concentrations and specific cytolytic activity (ρ = 0.84; P = .008).

DISCUSSION

The mechanisms responsible for the increased susceptibility to
malaria during pregnancy, particularly during first pregnancies,
have not been elucidated, and several theories have been sug-
gested. Cellular immune responses to P. falciparum antigens are
depressed in pregnant women, compared with nonpregnant
women [16, 17]. Antiadhesion antibodies against chondroitin
sulfate A–binding parasites are associated with protection from
maternal malaria, and these antibodies develop over successive
pregnancies, accounting for the susceptibility of primiparous
women to infection [18–20]. We recently observed that malaria
during pregnancy, particularly during first pregnancies,
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Figure 1. Box plot of plasma cortisol and prolactin concentrations. A, Comparison of cortisol concentrations between primiparous women (median, 287.2 ng/mL; range, 230–346 ng/mL), multiparous women (median, 187 ng/mL; range, 149.2–256.8 ng/mL), and nonpregnant women (median, 95.9 ng/mL; range, 81.3–115 ng/mL). *P < .001 for comparison with controls; †P = .001 for comparison with multiparous woman. B, Comparison of cortisol concentrations between P. falciparum–infected primiparous women (median, 310.1 ng/mL; range, 240–358.6 ng/mL) and uninfected primiparous women (median, 252.6 ng/mL; range, 221.7–301.3 ng/mL). *P = .030 for comparison with uninfected primiparous women. C, Comparison of prolactin concentrations between primiparous women (median, 102 ng/mL; range, 74.4–140 ng/mL), multiparous women (median, 148.6 ng/mL; range, 109.6–200 ng/mL), and nonpregnant women (median, 8.8 ng/mL; range, 6.7–13.3 ng/mL). *P = .001 for comparison with multiparous women. D, Comparison of ratios of prolactin level to cortisol level between primiparous women and multiparous women. **P = .001 for comparison with ratio of primiparous women. Box plots illustrate medians with 25th–75th percentiles, with lines extending to 10th and 90th percentiles.

rocytes [14, 22]. Studies with murine models of malaria do lend strong support the idea that NK cells contribute to protective immune responses against plasmodia [23–25]. In this study, we assessed the plasma concentrations of 2 immunoregulatory hormones (cortisol and prolactin), as well as the NK cell cytolytic effect against P. falciparum–infected erythrocytes, in Gabonese pregnant women. We found that prolactin concentrations were significantly higher in multiparous women than in primiparous women, whereas cortisol concentrations were significantly lower. A correlation was found between cortisol concentrations and malarial infection. Multiparous women had higher NK cell cytotoxic activity against P. falciparum–infected erythrocytes than did primiparous women. This cytolytic activity was inversely correlated with cortisol level and positively correlated with prolactin concentration.

Nonspecific immunosuppression may be caused by the presence of pregnancy-associated hormones. It has been proposed that reduced levels of immunosuppressive hormones [26] and higher levels of antimalarial antibodies [27] in multiparous women are associated with protection; however, strong supporting evidence is lacking. Corticosteroids suppress cell-mediated immunity, and corticosteroid concentrations are substantially increased during the third trimester of pregnancy. They are also increased in primiparous women and in P. falciparum–infected pregnant women, compared with other pregnant women [15], in keeping with our results. Prolactin stimulates the immune system acting as a growth factor and as an immune comedogenic cytokine via autocrine, paracrine, and endocrine mechanisms [3]. In addition, prolactin is secreted locally by immune cells, and its pituitary production is partially controlled by proinflammatory cytokines [28]. Increased pulsatile levels of prolactin were observed from the second trimester to the postpartum period [29, 30]. Interestingly, the highest prolactin concentrations were found in multiparous women, who represent the least susceptible group of pregnant women.

NK cells can directly lyse P. falciparum–infected erythrocytes [14, 22]. As expected, the percentage of pRBCs lysed was sig-
Figure 2. Cytolytic activity of natural killer (NK) cells. A, NK cell–mediated cytolysis of K562, *P < .05 with either pRBCs or K562. B, Comparison of ex vivo NK cell cytotoxicity from primiparous women, multiparous women, and nonpregnant women against *P < .002 for comparison with NK cells from nonpregnant women; † for comparison with NK cells from multiparous women. Results are expressed as means of absolute values ± SD of 17 individual experiments at an effector/target ratio of 25:1.

Significantly higher than the percentage of uRBCs and similar to the percentage of K562 lysed. NK cell cytolytic activity decreases significantly during pregnancy; an NK cytotoxic activity defect has been also reported in pregnant women by Baines et al. [31] and by Goldsobel et al. [32]. However, to our knowledge, this is the first time that NK cytotoxicity has been compared in primiparous and multiparous women. The NK cytolytic effect was significantly lower in primiparous women than in multiparous women. Furthermore, low NK cell cytotoxic activity was correlated with high cortisol concentrations. Some studies have found that exogenous cortisol suppresses NK cell cytotoxicity [33] and that subphysiological concentrations of cortisol result in direct functional inactivation [10, 34]. Thus, it seems reasonable to speculate that the difference in NK cell cytotoxic activity against *P < .001 for comparison with NK cells from nonpregnant women; † for comparison with NK cells from multiparous women. Results are expressed as means of absolute values ± SD of 17 individual experiments at an effector/target ratio of 25:1.

tween primiparous and multiparous women can be partially explained by the difference in cortisol concentrations found in these 2 groups of pregnant women.

NK cells have been shown to contribute to resistance to malaria in the preerythrocytic stage [35], as well as the erythrocytic stage [24], through the lysis of infected cells. Absence of NK cells in intervillous tissue is associated with the high susceptibility of placental malaria [36]. The mechanism by which NK cells combat infection remains poorly understood, but it may involve IFN-γ [37]. The induction of NK cell IFN-γ production by pRBCs was recently proposed to be an anti-parasitic effector mechanism [38]. In our earlier work [14], we proposed an alternative mechanism in which sFasL/Fas interactions at the NK-pRBC level and cytolytic granzyme B activity inhibit growth of asexual blood stages.

In conclusion, the NK cell cytotoxic activity against pRBCs is lower in primiparous women than in other women, and there is a positive correlation between cortisol concentrations and the presence of *P < .001 for comparison with NK cells from nonpregnant women; † for comparison with NK cells from multiparous women. Results are expressed as means of absolute values ± SD of 17 individual experiments at an effector/target ratio of 25:1.

significant malaria susceptibility of primiparous women. Because NK cells as well as cytotoxic T lymphocytes are major components of the cellular mechanism by which an immune response leads to the destruction of foreign, tumor, or infected tissue, it is important to know whether high cortisol levels also suppress cytotoxic T lymphocyte activity and antigen-specific cytokine release. The ability of NK cells to interact with transplacentally transferred maternal antibodies may represent an important first line of defense for neonates, and this is an aspect we are actively investigating. Ongoing work in our laboratory seeks to further delineate human NK cell responses both in nonexposed individuals and during and after naturally acquired *P. falciparum* infections in exposed populations. Future research activities may include the development of vaccines for pregnant women by induction of NK cell activity. Basic research should include efforts to define structural requirements for interaction between NK cell–specific proteins and *P. falciparum* variant proteins expressed on the surface of erythrocytes infected with mature forms of *P. falciparum*.

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References

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