Impact of Intermittent Preventive Treatment with Sulfadoxine-Pyrimethamine on Antibody Responses to Erythrocytic-Stage Plasmodium falciparum Antigens in Infants in Mozambique

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We evaluated the impact of intermittent preventive treatment in infants (IPTi) with sulfadoxine-pyrimethamine (SP), which was given at ages 3, 4, and 9 months through the Expanded Program on Immunization (EPI), on the development of antibody responses to Plasmodium falciparum in Mozambique. Immunoglobulin M (IgM) and IgG subclass antibodies specific to whole asexual parasites and to recombinant MSP-119, AMA-1, and EBA-175 were measured at ages 5, 9, 12, and 24 months for 302 children by immunofluorescence antibody tests and by enzyme-linked immunosorbent assays. Antibody responses did not significantly differ between children receiving IPTi with SP and those receiving a placebo at any time point measured, with the exception of the responses of IgG and IgG1 to AMA-1 and/or MSP-119, which were significantly higher in the SP-treated group than in the placebo group at ages 5, 9, and/or 24 months. IPTi with SP given through the EPI reduces the frequency of malarial illness while allowing the development of naturally acquired antibody responses to P. falciparum antigens.

Malaria remains one of the major infectious diseases globally, causing up to 3 million deaths and close to 5 billion episodes of clinical illness per year (7). In areas characterized by hyperendemic transmission, the greatest burden of malaria occurs in children less than 12 months of age (38); consequently, infants in sub-Saharan Africa are the main target population for any malaria control tool.

Intermittent preventive treatment in infants (IPTi) that consists of the administration of a full dose of an antimalarial within the Expanded Program on Immunization (EPI) has proven to reduce the risk of malaria in this vulnerable group (37). This strategy has gained increasing interest, and several intervention trials evaluating the efficacy of IPTi in the reduction of malaria morbidity have been and are still being carried out in several sub-Saharan countries (Tanzania, Ghana, Senegal, Mozambique, Gabon, and Kenya) as part of an international consortium (www.ipti-malaria.org). However, before setting any policy recommendation for the large-scale implementation of IPTi for malaria control, it is necessary to fully evaluate the consequences that this early preventive intervention may have later in life.

An important issue that needs to be considered is the impact that IPTi may have on the development of naturally acquired immunity to malaria. Early studies of continuous malaria chemoprophylaxis raised concerns regarding the loss of or delay in the acquisition of protective immunity (16, 23, 34). Weekly chemoprophylaxis between 2 and 11 months of age in infants in Tanzania significantly reduced the incidence of malaria and anemia during the first year of life, but the risk increased in the second year after stopping the intervention (26), suggesting that protection against Plasmodium falciparum infection during infancy had delayed the development of immunity to malaria. However, subsequent studies of IPTi in Tanzania (37) and Mozambique (24) showed that, as opposed to continuous chemoprophylaxis, intermittent prevention reduced the risk of malaria without being followed by a clinical rebound once the intervention was stopped. Furthermore, IPTi resulted in a sustained protective effect during the second year of life after the cessation of treatment (36), suggesting that the intervention had unanticipated beneficial effects in the acquisition of immunity.

Nevertheless, studies completed so far have been limited to the evaluation of the safety and efficacy of IPTi without the parallel assessment of immune responses to P. falciparum. Therefore, in the context of a randomized, placebo-controlled trial of IPTi in Mozambique that resulted in a 22.2% (95% confidence interval [CI], 3.7 to 37.0%; P = 0.020) reduction in the incidence of clinical malaria in the first year of life (24), we evaluated whether IPTi with sulfadoxine-pyrimethamine (SP), administered alongside the EPI system, could affect the qualitative and/or quantitative immune responses to malaria antigens. As surrogates of protective immunity, we measured the type and quality of antibodies to the blood-stage antigens mero-
zoite surface protein (MSP-1) (27), apical membrane antigen (AMA-1) (32), and erythrocyte binding antigen (EBA-175) (8, 40). These antigens play critical roles in erythrocyte invasion and are leading vaccine candidates (9). Antibodies raised against these parasite proteins inhibit the invasion of erythrocytes in vitro (11, 27, 30). Immunoglobulin G (IgG) antibodies are important in protection against blood-stage malaria infection, as demonstrated by the classical Ig passive-transfer studies (5, 10), and their protective effect has been attributed to the cytophilic (IgG1 and IgG3) rather than the noncytophilic (IgG2 and IgG4) subclasses (28). We hypothesized that the levels of P. falciparum-specific antibodies during the first 2 years of life would not significantly differ between children who received IPTi with SP and those who received a placebo.

MATERIALS AND METHODS

Study area and design. The study was conducted at the Centro de Investigação em Saúde da Manbiça, Manbiça District, southern Mozambique. The characteristics of the area have been described in detail elsewhere (21). The most recent data on the efficacy of SP in children in this area showed a combined (early and late) therapeutic efficacy rate of 83%, with an in vivo parasitological sensitivity of 78.6% at day 14 (1). The efficacy study was an individually randomized, placebo-controlled trial (24). Infants were recruited from those attending the EPI clinic to receive dose 2 of the diphtheria/acellular polio/hepatitis B vaccine between September 2002 and February 2004. Treatment with SP or a placebo was administered at 3, 4, and 9 months of age alongside the routine vaccinations. Cross-sectional visits were scheduled at 5, 9, 12, and 24 months of age. For the immunological studies of IPTi, we included the last 501 children recruited in the main trial. Among those, 302 were selected for the analysis of antibody responses, because they fulfilled the following criteria: (i) having received all three doses of SP or placebo, (ii) having plasma available to conduct all the serological determinations, and (iii) having an equal representation of SP and placebo recipients. Those children who came for all four visits were prioritized. At each cross-sectional visit, 1 ml of blood was collected by finger prick into EDTA microtainers to obtain plasma and an erythrocyte pellet. All immunological assays were performed by personnel in a blind manner. Clinical surveillance for malaria morbidity was done through passive case detection. Ethical approval for the protocol was obtained from the ethics review committees of Mozambique and the Hospital Clinic, Barcelona, Spain. The trial registration number is NCT00209795 (http://clinicaltrials.gov).

IFAT. In vitro cultures of P. falciparum (strain 3D7) were used at 3.5% parasitemia and 3 to 5% hematocrit to prepare immunofluorescence antibody test (IFAT) slides. Infected erythrocytes were resuspended in phosphate-buffered saline (PBS), and droplets of 25-μl cell suspensions were loaded onto 12-well Nunc microscope slides (Nunc-Immuno,4 Roskilde, Denmark) and fixed with 100% acetone. Twenty-five microliters of test plasma (twofold serial dilutions from 1/200 to 1/163,840) was placed in each well and incubated with the parasites for 30 min. Positive and negative control plasma pools were used in each slide. After the samples were washed, 15 μl of fluorescein isothiocyanatelabeled anti-human IgG antibody (1:120) in Evans Blue solution (0.01% [w/vol] in PBS) (Sigma, St. Louis, MO) was loaded in each well for 30 min. To stain parasite DNA, slides were incubated with a drop of 4',6-diamidino-2-phenylindole–PBS (300 mM) for 1 to 5 min and rinsed with PBS before being mounted with glycerol and covered with a coverslip. Antibody binding and DNA staining were assessed by fluorescence microscopy. The highest dilution giving positive green fluorescence was scored. Data are presented as endpoint IgG titers, i.e., the reciprocal of the last plasma dilution causing positive fluorescence above the negative-control level.

ELISA. For enzyme-linked immunosorbent assays (ELISA), all samples were assayed for IgG and IgM to the recombinant proteins MSP-1 (19-kDa C-terminal fragment, 3D7 strain), AMA-1 (3D7), and EBA-175 (region II, fragment II, CAMP strain) from the ICGEB (New Delhi, India). High-binding 96-well microplates (Nunc Maxisorp, Denmark) were coated with 200 ng per well of antigen diluted in 0.05 M carbonate-bicarbonate buffer and incubated overnight at 4°C. Plates were washed with 0.05% Tween 20 in PBS (PBS-Tween 4%) blocked with 2% bovine serum albumin in PBS-Tween for 8 h at 4°C, and washed with PBS-Tween, and then plasma samples (1:200) were added in duplicate along with positive (a pool from eight adults with lifelong exposure to malaria) and negative (nine nonexposed adults)-control plasma samples. Plates were incubated overnight at 4°C and washed, and peroxidase-conjugated goat anti-human IgG or IgM secondary antibodies (Sigma, St. Louis, MO) were added at 1:3,000 and 1:2,000 dilutions, respectively. After 1 h of incubation and washing, 100 μl of a phosphate solution with 0.012% H2O2 substrate and p-phenylenediamine chromogen was added per well for 5 min, and the colorimetric reaction was stopped with 25 μl of 3 M H2SO4. The specific reactivities of plasma samples were obtained as optical density (OD) values (absorbance measured at 492 nm using a Multiskan EX; Labsystems, Finland), normalized against a positive control (1:200) run in the same experiment, and used as continuous variables (in arbitrary units and percentages) or converted to a categorical variable (positive versus negative) using a cutoff OD value (the arithmetic mean of negative controls plus three standard deviations) for statistical analyses. IgG isotypes were analyzed in the samples in which a positive IgG response was detected for the corresponding antigen. Identical sets of antigen-coated plates were prepared for the determination of IgG1, IgG2, IgG3, or IgG4 in assays performed with any one plasma sample (1:200, duplicates) in parallel on the same day. Wells were incubated for 3 h with peroxidase-conjugated sheep anti-human IgG1 (1:6,000), IgG2 (1:3,000), IgG3 (1:6,500), or IgG4 (1:5,000) (Binding Site, Birmingham, United Kingdom) or peroxidase-conjugated rabbit anti-human IgG specific for gamma chains (1:8,000; DAKO, Glostrup, Denmark). In parallel, purified human myeloma proteins IgG1, IgG2, IgG3, and IgG4 (Binding Site) were coated on plates at twofold dilutions from 2 to 0.001 μg/ml. Peroxidase-conjugated antibodies to each IgG subclass (Binding Site), at the same dilutions as those used for plasma samples, were reacted with the myeloma proteins and used as positive controls. All plates were developed and read as described above. IgG isotype data was reported as the OD at 492 nm (OD492).

Malaria parasitemia. P. falciparum infections were detected by microscopy at 12 and 24 months of age. Thick and thin blood films were stained and read according to quality control procedures (2). Submicroscopic infections were assessed by PCR from erythrocyte pellets collected at all visits (5, 9, 12, and 24 months). Parasite DNA was extracted using the QIAamp 96 DNA blood kit (Qiagen, Venlo, The Netherlands). The amplification of genus- and species-specific P. falciparum DNA was done as described previously (42) in an MJ Research DYAD 96-well thermocycler, and PCR products were visualized on a 1.5% agarose gel in 1× Tris-borate-EDTA buffer.

Definitions and statistical methods. Malaria infection was defined as the presence of asexual P. falciparum parasites of any density in a blood smear. A clinical malaria episode was defined as the latter plus an axillary temperature of ≥37.5°C. Children were not considered to be at risk for 28 days after the start of each episode of clinical malaria.

Antibody values (IFAT IgG endpoint titers, ELISA-normalized OD values [given as percentages] for total IgM and IgG, and raw OD values for IgG isotypes) were logarithmically transformed, and averages within groups are presented as geometric means (GM) plus 95% CIs. Differences in antibody levels between children receiving placebo versus SP were tested with the Wilcoxon rank-sum test for 2 samples and the Kruskal-Wallis test for more than 2 samples. The distributions of antibody responses to each antigen at each time point in SP and placebo recipients were presented as (i) weighted scattered plots, with significance tests performed using linear regression models and adjusted for the t test, and differences in the frequencies of positive responses were estimated with Fisher’s exact test. The distributions of antibody responses to each antigen at each time point in SP and placebo recipients were presented as (i) weighted scattered plots, with significance tests performed using linear regression models and adjusted for previous malaria episodes and present infections, and (ii) reverse cumulative distribution plots (35), with significance tests performed using the Kruskal-Wallis test corrected for continuity.

To correct for the multiple comparisons performed, we used the Monte Carlo permutations test (17), applying 1,000 random permutations; the cases in which Monte Carlo correction altered the significance of P values are indicated in the text. Multivariate regression models using a stepwise procedure were estimated to identify variables independently associated with antibody measures. In a first model that included all children, the variables were intervention group (SP or placebo), age at visit, and the occurrence of previous clinical episodes (yes/no). In a second model, including only children with at least one clinical malaria episode, the variables were intervention group, age at visit, number of previous malaria episodes, age at first episode, parasite density at first and last episode, maximum parasite density before visit, time from first and last episode, and time from the episode of maximum density. 

In all of these analyses, intraindividual and interindividual variabilities were taken into account and adjusted for. Data analysis was performed using Stata 9.2 (Stata Corporation, College Station, TX). Statistical significance was defined as a P of <0.05.
RESULTS

Crude analysis of antibody responses to *P. falciparum* blood-stage antigens in relation to IPTi with SP. We found no significant differences in IgG IFAT titers (given in parentheses as GM [95% CI]) between the SP and the placebo groups at any of the following time points: 5 months, SP (11,192 [8,946 to 14,000]) and placebo (11,880 [9,608 to 14,689]), \( P = 0.702; 9 \) months, SP (10,364 [8,101 to 13,259]) and placebo (10,240 [8,153 to 12,861]), \( P = 0.943; 12 \) months, SP (9,132 [6,922 to 12,048]) and placebo (11,880 [9,608 to 14,689]), \( P = 0.612; 24 \) months, SP (4,45 [286 to 692]) and placebo (736 [450 to 1,204]), \( P = 0.131.\)

Similarly, crude IgG responses to *P. falciparum* merozoite antigens MSP-1\(_{19}\), AMA-1, and EBA-175, measured by ELISA, did not significantly differ overall between children receiving IPTi with SP or those receiving a placebo at any of the cross-sectional visits, when analyzed either as continuous or as discontinuous variables (Table 1). There was one exception: IgG responses to AMA-1 at age 5 months (after two IPTi doses at 3 and 4 months), which were significantly higher in children who received SP than those who received the placebo, as determined by reverse distribution cumulative plots (\( P = 0.032; \) data not shown) and by \( t \) test of the GM (\( P = 0.050 \) in Table 1; the corrected \( P \) value by Monte Carlo permutations test was 0.056). Correction for multiple tests slightly altered the significance of only five comparisons, in which \( P \) values already were borderline significant; these are indicated in the text. For all other cases, permutations did not significantly change the \( P \) values and, thus, the correction is not reported. IgM responses to the same antigens did not significantly differ between the two treatment groups (data not shown).

Likewise, IgG subclass responses to MSP-1\(_{19}\), AMA-1, or EBA-175 did not significantly differ between SP and placebo recipients for most of the cross-sectional visits when analyzed either as continuous (Table 2) or as categorical (data not shown) variables. There were, however, three exceptions in which the levels of cytophilic IgG1 antibodies were significantly higher in the SP than in the placebo group, as determined by reverse distribution cumulative plots (data not shown): at 5 months the \( P \) value for AMA-1 was 0.035 and the \( P \) value for EBA-175 was 0.050 (corrected \( P = 0.060 \)), and at 9 months the \( P \) value for MSP-1\(_{19}\) was 0.033.

Factors affecting IgG antibody responses after IPTi with SP-adjusted analysis. The occurrence of previous clinical malaria episodes was strongly associated with IgG levels. The number of children who had previous clinical episodes was 18 at 5 months (3 SP, 15 placebo), 54 at 9 months (25 SP, 29 placebo), 60 at 12 months (26 SP, 34 placebo), and 82 at 24 months (34 SP, 48 placebo). Children with a previous clinical malaria episode had, on average, 2.01 times higher IgG responses to MSP-1\(_{19}\) than children without a previous episode (CI, 1.72 to 2.34; \( P < 0.001 \)). Comparable associations were found for AMA-1 and EBA-175. Similarly, children with current parasitemia (36 children at 5 months, 62 at 9 months, 37 at 12 months, and 78 at 24 months), detected by microscopy and PCR, had 1.34 times higher IgG responses to MSP-1\(_{19}\) than did children without current parasitemia (CI, 1.17 to 1.54; \( P < 0.001 \)). Present infections also were significantly associated with IgM levels (\( P = 0.001 \) for MSP-1\(_{19}\), \( P < 0.001 \) for AMA-1, and \( P = 0.055 \) for EBA-175).

Adjusted IgG responses to whole parasites (data not shown), MSP-1\(_{19}\), AMA-1, and EBA-175 (Fig. 1 and 2) generally did not differ between SP and placebo groups, except for a few cases in which IgG levels were significantly higher in the SP group than the placebo group, namely, with the (i) IgG response to MSP-1\(_{19}\) at 24 months, adjusting for previous clinical malaria episodes (\( P = 0.041 \)) and present malaria parasitemia (\( P = 0.053 \)); (ii) IgG and IgG1 responses to AMA-1 at 5 months, adjusting for previous clinical episodes (\( P = 0.012 \) and \( P = 0.004 \), respectively) and present parasitemia (\( P = 0.049 \)).

### Table 1. IgG responses to erythrocytic-stage antigens after IPTi with SP

| Age\(^a\) | Antigen | SP group | Placebo group | \( P \) value\(^c\) | \( P \) value\(^c\) |
|-------|---------|---------|---------------|----------------|-----------------|
|       |         | GM\(^b\) | 95% CI        | \( n \) | % Pos | GM\(^b\) | 95% CI | \( n \) | % Pos |                      |                      |
| 5\(^f\) | MSP-1\(_{19}\) | 11.19 | 9.70–12.90 | 30 | 20 | 12.13 | 10.18–14.45 | 43 | 30 | 0.479 | 0.079 |
|        | AMA-1   | 32.49 | 27.89–37.86 | 93 | 63 | 26.12 | 22.32–30.55 | 86 | 61 | 0.050 | 0.809 |
|        | EBA-175 | 19.07 | 17.16–21.19 | 35 | 24 | 17.57 | 15.53–19.89 | 36 | 25 | 0.321 | 0.892 |
| 9\(^g\) | MSP-1\(_{19}\) | 13.74 | 11.45–16.50 | 43 | 29 | 12.41 | 10.38–14.83 | 45 | 31 | 0.429 | 0.703 |
|        | AMA-1   | 18.87 | 16.34–21.80 | 62 | 42 | 17.52 | 15.07–20.35 | 54 | 38 | 0.480 | 0.473 |
|        | EBA-175 | 17.05 | 15.29–19.01 | 26 | 18 | 17.21 | 15.27–19.40 | 31 | 22 | 0.907 | 0.460 |
| 12\(^h\) | MSP-1\(_{19}\) | 12.26 | 10.44–14.40 | 35 | 24 | 12.66 | 10.65–15.04 | 42 | 29 | 0.789 | 0.355 |
|        | AMA-1   | 15.32 | 13.36–17.56 | 43 | 29 | 15.42 | 13.29–17.88 | 51 | 35 | 0.950 | 0.318 |
|        | EBA-175 | 17.61 | 15.88–19.52 | 29 | 20 | 17.42 | 15.54–19.53 | 29 | 20 | 0.891 | 1.000 |
| 24\(^i\) | MSP-1\(_{19}\) | 16.93 | 14.04–20.41 | 25 | 21 | 13.64 | 11.71–16.36 | 27 | 24 | 0.114 | 0.753 |
|        | AMA-1   | 21.39 | 18.76–24.39 | 27 | 23 | 23.90 | 20.88–27.35 | 37 | 32 | 0.244 | 0.109 |
|        | EBA-175 | 18.33 | 16.48–20.38 | 20 | 17 | 18.67 | 16.94–20.58 | 20 | 18 | 0.798 | 1.000 |

\(^a\) Age in months.

\(^b\) GM of ELISA antibody levels, expressed as arbitrary units of OD normalized against results for the positive control (in percentages).

\(^c\) \( n \) and frequency (% of positive responders. Average GM values for negative controls were the following: AMA-1, 15.96%; MSP-1\(_{19}\), 15.17%; and EBA-175, 21.75%.

\(^d\) Determined by Fisher’s exact test for the comparison of the frequency of positive antibody responses.

\(^e\) Determined by \( t \) test for the comparison of the magnitude of antibody responses.

\(^f\) Number of samples at 5 months: SP (\( n = 148 \)) and placebo (\( n = 145 \)).

\(^g\) Number of samples at 9 months: SP (\( n = 147 \)) and placebo (\( n = 143 \)).

\(^h\) Number of samples at 12 months: SP (\( n = 146 \)) and placebo (\( n = 145 \)).

\(^i\) Number of samples at 24 months: SP (\( n = 118 \)) and placebo (\( n = 114 \)).
We next stratified children into those with and those without previous malaria/present infection and compared their IgG levels by a reverse cumulative distribution function. In this subgroup analysis, children with malaria exposure had significantly higher antibody responses after IPTi with SP for some antigens and at some time points. In particular, in children who [corrected $P = 0.056$], and $P = 0.045$ [corrected $P = 0.054$], respectively) (data not shown for IgG1), consistently with the initial crude analysis; (iii) IgG1 response to MSP-1$_{19}$ at 9 months, adjusting for the presence of parasitemia ($P = 0.013$); and (iv) IgG1 response to EBA-175 at 5 months, adjusted for previous clinical malaria episodes ($P = 0.038$).
have had previous clinical malaria episodes, IgG IFAT titers were significantly higher in the SP than in the placebo group at the age of 9 months ($P = 0.018$; data not shown), and ELISA antibody levels also were significantly higher in the SP than in the placebo group for MSP-1 at 9 (IgG, $P = 0.001$ [Fig. 3A]; IgG1, $P = 0.024$ [data not shown]) and 24 (IgG, $P = 0.022$; Fig. 3A) months of age and for AMA-1 at 9 months (IgG, $P = 0.010$; Fig. 3B). In children with present infection, the levels of IgG1 response to MSP-1 were significantly higher in the SP than in the placebo group at 9 months ($P = 0.011$; data not shown). For children with no documented previous clinical episodes, significant differences were observed only at 5 months, when only a few malaria episodes had occurred due to their young age, particularly in the SP group. At that time, the levels of the IgG and IgG1 responses to AMA-1 were higher in the SP than in the placebo group ($P = 0.010$ [Fig. 3B]; $P = 0.006$ [data not shown]), as found in the initial crude analysis. Similarly, the levels of the IgG1 response to EBA-175 were higher in the SP than in the placebo group at age 5 months ($P = 0.014$; corrected $P = 0.056$; Fig. 3C), also consistent with the above analyses.

FIG. 1. IgG responses to MSP-1 (PfMSP-1) (A), AMA-1 (PfAMA-1) (B), and EBA-175 (PfEBA-175) (C) in Mozambican infants receiving IPTi with SP or the placebo, adjusted for previous clinical malaria episodes (left) or present malaria infection (right) by linear regression analysis. IgG levels (y axes) are expressed as normalized OD values (as percentages). In the weighted scatter plots, the area of the symbol is proportional to the number of observations. Geometric (Geom) mean IgG levels and 95% confidence (Conf) intervals are indicated by horizontal red and blue lines. Red symbols correspond to IgG levels in children with previous/present infection.
To investigate why IPTi with SP would result in enhanced antibody levels to some antigens in children with previous \textit{P. falciparum} exposure, we used a multilevel regression model that included only children with at least one clinical episode of malaria and looked at whether intervention group status, age, number of previous episodes, time from past episodes, parasite density, and infection status were independently associated with IgG levels. On average, the levels of IgG in response to MSP-1\(_{19}\) were significantly higher in the SP than in the placebo group: according to the estimated models, they were 0.55 times higher (CI, 0.40 to 0.74; \(P < 0.001\)) after being adjusted for all of the other variables and taking into account repetitive measures. In addition, in these same children, the levels of IgG in response to MSP-1\(_{19}\) in parasitemic children were 1.65 times higher than in those who were aperasitemic (CI, 1.27 to 2.13; \(P < 0.001\)). Figure 3A illustrates that children who received IPTi with SP and who had had previous exposure to malaria have higher anti-MSP-1\(_{19}\) IgG levels than those who received placebo, particularly during the first year. Different ages at first episode or different parasite densities did not seem to explain...
the higher IgG levels after IPTi with SP. No other significant associations in relation to SP treatment were found with the other antigens and antibodies studied.

**DISCUSSION**

This study shows that IPTi with SP administered at 3, 4, and 9 months of age does not modify the levels of antibodies to *P. falciparum* erythrocytic-stage antigens in the first 2 years of life, indicating that this intervention does not negatively affect the development of naturally acquired antibody responses to malaria. These results contrast with those of previous studies of continuous chemoprophylaxis, in which a significant reduction of IFAT malaria antibody responses was observed (4, 6, 18–20, 29, 45). In some cases, the decrease in antibody titers was accompanied by a rebound in clinical and/or parasite prevalence (18, 29). Moreover, we found some evidence indicating that IPTi with SP is associated with higher IgG responses to certain antigens at certain time points. This was the case for IgG and/or IgG1 responses to AMA-1 and EBA-175 at 5 months and for IgG and/or IgG1 to MSP-1 and AMA-1 at 9 and/or 24 months. Furthermore, it appeared that the pattern of responses in the SP and placebo groups was different according to past/present malaria history. These analyses took into account the confounding effect of having had previous clinical episodes of malaria and being parasitemic at the visits; both variables were independently associated with high levels of antibodies to all antigens. Despite the multiple comparisons performed, results were always internally consistent, and in no case was the antibody level superior in placebo recipients than in SP recipients. When analyses were corrected for multiple comparisons, the overall outcome did not vary significantly.

Previous studies by Schellenberg et al. (36) show that subjecting Tanzanian infants to IPTi with SP may result in a sustained protection from malaria during the second year of life after the therapeutic effect of the drug had ceased. This indicates that IPTi facilitates the development of protective immunity to malaria. It has recently been hypothesized that the induction of effective and sustained immunity against malaria by IPTi could be due to the generation of low-dose blood-stage inocula and attenuated infections because of the long serum half-life of SP and its activity against developing hepatic parasite stages (43). It is possible that higher IgG and cytophilic IgG1 responses in SP recipients, particularly to highly immunogenic antigens, are associated with the enhanced acquisition of clinical immunity.

As pointed out by Schofield and Mueller (39), current thinking concerning rebound versus sustained protection hinges on concepts of an immunological nature, but to date there have been no direct measurements of the immunological impact of IPTi. It is possible that the consistently lower levels of IgG in response to AMA-1 at 5 months in placebo recipients is explained by the higher clearance of antibodies, probably of maternal origin, due to the higher incidence of infection. In addition, the significant differences in IgG levels between SP and placebo recipients at 5, 9, and 24 months of age may be related to immunological processes resulting from SP administration. We speculate that subtherapeutic drug concentrations due to partial SP resistance could have attenuated parasites in vivo, resulting in subpatent infections of very low densities between 3 and 5 months. Low-antigenic-dose stimulation in SP recipients may have more adequately primed the immune system, resulting in the induction of cytophilic IgG1 antibodies at higher titers later on. Conversely, high-density parasitemia and clinical malaria episodes between 3 and 5 months in placebo recipients may result in immune suppression and a less efficient mounting of antibody responses. This interpretation is consistent with a study from Kenya showing that children with a low intensity of malaria exposure during the first 2 years of life had higher subsequent IgG responses to MSP-1(19) (41). A trial of insecticide-treated bed nets in the same area also found a significantly higher prevalence of IgG responses to MSP-1(19) in children from bed net areas, concluding that in areas of intense malaria transmission, interventions that reduce the number of asexual parasitic episodes do not delay the development of antibody responses to blood-stage malarial antigens (22).

The importance of low doses of parasite antigen in the induction of protective immunity also is supported by other studies of animal models and humans. Subpatent infections with blood-stage *Plasmodium chabaudi* or *Plasmodium yoelii* rodent malaria stimulate a good level of immunity, which differed from that induced by patent infection (3, 14, 15, 44). In humans, subpatent infections with a very low inoculum of *P. falciparum* induce protective immunity (33). Low-level exposure related to subtherapeutic drug concentrations of antimalarials due to moderate resistance also has been shown to have important effects on immune responses (25). Higher titers of IgG responses to MSP-1(19) enhanced the likelihood of parasitological clearance in individuals treated with a suboptimal drug regimen, and it was suggested that recovery from uncomplicated malaria in patients carrying drug-resistant *P. falciparum* is a phenotypic marker of acquired functional immunity (12, 13, 31).

In addition to parasite density, the age at which the infant’s immune system first encounters an infection also may play an important role in determining the magnitude and quality of subsequent immune responses. It is probable that infants who receive SP have their first clinical episode 2 months later, on average, than those who receive the placebo due to the protection granted by the treatment. However, our study was not

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**FIG. 3.** IgG responses to MSP-1(19) (PMSP-1) (A) and AMA-1 (PIAMA-1) (B) and IgG1 responses to EBA-175 (PIEBA-175) (C) in Mozambican infants receiving IPTi with SP (red) or the placebo (blue), stratified by those with previous clinical malaria episodes (discontinuous lines) and those without (continuous lines) and illustrated as reverse cumulative distribution functions. IgG levels (x axes) are expressed as normalized OD values (as percentages) (A and B), and IgG1 levels (y axis) are expressed as OD values (C). The y axes represent the proportion of children from each group that has a given OD value or higher. Statistical significance between the two treatment groups was analyzed by a Kruskal-Wallis test adjusted for ties. P values shown correspond to the comparison between SP and placebo groups of children with previous episodes (right) and without previous episodes (left).
designed or powered to test this hypothesis, and further studies are now under way in Manhiça that will help clarify this possibility.

Finally, currently we are investigating whether IPTi with SP also has an effect on other types of antibody responses that are thought to be involved in the acquisition of protective immunity to malaria, such as IgG to P. falciparum variant surface antigens and functional growth-inhibitory antibodies.

In conclusion, IPTi with SP is safe and protects children against clinical malaria without a rebound and without negatively affecting the development of P. falciparum-specific antibody responses to blood-stage antigens considered targets of immunity to malaria. Furthermore, IPTi with a partially effective drug such as SP may have unanticipated benefits by allowing immune priming with lower parasite densities at earlier ages, which may result in higher levels of cytophilic IgG subclass responses to some antigens, which may contribute to the acquisition of protective immunity. It remains to be established whether IPTi with more efficacious drug combinations will have a similar impact on the development of naturally acquired immune responses.

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