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Transplacentally Transferred Functional Antibodies against *Plasmodium falciparum* Decrease with Age

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1. Introduction

Although malaria is a leading cause of death in children, it rarely causes clinically significant disease in those less than six to eight months of age. A leading hypothesis for this observation is infants are protected by maternal antibodies (Macdonald, 1950; McGuinness et al., 1998). Previous studies have attempted to test this hypothesis by examining cord blood for the presence of malaria-specific antibodies by ELISA. Antibodies detected by this method have not correlated with protection (Ahlborg et al., 2000; Dodoo et al., 2008; Zhou et al., 2002). It has been demonstrated however, that such ELISA assays may not always detect functional antibodies, which can inhibit merozoite invasion or growth.

Antibodies likely protect infants from clinical malaria by multiple mechanisms including the ability to bind to merozoite surface proteins inhibiting erythrocyte invasion and intraerythrocytic maturation (Marsh and Kinyanjui, 2006). Such antibodies would be expected to result in invasion/growth inhibition of blood stage *Plasmodium falciparum* (Pf) parasite in vitro, as measured by a Growth Inhibition Assay (GIA). This assay quantifies antibody-mediated activity against blood stage parasites by measuring parasite growth in the presence of malaria exposed plasma in comparison with non-malaria exposed control plasma and thought to be mediated primarily by IgG (Crompton et al., 2010; Miura et al., 2008). GIA has been used in vaccine studies (Darko et al., 2005; Singh et al., 2003; Singh et al., 2006) (Dicko et al., 2007; Thera et al., 2006; Withers et al., 2006) and found in persons with

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Conflict of interest

The authors declare that have no conflict of interest.

Author's Contributions

AED, CLK, and PTW designed the study. PTW and AED performed the experiments. PTW and AED performed data analysis. PM and IM performed sample collection. PTW wrote the first draft of the manuscript with contributions from all authors.

naturally acquired malaria immunity (Bolad et al., 2003; Dent et al., 2008; Perraut et al., 2005).

This is of importance since transplacental transport is restricted to IgG isotype and the efficiency of transplacental transport varies by subclass with IgG1>IgG4>IgG3>IgG2 (Costa-Carvalho et al., 1996). Thus if functional antibodies are not IgG1 or IgG4 or are primarily IgG3 or IgG2 subclasses, transplacental transfer of putatively protective antibodies may be diminished relative to total. Because many malaria antigens show antigen-specific subclass distribution (Dodoo et al., 2008) antibodies to certain antigens may be underrepresented in transplacental IgG. Other factors important in transplacental antibody transfer are maternal antibody levels and gestational age (Palmeira et al., 2012). Most transplacentally transferred IgG has a half-life of 21 days and is undetectable by six months of age, but the duration of functional activity exerted by these antibodies against malaria is unknown.

We hypothesized that functional antibodies would be demonstrable in cord blood of infants born to women in a malaria endemic area using growth inhibition assays, and that they would wane over time. We investigated this hypothesis by examining functional antibodies in cord blood, and at six and 12 months of life.

2. Materials and Methods

2.1 Study Participants

Healthy pregnant women from the area served by the Msambweni District Hospital on the South Coast of Kenya were recruited from 2005–2007 as part of a larger study (Malhotra et al., 2009) that was approved by the Case Western Reserve University Institutional Review Board and the Kenyan Medical Research Institute/National Ethical Review Committee. At the time of delivery, cord blood was collected and infants were followed every six months of age with a clinical assessment and venous blood draw. Only HIV negative women with term deliveries (37 weeks gestation or later) were included in the study. The average age of mothers in our cohort was 25.5 years with an average parity of 2.3. Any participants with malaria infections were treated according to Kenya Ministry of Health guidelines.

2.2 Treatment of Plasma Samples

All plasma samples were stored at -80°C with minimal freeze/thaw cycles. 300 μl of each plasma sample was dialyzed with two buffer exchanges of sterile PBS and 100,000 molecular-weight-cutoff dialysis tubes (Spectrum Lab, Rancho Dominguez, CA) at 4°C then reconstituted to the original 300 μl starting volume using 100 kilo-Dalton molecular-weight-cutoff centrifugal concentration tubes (Pall Corporation, Ann Arbor, MI) to retain antibodies and remove drugs or other potential factors that could augment or inhibit parasite growth (Sy et al., 1990). Non-malaria exposed negative control cord plasma was obtained from four North American neonates at University Hospitals in Cleveland, Ohio, USA that were then pooled and dialyzed as described above.

2.3 Growth Inhibition Assays

Laboratory strains of Pf (W2Mef, D10, 3D7) were maintained in 10 ml plastic Petri dishes at 4% hematocrit of O + erythrocytes in RPMI-HEPES medium with 0.2 % sodium bicarbonate supplemented with 200 mM hypoxanthine, 200 mM L-glutamine, 10% Albumax, and 50 mg per ml gentamicin (Dent et al., 2008; McCallum et al., 2008; Persson et al., 2006). Parasite strains were cultured at 1% O₂, 5% CO₂, and 95% Nitrogen atmosphere and at 37°C (Beeson et al., 1999). Parasites were synchronized at the ring stage with pre-warmed 5% D-Sorbitol (Sigma, St Louis, MO) two times per week. No genotypic analysis was performed to verify the identity of the different parasites used at different times. 10 µl of test plasma and 40 µl of complete culture media were added to the 96-well flat bottom plates (Falcon; Becton Dickinson, Franklin Lakes, NJ) in duplicate. 50 µl of parasite culture at the mature trophozoite stage (confirmed by microscopy) was added to each well. The final plasma dilution was 1:10. Starting parasitemias were between 0.3–0.8%. Plates were covered, gassed, and incubated at 37°C until one invasion cycle was completed as observed by microscopy of parallel cultures (approximately 24 hours). 25 µl of re-suspended cultures were removed and fixed with 0.25% gluteraldehyde in PBS for 45 minutes and parasitemia measured with a BD LSRII flow cytometer (Becton-Dickinson, Franklin Lakes, NJ)(Dent et al., 2008) using 10× Sybr Green (Invitrogen, Eugene, OR) and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR) by gating on 50,000 red blood cells using forward and side scatter then determining the number of Sybr Green staining ring invaded red blood cells (Bei et al., 2010; Izumiyama et al., 2009; Johnson et al., 2007). Difference in parasite growth between the test plasma and non-malaria exposed control plasma is reported as percent inhibition attributable to functional antibodies.

2.4 Msambweni 2006 field isolate

Blood was obtained from a pediatric patient admitted to Msambweni District Hospital for treatment of severe malaria. Parasitemia was confirmed by microscopy and red blood cells separated using Histopaque (Sigma, St. Louis, MO) density gradient, centrifuged for 30 minutes at 400g and washed three times with RPMI complete media. The red blood cell pellet was frozen in glycerolyte (Diggs et al., 1975), placed at –80°C for 24 hours then transferred to liquid nitrogen. The isolate was shipped to the United States, thawed, established in laboratory culture for approximately two weeks prior to use in the GIA described above.

2.5 Maternal Pf Infection Status

Maternal venous blood, intervillous placental blood, and cord blood were examined for malaria infection status by PCR/ligase detection reaction-fluorescent microsphere assay specific for Pf (Kasehagen et al., 2006).

2.6 Statistical Analysis

Wilcoxon signed-rank test was used to compare differences in GIA levels of cord blood with four different Pf strains and changes in GIA from birth to six months to one year. A linear mixed model with random intercept was used to estimate rate of decline of GIA levels. Spearman's rank coefficient (r_s) was used to measure GIA correlation between parasite

strains. Mann-Whitney U test was used to compare GIA levels between infected and non-infected mothers at time of birth. Analyses were conducted with R-Commander, SAS and GraphPad Prism 4.

3. Results

3.1 GIA are transferred to the fetus and levels vary against different Pf strains

Growth inhibitory antibody responses likely target merozoite surface antigens, proteins involved in invasion and those necessary for intraerythrocytic growth. These pathways vary among Pf strains thus making it important to examine GIA responses to multiple strains. Therefore we examined GIA against strains representing sialic acid dependent (W2Mef) and sialic acid independent (3D7 and D10) erythrocyte invasion pathways as well as strains that vary considerably on their surface proteins, 3D7 and D10. We also examined GIA to a parasite strain isolated from the endemic area referred to as Msambweni 2006. The median (ranges) GIA levels of cord blood were: D10, 0% (0–81); W2mef, 6% (0–80); 3D7; 18% (0–88) inhibition for N= 270 samples (a composite of three different experiments: N=104, N=54, and N=112) and significantly differed among the laboratory lines ($P < 0.001$, Wilcoxon signed-rank test) (Figure 1A). The prevalence of positive responders (≥ 5% inhibition) for D10, W2Mef, and 3D7 was 46%, 55% and 64% respectively. GIA levels correlated between the sialic acid independent and dependent strains; W2Mef and D10 $r_s = 0.54$, W2Mef and 3D7 $r_s = 0.83$ and less between the sialic independent strains 3D7 and D10 $r_s = 0.52$, although many individuals had antibodies that inhibited only one Pf strain. Thus transplacental transfer of growth inhibitory antibodies occurs against both sialic-acid independent and dependent invasion pathways in Kenyan mothers. Of note, maternal infection status by PCR at time of birth was not associated with GIA levels in cord blood of their offspring ($P = 0.42$, Mann-Whitney U test). The Msambweni 2006 field isolate GIA differed from W2mef ($P < 0.010$, Wilcoxon signed-rank test) in the subset of 112 samples (Figure 1B) and had a 32% prevalence of positive responders. GIA to the Msambweni 2006 correlated poorly with GIA to D10 $r_s = 0.25$ but better with GIA to 3D7 $r_s = 0.48$ and W2Mef $r_s = 0.73$).

3.2 GIA levels to the four Pf strains decrease longitudinally from birth to one year of age

To examine whether GIA levels persist during infancy following transplacental antibody transfer, a subset of matched cord and infant samples were examined at birth, six months (N=86) and 12 months of age (N=65). GIA levels against all Pf strains were found to decline in infants from birth to six months (Figure 2) ($P < 0.01$, Wilcoxon signed-rank test). GIA levels decreased by 1.9%, 1.0%, 0.9% and 0.8% inhibition per month for 3D7, Msambweni 2006, W2Mef, and D10 respectively (linear mixed model). GIA levels remain low at 12 months. The drop in levels was over 5-fold which is consistent with expected half-life of transplacentally transferred IgG antibodies indicating lack of rapid antibody consumption of GIA.

4. Discussion

The current study demonstrates transplacental transfer of growth inhibiting antibodies to four different blood stage parasites strains that are present at birth and decrease to non-detectable levels over the first year of life. These functional antibodies may contribute to the well-established observation that infants less than six month of age are relatively resistant to clinical malaria and high parasitemia compared with older infants and children (Macdonald, 1950; McGuinness et al., 1998). Other studies have demonstrated no change in levels of antigen-specific growth inhibition from birth to six months of age but show an increase from six months to one year, which may represent the beginning of acquired immunity (Dent et al., 2006). Functional antibodies from immune mothers may offer protection from clinical malaria until they wane in the infant making them vulnerable to clinical infections until they develop sufficient acquired immunity.

We and others have shown that children and adults with higher levels of GIA are associated with protection from malaria infection or clinical disease (Crompton et al., 2010; Dent et al., 2006; Rono et al., 2012a). Interestingly, adults can have lower levels of GIA than children (Dent et al., 2006; McCallum et al., 2008). It may be that inhibitory antibodies are more important in children and that with age and repeated malaria exposure; different antibodies develop, such as opsonizing antibodies that are more important. Measured GIA levels may be affected by the individual's age and malaria exposure in addition to whether malaria transmission is holoendemic or seasonal. The difference in GIA activity in the subset of 112 samples may be at least partially accounted for by seasonal variation in IgG levels in mothers. Measured GIA levels can also be affected by the Pf strain used and variations in assay methodology. Comparing absolute levels of growth inhibition among studies is problematic. Of greater importance is the pattern of response and the relative Pf strain inhibition when multiple strains are examined.

Growth inhibitory activity varied considerably among the four blood stage parasite strains used. Interestingly most inhibitory antibodies were directed against the 3D7 parasite, which has also been noted in growth inhibitory studies of older people (Rono et al., 2012b). This shows the breadth of transplacental transfer of functional antibodies to the newborn. This is not surprising as acquired immunity to blood stage malaria likely involves generation of antibodies that target multiple merozoite surface antigens and invasion pathways that may not be adequately represented by total antibody levels. *P. falciparum* has developed the ability to invade red cells using multiple parasite ligand-erythrocyte receptor interactions that have become known as alternative invasion pathways (Hadley et al., 1987). Two major invasion pathways have been described in *P. falciparum*: a sialic acid dependent (W2Mef) pathway and a sialic acid independent (3D7 and D10) pathway (Duraisingh et al., 2003; Ord et al., 2012). The different growth inhibition levels among our laboratory strains may be secondary to parasite antigenic diversity and pathways involved in merozoite invasion as reported by others (Dent et al., 2008; McCallum et al., 2008; Mu et al., 2007; Wahlin Flyg et al., 1997). Using three different laboratory strains with varying invasion pathways in the assay strengthens the validity of our results.

Limitations of our study include 1) measuring total functional antibodies without differentiating IgG subclasses; 2) fewer paired samples at the one year follow-up compared with cord blood and six month follow-up. However, the trend of decreasing levels with time is apparent; 3) as this cohort experienced very few clinical malaria infections by 12 months of age, we were unable to correlate GIA levels with potential protection from disease; 4) using only one Pf field isolate for GIA which may not truly represent the antigenic diversity of the parasite population found in this as has been demonstrated by others (Wahlin Flyg et al., 1997); and 5) not examining the GIA levels in maternal blood.

In conclusion we demonstrated functional antibodies as measured by GIA are transferred to the fetus and wane in the infants over time. Infant protection from clinical malaria disease may in part be mediated by these functional anti-malaria antibodies. It will be important to determine if a significant correlation with infection status and overall amount of GIA present in the individual exists.

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Highlights

- Growth inhibition assays measured functional antibodies against *P. falciparum*.
- Three laboratory malaria strains and a field isolate were used in the assay.
- Functional antibodies were present in cord blood.
- Functional antibodies waned in infants from birth to six months to one year.

A

B

Figure 1.

A: GIA results for N=270 samples of cord blood for each laboratory parasite line (D10, W2Mef, and 3D7). The results are a composite of three separate experiments (N=104, N=54 and N=112; Wilcoxon signed-rank test).

B: GIA results for N=112 samples of cord blood for each laboratory parasite line (D10, W2Mef, and 3D7) and Msambweni 2006 (Wilcoxon signed-rank test).

Figure 2.

GIA results from paired cord blood (N=86), six month follow-up (N=86), and 12 month follow-up (N=65) with laboratory parasite strains (3D7, W2Mef, and D10) and Msambweni 2006 field isolate (Wilcoxon signed-rank test).