Impaired *Haemophilus influenzae* Type b Transplacental Antibody Transmission and Declining Antibody Avidity through the First Year of Life Represent Potential Vulnerabilities for HIV-Exposed but -Uninfected Infants

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To determine whether immune function is impaired among HIV-exposed but -uninfected (HEU) infants born to HIV-infected mothers and to identify potential vulnerabilities to vaccine-preventable infection, we characterized the mother-to-infant placental transfer of *Haemophilus influenzae* type b-specific IgG (Hib-IgG) and its levels and avidity after vaccination in Ugandan HEU infants and in HIV-unexposed U.S. infants. Hib-IgG was measured by enzyme-linked immunosorbent assay in 57 Ugandan HIV-infected mothers prenatally and in their vaccinated HEU infants and 14 HIV-unexposed U.S. infants at birth and 12, 24, and 48 weeks of age. Antibody avidity at birth and 48 weeks of age was determined with 1 M ammonium thiocyanate. A median of 43% of maternal Hib-IgG was transferred to HEU infants. Although its level was lower in HEU infants than in U.S. infants at birth ($P < 0.001$), Hib-IgG was present at protective levels (>1.0 μg/ml) at birth in 90% of HEU infants and all U.S. infants. HEU infants had robust Hib-IgG responses to a primary vaccination. Although Hib-IgG levels declined from 24 to 48 weeks of age in HEU infants, they were higher than those in U.S. infants ($P = 0.002$). Antibody avidity, comparable at birth, declined by 48 weeks of age in both populations. Early vaccination of HEU infants may limit an initial vulnerability to Hib disease resulting from impaired transplacental antibody transfer. While initial Hib vaccine responses appeared adequate, the confluence of lower antibody avidity and declining Hib-IgG levels in HEU infants by 12 months support Hib booster vaccination at 1 year. Potential immunologic impairments of HEU infants should be considered in the development of vaccine platforms for populations with high maternal HIV prevalence.
centrations than those derived from vaccine responses, an observation that may relate to antibody avidity (14).

In this study, we characterized the development of Hib-specific IgG in Ugandan HEU infants by quantification of transplacental transfer, responses to primary Hib vaccination, and evolution of the avidity of Hib- and Hib vaccine-associated diphtheria toxoid-specific IgG through their first year of life.

MATERIALS AND METHODS

Study populations. This analysis was part of a prospective study of the impact of breast-feeding practices on a cohort of uninfected Ugandan infants born to HIV-infected mothers between 2010 and 2013. One hundred one mother-infant dyads were recruited from the Mulago Hospital Antenatal Clinic in Kampa, Uganda. Of these, 57 had previously undergone a stool microbiome assessment; these same 57 were selected for the present study. The enrollment criteria for women were HIV infection, an age of ≥18 years, 32 to 38 weeks of gestation at enrollment, and planning to breastfeed for 6 months. The eligibility criteria for infants were a singleton birth weight of >2,500 g and the absence of life-threatening conditions. All pairs received perinatal prophylaxis preventing mother-to-child transmission. One infant was infected perinatally and was not included in this study. Clinical and anthropometric data, infant blood and stool, and maternal breast milk samples were obtained at birth (within 72 h) and 12, 24, and 48 weeks later. Hib polysaccharide conjugate vaccine (Tritanrix HepB/Hib [DTwP/HBV/PRP-T]; GlaxoSmithKline) was given to all subjects at 6, 10, and 14 weeks of age, according to Uganda Ministry of Health guidelines. Written informed consent was obtained for all subjects with protocols approved by the Colorado Multiple Institutional Review Board, the Johns Hopkins University School of Medicine, the Makere University School of Medicine and Ethics Committee, and the Uganda National Council for Science and Technology.

In addition, serum samples from 14 healthy infants (30% female; birth weight, 3.6 ± 3 kg) born to HIV-negative women 18 to 40 (mean, 31.6 ± 5.0) years old in Aurora, CO, with enrollment criteria comparable to those of the Ugandan cohort were collected at equivalent time points. This cohort was included to put certain features of Hib responses in the Ugandan HEU infants into context (e.g., levels at birth, avidity and decline at 1 year) rather than as a formal control population, particularly given the later vaccination schedule (Hib vaccines were administered at 2, 4, and 6 months) and different vaccine formulation (U.S. infants received Hib conjugate vaccine combined with acellular pertussis vaccine). Furthermore, maternal plasma was not obtained at enrollment from the U.S. mothers and the maternal Hib vaccination status was unknown (the cohort included women born both before and after the introduction of routine childhood Hib vaccination in the United States). Thus, the two populations were evaluated in a side-by-side descriptive analysis of Hib immunity through the first year of life in the two populations.

Laboratory methods. (i) ELISA for Hib-specific IgG. Hib IgG concentrations were determined by indirect enzyme-linked immunosorbent assay (ELISA) as previously described, by using Hib polyribosylribitol phosphate (PRP) linked to tyrosine hydrochloride (Connaught Labs, Toronto, Canada) as the capture antigen, a horseradish peroxidase-conjugated anti-IgG antibody (Jackson Laboratories, Bar Harbor, ME), and a 3,3',5,5'-tetramethylbenzidine-based developer (BD Biosciences, San Jose, CA) (16). Antibody concentrations in serial dilutions were determined with a reference serum standard with a known PRP-specific IgG antibody concentration (Connaught Labs). All samples were run in duplicate and included an internal control sample with a known concentration. The coefficient of variation of the controls on each plate was 10% ± 5%.

(ii) DT-specific IgG ELISA. We measured diphtheria toxin (DT)-specific IgG by ELISA as previously described (17). DT (Massachusetts Department of Public Health Biolabs), which differs from the Hib vaccine protein conjugate CRM197 (cross-reactive material 197) by only one amino acid, was bound as the capture antigen (1 μg/ml). Other steps were as described above but with the serum IgG concentration as the assay standard. The coefficient of variation was 10.5% ± 5%.

(iii) Hib- and DT-specific IgG subtype ELISA. Hib- and DT-specific IgG1 and IgG2 concentrations were measured by using similar ELISA methods, substituting biotin-conjugated anti-IgG1 HP6069 (EMD Millipore, Darmstadt, Germany) or anti-IgG2 HP6002 (Invitrogen, Grand Island, NY) as conjugates.

(iv) Hib-IgG avidity assay. The avidity of Hib-IgG was determined with the chaotropic agent ammonium thiocyanate (NH4SCN) to disrupt weaker antibody-antigen binding, by a modified form of the method described by Goldblatt et al. (18). After incubating serum samples diluted to produce an optical density (OD) of 0.8 to 1.0 by ELISA and washing, 100 μl of NH4SCN was added to serial wells at 0.1 to 6 M for 30 min, washed, and developed as described above. The avidity index was the molar concentration of NH4SCN required to reduce the OD by 50%. Because the amounts of infant serum samples available were limited, we adapted the avidity assay to the use of only 1 M NH4SCN, with avidity (percent retention on the plate with 1 M NH4SCN) defined as (OD with 1 M NH4SCN/OD at baseline) × 100. The log10-transformed results of this proxy assay correlated closely with the serial dilution avidity index by linear regression (r = −0.8986; P < 0.001; see Fig. S1 in the supplemental material).

(v) Clinical immune assays. We measured total serum IgG, IgM, and IgA levels at Children’s Hospital Colorado by nephelometry (Siemens, Erlangen, Germany) and determined complete and differential blood counts by using standard automated hematology techniques in Uganda. Lymphocyte lineages and subsets were characterized by whole-blood flow cytometry with fluorescently labeled monoclonal antibodies to CD19 for B cells; CD3, CD4, and CD8 for T cells; and CD45RA andCCR7 for naive/memory cells analyzed on a FACSCanto (BD Biosystems) using FlowJo software.

Statistical analysis. Statistical analysis was done with Stata, version 11.2 (StatCorp, College Station, TX). Geometric mean Hib-specific IgG concentrations in mothers at enrollment and in infants at birth and 12, 24, and 48 weeks of age as primary outcomes were compared at different time points within groups with paired t tests and comparisons of continuous variables between HEU and HIV-unexposed uninfected (HUU) populations with nonparametric Mann-Whitney tests given the low number of HUU subjects. Secondary outcomes included change in Hib-IgG over time and avidities at each time within groups and between groups and were assessed by using paired t tests. No correction was made for multiple comparisons, as the overall number of analyses was low. To compare placental transfer of Hib- and DT-specific IgG (paired, nonparametric data) and proportions of IgG subtypes, we used Mann-Whitney tests. Simple linear regression was used to correlate the proportions in the 1 M NH4SCN avidity assay with the full avidity index and to assess the correlation between the maternal antibody concentration and placental transfer. Correlations among antibody levels, changes in antibody levels between time points, and continuous variables such as hematologic parameters and total immunoglobulins were assessed by using linear regression. Antibody levels and avidity indexes were log10 transformed for regression analysis. The P value for statistical significance was set at <0.05.

RESULTS

Study population characteristics. Baseline demographic, clinical anthropometric, and clinical data on the subjects enrolled in this study are summarized in Table 1. All of the enrolled Ugandan mothers had a CD4 cell count of >350/μl, and 77% had HIV RNA loads of <10,000 copies/ml. Only infants of adequate birth weight (>2,500 g) were enrolled; however, poor weight gain was commonly observed through the study period, with 10% of the subjects showing severe malnutrition by 1 year (z-scores of <−2 for weight according to World Health Organization [WHO] standard growth parameters; data not shown). All of the infants in the United States and Uganda were exclusively breast fed after birth.
and two-thirds of those at both sites were exclusively breast fed in the first 24 weeks of the study.

**Mother-to-infant transplacental antibody transfer.** Among 52 Ugandan infants whose serum was available at birth, the geometric mean Hib IgG level was significantly lower than the maternal values (median, 2.6 versus 5.7 μg/ml, respectively; \( P = 0.01 \) [paired \( t \) test]) (Fig. 1). Nevertheless, all of the mothers and 90% of the infants had Hib-IgG levels above the threshold for long-term protection (>1.0 μg/ml). Among Ugandan newborns, the median transplacental transfer of Hib-IgG was 43% (range, 12 to 173%). In contrast, transfer of IgG to the DT protein in the same subjects was more efficient (median, 68% [range, 6 to 177%]; \( P = 0.001 \) [Mann-Whitney test]) (Fig. 1B and C). We identified no significant correlation in the proportional transfer of Hib- and DT-specific IgG between mother-infant dyads. Serum samples were not available from U.S. mothers to assess transfer. Specific IgG1 and IgG2 subclasses comprised comparable proportions of IgG to the polysaccharide Hib and to the protein DT in 20 mother-infant pairs (Table 2). The majority of the IgG to both vaccine antigens was of the IgG1 subclass in both mothers and infants. The proportion of specific IgG transferred did not correlate with either the subclass or the level of these antibodies in maternal serum samples (Fig. 2).

**Antibody responses to primary vaccine series.** All of the infants in our HEU cohort mounted robust antibody responses at 24 weeks after receiving three doses of Hib conjugate vaccine at 6, 10, and 14 weeks (Fig. 3A). By 12 weeks of age (following two Hib vaccinations), 91% had protective levels (>1.0 μg/ml), as did all of the infants by 24 weeks after three doses. Levels of Hib-specific IgG at birth did not predict or correlate with those at 12 or 24 weeks of age. From a peak geometric mean titer (GMT) of 13.7 μg/ml at 24 weeks, Hib-specific IgG levels declined in 86% of the subjects to 7.5 μg/ml at 48 weeks (\( P < 0.001 \), paired \( t \) test), although 98.3% still retained protective levels.

Among the U.S. infants, the GMT of Hib-specific IgG was significantly higher than that in the Ugandan HEU infants at birth (8.1 versus 1.7 μg/ml, respectively; \( P < 0.001 \) [Mann-Whitney test]) (Fig. 3B). After receiving three vaccine doses on an accelerated schedule, Ugandan HEU infants had generated greater responses than U.S. infants, who had received only two doses by that time. This difference persisted at 48 weeks (7.5 versus 3.3 μg/ml, respectively; \( P = 0.002 \) [Mann-Whitney test]).

| TABLE 1 Clinical characteristics of 57 HIV-infected mothers and uninfected infants in Uganda |
|---------------------------------------------|----------------------|-----------------|
| Group and characteristic                  | Value               | Range           |
| Mothers                                    |                      |                 |
| Mean age (yr)                              | 25.1                | 4.9             |
| Median no. of HIV copies/ml                | 1,538               | 20–73,900       |
| Median CD4 cell count/cm²                  | 641                 | 367–1,301       |
| % with spontaneous vaginal delivery        | 100                 |                 |
| Infants                                    |                      |                 |
| Mean birth wt (kg)                         | 3.71                | 3.1–4.4         |
| Mean birth wt for age (z-score)            | −0.08               | −1.65–1.08      |
| Mean 48-wk wt (kg)                         | 8.7                 | 5.7–10.6        |
| Mean 48-wk wt for age (z-score)            | −0.68               | −3.52–1.45      |
| % of females                               | 50                  |                 |
| % Exclusively breast fed at 24 wk          | 67                  |                 |

The pattern of DT antibody levels through the first year of life in the Ugandan infants was similar to that of Hib antibody levels. The lowest levels were seen at birth (median, 3.5 [range, 1.2 to 31.8] μg/ml), followed by a peak at the 24-week time point (me-
TABLE 2 Subclass distribution of IgG to Hib and DT in 20 mother-infant pairs

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean % IgG1a (range)</th>
<th>P valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mothers</td>
<td>88.7 (14.8–98.2)</td>
<td>0.98</td>
</tr>
<tr>
<td>Infants</td>
<td>84.4 (30.3–96.0)c,d</td>
<td>0.14</td>
</tr>
</tbody>
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a Percent IgG1 = [concentration [μg/ml] of specific IgG1/specific IgG1 + IgG2] × 100.

b Determined by Wilcoxon rank sum test (Hib versus DT).

c P = 0.64 by Wilcoxon rank sum test (mother versus infant).

d P = 0.39 by Wilcoxon rank sum test (mother versus infant).

Correlates of Hib antibody responses. We determined whether the hematologic (lymphocyte numbers and hemoglobin levels), immunologic (numbers of CD19+ B cell and CD3+ and CD4+ T cells), feeding (duration of all or exclusive breastfeeding), or anthropometric (length for age, weight for length) characteristics of each child correlated with the levels of or changes in Hib-IgG levels or avidity in the HEU cohort. In this context, the total lymphocyte count (r = 0.308; P = 0.03), CD3+ T cell count (r = 0.357; P = 0.01), and CD4+ T cell count (r = 0.283; P = 0.05), and total serum IgG level showed a weak positive correlation with the Hib-IgG level at 48 weeks (r = 0.300; P = 0.03 [linear regression]), but growth, nutritional status, and breastfeeding status did not.

Avidity of Hib-specific IgG. The avidities (binding strengths) of Hib-IgG in Ugandan HEU infants and U.S. HIV-unexposed infants at birth were comparable (Fig. 4). These avidities remained comparable in the two groups at 48 weeks. However, despite the administration of three doses of the Hib conjugate vaccine in the intervening year, the avidities declined significantly from birth to 48 weeks of age (P < 0.001 for both groups [paired t tests]).

FIG 2 Correlation of percent transfer of Hib- and DT-specific IgG and concentration of maternal antibody (n = 20 mother-infant pairs; P values from linear regression for each antigen and analysis of covariance for comparison between antigens).

FIG 3 H. influenzae type b antibody concentrations at four time points in the first year of life in Ugandan HIV-exposed but-uninfected (A) and U.S. HIV-unexposed (B) infants (n = 57 Ugandan and 14 U.S. infants).

DISCUSSION

In this cohort of maternally HIV-exposed but-uninfected Ugandan infants who are at increased risk for infectious complications (8), we identified a relatively low proportion of transplacental passage of Hib antibody. Hib-specific IgG responses following primary vaccination were robust in the fully vaccinated Ugandan infants despite the low levels at birth, declining in 65 versus 86% of the infants, respectively. Antibody levels at birth did not directly or inversely correlate with vaccine responses at subsequent time points.

A striking difference in the Ugandan HEU infants compared with the U.S. infants was the relatively lower level of Hib IgG at birth, which may result in a period of increased vulnerability to Hib disease in these HEU newborns. Ten percent of the newborns were below the putative protective Hib IgG level of 1 μg/ml at
One theory is that the reactive with Hib polysaccharide (43% transferred) than for diphtheria toxoid protein (68% transferred). The avidity in 1 M ammonium thiocyanate of H. influenzae type b IgG from Ugandan HIV-exposed but -uninfected and U.S. HIV-unexposed infants at birth and 1 year of age. The avidity percentage represents the percentage of Hib-specific IgG persistently bound in the presence of 1 M ammonium thiocyanate. A higher percentage represents greater avidity for and strength of binding to the solid-phase Hib capsular PRP antigen. Statistical comparisons are represented by dark horizontal lines; P values are for unpaired t tests (n = 23 Ugandan and 14 U.S. infants). NS, not significant.

Birth, a proportion that would likely increase prior to the emergence of vaccine-induced immunity as a result of postnatal decay in maternal IgG. The birth levels of Hib IgG in the U.S. population are consistent with those observed in previous studies of Hib-vaccinated women (19). Thus, much of the disparity at birth between the Ugandan and U.S. babies may be due to receipt of Hib vaccination in at least some U.S. mothers, resulting in increases in maternal IgG. However, impaired transplacental transmission in association with HIV infection may also have contributed. The 43% placental transfer of Hib IgG in the 52 HEU infants in our study is consistent with a 61% reduction relative to HUU infants found in 12 HIV-infected mothers in the United Kingdom and 57% Hib-IgG transfer in 46 HIV-exposed but -uninfected infants in South Africa (12, 20). Each of these proportions of transplacental transfer is notably lower than those reported in healthy pregnancies in both developed and developing countries, which are consistently above 90% (21–23). This limited transfer with resulting low levels of Hib-specific IgG in HIV-exposed infants supports the earlier timing of vaccinations advanced by the WHO Expanded Program on Immunization for this population (6, 10, and 14 weeks) compared with the later 8-, 16-, and 24-week schedule common in many developed nations. Decreased transplacental antibody transfer in HIV-infected mothers has also been described for varicella-zoster virus, tetanus toxoid, measles virus, streptolysin O, pertussis, and Streptococcus pneumoniae capsular antigens (24–28).

The low level of mother-to-child transfer of specific IgG in our HIV-infected cohort was also more pronounced for antibodies reactive with Hib polysaccharide (43% transferred) than for diphtheria toxoid protein (68% transferred). One theory is that the efficiency of transfer may differ among IgG subclasses (29). Maternal antibodies to polysaccharide antigens such as Hib after natural exposure have been shown to contain a higher proportion of the IgG2 subtype, which may not be transported across the placenta as efficiently as IgG1, the subclass that makes up the majority of the IgG to protein antigens such as DT (30–32). However, we found that the IgG1, rather than the IgG2, subclass made up the majority of the antibodies to both Hib and DT in our HIV-infected Ugandan mothers and their infants and that the proportions of each subclass were similar in both groups and in both mothers and infants (Table 2). To ensure that this proportion was not mediated by poor IgG2 assay sensitivity, we confirmed the predominance (87%) of IgG2 to pneumococcal capsular polysaccharides relative to IgG1 in five previously pneumococcal-vaccine-treated healthy adults (data not shown), as previously reported (33). Our data raise the possibility that the ability of HIV-infected adults to produce IgG2 may also be compromised.

Other potential mechanisms underlying the decrement in placental transfer of IgG during maternal HIV infection may include competitive inhibition with high levels of circulating total IgG (hypergammaglobulinemia), IgG glycosylation, malaria exposure, malnutrition, and placental inflammation, including its effect on the expression and transport of IgG by the neonatal IgG receptor (FcRN) (13, 25, 27). Transplacental passage of IgG was not directly dependent on maternal concentrations. However, we identified significant heterogeneity of transfer in our population (e.g., several infants showed higher concentrations of specific IgG at birth than their mothers), a phenomenon that may relate to both variance in the assay and high placental efficiency in certain individuals (34).

Maternal HIV exposure did not appear to compromise infant responses to the primary Hib vaccine series, which were robust in all subjects, consistent with previous reports (35, 36). Indeed, the vaccine responses in our study are typical of observations in non-HIV-exposed populations in developing countries, where initial Hib vaccine responses may be more vigorous than in higher-income populations (37), as observed in Nepal, South Africa, and Latin America, where 93 to 100% of the infants generate protective levels of Hib-specific IgG (>1 μg/ml) after a primary series. Indeed, even fractional doses (a fraction of the typical dose) of vaccine have been highly immunogenic in developing countries (38–41). Furthermore, Hib vaccine responses do not appear to be affected by combination with whole-cell pertussis vaccine, such as that used in our Ugandan cohort and many low- and middle-income nations, whereas acellular pertussis vaccines are associated with decreased Hib antibody responses when used in combination, a phenomenon that may explain the higher levels of Hib IgG at 1 year among the Ugandan infants in this study (42).

Despite the adequate vaccine responses at 24 weeks of age in Ugandan infants, two observations at the 48-week time point raise concerns for waning protection in the second year of life. The first, the decrease in Hib IgG concentrations from 24 to 48 weeks of age, may lead to levels below the protective threshold in the ensuing months, consistent with previous findings in both HIV-exposed and -unexposed infants (35, 38, 43). Such data have been invoked in support of a recommendation for a booster dose at 1 year. The second observation, decreased antibody avidity relative to birth values, may further complicate the clinical significance of declining antibody levels. At birth, Hib-specific IgG is essentially all of maternal origin and, in the case of both of our study populations, likely derived primarily from natural exposure. Conversely by 48 weeks of age, the antibody will be the infant’s own, derived in large part from vaccine, which may be less effective than the naturally derived antibody. In Scandinavia, an antibody level of 0.15 μg/ml...
was adequate for protection from Hib disease in populations with naturally derived Hib immunity, whereas in populations with vaccine-derived immunity, a much higher level of 1.0 μg/ml was required for protection (44). Thus, the risk of underprotection in the newborn period because of low maternal placental transfer may be ameliorated to some extent by the higher-quality antibody, but at 1 year, adequate levels of specific IgG may overestimate protection in the setting of low antibody avidity. Thus, infants with lowest-avidity antibodies may even be vulnerable at antibody levels greater than the 1.0-μg/ml threshold. Planned future work characterizing functional antibody assessment including bactericidal assays may more clearly define these potential vulnerabilities.

Limitations of our study include the absence of a Ugandan HIV-unexposed control group which limits our ability to analyze the impact of HIV infection on impaired placentation transfer. The relevance of a small cohort of U.S.-borne HIV-unexposed infants as a reference population is limited because of a number of differences from our study population, including reduced environmental exposure to infectious agents, better nutrition, and, importantly, the Hib vaccination schedule. The unknown Hib vaccination status of the U.S. mothers and the lack of a maternal serum sample in this cohort prevent comparison of transplacental transmission and do not allow a full understanding of the differences in Hib IgG at birth. However, the U.S. cohort does allow direct comparison of Hib antibody levels at 1 year and differences over time in antibody avidity, data that will inform decisions regarding the use of a booster dose of Hib vaccine after the first year of life.

Current WHO recommendations, adopted by most sub-Saharan African countries, often result in complete Hib vaccination by as early as 14 weeks of age (45, 46). The booster dose administered at 12 to 15 months in many higher-income countries is not formally included in the WHO recommendations. Our findings provide an additional rationale for previous evidence that residual Hib disease in older children will continue to occur and suggest that the advent of conjugate vaccines is highly effective in the Ugandan routine immunization program: a case-control study. Trop. Med. Int. Health 13:495-502. http://dx.doi.org/10.1111/j.1365-3156.2008.02027.x.


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