Fetal Immune Responses to Plasmodium falciparum Antigens in a Malaria-Endemic Region of Cameroon

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Fetal Immune Responses to \textit{Plasmodium falciparum} Antigens in a Malaria-Endemic Region of Cameroon\footnote{This work was supported by the National Institutes of Health Grant U01 AI43888. S.M. was supported by a training grant from the Fogarty International Center (5 D43 TW01264).}

Simon Metenou,\textsuperscript{*} Amorsolo L. Suguitan, Jr.,\textsuperscript,* Carole Long,\textsuperscript{†} Rose G. F. Leke,\textsuperscript{‡} and Diane Wallace Taylor\textsuperscript{2*}

\textit{Plasmodium falciparum} infection during pregnancy can lead to the transplacental passage of malarial Ags that are capable of inducing acquired immune responses in the fetus. Studies have identified cytokines produced by malaria-specific cord blood (CB) T cells, but information on fetal B cells is limited. Thus, CB mononuclear cells from 120 Cameroonian newborns were cultured for 7 days in vitro and supernatants were assessed by ELISA for Abs to an extract of malarial schizonts (MA), recombinant apical merozoite Ag 1 (AMA-1), the 42-kDa C-terminal region of merozoite surface protein 1 (MSP-1\textsubscript{42}), a B epitope of ring-infected erythrocyte surface Ag (RESA), and the dominant B epitope of the circumsporozoite protein (CSP). Only 12\% of supernatants contained IgM to MA but 78\% had IgG to one or more malarial Ags, with 53\% having IgG to AMA-1, 38\% to MSP-1\textsubscript{42}, 3\% to RESA, and 0\% to CSP. The Abs to AMA-1 and MSP-1\textsubscript{42} were predominantly IgG1 and IgG3. CB mononuclear cells were also tested for the ability to secrete cytokines in response to MA and a pool of conserved MSP-1 T cell epitopes. Among the Ag-reactive samples, 39.3\% produced only Th2-type cytokines, whereas 60.6\% produced a combination of Th1- and Th2-type cytokines. Although a Th2 bias was observed, the in utero cytokine environment was adequate to support isotype switching to cytophilic IgGs, the isotypes that are protective in adults. Because many infants living in a low transmission area are born with malaria-specific B and T cells, the influence of in utero priming on neonatal immunity merits further investigation. \textit{The Journal of Immunology}, 2007, 178: 2770–2777.

In humans, the immune system of the fetus develops early in gestation and is able to respond to foreign Ags (1–3). By the 18th week of gestation, fetal splenic T cells can produce cytokines and express costimulatory molecules as well as induce Ig switching in B cells (3). B cells expressing CD19, CD20, CD21, CD22, IgM, IgD, and HLA-DR have been found in human fetuses as early as the 12th week of gestation, with \textsim 90\% of mature B cells being CD5\textsuperscript{+} B positive (B-1 B cells). The ratio of B-1 to B-2 B cells decreases with gestational age, such that by birth only 35–40\% of B cells are B-1 (3, 4). In humans, IgM and IgG synthesis can start as early as the 10th week of gestation and increases with gestational age (3). At birth, fetal cord blood (CB)\footnote{Abbreviations used in this paper: CB, cord blood; AMA-1, apical merozoite Ag 1; CSP, circumsporozoite protein; IFA, indirect fluorescent antibody assay; IRBC, infected red blood cells; MA, extract enriched for schizont-stage malarial parasites; MC, mononuclear cell; MSP-1, merozoite surface protein-1; MSP-1\textsubscript{19}, 19-kDa fragment of MSP-1; MSP-1\textsubscript{42}, 42-kDa fragment of MSP-1; NRBC, noninfected RBC; RESA, ring-infected erythrocyte surface antigen; SI, stimulation index.} mononuclear cells (MC) (CBMC) are generally less responsive than T cells, but information on fetal B cells is limited. Thus, CB mononuclear cells from 120 Cameroonian newborns were cultured for 7 days in vitro and supernatants were assessed by ELISA for Abs to an extract of malarial schizonts (MA), recombinant apical merozoite Ag 1 (AMA-1), the 42-kDa C-terminal region of merozoite surface protein 1 (MSP-1\textsubscript{42}), a B epitope of ring-infected erythrocyte surface Ag (RESA), and the dominant B epitope of the circumsporozoite protein (CSP). Only 12\% of supernatants contained IgM to MA but 78\% had IgG to one or more malarial Ags, with 53\% having IgG to AMA-1, 38\% to MSP-1\textsubscript{42}, 3\% to RESA, and 0\% to CSP. The Abs to AMA-1 and MSP-1\textsubscript{42} were predominantly IgG1 and IgG3. CB mononuclear cells were also tested for the ability to secrete cytokines in response to MA and a pool of conserved MSP-1 T cell epitopes. Among the Ag-reactive samples, 39.3\% produced only Th2-type cytokines, whereas 60.6\% produced a combination of Th1- and Th2-type cytokines. Although a Th2 bias was observed, the in utero cytokine environment was adequate to support isotype switching to cytophilic IgGs, the isotypes that are protective in adults. Because many infants living in a low transmission area are born with malaria-specific B and T cells, the influence of in utero priming on neonatal immunity merits further investigation. \textit{The Journal of Immunology}, 2007, 178: 2770–2777.

In malaria-endemic regions of sub-Saharan Africa, pregnant women and their newborns are at a high risk of developing the severe effects of malaria caused by \textit{Plasmodium falciparum}. The severity of malaria during pregnancy has been attributed to both the modulation of maternal immune responses and the sequestration of malarial parasites in the intervillous space of the placenta (8, 9). The sequestration of parasites is thought to cause alterations to the syncytiothrophoblastic layer that could increase fetal exposure to parasites or malarial Ags (10). Although exposure in utero to foreign Ags was initially thought to induce tolerance or anergy, recent data suggest that exposure of the human fetus to foreign Ags can lead to the activation, priming, and differentiation of Ag-specific lymphocytes into effector and memory T cells (1, 11). In 1988, Desowitz (1) reported that CBMC from newborns in Papua New Guinea proliferated when cultured with an extract of malarial parasites, whereas babies born in a malaria-free area did not. Subsequently, malaria-specific T cell proliferation and cytokine responses were found in CBMC of newborns in Kenya, Gabon, Togo, and Cameroon (12–15). Thus, there is substantial evidence for in utero priming of T cells to malaria.

The presence of malaria-specific IgM and/or IgE in cord blood has been used as evidence of in utero activation (priming) of fetal B cells (1, 15, 16), because these subclasses do not cross the placenta from maternal circulation. To date, only one study has attempted to assess B cell priming to malarial Ags in utero (12). In a malaria-endemic area of Kenya, 18\% of CBMC from newborns produced IgG in vitro when stimulated with the C-terminal 19-kDa fragment of merozoite surface protein 1 (MSP-1), designated MSP-1\textsubscript{19} (12). Because Abs play a critical role in immunity to malaria (17), data on the extent and nature of malaria-specific humoral responses induced in utero are of capital importance for...
understanding the acquisition of immunity in infants and designing vaccines for young children.

Thus, this study sought to determine whether newborns in Yaoundé, Cameroon, an area where the transmission of *P. falciparum*-malaria is low throughout the year, are born with primed B cells. CBMC were cultured in vitro and the supernatants were tested for IgG Abs to apical merozoite Ag 1 (AMA-1), the 42-kDa fragment of MSP-1 (MSP-142), the ring-infected erythrocyte surface Ag (RESA), and the circumsporozoite protein (CSP) by ELISA. Supernatants were also tested by ELISA and indirect fluoroescence Ab assay (IFA) for Abs to asexual stage parasites. Results showed that CBMC from 78% of newborns produced IgG in vitro to at least one malarial Ag and that IgG1 was the predominant isotype. In addition, most newborns had CBMC that secreted only Th1 (e.g., IL-2 and/or IFN-γ) and Th2 cytokines (e.g., IL-4, IL-5, and IL-13) or a combination of both cytokines. Most newborns had CBMC that secreted at least one malarial Ag and that IgG1 was the predominant isotype. In addition, most newborns had CBMC that secreted only Th2 cytokines (e.g., IL-4, IL-5, and IL-13) or a combination of both cytokines. Most newborns had CBMC that secreted at least one malarial Ag and that IgG1 was the predominant isotype.

**Materials and Methods**

**Study population and sample collection**

Ethical approval was obtained from the Institutional Review Board of Georgetown University (Washington D.C.) and the National Ethical Committee of Cameroon. The study was conducted at Central Maternity and Biometry Assi Hospitals in Yaoundé, Cameroon. Between June and November 2002, women were informed about the project and, after receiving informed consent, were 120 paired maternal peripheral and fetal CB samples collected. At delivery, information on the mother and newborn were obtained; ~8 ml of heparinized maternal blood and 20 ml of CB were collected for immunological studies, 3 ml of maternal blood and 5 ml of fetal CB were collected in EDTA for molecular studies, and a small biopsy of placental tissue was excised for parasitological analysis. All blood samples were stored on ice and transported to the Immunology Laboratory, Biotechnology Center, Nkolbisson, Cameroon, where laboratory studies were performed. A 1-ml aliquot of the EDTA-treated whole blood was stored at −70°C until it was used for the detection of *P. falciparum* and human microsatellite analysis by PCR.

**Malaria Ag**

To produce infected RBC (IRBC), the 3D7 strain of *P. falciparum* was grown in vitro in O positive RBC using standard culture conditions. An impression smears of placental tissue, were prepared and stained with Diff-Quick solutions (Baxter Healthcare). Slides were examined for malarial parasites by two microscopists. Maternal venous and CB samples were also screened for *P. falciparum* DNA by nested PCR as described by Snounou et al. (21).

**Microsatellite DNA genotyping**

To confirm that CB samples were not contaminated with maternal leukocytes, polymorphisms in the D1S80 and Alu TPA25 loci were examined. Recent studies have shown that D1S80 is highly polymorphic in Cameroonian and is a valuable method for discriminating among ethnic groups in Africa (22). Alu diversity is high throughout Africa compared with Europe (23). Thus, these microsatellite markers were used to detect possible contamination of CB with maternal blood. DNA was purified from CB and maternal blood using a commercial kit (Generation capture column kit; Gentra). In the D1S80 amplification, the forward primer 5′-GAAAAC TGCCCTCCAACACTGGCGCG-3′ and reverse primer 5′-GTGTTG TTGGAAGATGACGTCGCCCCCTTGC-3′ were used. In the Alu (TPA-25) reaction the forward primer used was 5′-GTAAGAGTTCGTTAAACAGCAGC-3′ and the reverse primer was 5′-CCCCCCTTAGGAAAC TTCTCTTT-3′ (22). The reaction mix contained 5 μg of DNA, 0.2 mM each dNTP, 1× Mg-free PCR buffer, 2 mM MgCl2, 200 ng of each primer, and 1.25 U of Taq polymerase in a total volume of 50 μl and was amplified under the following conditions: one cycle of denaturation at 95°C for 2 min, 5 cycles of denaturation (95°C for 30s), annealing (65°C for 30s), and extension (65°C for 1 min), and 25 cycles of denaturation (95°C for 30s), annealing (65°C for 30s), extension (72°C for 1 min), and a final elongation (72°C for 5 min). The amplified products were stored at 4°C until visualized on a 1.5% agarose gel. The presence of more than two bands in CB demonstrated maternal blood contamination.

**Isolation of MC**

Heparinized maternal peripheral blood and CB samples were centrifuged at 447 × g for 10 min and plasma was removed. MC were purified by density gradient centrifugation at 1006 g for 25 min using Ficoll-Paque solution (Amershams Bioscience).

**In vitro B cell cultures**

To determine whether Ag-specific B cells were present in CB, purified CBMC were cultured for 7 days in serum-free, B cell complete medium (RPMI 1640 supplemented with 10% Controlled Processed Serum Replacement-3 (Sigma-Aldrich), 0.02 M HEPES, 0.2 M l-glutamine, 0.1 M sodium pyruvate, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate). CBMC were diluted to 10^6 cells/ml in B cell complete medium and 200 μl (2 × 10^5 cells/well) were plated in 96-well culture plates in penicillin G (100 U/ml). Cells were stimulated with 1 μg of PWM per well and/or 20 μl of RPMI 1640 per well (unstimulated). The plates were incubated at 37°C for 7 days and the supernatants from the replicates were pooled and stored at −70°C.

**Quantitation of total IgG and malaria-specific IgG and IgM in culture supernatants**

To measure the total amount of IgG present in culture supernatants, wells of microtiter plates (Maxisorp; Nunc) were coated with 0.1 μg of purified goat anti-human IgG (Southern Biotechnology) in 100 μl of coating buffer (0.1 M carbonate buffer (pH 9.5)) and incubated overnight at 4°C. Plates were washed five times with PBS containing 0.05% Tween20 and blocked with PBS, 0.05% Tween 20, and 10% nonfat milk for 1 h at 37°C. After washing, 100 μl of the IgG standard containing various amounts of recombinant human IgG (0–1000 pg/ml) (The Binding Site) was added to the plate. The culture supernatants were added undiluted to the remaining wells. The plates were incubated for 1 h at 37°C and washed, 100 μl of the mouse anti-human IgG-alkaline phosphatase (Sigma-Aldrich) diluted at 1/4000 was added, and the plates were incubated for 1 h at 37°C. After washing, 100 μl of the substrate (p-nitrophenylphosphate; Sigma-Aldrich) was added at 1 mg/ml, plates were incubated at room temperature for 30 min, and OD values were then read at λ = 405/630 nm using a dual wavelength spectrophotometer. Ab concentrations were determined by linear regression using SPSS software.

**ELISA for antimalarial Ab**

To measure Ab responses to AMA-1 and MSP-142, microtiter plates (Maxisorp; Nunc) were coated with 100 μg/well recombinant Ag diluted in coating buffer (final concentration 1 μg/ml). To determine Ab titers to RESA and CSP, which were conjugated to BSA, alternate rows of microtiter plates were coated with 2 μg/well RESA-BSA or 2 μg of BSA per
well (non-specific control). To measure Ab against MA, alternate rows of the plates were coated with an extract of $10^5$ NRBC or $10^5$ schizont-enriched IRBC per well. After overnight incubation at 4°C, the plates were washed, blocked, and 100 μl of undiluted culture supernatant was added and incubated for 1 h at 37°C. Pools of plasma from Cameroon and American adults were used at 1/500 dilutions as positive and negative controls. After washing, 100 μl of diluted (1/4000), alkaline phosphatase-conjugated mouse anti-human IgM or mouse anti-human IgG (Sigma-Aldrich) was added to each well, and the plates were incubated at 37°C for 1 h. The substrate was added and the plates were read following incubation as described above. Appropriate background values were subtracted from the test values. The cutoff for positivity for both the IgM and IgG assays was the mean of the negative control plus 3 SD.

**IFA assay for antimalarial Ab**

Thin blood smears of 3D7 parasites obtained from asynchronous in vitro cultures were prepared, air dried, and stored at −70°C until use. Slides were defrosted in the presence of desiccant, fixed for 5 min in cold acetone, and then incubated with 15 μl of undiluted culture supernatant along with the Ab-positive plasma from Cameroonian adults diluted 1/500 and negative control plasma from unexposed Americans diluted 1/500 for 30 min at room temperature. Slides were washed, incubated with goat anti-human IgG, and IgM labeled with FITC (Kirkegaard & Perry Laboratories) for 30 min at room temperature, washed, mounted in mounting medium (1 M barbital buffer plus 50% glycerol (pH 8.6)), and examined using a Zeiss Axioshot fluorescence research microscope (Carl Zeiss). The intensity of each sample was compared with that of the positive and negative controls. Results were classified as strongly positive (+ + +), positive (+ +), or weakly positive (+). All of the borderline (±) results were considered negative.

**T cell proliferation assays**

PBMC and CBMC were diluted to $10^5$ cells/ml in complete medium RPMI-1640 (Invitrogen Life Technologies) supplemented with 10% human serum (Sigma-Aldrich), 1% of 100× nonessential amino acids, 0.02 M HEPES, 0.2 M l-glutamine, 0.1 M sodium pyruvate, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate (Invitrogen Life Technologies). CBMC and PBMC were plated at $2 \times 10^4$ CBMC/well in 200 μl of complete medium in 96-well culture plates (Costar; Corning). PBMC and CBMC were stimulated with the following Ags: 1) 2 μg/well (20 μl) MSP-1 peptide pool; 2) 2 μg/well (20 μl) CSP peptide pool; 3) 1 μg/well (20 μl) PHA; 4) an aliquot of MA equivalent to $10^4$ IRBC/well; and 5) $20 \mu$l/well RPMI 1640 (unstimulated cultures) in quadruplicate for 4 days. Cells were pulsed with 0.5 μCi/ml [3H]TdR (Sigma-Aldrich) for 16 h, harvested, and counted. Results are expressed as the stimulation index (SI), i.e., mean counts per minute of stimulated wells divided by the mean counts per minute of the unstimulated wells. A sample was considered positive for T cell proliferation if the SI was ≥3 for MA and PHA or ≥1.5 for MSP-1 and CSP.

**Cytokine cultures**

To compare cytokine production between maternal and fetal cells, PBMC and CBMC were cultured in quadruplicate at a concentration of $2 \times 10^5$ cells/well with 1 μg/well (20 μl) PHA and 20 μl/well RPMI 1640 (for unstimulated cells). Following incubation at 37°C for 4 days, culture supernatants were collected and stored at −70°C until used. In addition, the ability of CBMC to produce cytokines in response to malaria Ags was tested. CBMC were cultured in quadruplicate as described above with the pools of MSP-1 and CSP peptides and the MA extract containing the equivalent of $10^4$ IRBC/well. After 4 days, supernatants were collected and stored at −70°C until used.

**Cytokine assays**

Supernatants were screened using the LumiChip Human 10-Cytokine kit from Qiagen. The protocol provided by the manufacturer was followed and samples of the manufacturer’s instructions. A total of 50 μl of supernatant was incubated with microspheres coated with Abs specific to IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 (p70), IFN-γ, TNF-α, and GM-CSF. In addition, supernatants were incubated with microspheres for the TNF-α, IL-5, and IL-12 cytokines (Linco Research) following the manufacturer’s instructions. The supernatants stimulated were considered to be positive for a specific cytokine if the cytokine concentrations in the stimulated wells were greater than the mean + 3 SD in the unstimulated wells.

**Statistical analysis**

A Mann-Whitney U test was used to compare cytokine levels in culture supernatants of CBMC and maternal PBMC using SPSS software.

**Results**

**Description of study population**

A total of 120 paired maternal and fetal cord blood samples were evaluated. Among the women, 30 were primigravida, 25 were secundigravida, and 65 were multigravida. By microscopy, 14.2% of the women were malaria-positive with parasites detected in either peripheral and/or placental blood smears. However, 37% of the women were positive for *P. falciparum* by PCR. No parasites were detected by microscopy in CB samples, but 11% were positive for malaria by PCR.

**Assessing the level of maternal leukocyte contamination in CB**

Because the objective was to determine whether CBMC were primed in utero, it was important to determine whether CB samples were contaminated with maternal PBMC. According to polymorphisms in the microsatellite loci D1S80 and TPA-25 were assessed. Initially, the sensitivity of the assay was determined by spiking a sample of CB with aliquots of blood from an unrelated donor to create samples with 1, 2, 5, and 10% contamination. Detection of more than two bands demonstrated contamination. Four bands were found in samples containing 1–10% donor blood. Thus, the assay was able to detect at least 1% maternal contamination of CB samples. Results showed that all of the cord samples tested had only two alleles. Thus, the CB samples used in this study were contaminated with <1% maternal cells or DNA.

**CBMC secreted Ab against asexual stage parasite Ags in vivo**

Supernatants from B cell cultures were screened by IFA for anti-parasite IgG and 28% were found to be positive. The IFA pattern produced was similar to that seen using plasma from Cameroonian adults. IgG immunoreactivity was seen in the cytosols of ring, trophozoite, and segmenter parasites, was associated with Maurer’s clefts in the erythrocyte cytosol, and was localized on freed merozoites. A few supernatants were strongly positive (i.e., +4 fluorescence on a scale of 1 to 4), but most had lower fluorescence intensities (i.e., +2 to +3). Thus, the fetal humoral response was polyclonal in nature and produced IgG to Ags in multiple stages and intracellular sites of asexual stage parasites.

Next, supernatants of CBMC cultures were screened by ELISA for IgM against MA and AMA-1. Results showed that 12% of the cultures contained IgM that was specific for the MA extract but none contained Ab against AMA-1. In contrast, among 120 supernatants 52% had IgG to recombinant AMA-1, 38% to the MA extract, 32% to MSP-142, 3% to RESA, and 0% to CSP (Fig. 1). Taking ELISA and IFA results together, 78% of CBMC cultures were found to have IgG Abs to one or more of the Ags. Thus, although the prevalence of IgM-secreting B cells was low, many newborns had B cells capable at birth of producing IgG to at least one of the malarial Ags tested.
CBMC secrete different IgG isotypes in response to malaria Ags

Because the cytokine environment influences IgG isotope switching, the remaining amount of the supernatant from the CBMC cultures was tested in an isotype-specific IgG assay using AMA-1 and MSP-142. The results showed that the predominant isotype produced by most of the CBMC was IgG1 (Fig. 2). Of the 52 cultures with IgG to AMA-1, 64% contained Ag-specific IgG1 and 6% had IgG3. No IgG2 was detected. In contrast, of the 16 cultures with IgG for MSP-142, 63% contained IgG1, 31% had IgG3, and 10% had IgG2. Thus, IgG isotype-switching occurred in utero with different isotype patterns for the two vaccine candidate Ags.

Malarial Ags induced CBMC to proliferate in vitro

CBMC were cultured for 7 days with PHA, the MA schizont extract, and pools of MSP-1 and CSP peptides. Overall, 93% of the 120 CBMC cultures responded to PHA, with a mean SI for positive samples of 11.8 ± 15.4 (Fig. 3). A positive response was also found in 54% of the CBMC cultures incubated with MA (mean SI = 4.8 ± 3.0 for positive wells), 9% with the MSP-1 pool (mean SI = 1.6 ± 0.2), and 0% with the pool of CSP peptides (mean SI = 0.9 ± 0.2). In contrast, 97, 74, 18, and 12% of PBMC from their mothers proliferated in response to PHA, MA, MSP-1, and CSP, respectively. These results show that, although CBMC from many newborns proliferated in vitro, the prevalence was lower than that in their mothers.

CBMC produced less IL-4, IL-5, and IFN-γ than maternal PBMC in response to PHA

The cytokine profile in supernatants of paired maternal and CBMC stimulated with PHA was determined (Table I). No significant differences were observed in the amounts of IL-2 and IL-12 produced by maternal and CBMC, however, CBMC produced significantly lower amounts of IFN-γ (p = 0.001), IL-4 (p < 0.001), and IL-5 (p = 0.001). In contrast, supernatants from CBMC cultures contained higher amounts of IL-10 and IL-13 than maternal MC (p < 0.001 and p < 0.05, respectively). No difference in the levels of the inflammatory-type cytokines was found in the supernatants of cultures of CB and maternal MC stimulated with PHA (Table I).

CBMC produced cytokines when stimulated with MA and MSP-1

Because CBMC produced Th1-, Th2-, and inflammatory-type cytokines in response to PHA, the cytokine profiles induced by MA and MSP-1 peptides were assessed in supernatants of the first 45 samples collected (Fig. 4). The most striking response to MA was that of the inflammatory cytokines, with all cultures containing IL-1β and IL-6, most having TNF-α (100% by Linco, 67% by Qiagen), 87% containing GM-CSF, but only 11% having IL-8 (Fig. 4). A strong Th2-type response was seen in response to MA, because IL-4 was detected in 11%, IL-5 in 69%, and IL-13 in 87% of the cultures. IL-10 was detected in 89% of the MA-stimulated cultures. A weaker Th1-type response was found with IL-2 and/or IFN-γ being detected in 58.4% of the MA-stimulated cultures. A weaker Th1-type response was found with IL-2 and/or IFN-γ being detected in 57.8% of the MA-stimulated cultures. Thus, overall newborn CBMC demonstrated strong inflammatory and Th2-type responses and a solid Th1-response to MA. In comparison, the MSP-1 peptide pool stimulated production of two or more inflammatory-type cytokines in 13.3% of the cultures (7% had GM-CSF, 9% IL-8, 13% TNF-α, 16% IL-1β, and 18% IL-6) (Fig. 4). None of the MSP-1-stimulated cultures contained positive samples of 11.8 ± 15.4 (Fig. 3). A positive response was also found in 54% of the CBMC cultures incubated with MA (mean SI = 4.8 ± 3.0 for positive wells), 9% with the MSP-1 pool (mean SI = 1.6 ± 0.2), and 0% with the pool of CSP peptides (mean SI = 0.9 ± 0.2). In contrast, 97, 74, 18, and 12% of PBMC from their mothers proliferated in response to PHA, MA, MSP-1, and CSP, respectively. These results show that, although CBMC from many newborns proliferated in vitro, the prevalence was lower than that in their mothers.

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Th2-type cytokines, 4% had IL-10, but 7% contained the Th1-cytokine IFN-γ.

Previous studies have reported that some newborn CBMC produced only Th1-type and some produced only Th2-type cytokines, whereas other babies produced a combination of Th1- and Th2-type cytokines (14, 24). In the current study, among the infants who responded to MA (n = 33) none had a Th1-restricted response (i.e., only produced IL-2 or IFN-γ), 39.3% produced only Th2 cytokines.

Table I. Cytokine response of maternal PB and fetal CB MC stimulated with PHA

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Percentage of responders (%)</th>
<th>Mean pg/ml (\pm SD) (median)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unstimulated</td>
<td>PHA</td>
</tr>
<tr>
<td>IL-2</td>
<td>Mother 15</td>
<td>4.4 (\pm 3.9) (4.2)</td>
</tr>
<tr>
<td></td>
<td>Cord 20</td>
<td>6.7 (\pm 5.6) (6.3)</td>
</tr>
<tr>
<td>IL-12</td>
<td>Mother 0</td>
<td>5.3 (\pm 5.1) (3.1)</td>
</tr>
<tr>
<td></td>
<td>Cord 0</td>
<td>7.5 (\pm 4.3) (7.9)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Mother 100</td>
<td>22.6 (\pm 8.8) (22.6)</td>
</tr>
<tr>
<td></td>
<td>Cord 80</td>
<td>17.5 (\pm 7.8) (13.0)</td>
</tr>
<tr>
<td>IL-4</td>
<td>Mother 75</td>
<td>4.1 (\pm 3.0) (3.0)</td>
</tr>
<tr>
<td></td>
<td>Cord 0</td>
<td>4.6 (\pm 3.4) (4.0)</td>
</tr>
<tr>
<td>IL-5</td>
<td>Mother 75</td>
<td>5.6 (\pm 4.9) (6.1)</td>
</tr>
<tr>
<td></td>
<td>Cord 0</td>
<td>3.4 (\pm 7.2) (5.3)</td>
</tr>
<tr>
<td>IL-10</td>
<td>Mother 95</td>
<td>8.3 (\pm 14.0) (3.4)</td>
</tr>
<tr>
<td></td>
<td>Cord 100</td>
<td>7.1 (\pm 6.8) (4.2)</td>
</tr>
<tr>
<td>IL-13</td>
<td>Mother 100</td>
<td>19.7 (\pm 7.8) (18.6)</td>
</tr>
<tr>
<td></td>
<td>Cord 100</td>
<td>26.2 (\pm 3.5) (25.3)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Mother 100</td>
<td>25.3 (\pm 55.6) (13.6)</td>
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<tr>
<td></td>
<td>Cord 100</td>
<td>8.4 (\pm 8.6) (4.9)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Mother 85</td>
<td>6.5 (\pm 4.8) (7.0)</td>
</tr>
<tr>
<td></td>
<td>Cord 100</td>
<td>4.7 (\pm 3.3) (5.5)</td>
</tr>
<tr>
<td>IL-2</td>
<td>Mother 0</td>
<td>4.4 (\pm 3.9) (4.2)</td>
</tr>
<tr>
<td></td>
<td>Cord 0</td>
<td>6.7 (\pm 5.6) (6.3)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Mother 75</td>
<td>220 (\pm 338) (57.8)</td>
</tr>
<tr>
<td></td>
<td>Cord 85</td>
<td>132 (\pm 179) (24.2)</td>
</tr>
<tr>
<td>IL-8</td>
<td>Mother 0</td>
<td>512 (\pm 142) (525)</td>
</tr>
<tr>
<td></td>
<td>Cord 0</td>
<td>524 (\pm 115) (519)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Mother 100</td>
<td>7.0 (\pm 12.5) (0)</td>
</tr>
<tr>
<td></td>
<td>Cord 100</td>
<td>8.9 (\pm 14.4) (1.8)</td>
</tr>
</tbody>
</table>

\(a\) Comparison of medians between mother and cord mononuclear cells.

FIGURE 4. Cytokine response of CBMC following in vitro culture with MA and MSP-1. Supernatants from CBMC stimulated with MA and a pool of MSP1 peptides were screened for the cytokines shown. Results are based on 45 CB samples.
(IL4, IL-5 and/or IL-13), and 60.6% had a combination of Th1 and Th2 cytokines.

Maternal infection and frequency of in utero priming

Previous studies found a higher prevalence of primed MC cells in primigravida women (24) and in women who have placental malaria at delivery (25). In the current study, the prevalence of IgG to one or more malaria Ags was significantly higher in cultures of CBMC from babies born to primigravida and secundigravida (89%) compared with multigravida women (69%) (p < 0.001). However, the prevalence IgG Abs was not significantly higher in infants born to mothers who had placental malaria at delivery (81.3 vs 73.0%; p = 0.65) or between low birth weight and normal birth weight babies (75% low birth weight vs 71% normal birth weight; p = 0.4).

Discussion

Originally, it was thought that in utero exposure to foreign Ags resulted in immunological tolerance, but accumulating evidence now shows that exposure to small doses of microbial Ags can lead to the priming of fetal lymphocytes (12, 14, 26, 27). In developing countries, women are often infected with parasites during pregnancy and expose the developing fetus to foreign Ags. Mechanisms underlying transplacental passage of parasite Ags are unclear, but malarial, filarial, and schistosomal Ags have been detected in CB (2, 10, 26, 28).

Developing germinal centers have been detected in human neonates at birth (29) and neonatal B cells have the ability to produce IgM and different IgG subclasses in vitro (6). In the current study, malaria-specific IgM was detected in 12% of 120 CBMC cultures. Previously, Xi et al. (30) detected IgM in 14% of CB samples collected in Yaoundé and demonstrated by Western blotting that the Abs reacted with a wide range of asexual stage Ags, with each newborn having its own unique pattern of IgM reactivity.

IgG to one or more malarial Ags was identified in 78% of the CBMC cultures. The pattern of IFA produced demonstrates that the IgG response is also polyclonal in nature, because the Abs recognized Ags located in the cytosol of rings, trophozoites, and schizonts, as well as Ags associated with merozoites and Maurer’s clefts. Further screening showed that 52% of the 120 CBMC samples had plasma cells capable of producing IgG to AMA-1, and 32% to MSP-1\(_{42}\), two merozoite-associated Ags (Fig. 1). Large numbers of merozoites are present in the intervillous space of the placenta, and these molecules are strong immunogens, with most adults developing high Ab titers to them. Only 9% of CBMC cultures contained IgG Ab to RESA, an Ag expressed on the surface of erythrocytes infected with ring-stage parasites. Few rings are present in the placenta because they lack erythrocyte membrane protein 1 and thus cannot sequester in the intervillous space. The prevalence of Abs to RESA is relatively low in adults (31, 32). None of the cultures has Abs to CSP, a sporozoite-associated Ag. Because sporozoites do not reside in the placenta, their Ags are unlikely to cross the placental barrier. Thus, the order of IgG Ab responses by CB B cells was directly related to the risk of exposure in utero and the immunogenicity of the Ag. Because CBMC samples contained <1% maternal cells as determined by microsatellite analysis, the malaria-specific IgM and IgG found in CB supernatants must have been produced by primed fetal B cells that underwent isotype-switching in utero and continued to produce Ab when cultured in vitro.

As noted previously, only one study has investigated malaria-specific priming of B cells in utero. In 2002 King et al. (12) reported that 18% of CBMC from Kenyan newborns secreted IgG specific to MSP-1\(_{19}\) when restimulated in vitro. IgG was not produced by CBMC from American newborns. In the current study, IgG to the 42 kDa C-terminal region of MSP1 was detected in 32% of the 120 cultures without additional stimulation. Because germinal centers are not well formed in newborns, it would not be surprising to find relatively high numbers of plasma cells circulating in CB. MSP-1 is a major vaccine candidate and it will thus be important to take in utero priming into consideration when testing the efficacy of MSP-1 vaccines in infants. Infants born with primed cells may produce secondary responses upon vaccination, whereas those who were not exposed may produce primary responses.

This is the first study to evaluate Ab production by CBMC to malarial Ags other than MSP-1\(_{19}\) and to assess the subclass of IgG produced. IgG1 and IgG3 were the predominant IgG subclasses produced to both AMA-1 and MSP-1\(_{42}\), although some MSP-1-specific IgG2 was detected (Fig. 2). Isotype-switching from IgM to IgG1 and IgG3 is directed by Th1-type cytokines. A combination of Th1- and Th2-type cytokines was detected in 60.6% of Ag-responsive CBMC cultures. Thus, at term a number of newborns had T cells that supported isotype switching to IgG1 and IgG3. Interestingly, IgG1 is also the predominant isotype produced by adults against AMA-1 and MSP-1 (33, 34). Therefore, although the uterine environment is Th2-biased (35), Th1-type responses were adequate in utero to switch to the same isotypes as adults.

Clearly, many questions remain about the humoral immune response to *P. falciparum* in utero, including when switching occurs during gestation.

The results from this malaria-endemic population are different from those reported from developed countries. In western countries, CB cells produce more IgM than IgG compared with adults B cells (5). The high prevalence of malaria-specific IgG produced by the CBMC of Cameroonian newborns suggests that malarial Ags commonly cross the placental barrier inducing T cell-dependent Ab responses and that plasma cells circulating in CB are similar to the newly produced B cells found in the circulation of individuals following primary exposure to Ag.

Previous studies on in utero priming have focused on memory T cell responses to asexual stage Ags. These studies have measured cytokine responses of CBMC using ELISPOT (12, 24), intracellular staining (13, 25, 36), or in culture supernatants following stimulation with mitogen, MA extract, MSP1, and RESA (13, 15, 25). All of the studies concluded that malaria-specific T cell priming occurs in utero. Results from the current study for 12 cytokines support and extend the previous results. The most impressive finding was that MA induced cytokines associated with innate immunity, namely GM-CSF, IL-1β, IL-6, and/or TNF-α, in essentially all CBMC cultures (Fig. 4). The schizont extract contains molecules that are known to stimulate innate cells via TLRs. For example, hemozoin pigment activates monocytes through TLR-9 to produce IL-1β, IL-6, and TNF-α (37, 38), and parasite GPI can induce high levels of TNF-α, IL-1β, and IL-6 in vitro through the activation of TLR-2 and TLR4 (37, 39, 40). The ability of innate immune cells to produce inflammatory cytokines depends on their maturity (41). CB innate cells are generally considered to be immature (42). However, MC from neonates exposed in utero to *Trypanosoma cruzi* Ags from their infected mothers produced higher levels of proinflammatory IL-1β, IL-6, and TNF-α compared with MC from unexposed neonates (43, 44). Thus, maternal infection may not only modulate the fetal acquired immune response but also up-regulate the capacity of fetal mononuclear cells to produce proinflammatory cytokines.

CBMC also produced a variety of Th1 and Th2-type cytokines upon stimulation. When stimulated with PHA, Cameroonian CBMC produced levels of IL-2 and IL-12 similar to those of maternal PBMC, higher amounts of IL-10 and IL-13, but significantly...
lower amounts of IL-4, IL-5, and IFN-γ (Table I). Most cultures were positive for IL-10 (89% of controls) and IL-13 (87%) when stimulated with the MA extract. These results support previous findings of low IL-4 and IFN-γ and high IL-10 responses of CB cells to malaria in infants living in developing countries (14, 15).

Newborns in Yaoundé not only demonstrated IgG responses to MSP-1 but also T cells responses to a pool of conserved peptides (Figs 1 and 3). Nine percent of newborns compared with 18% of their mothers had positive T cell proliferative responses and Th1/Th2-type cytokines were detected in 11% of CBMC cultures. King et al. (12) also evaluated cytokine production by CBMC from Kenyan newborns using two of the MSP-1 peptides (P2 and P3) used in this study (12). They reported that two of 12 (16.7%) samples of CBMC produced IFN-γ and two of 12 (16.7%) produced IL-13 following stimulation in vitro, with IFN-γ being detected in two additional cultures following enrichment for CD4+ T cells. We detected IFN-γ in three of 45 (6.7%) and IL-10 in 2 of 45 (4.4%), but not IL-13, in Cameroonian CBMC cultures. Thus, results from these studies show that the fetus has T cells as well as primed B cells to MSP-1.

The number of babies who are born with malaria-specific T cells resulting from exposure to P. falciparum Ags in utero is difficult to determine, because women in developing countries are often infected with multiple pathogens during pregnancy. Thus, some of the T cell responses to MA could have been produced by cells exposed to cross-reactive viral, bacterial, and/or parasitic Ags in utero. That is, positive T cell responses to MA could have resulted from exposure to other pathogens. However, infants with Ag-specific memory T/B cells should still be able to mount a secondary response upon primary P. falciparum infection after birth.

In summary, this study shows that many infants living in Yaoundé, where transmission of malaria is low, are born with B cells capable of secreting IgM and IgG Abs to at least three different asexual stage Ags, i.e., AMA-1, MSP1, and RESA, but not to the sporozoite Ag CSP. Today it is generally accepted that the differentiation of conserved T- and B-cell epitopes in Plasmodium falciparum inactivated sporozoite-infected women and high concentrations of soluble E-selectin and a circulating Plasmodium falciparum protein in the cord sera. Immunology 93: 264–269.


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