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MALARIA AND PREGNANCY IN CAMEROONIAN PRIMIGRAVIDAE: HUMORAL AND CELLULAR IMMUNE RESPONSES TO *PLASMODIUM FALCIPARUM* BLOOD-STAGE ANTIGENS

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Abstract. To investigate the mechanisms underlying the increased susceptibility to malaria in pregnant women, we determined the level of malaria-specific immunity in primigravidae. Humoral and cellular in vitro responses to unpurified (a crude schizont extract and a gametocyte preparation) and purified (affinity-purified Pf155/ring-infected erythrocyte surface antigen [RESA]) *Plasmodium falciparum* proteins, an immunodominant 45/47-kilodalton antigen from *Mycobacterium bovis*, and leucoagglutinin were compared between 52 primigravidae and 52 nonpregnant women from a semirural area of Cameroon. In vitro cellular responses were investigated in terms of lymphocyte proliferation, as well as production of interleukin-2 (IL-2), interferon-gamma (IFN-γ), and IL-4. Cells from primigravidae exhibited a reduced proliferative response to schizont and gametocyte antigens, as well as to the *M. bovis* antigen. Conversely, the IL-2 response to Pf155/RESA was reduced. Interleukin-4 and IFN-γ production did not appear to be affected in primigravidae. Antibody levels were also similar between pregnant and nonpregnant women. Our results underline the importance of examining several parameters of T cell activation with different types of antigens for a correct evaluation of the ability of lymphocytes to respond to malaria.

Malaria is known to cause serious problems during pregnancy. Previously immune pregnant women living in malaria-endemic areas are more likely to develop clinical attacks of malaria and serious complications than nonpregnant women. This increased risk of malaria in pregnant women is associated with abortions, stillbirths, and placenta infection that is responsible for low birth weight. Several studies have demonstrated that these effects are most frequent and marked in primigravidae.[1,2] The reasons why pregnant women, and in particular primigravidae, are more likely to present with malaria-related morbidity are not fully understood. Pregnancy is characterized by a transient depression of cell-mediated immunity. Recently, it has been suggested that the maternal immune system during pregnancy is dominated by a T helper cell 2 (Th2)–type response.[3–5] These Th2 cytokines may inhibit Th1 responses, protecting the fetus from rejection,[5,6] but also increasing the incidence of several infectious diseases that are usually under the control of $CD4^+$ T lymphocytes.[7] Therefore, we evaluated the influence of gestation on the peripheral blood T cells response to malaria. We compared antibody levels and in vitro proliferation as well as interleukin-2 (IL-2), interferon-gamma (IFN-γ), and IL-4 production in response to stimulation by three *Plasmodium falciparum* antigens, by a *Mycobacterium bovis* antigen, and by a mitogen in primigravidae and nonpregnant women from a semirural area of Cameroon.

SUBJECTS AND METHODS

Subjects. One hundred four women were studied in Ebolowa, a town of 35,000 inhabitants located 160 km south of Yaounde, Cameroon. In this rain forest area, *P. falciparum* malaria is hyperendemic with perennial transmission. Two matched groups of 52 women were enrolled from March to September 1992. The first group consisted of primigravidae from the control group (not receiving prophylaxis) of a randomized trial of chloroquine prophylaxis efficacy.[8] At six (range 4–8) months of pregnancy, heparinized venous blood samples (20 ml) were drawn. The second group consisted of nonpregnant women matched for day of sampling, ethnic group, living conditions, and age to each pregnant woman. Nonpregnant status of control women was confirmed by a urine test (Sero-UCG®; Fumouze Laboratories, Ile Saint-Denis, France).

Hematologic measurements. At enrollment, whole blood cell counts and hemoglobin measurements were done. Malaria parasites were searched for on Giemsa-stained thick blood smears against 1,000 leukocytes.

Antigens. A crude preparation of asexual *P. falciparum* components was obtained by sonication of an in vitro culture of the Palo Alto strain of *P. falciparum* (35% parasite density, 55% late stages). This preparation, referred to as schizont antigen, was used at a final concentration of 5 μg/ml. The lymphocyte response to Pf155/ring-infected erythrocyte surface antigen (RESA),[9] a major *P. falciparum* antigen, was tested using an affinity-purified Pf155/RESA preparation. Briefly, culture supernatants were concentrated by vacuum dialysis and run through a column of cyanogen bromide–activated sepharose coupled to Pf155/RESA antibodies. These antibodies were purified from a pool of hyperimmune sera by absorption on a monolayer of glutaraldehyde-fixed and air-dried ring-infected erythrocytes.[10] When this Pf155/

RESA preparation was subjected to electrophoresis, nitrocellulose-blotted, and reacted with the monoclonal antibody 33G2 (an antibody reacting with Pf1555/RESA),[11] a doublet was seen in the 155–135-kilodalton (kD) region. This Pf155/RESA antigen was used at final concentrations of 1, 5, and 10 μg/ml and the highest stimulation index (SI) obtained was considered. A third *P. falciparum* antigen consisted of gametocytes from an in vitro culture of the NF54 isolate of *P. falciparum*. Gametocytes were harvested over a biphasic gradient (Nycodenz BV, Haarlem, The Netherlands), frozen, and used at a final concentration equivalent to 5×10^5 gametocytes/ml.

Cellular responses to nonmalarial proteins were also assessed. A fraction referred to as Bacille Calmette Guerin (BCG) 45/47, which is highly enriched in a proline-rich 45/47-kD antigen complex from *M. bovis* and elicits delayed-type hypersensitivity reactions in immunized guinea pigs,[12] was used at a final concentration of 10 μg/ml. Leucoagglutinin (Sigma, St Louis, MO) was used at a final concentration of 10 μg/ml.

Antibody measurement. Plasma anti-*P. falciparum* antibody was measured by indirect immunofluorescent assay using air-dried smears of *P. falciparum* late stages. Anti-Pf155/RESA antibody was measured by erythrocyte membrane immunofluorescent assay using glutaraldehyde-fixed and air-dried monolayers of ring-infected erythrocytes.[9]

Lymphocyte proliferative assay. Within 16 hr after bleeding, peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-Paque® (Pharmacia, Uppsala, Sweden), and cell viability was confirmed by trypan blue staining. Purified PBMC were suspended at a concentration of 10^6 cells/ml in buffered RPMI 1640 medium containing 10% human serum, and 100-μl aliquots were plated in triplicate in 96-well, round-bottom plates. Mitogen, antigens, or RPMI 1640 medium alone were added in 100-μl amounts. Plates were incubated at 37°C in a humidified chamber with 5% CO_2. After six days, 110-μl culture supernatants were removed and 50 μl of fresh medium containing 0.5 μCi of methyl-^{3}H-thymidine (specific activity = 2 Ci/mmole; Amersham, Les Ulis, France) were added to each well. After 16 additional hr, cells were collected on glass-fiber filter paper and radioactivity was counted. Stimulation indices were calculated by dividing the geometric mean counts per minute (cpm) of antigen-stimulated cultures by the geometric mean cpm of unstimulated cultures. The threshold of positivity for all antigens was an SI $\geq$ 2.5.[13]

In vitro production of IFN-γ, IL-2, and IL-4. The six-day culture supernatants from each triplicate were pooled and stored at −80°C. The IFN-γ was assayed by a commercial two-site enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Genzyme, Cambridge, MA). The absorbance was read at 405 nm. Interferon-gamma concentrations were determined by reference to a human IFN-γ standard (Gg 23-901-530, 4,000 U/ampule; National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). The threshold of sensitivity was 2.5 U/ml. For statistical purposes, values less than this threshold were assigned a concentration of 1.25 U/ml. Mitogen- or antigen-induced IFN-γ production was derived from the difference between the IFN-γ content in stimulated cultures and the spontaneous IFN-γ content in unstimulated cultures. Due to material shortage, this assay was not done on gametocyte antigen.

Similar cultures were incubated for 72 hr, and triplicate supernatants were pooled and stored at −80°C for assaying the IL-2 and IL-4 contents. Cells from an IL-2–dependent mouse cytotoxic T cell line (CTLL2) were used as responder cells. Briefly, 10^4 CTLL2 cells/well were plated in 96-well, round-bottom plates in 100-μl volumes and 100 μl of undiluted supernatants were added. After 24 hr, cells were pulsed for 12 hr with 0.5 μCi of ^{3}H-thymidine/well, harvested, and processed as described above. Data were expressed as an SI as for lymphoproliferation. The threshold of positivity for all antigens was an SI $\geq$ 1.77.[14]

Interleukin-4 was assayed by a two-site immunometric assay as described for IL-1α, IL-1β, and IL-2.[15,16] A mouse monoclonal antibody (IL4-38) was used as capture antibody while a second monoclonal antibody (IL4-3, Fab$^+$-acetylcholinesterase conjugate) was used as tracer antibody. Bound enzyme was detected with Ellman's reagent and absorbance was read at 412 nm. The IL-4 concentrations were determined by reference to a standard (recombinant IL-4 produced in Chinese hamster ovary cells, a generous gift from Dr. J. Banchereau, Schering-Plough, Dardilly, France). The threshold of sensitivity was 12 pg/ml. As for IFN-γ, values less than this threshold were assigned a concentration of half this value (6 pg/ml). Mitogen- and antigen-induced IL-4 production were calculated as for IFN-γ (IL-4 content in stimulated cultures minus IL-4 content in unstimulated cultures).

Statistical analysis. All immunologic variables were log-transformed for analysis. When variable distribution was normalized, differences between matched pairs of pregnant women and nonpregnant women were tested by the paired Student's *t*-test. Otherwise, differences were tested by the Wilcoxon signed rank test. Differences between parasitized subjects and nonparasitized subjects were tested by the Student's *t*-test or the Mann-Whitney U test. Relationships at the individual level were tested by the Spearman ranked correlation test. All tests were two-tailed and P values < 0.05 were considered significant. Statview 4 (Abacus Concept, Berkeley, CA) and BMDP (BMDP Statistical Software Inc., Los Angeles, CA) statistical software were used.

RESULTS

Fifty-two primigravidae and 52 nonpregnant women were studied. Among nonpregnant women, 34% were nulligravidae, while others had one (25%), two (16.5%), or more (24.5%) pregnancies. In primigravidae, the main ethnic groups were Boulou (56%), Bamileke (17%), and Mbam (4%), while other ethnic groups represented 23%. Distribution of the ethnic groups in nonpregnant women was similar. The mean age of primigravidae and nonpregnant women was similar (P = 0.07) (Table 1). The nonsignificant difference in the age of the two groups was related to the fact that in two instances, we were unable to identify a nonpregnant woman of same ethnic group and living area younger than 39 and 43 years of age.

Hematology and parasitology. Hematologic and parasitologic data are given in Table 1. Hemoglobin levels were lower in primigravidae than in nonpregnant women (P = 0.001). Asexual *P. falciparum* parasitaemia was more fre-

TABLE 1
Hematologic and parasitologic data according to pregnancy status*

	Primigravidae (n = 52)	Nonpregnant women (n = 52)	P
Age (years)	18.9 ± 0.4	21.2 ± 0.8	0.07
Hemoglobin (g/L)	10.6 ± 0.3	11.9 ± 0.3	0.001
Plasmodium falciparum parasitemia, no. (%)	23 (42)	16 (29)	0.16
P. falciparum density/ml	684 (345–1,356)	422 (155–1,148)	0.41
IFA	2,193 (1,362–3,531)	2,279 (1,521–3,411)	0.90
EMIF	15 (9–25)	36 (20–65)	0.09

*Values are the mean ± SEM, the geometric mean density (95% confidence interval) of positive women, or the geometric mean positive antibody titer (95% confidence interval). IFA = indirect immunofluorescent antibody assay; EMIF = erythrocyte membrane immunofluorescent assay. All women were serologically positive by IFA; 25 primigravidae and 29 nonpregnant women were serologically positive by EMIF.

quent in primigravidae than in nonpregnant women (42% versus 29%), but the difference was not significant ($P = 0.16$). One pregnant woman presented with *P. falciparum* gametocytes and one nonpregnant woman presented with sexual and asexual *P. falciparum* parasites.

Malaria antibody levels. Total antimalaria antibody levels were similar in both groups (Table 1). More than half of the women (48% of primigravidae and 56% of nonpregnant women) had antibodies to Pf155/RESA. Mean levels of anti-Pf155/RESA antibodies were similar in both groups ($P = 0.14$).

Cellular responses. In vitro proliferative responses were measured after seven days. Cytokine production was assayed in three-(IL-2 and IL-4) and six-day (IFN-γ) culture supernatants. In response to leucoagglutinin, all cultures proliferated, while most produced IL-2 (81%), IFN-γ (97%), or IL-4 (97%) (Table 2). The mean proliferative and cytokine responses were similar in primigravidae and nonpregnant women.

Proliferation was observed in 74% of BCG 45/47-stimulated cultures, and in 22–65% of malaria antigen–stimulated cultures. The mean proliferative response to all antigens (except Pf155/RESA) was lower in primigravidae than in nonpregnant women (all $P < 0.05$). Interleukin-2 production was observed in 56% of BCG 45/47-stimulated cultures, and in 23–52% of malaria antigen–stimulated cultures. Overall, IL-2 production was lower in primigravidae than in nonpregnant women, but the difference was significant only for Pf155/RESA; ($P < 0.05$) and close to significance for schizont antigen ($P = 0.07$). The IFN-γ antigen-induced production was variable: cells from some women produced relatively large amounts of IFN-γ, while most produced very little or none. Interferon-gamma production was observed in 37% of BCG 45/47-stimulated cultures, in 6% of schizont antigen–stimulated cultures, and in 22% of Pf155/RESA-stimulated cultures. All but one individual producing IFN-γ in response to schizont antigen also responded to Pf155/RESA. Although BCG 45/47 and both malaria antigens seem to induce less IFN-γ production by cells from primigravidae than from nonpregnant women, the differences were not significant (all $P > 0.25$). Interleukin-4 production was observed in 22% of BCG 45/47-stimulated cultures and in 23–35% of malaria antigen–stimulated cultures. Amounts of IL-4 produced were usually rather low (IL-4 production was higher than 10 pg/ml in five BCG 45/47 supernatants, and in two, 10, and nine schizont, Pf155/RESA, and gametocyte antigen supernatants, respectively). Mean concentrations of IL-4 were low and similar in cultures from primigravidae and nonpregnant women.

To assess that the observed differences were indeed related to pregnancy, we investigated the influence of malaria infection and age in the measurements of immunologic parameters. In a two-way analysis of variance (ANOVA) taking into account group and malaria infection, the influence of the group factor remained significant in all instances (all $P < 0.05$). Similarly, in a two-way ANOVA taking into account group and age, the influence of the group factor remained significant in all instances but one, that being the proliferative response to the schizont extract, for which the difference between primigravidae and nonpregnant women appeared to be due to age ($P = 0.005$) and not to the pregnancy status ($P = 0.41$).

At the individual level, the production of IL-2 was related to both the proliferative and IL-4 responses following stimulation by the gametocyte antigen (both $P < 0.03$). There was no association between proliferative and lymphokine responses to other malaria antigens. Antibody levels and cellular responses of parasitemic women were compared with those of nonparasitemic women. Proliferative responses to schizont, Pf155/RESA, and gametocyte antigens were higher in nonparasitemic women than in parasitemic women (all $P < 0.04$). Similarly, IFN-γ production in response to schizont antigen was higher in nonparasitemic women than in parasitemic women ($P = 0.05$). No other difference could be demonstrated between both groups.

DISCUSSION

Humoral and cellular in vitro responses to leucoagglutinin, BCG 45/47, and three *P falciparum* antigens were compared between 52 primigravidae and 52 nonpregnant women. Cellular responses were investigated in terms of lymphocyte proliferation, as well as production of Th1-like (IL-1 and IFN-γ) and Th2-like (IL-4) cytokines following in vitro culture in the presence of different types of *P. falciparum* antigens: unpurified (schizont and gametocyte antigens) or purified (Pf155/RESA) proteins.

Titers of antibodies to *P. falciparum* and to Pf155/RESA did not differ between pregnant and nonpregnant women, confirming results of previous studies.[2, 17] Although earlier studies reported that anti-Pf155/RESA antibody titers were lower in pregnant than in nonpregnant women and in primigravidae than in multigravidae,[17, 18] we did not observe such a difference.

TABLE 2

Cellular responses to leucoagglutinin, BCG 45/47, and malaria antigens in 52 primigravidae and 52 nonpregnant women from Cameroon*

Antigen	Lymphoproliferation†		IL-2 production†		IFN-γ production (U/ml)‡		IL-4 production (pg/ml)‡	
	Primigravidae	Nonpregnant	Primigravidae	Nonpregnant	Primigravidae	Nonpregnant	Primigravidae	Nonpregnant
Leucoagglutinin	104.09 ± 26.99 (100%)	116.66 ± 28.66 (100%)	141.28 ± 43.60 (71%)	283.34 ± 91.81 (90%)	28.74 ± 2.25 (98%)	23.89 ± 2.27 (96%)	163.02 ± 28.32 (96%)	160.43 ± 19.36 (98%)
BCG 45/47	15.53 ± 4.20§ (65%)	28.35 ± 7.34 (83%)	3.03 ± 0.50 (54%)	5.09 ± 1.07 (57%)	2.62 ± 0.63 (37%)	2.98 ± 0.88 (37%)	1.36 ± 0.45 (20%)	1.84 ± 0.53 (24%)
Schizont	2.56 ± 0.72§ (16%)	5.59 ± 1.66 (27%)	1.64 ± 0.29 (21%)	1.99 ± 0.28 (33%)	0.13 ± 0.07 (6%)	0.18 ± 0.10 (6%)	1.13 ± 0.38 (22%)	1.73 ± 0.55 (24%)
Pf155/RESA	3.48 ± 1.42 (22%)	2.11 ± 0.19 (27%)	1.32 ± 0.09§ (17%)	1.53 ± 0.12 (29%)	0.56 ± 0.20 (19%)	0.76 ± 0.24 (25%)	3.88 ± 2.30 (24%)	4.25 ± 1.79 (37%)
Gametocyte	7.99 ± 3.36§ (57%)	17.94 ± 4.43 (74%)	3.03 ± 1.10 (44%)	3.73 ± 0.90 (59%)	ND	ND	2.78 ± 0.96 (31%)	3.61 ± 1.28 (39%)

*Values in parentheses are the percentage of positive response (see text). n = 49–52 in each group (primigravidae and nonpregnant women) for all antigens except gametocyte (n = 28–38). IL-2 = interleukin-2; IFN-γ = interferon-γ; BCG = Bacille Calmette Guerin; RESA = ring-infected erythrocyte surface antigen; ND = not done.
†Stimulation index ± SD (stimulation index = cpm of stimulated cultures/cpm of unstimulated cultures) (± SD).
‡IFN-γ (or IL-4) in stimulated cultures − IFN-γ (or IL-4) in unstimulated cultures.
§$P < 0.05$.

When cultured in presence of leucoagglutinin, in vitro cell proliferation and cytokine production were similar in primigravidae and nonpregnant women. The lack of changes in proliferative responses to various mitogens during pregnancy has been reported previously.[19–21] Moreover, Riley and others reported that following in vitro stimulation with phytoagglutinin, cells from all 30 tested pregnant women produced high amounts of IFN-γ.[21] The ability of cells from primigravidae to proliferate and to produce various cytokines in response to mitogens suggests that the immunosuppression process occurring during pregnancy does not involve an impairment in the antigen recognition mechanisms by lymphocytes, but rather a defect in the antigen processing or presentation mechanisms.

Following culture in the presence of BCG 45/47, the proliferative response was reduced in primigravidae, while the in vitro production of the three investigated cytokines was similar in both groups despite a rather high rate (22–56%) of responders in each read-out. As expected, this antigen is a relatively poor inducer of IL-4 responses. This is in line with previous reports using tuberculin-purified protein derivative (PPD), specific T cell clones,[22] or the enzyme-linked immunospot assay,[23] and is indicative of a Th1 type of response to mycobacteria. Suppressed proliferative responses to PPD during pregnancy have been previously reported.[7, 19, 20] Our results demonstrate that the impairment of the proliferative response to BCG 45/47 in primigravidae is not paralleled by a similar alteration of lymphocytes functional activity (as assessed by lymphokine production).

Following culture in the presence of schizont or gametocyte antigen, lymphocyte proliferation was reduced in primigravidae, while in vitro production of IL-2 remained unchanged. An opposite observation was made with Pf155/RESA purified protein: cell proliferation remained unchanged, while in vitro production of IL-2 was decreased in primigravidae. Reduced lymphocyte proliferation during pregnancy in the presence of crude asexual malaria antigen is in agreement with the results of a previous study in pregnant Gambian women.[21] Conversely, IL-4 and IFN-γ production in response to all three malaria antigens were not affected in primigravidae, demonstrating that various functional subsets of lymphocytes might be independently triggered,[22] and that alteration of the immune response may be restricted to a particular subset of lymphocytes,[24, 25] particularly during pregnancy. In primigravidae, the alteration of the response to unpurified malaria antigens mainly involves proliferation, while that to purified Pf155/RESA mainly involves IL-2–producing cells.

Overall, most differences between primigravidae and nonpregnant women are related to the capacity of lymphocytes to proliferate and to produce IL-2. However, proliferative response to the nonmalarial antigen BCG 45/47 is also affected. Thus, the alteration of malaria immunity might be part of the general frame of the depression of cellular immunity during pregnancy rather than a specific phenomenon. We did not observe a Th2 enhancement of the peripheral cell response; in mice this enhancement is higher at the maternal-fetal interface than at the peripheral level.[3, 4] Unprimed T cells, when first stimulated, proliferate and produce large amounts of IL-2, but no other T cell cytokines.[26, 27] Conversely, T cells producing multiple cytokines are considered

to be memory T helper cells.[28] Thus, response of naive T cells may be more suppressed during pregnancy than that of memory cells.

Several studies have demonstrated that the ability to respond to a given antigen is seriously underestimated unless multiple parameters of T cell activation are measured.[29,30] Our data confirm this observation; there was a limited relationship between the proliferative and cytokine responses at the individual level. This underlines the importance of examining several parameters of T cell activation with different types of antigens for a correct evaluation of the ability of lymphocytes to respond to malaria. Further investigations of the alterations of malaria-specific immune mechanisms during pregnancy will be useful in understanding malaria immunity in general.

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