HIGH INCIDENCE OF ASYMPTOMATIC MALARA INFECTIONS IN A BIRTH COHORT OF CHILDREN LESS THAN ONE YEAR OF AGE IN GHANA, DETECTED BY MULTICOPY GENE POLYMERASE CHAIN REACTION

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Abstract. The incidence of Plasmodium falciparum infection has been followed in a birth cohort of 71 infants in southern Ghana, an area of perennial malaria transmission. Parasite DNA detection established the presence of a high rate of infection in newborns (13.6%), a low level of infection from two to 26 weeks (1.5–9.7%) and a steadily increasing parasite rate from 26 weeks of age. The median age to first infection was 42 weeks. Five cases of fever (temperature ≥ 37.5°C) and parasite density greater than 1,000 parasites/μl of blood, all in children more than 18 weeks of age, were considered possible cases of clinical malaria. The risk of infection was almost three times higher in the wet season than in the dry season and increased significantly from the age of 18 weeks. The level of malaria-specific IgG at birth was positively correlated with risk of infection in children 6–12 months of age, indicating that maternally derived anti-malarial IgG is correlated with exposure to malaria infection. There was no association between malaria-specific IgG at birth and risk of infection in children 0–6 months of age. However, infants do appear to possess mechanisms to limit parasite growth and a role for maternal antibody cannot be ruled out.

In regions of endemic malaria transmission, children are continually exposed to malaria infection; it is believed that repeated exposure is required for the acquisition of protective immunity. However, epidemiologic studies have consistently found that children less than six months of age experience few or no episodes of clinical malaria. Parasite prevalence is lower in the first year of life than in subsequent years and is especially low in children less than 3–4 months old.

There are several factors that may account for the low incidence of malaria infection in infants and which, by inhibiting parasite growth, prevent the development of high parasitemia and thus protect against clinical disease. These include the presence of fetal hemoglobin (HbF), low intake of dietary p-aminobenzoic acid (PABA) in breast milk, and the transplacental transfer of maternal malaria-specific antibodies. Infants may also be at lower risk of infection per se due to reduced exposure to infective mosquito bites. All of these putative protective mechanisms are likely to become less effective with age; the rates of decrease in HbF and maternal IgG are similar, with both decreasing to negligible levels by 16–18 weeks, making it difficult to separate the effects of one from the other.

The role of passively acquired immunity in the protection of infants from malaria has been debated in meta-analyses of epidemiologic studies of infant malaria in Africa and there are few studies that have looked prospectively for evidence of an association between levels (or specificity) of maternally-derived antibody at birth and subsequent risk of malaria infection or malarial disease. Sehgal and others reported that in Papua New Guinea, parasite prevalence and the incidence of clinical malaria were lower in children who retained maternal IgG than in those who had completely lost it, but the sample size was small and the data could not be corrected for the effect of confounding factors such as age and season. In El Salvador, Campbell and others reported that the incidence of parasitemia was lower in antibody-positive infants than in antibody-negative infants but, again, the sample size was too small for detailed statistical analysis.

The aim of the current study was to determine the pattern of malaria infection and clinical malaria in children less than one year of age and to begin to investigate the protective efficacy of maternally derived antibodies against malaria infection in a prospective, longitudinal study of a birth cohort of 71 Ghanaian infants. We have used a highly sensitive polymerase chain reaction (PCR) technique to determine the prevalence of malaria parasites in blood samples collected at 2–4-week intervals over the first year of life. We have compared these results with parasite detection by microscopy and have related parasitologic data to clinical data to determine the relationship between malaria infection and disease in infants. Finally, we have measured maternally derived antimalarial antibodies by ELISA and related IgG levels at birth to subsequent risk of malaria infection.

METHODS

Study population. One hundred forty-three mother/child pairs from the village of Prampram, Ghana were recruited between April 1994 and August 1995 and the children were monitored throughout the first year of life; only those with good compliance (at least 13 of 17 possible clinic visits) were included in this analysis (n = 71 infants). Prampram is a small coastal fishing village (population ~7,000), approximately 30 miles east of the capital of Accra. Malaria in the region is predominantly Plasmodium falciparum (91.6%) with P. malariae (8.2%) and P. ovale (0.2%) also recorded. Transmission occurs throughout the year; however, peak transmission occurs after the rainy season in July/August. Entomologic inoculation rate calculations indicate an average 8.5 infective bites per person per year.

Ethical permission for the study was obtained from the Ghanaian Ministry of Health (ref. DMS-083).

Study design. Mothers were recruited into the study in the final trimester of their pregnancy and informed consent...
was obtained to take blood samples from themselves and their newborns. A maternal venous blood sample was collected approximately two weeks prior to delivery of the child. Infant samples were collected by heel prick, and later by venipuncture at birth, two weeks, four weeks, six weeks, and then at four-week intervals until 54 weeks of age; the maximum number of blood samples collected from each mother/child pair was 17 (one mother’s sample and 16 child samples). Thick and thin blood films were stained with Giemsa. Parasite density was determined based on the number of parasites per 300 white blood cells (WBCS). This was converted to parasites/µl on the basis of an average WBC count of 13,000/µl of whole blood in African infants.27 Slides were recorded as negative only after 1,000 WBCS had been counted. Erythrocytes were separated from plasma by centrifugation and both were stored at −20°C. Children were monitored every two weeks, from the age of two weeks, for symptoms of clinical malaria (fever, vomiting, rigors, chills, cough, diarrhea, and ability to feed) and a retrospective questionnaire was completed (maximum number of observations per child = 27). Whenever a fever was detected (axillary temperature ≥ 37.5°C or a history of fever reported by the mother) a blood film was made and examined for the presence of Plasmodium parasites.

Parasite DNA detection. Parasite DNA was extracted from red blood cells following the method of Foley and others.28 The presence of P. falciparum infection was detected by PCR amplifying the 7H8/6 P. falciparum multicopy gene29 using primers kindly donated by Professor Allan Saul (Queensland Institute for Medical Research, Brisbane, Australia). The primer sequences were ACATTATCATAATTGAC(T)CCAGAACT and GTTTCACAATTTCTTTTCTATC. One microliter of extracted DNA was amplified in a 20-µl reaction mixture of 1× PrimeZyme buffer (50mM KCl, 10mM Tris-HCl [pH 8.8], 1.5 mM MgCl2, 0.1% Triton X-100) (Biometra, Kent, United Kingdom), 2.5 mM additional MgCl2, 100 nM of each primer, 75 mM each dinucleotide (dATP, dCTP, dGTP, and dTTP; Boehringer Mannheim, Mannheim, Germany), and 0.5 units of PrimeZyme DNA Polymerase (Biometra) on a Hybaid (Teddington, United Kingdom) thermocycler (35 cycles of 94°C for 30 sec, 55.8°C for 30 sec, and 72°C for 30 sec). The PCR products were subjected to electrophoresis on 2% Tris-borate-EDTA (TBE) agarose gels containing ethidium bromide (2.6 mg/ml of agarose) and gels were examined under UV illumination.

Hemoglobin typing. Hemoglobin variant genes (S and C) were detected using an Acid Haemoglobin Titan Gel Agarose Electrophoresis kit (Helena Laboratories, Tyne and Wear, United Kingdom).

Enzyme-linked immunosorbent assay analysis. The ELISA plates (Inmulon 4; Dynatech, Chantilly, VA) were coated with an optimal dilution (determined by titration) of crude P. falciparum schizont extract (from in vitro cultures of parasite clone 3D730) in carbonate coating buffer (15mM Na2CO3, 35mM NaHCO3, 0.02% NaN3) and incubated a minimum of 24hr at 4°C. The plates were washed six times in phosphate-buffered saline (PBS) and 0.05% Tween 20 (PBST), blocked with 200 µl/well of PBST and 1% low fat milk, incubated for 5 hr at room temperature, and washed as before. The sera had been previously diluted 1:10 with PBST and milk powder to give a final dilution of 1:1,000. Duplicate wells were coated with 100 µl/well of rabbit anti-human IgG conjugated to horseradish peroxidase (HRP) (Dako, High Wycombe, United Kingdom) at a 1:1,000 dilution in PBST. The plates were incubated for 3 hr at room temperature, washed, and finally developed at room temperature (10 min) using 100 µl/well of an o-phenylenediamine (Sigma, Poole, Dorset, United Kingdom) solution. Optical density (OD) was measured at 492 nm. Sera from 21 nonimmune European controls were included in each assay as negative controls. A positive control from pooled immune Gambian sera was also included. A cut-off level for positive sera was defined as an OD > the mean + 2 SD of the OD values for the nonimmune controls.

Statistical procedures. Data processing and statistical analysis were conducted using SPSS for Windows version 6.1 (SPSS, Inc., Chicago, IL), EGRET version 1.0 (SERC, Seattle, Washington), and STATA version 5.0 (Timberlake Consultants, Kent, United Kingdom) software. Infection status was defined as positive if either the PCR or blood film were positive. We estimated the effects of season, age, hemoglobin type, and malaria-specific IgG on the risk of first infection and on the risk of any infection during the year.

Risk of first infection was modeled using a Cox proportional hazards regression model that models the hazard or age-specific incidence of first infection among those in the population not yet infected, and estimates the relative risk for age-dependent covariates (season) and age-constant covariates (hemoglobin genotype, IgG at birth). To determine whether the hazard increased with age, a parametric Weibull regression model31 was fitted. Children who missed two or more visits were considered to be censored at the age prior to the first missing data point (i.e., status unknown after that time point).

For analysis of risk of any infection, a binary response variable (infection or no infection) was defined at each measured time point. Logistic regression with random effects was used to model the relationship between the probability of infection and season, age, hemoglobin type, and IgG at birth. The random effects component in the model accounted for correlation between repeated observations within child. Likelihood ratio tests (LR χ2) were used to test for covariate effects on the risk of infection.

RESULTS

Demographic data and compliance. Dates of birth for the 71 children covered a period of 15 months; births were relatively evenly distributed over this period with 20 births in the first 3 months, and 14, 13, 10, and 14, respectively, in the subsequent three-month periods. There were 41 (57.7%) boys in the cohort. Birth weights were available for 69 children (maximum = 4.2 kg, median = 3 kg, minimum 2.3 = kg, SD = 0.42 kg). Fifty children (70.4%) were of the hemoglobin AA genotype, 16 (22.5%) were AS, and five (7%) were AC. Compliance rates (samples collected/expected) among the 71 children were 89.1% (1,076 of 1,207) for PCR data, 88.6% (1,069 of 1,207) for blood film data, and 87.5% (1,678 of 1,917) for morbidity data.
Sensitivity of parasite detection methods. Serial dilutions of cultured *P. falciparum* parasites were made to test the sensitivity of the 7H8/6 *P. falciparum* gene PCR assay. The lowest dilution of parasite material that could be amplified was equivalent to 1.9 infected erythrocytes/PCR, which is equivalent to 2.02 infected erythrocytes/μl of whole blood (assuming $5 \times 10^6$ erythrocytes/μl of whole blood). Since African children tend to have a lower hematocrit (30–35%) than healthy Europeans (40–45%), the adjusted lower limited of detection of parasites in African children’s blood is approximately 1.5 parasitized erythrocytes/μl of whole blood. This is very similar to levels of parasite detection seen in other sensitive PCR-based parasite detection studies, but has the benefit of only requiring a single, rather than a nested, PCR procedure. We estimate that using our protocol, the lower limit of parasite detection by microscopy is 10–20 parasites/μl of blood, making the PCR technique 10 times more sensitive than microscopy.

There were 1,039 samples with both PCR and blood film data available. One hundred fourteen of these samples were positive by PCR and of these, only 59 (51.8%) were also positive by blood film. The sensitivity of blood film relative to PCR increased with age (Figure 1): the sensitivity up to and including week 14 was 15.8% (three blood film–positive samples of 19 PCR positive samples) compared with 76% (19 of 25) for weeks 50 and 54. The PCR method was also more sensitive than microscopy for detecting maternal infection: of those maternal samples that were tested by both methods, the blood film detected 8.3% (1 of 12) of PCR-positive samples.

Rates of malaria infection. Age-specific parasite prevalence. Including extra samples taken outside the normal sampling schedule, there were 1,098 PCR samples and 1,084 blood films available. The PCR method detects only *P. falciparum* infections. Of 1,084 blood films, two were positive for *P. malariae* and one sample was a mixed *P. falciparum*/*P. malariae* infection. All remaining positive blood films were *P. falciparum* only. Figure 1 shows the estimated prevalence of *P. falciparum* infection by age detected by PCR and blood film. In mothers, 25% (13 of 52) of samples were positive by PCR and 10% (6 of 60) were positive by microscopy. For birth samples (week 0), rates of infection were 13.6% (9 of 66) for PCR and 2.5% (1 of 40) for blood film. From week 2 to week 54 the rates of infection were broadly similar for the two methods, although the PCR consistently gave slightly higher rates. Parasite prevalence was highest at week 54: 28.1% (16 of 57) by PCR and 25% (15 of 60) by blood film.

Seasonal variation in parasite prevalence. Samples collected during the wet season (July to September), during which most transmission occurs, were compared with those collected during the dry season (October to June) by comparing age-specific parasite prevalence (Figure 2). The overall rates of infection by PCR were 15.1% (41 of 272) in the wet season and 9.4% (74 of 785) in the dry season; by microscopy rates were 12.3% (36 of 292) in the wet season and 6.5% (54 of 837) in the dry season.

Risk of first infection. The cumulative number of first infections by age is shown in Figure 3. By age 54 weeks, a first infection (PCR or blood film positive) had occurred in 40 of the 71 children. Of those children not observed to have a first infection, 16 children were observed for the full year and 15 children were censored before 54 weeks. The median time to first infection was 42 weeks (95% confidence interval [CI] = 31, 53 weeks) and there was a 75% probability of remaining free of infection up to age 18 weeks. The effect of age on the risk of first infection among those not yet infected (the hazard) was investigated by fitting a parametric Weibull regression model. This model included season (dry/wet) as an age-dependent covariate that varied independently for each child. The estimated Weibull shape parameter was greater than one (1.29, 95% CI = 0.988, 1.70, $P = 0.07$), providing marginal evidence that the hazard of first infection is increasing with age. A Cox proportional
hazards model showed that the risk of first infection was almost three times higher in the wet season than in the dry season (Table 1). Hemoglobin genotype (AA, AS, or AC) was not significantly related to risk of first infection (LR $\chi^2 = 3.21$, degrees of freedom [df] = 2, $P = 0.20$).

We examined the relationship between malaria-specific IgG at birth (week 0) and the subsequent risk of infection in the child. The IgG antibody response was analyzed on a continuous scale (OD) or as a binary variable (OD above/below the median OD). Neither analysis showed a significant effect of IgG on risk of infection (Table 1) but this analysis lacked statistical power because the age of first infection was known for only 36 of the 60 children for whom IgG data was available.

Risk of any infection. The number of infections per child ranged from zero to nine, with the overall frequencies being: no infections (17 children), one infection (23 children), two (13), three (5), four (3), five (6), six (3), and nine infections (1 child). To determine whether this variation in susceptibility to infection was greater than would be expected by chance, we fitted a logistic regression model with a random effect covariate relating to each child. This confirmed that some children have a significantly higher risk of being infected than others (LR $\chi^2 = 29.0$, df = 1, $P < 0.001$).

After adjusting for season, age had a highly significant effect on the risk of infection (LR $\chi^2 = 60.6$, df = 15, $P < 0.001$). Relative to a baseline at four weeks of age, seasonally adjusted odds ratios (ORs) were significantly higher at birth (OR = 10.8) and from 18 weeks of age onwards (except at 34 weeks) (OR ranged from 5.3 at 18 weeks to 27.8 at 54 weeks).

During the wet season infection was about three times more likely than during the dry season (Table 2). Overall, hemoglobin genotype (AA, AS, or AC) was significantly related to risk of infection (LR $\chi^2 = 6.2$, df = 2, $P = 0.045$). Children with the AS hemoglobin genotype were almost twice as likely to be infected as AA children, but this difference was not significant. However, AS children did have a significantly higher risk of infection than AC children (OR = 6.5, 95% CI = 1.2–34.3, $P < 0.03$).
When IgG at birth was defined as a continuous variable there was no significant association with the risk of any infection (LR $\chi^2 = 2.46$, df = 1, $P = 0.12$). When defined as a binary variable, there was a significant positive association between IgG and the risk of infection (Table 2), i.e., children with higher than average ODs were twice as likely to become infected as children with lower than average ODs; this effect is in addition to the effect of age. However, when the risk of infection is stratified by age, it is clear that the increased risk of infection with increasing IgG at birth is seen only in children 6–12 months of age and not in children less than six months of age (see legend to Table 2).

Positive parasite density. The geometric mean parasite density was determined for 83 positive blood films as 533 parasites/$\mu l$ of blood (range = 43–173,333 parasites/$\mu l$). Average parasite density increased with age but the sample size was low and the trend was not statistically significant (one-way analysis of variance of log parasite density, $F = 0.89$, $P = 0.45$).

Clinical data. Risk of clinical symptoms. Table 3 shows the relationship between the risk of each clinical symptom and age after adjusting for season and parasite density. The risk of cough, diarrhea, and refusing to feed were all significantly higher in children greater than three months of age than in children less than three months of age. The risk of fever, as reported by the mother, was significantly higher at 6–9 months than at 0–3 months.

Table 4 shows the relationship between risk of symptoms and parasite density, after adjusting for the effects of age and season. The risk of fever (either definition), but not other symptoms, was significantly associated with increasing parasite density.

Episodes of clinical malaria. There were 956 observations with both clinical and parasitologic observations recorded. There were 34 episodes of a temperature ≥ 37.5°C and 120 parasite positive blood samples (PCR or blood film). Among these, there were 14 episodes of coincident fever and parasitemia occurring in 11 children between the ages of two

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>RR†</th>
<th>CI for crude RR†</th>
<th>Adjusted RR‡</th>
<th>CI for adjusted RR‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Season (n = 71)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry</td>
<td>1.00</td>
<td>(1.43, 5.40), $P &lt; 0.01$</td>
<td>2.77§</td>
<td>(1.43, 5.35), $P &lt; 0.01$</td>
</tr>
<tr>
<td>Wet</td>
<td>2.78</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hemoglobin type (n = 71)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA (n = 50)</td>
<td>1.00</td>
<td></td>
<td></td>
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<tr>
<td>AS (n = 16)</td>
<td>1.30</td>
<td>(0.64, 2.64), $P = 0.47$</td>
<td>1.28§</td>
<td>(0.63, 2.61), $P = 0.50$</td>
</tr>
<tr>
<td>AC (n = 5)</td>
<td>0.28</td>
<td>(0.04, 2.07), $P = 0.21$</td>
<td>0.27§</td>
<td>(0.04, 2.03), $P = 0.20$</td>
</tr>
<tr>
<td>IgG at birth (n = 60)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OD ≤ median#</td>
<td>1.24</td>
<td>(0.65, 2.41), $P = 0.51$</td>
<td>1.29</td>
<td>(0.66, 2.51), $P = 0.46$</td>
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<tr>
<td>OD &gt; median</td>
<td>3.02</td>
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</tr>
<tr>
<td>OD value</td>
<td>1.56#</td>
<td>(0.75, 3.23), $P = 0.23$</td>
<td>1.61</td>
<td>(0.77, 3.35), $P = 0.20$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Rate of infection</th>
<th>Crude OR*</th>
<th>CI for crude OR†</th>
<th>Adjusted OR‡</th>
<th>CI for adjusted OR‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Season (n = 71)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry</td>
<td>11.1% (82/742)</td>
<td>1.00</td>
<td></td>
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</tr>
<tr>
<td>Wet</td>
<td>18.4% (47/255)</td>
<td>1.82</td>
<td>(1.34, 3.12), $P &lt; 0.001$</td>
<td>2.91§</td>
<td>(1.80, 4.68), $P &lt; 0.001$</td>
</tr>
<tr>
<td>Hemoglobin type (n = 71)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>12.2% (86/705)</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS</td>
<td>17.9% (40/223)</td>
<td>1.57</td>
<td>(0.88, 3.33), $P = 0.11$</td>
<td>1.89§</td>
<td>(0.90, 3.94), $P = 0.09$</td>
</tr>
<tr>
<td>AC</td>
<td>4.3% (3/69)</td>
<td>0.33</td>
<td>(0.07, 1.43), $P = 0.14$</td>
<td>0.29§</td>
<td>(0.06, 1.40), $P = 0.12$</td>
</tr>
<tr>
<td>IgG at birth (n = 60)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OD ≤ median#</td>
<td>10.0% (42/421)</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OD &gt; median</td>
<td>17.5% (74/424)</td>
<td>1.91</td>
<td>(1.06, 3.63), $P = 0.03$</td>
<td>2.09</td>
<td>(1.10, 4.00), $P = 0.02$</td>
</tr>
<tr>
<td>OD value</td>
<td>1.72**</td>
<td>(0.83, 3.54), $P = 0.14$</td>
<td>1.86</td>
<td>(0.86, 4.02), $P = 0.11$</td>
<td></td>
</tr>
</tbody>
</table>

* Odds ratio (OR) based on raw data.
† 95% CI via logistic regression with no other covariates added.
‡ Adjusted ORs do not change significantly when IgG at birth is included as a covariate.
§ Stratifying the data into three-month age groups gave crude ORs: 1.15 (0–3 months); 1.13 (3–6 months); 3.09 (6–9 months); 2.73 (9–12 months). After adjusting for season and hemoglobin type, there was a significant ($P < 0.05$) interaction between IgG and age in their effect on the risk of infection.
# Relative risk for a unit change in OD value.
of 2,773, 4,853, 6,408, 8,573, and 65,260 parasites/µl; five were >1,000 parasites/µl (with densities of 2,773, 4,853, 6,408, 8,573, and 65,260/µl); data were missing for one PCR-positive sample. All cases of fever with parasitemia greater than 100 parasites/µl occurred in children 22 weeks of age or older.

**DISCUSSION**

Infants in malaria-endemic areas are reported to have lower than expected parasite prevalence and few episodes of clinical malaria (reviewed by Brabin7), and it is postulated that this is due to the cumulative effects of the presence of maternal antibodies. However, much of the data is anecdotal and few detailed studies have been performed to assess the relative importance of the different factors. Analysis of multiple data sets from malaria-endemic areas showed that age-specific parasite prevalence rates in infants were not significantly different from rates predicted for nonimmune individuals, assuming a mean duration of parasite infection of 200 days.3,7 These studies concluded that infants born to immune mothers were as susceptible to malaria infection as children born to nonimmune mothers and that maternal antibodies did not protect infants against infection.3,7 However, the prevalence of clinical symptoms of malaria was lower than expected and it was postulated that maternally derived antibody may limit parasite clearance, leading to a shorter mean duration of fever and parasitemia occurring together more often than expected by chance (expected = 4, observed = 14, z = 2.19, P < 0.05) suggesting that there were several cases of expected malaria. Five episodes of fever and parasitemia were associated with parasite densities less than 100 parasites/µl; three were in the range 100–1,000 parasites/µl; five were >1,000 parasites/µl (with densities of 2,773, 4,853, 6,408, 8,573, and 65,260/µl); data were missing for one PCR-positive sample. All cases of fever with parasitemia greater than 100 parasites/µl occurred in children 22 weeks of age or older.

**TABLE 3**

Risk of clinical symptoms: effects of age*

<table>
<thead>
<tr>
<th>Symptom</th>
<th>0–3 months (n = 331)</th>
<th>3–6 months (n = 199)</th>
<th>6–9 months (n = 201)</th>
<th>9–12 months (n = 259)</th>
<th>All (n = 990)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T ≥ 37.5°C</td>
<td>1.8% (6)</td>
<td>4.5% (9)</td>
<td>4.5% (9)</td>
<td>4.6% (12)</td>
<td>3.6% (36)</td>
</tr>
<tr>
<td>Fever (mother)</td>
<td>1.5% (5)</td>
<td>4.5% (9)</td>
<td>5.5% (11)</td>
<td>5.4% (14)</td>
<td>3.9% (39)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>1.8% (6)</td>
<td>3.0% (6)</td>
<td>1.5% (3)</td>
<td>1.9% (5)</td>
<td>2.0% (20)</td>
</tr>
<tr>
<td>Coughing</td>
<td>4.8% (16)</td>
<td>10.6% (21)</td>
<td>10.4% (21)</td>
<td>8.1% (21)</td>
<td>8.0% (79)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>3.0% (10)</td>
<td>8.0% (16)</td>
<td>10.0% (20)</td>
<td>8.9% (23)</td>
<td>7.0% (69)</td>
</tr>
<tr>
<td>Refusing to feed</td>
<td>0.3% (1)</td>
<td>1.0% (2)</td>
<td>3.5% (9)</td>
<td>1.2% (12)</td>
<td><strong>0.00%</strong></td>
</tr>
</tbody>
</table>

* n = total number of observations in age group; T = axillary temperature.
† Odds ratio for symptom to occur relative to the baseline category (age 0–3 months), obtained from a logistic regression model (including random effects) including season (wet, dry) age (4 levels, as above), and parasite density (3 levels) as covariates.
§ Likelihood ratio test for an overall parasite density effect obtained from logistic regression (as above).
² Odds ratio for symptom to occur relative to the baseline category (age 0–3 months), obtained from a logistic regression model (including random effects) including season (wet, dry), age (4 levels, as above), and parasite density (3 levels, as above) as covariates.
³ Odds ratio for symptom to occur relative to the baseline category (no parasites), obtained from a logistic regression model (including random effects) including season (wet, dry) age (4 levels, as above), and parasite density (3 levels) as covariates.
# Likelihood ratio test for an overall age effect obtained from logistic regression (as above).
¶ Fever reported verbally by mother.
** Baseline category taken as 0–6 months.
* n = total number of observations in group; T = axillary temperature.
† Parasite density per microliter.
‡ Odds ratio for symptom to occur relative to the baseline category (no parasites), obtained from a logistic regression model (including random effects) including season (wet, dry) age (4 levels), and parasite density (3 levels, as above) as covariates.
§ 95% confidence interval for odds ratio and P value.
¶ Likelihood ratio test for an overall age effect obtained from logistic regression (as above).
** Baseline category taken as 0–6 months.
³ Odds ratio for symptom to occur relative to the baseline category (no parasites), obtained from a logistic regression model (including random effects) including season (wet, dry) age (4 levels, as above), and parasite density (3 levels, as above) as covariates.
²² Odds ratio comparing parasitemic samples with aparasitemic samples.
²²² Fever reported verbally by mother.

**TABLE 4**

Risk of clinical symptoms: effects of parasite density*

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Not infected (n = 882)</th>
<th>Density &lt; 2,000† (n = 58)</th>
<th>Density ≥ 2,000 (n = 21)</th>
<th>All (n = 961)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T ≥ 37.5°C</td>
<td>2.3% (20)</td>
<td>12.1% (7)</td>
<td>14.3% (3)</td>
<td>3.1% (30)</td>
</tr>
<tr>
<td>Fever (mother)**</td>
<td>2.7% (24)</td>
<td>5.69% (2.21, 14.64)§, P &lt; 0.001¶</td>
<td>7.96% (1.99, 31.81), P &lt; 0.01¶</td>
<td>3.3% (32)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>1.9% (17)</td>
<td>4.60% (1.47, 13.47), P &lt; 0.01¶</td>
<td>5.11% (0.87, 30.05), P = 0.07</td>
<td>9.5% (2)</td>
</tr>
<tr>
<td>Coughing</td>
<td>8.3% (73)</td>
<td>1.84% (0.40, 8.51), P = 0.43</td>
<td>2.50% (0.29, 21.22), P = 0.40</td>
<td>5.2% (3)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>6.3% (56)</td>
<td>0.48%†, (0.13, 1.76), P = 0.27</td>
<td>14.3% (3)</td>
<td>5.2% (3)</td>
</tr>
<tr>
<td>Refusing to feed</td>
<td>1.1% (10)</td>
<td>1.48% (0.54, 4.04), P = 0.45</td>
<td>2.24% (0.54, 9.18), P = 0.26</td>
<td>0.99%†, (0.11, 8.62), P = 0.99</td>
</tr>
</tbody>
</table>

* n = total number of observations in group; T = axillary temperature.
† Parasite density per microliter.
‡ Odds ratio for symptom to occur relative to the baseline category (no parasites), obtained from a logistic regression model (including random effects) including season (wet, dry) age (4 levels), and parasite density (3 levels, as above) as covariates.
§ 95% confidence interval for odds ratio and P value.
¶ Likelihood ratio test for an overall parasite density effect obtained from logistic regression (as above).
** Fever reported verbally by mother.
†† Odds ratio comparing parasitemic samples with aparasitemic samples.
infections, but concluded that detailed longitudinal studies of infants living under conditions of seasonal malaria transmission were required to test either of these hypotheses.

As far as we are aware, this is the first report of a longitudinal cohort study in infants where subpatent malaria infection has been detected by PCR and where the relationship between malaria-specific antibodies measured at birth and the subsequent risk of infection has been assessed. Seventy-one newborns were followed for one year with morbidity surveillance every two weeks and monthly blood samples to determine the incidence of malaria infection and clinical malaria and to relate these findings to levels of maternally derived anti-malarial IgG. Accurate age-specific prevalence curves depend on having a sensitive method of parasite detection. The poor sensitivity of microscopy in detecting very low density malaria infections in infants was alluded to in earlier studies (Metselaar D, 1957. A Pilot Project of Residual Insecticide Spraying in Netherlands New Guinea. Contribution to the Knowledge of Holoendemic Malaria. Ph.D. thesis, University of Leiden, Leiden, The Netherlands), in which spleen rates were found to increase earlier and faster than parasite rates, indicating that a significant proportion of infections were not being detected by microscopy. This problem is particularly acute in infants in whom parasite densities are very low. Using a highly sensitive PCR method, we have detected asymptomatic \textit{P. falciparum} infection in newborns, at two weeks after birth, and in all subsequent four-week periods throughout infancy. The PCR was found to be at least six-fold more sensitive than microscopy in children less than 18 weeks of age. In children greater than 18 weeks of age, the sensitivity of microscopy increased, detecting more than 75\% of the PCR-positive samples. We suspect that this is because the PCR method is detecting very low level infections that would easily be missed by microscopy. In some children, infections detected by PCR but not detected by microscopy persist for several months (Franks S, Riley E, unpublished data), indicating that parasites detected by PCR are viable. These data indicate that very low level malaria infections are common in Ghanaian children less than four months of age and suggest that previous studies may have seriously underestimated the incidence of malaria infection in very young children.

Our data provide considerable support for the theory of McGregor and Brabin: subclinical asymptomatic infections were common but clinical symptoms of malaria were rare and absent in children less than five months of age. In addition, the longitudinal nature of our data shows that most infections are transient; at least 70\% (47 of 67) of the episodes were transient (i.e., the preceding samples and the subsequent samples were both negative). Similar findings have recently been reported from Tanzania where the average duration of infection in infants was 64 days and infections in older infants lasted longer than infections in children less than two months of age.\textsuperscript{36} Thus, infections in infants born to immune mothers last much less than the 200 days assumed to be the duration of infection in nonimmunes individuals.\textsuperscript{3} This supports the hypothesis of rapid parasite clearance in the presence of maternal antibody.

One aim of the study was to evaluate the effect of maternal antibody on the risk of malaria infection. Since only just more than half of the children in this cohort experienced any malaria infections in their first year, the number of documented first infections was too small to draw any firm conclusions about the effects of maternal IgG, or indeed hemoglobin genotype, on the risk of first infection. Analysis of data from a larger cohort of children is underway and may allow us to obtain more precise answers to this question. However, the effect of season was strong enough to be detected, with first infections approximately three times more likely to occur during the wet season than during the dry season.

In contrast, the total number of infections recorded in the cohort was large enough to draw confident conclusions regarding the effect of age, season, and malaria-specific maternal IgG on the risk of any malaria infection. After adjusting for the effect of season, the risk of infection increased significantly with age from the age of 18 weeks onwards. This provides support for the alternative hypothesis of MacDonald\textsuperscript{1} that if passive immunity is protecting the infant from infection \textit{per se}, then the age-specific parasite prevalence curves will become steeper as the effects of maternal antibody wane. Similar increases in parasite prevalence at 16 weeks of age were recorded in Nigerian\textsuperscript{9} and Liberian\textsuperscript{15} infants. However, the period of 16–20 weeks coincides with the time at which both HbF\textsuperscript{29, 34} (Wagner G and others, unpublished data) and maternal IgG (Kramer K and others, unpublished data)\textsuperscript{5, 6, 22–24} decrease to undetectable levels, and it is not possible from this or other studies to disentangle the effects of HbF and IgG on the risk of infection. In any case, an apparent association between a decrease in these parameters and increasing prevalence of infection is not sufficient to infer a causal relationship. The gradual increase in risk over the whole age range from 18 weeks to one year suggests that other factors, such as increased exposure to infected mosquitoes with increasing body size\textsuperscript{31} and changes in diet, may also be important.

There was no association (either positive or negative) between levels of maternally derived anti-malarial IgG at birth and risk of malaria infection within the first six months of life. Thus, our study offers no evidence that maternal antibodies protect against infection. However, the measure of anti-malarial antibody used in this study, IgG to schizont lysate, is a relatively crude parameter and antibodies to such antigens have not previously been shown to be associated with protection. Antibodies to defined merozoite antigens such as merozoite surface antigen 1 (MSP1)\textsuperscript{37, 38} and MSP2\textsuperscript{29, 40} have been shown to correlate with protection; experiments are underway to test these specific antibodies in this cohort. Importantly, however, serologic studies in older children have not shown a link with protection from infection \textit{per se}, but with resistance to clinical malaria. The lack of clinical cases in children less than five months of age is consistent with a role for maternal antibody in protection against clinical disease, but in the absence of an antibody-negative population of children, it is not possible to test this association.

In contrast, the risk of a child becoming infected from six to 12 months of age was positively correlated with levels of malaria-specific maternal IgG at birth. This is consistent with previous studies in infants in whom high levels of maternal antibody to the circumsporozoite protein and to PI155, ring-infected erythrocyte surface antigen were indicative of exposure
to, rather than protection from, malaria infection. Antibody levels in the newborn are correlated with levels in the mother (Kitua AY, 1996. *Incidence of Plasmodium falciparum Infection and Disease Among Infants Living in a Rural Area Under Intense and Perennial Malaria Transmission*. Ph.D thesis, University of Basel, Basel, Switzerland) used cut-off values of parasitemia (21,000-173,000/μl), of which only one was symptomatic. High levels of malaria-specific antibody in the mother may reflect boosting following infection during pregnancy. This would be likely if the mother lives in an area of the village with high transmission, in which case the child would also be at increased risk of infection once innate protection had decreased.

Few previous studies have attempted to obtain precise definitions of clinical malaria for children less than one year of age. It is clear that the threshold of parasite density required to trigger a febrile response changes with age. Preliminary analysis of data from a larger cohort of infants in this study suggests that the threshold is likely to be somewhere between 300 and 1,000 parasites/μl (McGuinness D and others, unpublished data). In a similar age group, in Tanzania, Kitua (Incidence of Plasmodium falciparum Infection and Disease Among Infants Living in a Rural Area Under Intense and Perennial Malaria Transmission. Ph.D thesis, University of Basel, Basel, Switzerland) used cut-off values of 10,000/μl and 20,000/μl, suggesting that thresholds are affected by transmission intensity as well as age. Regardless of the precise definitions of clinical malaria, it is evident that the vast majority of infections were of very low parasite density and were not accompanied by symptoms of ill health. This implies not only that infants possess effective mechanisms for controlling and clearing parasites, but also suggests that infants may be less susceptible to the pyrogenic effects of malaria parasites. For example, eight episodes of parasitemia > 15,000/μl were detected (densities from 21,000 to 173,000/μl), of which only one was symptomatic. Infants are not particularly resistant to fever and will readily develop fever in response to other infections, suggesting that the failure to have a febrile response to malaria parasites may be due to a specific mechanism. It has been proposed that priming of T cells to produce proinflammatory cytokines in response to malaria antigens may increase the production of endogenous pyrogens. If so, then the lack of immunologic priming in infants may explain their failure to become pyrexic and second or subsequent infections might be more likely to be accompanied by disease.

Finally, this study revealed a high incidence of congenital malaria infections (13.6%). This figure is similar to that reported from Papua New Guinea, where 15% of cord blood samples and 8% of neonatal heelprick samples were slide positive, but given the low sensitivity of microscopy compared with the PCR, true rates of congenital infection in Papua New Guinea are probably higher. None of the children with congenital infections were still parasite positive at two weeks of age, indicating that parasites transferred across the placenta at birth are unable to establish themselves in the infant. This further strengthens the argument that innate immune mechanisms control parasite proliferation in neonates.

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