Antibody Avidity as a Surrogate Marker of Successful Priming by *Haemophilus influenzae* Type b Conjugate Vaccines following Infant Immunization

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Evaluation of the new generation of conjugate vaccines is hampered by the absence of reliable surrogate markers of immunologic memory. Memory responses are characterized by rapid production of relatively high-avidity antibody; thus, a solid-phase ELISA was adapted for the measurement of anti-"Haemophilus influenzae" type b (Hib) IgG avidity. In a cohort of infants vaccinated at 2, 3, and 4 months of age with Hib conjugate vaccines, avidity increased in the period following vaccination, while antibody titer fell. After a booster dose at 1 year of age, both antibody titer and avidity increased. In a cohort with anti-Hib IgG <1.0 μg/mL following primary immunization, antibody avidity after booster was low, indicating an absence of priming. Antibody avidity may help distinguish, in persons with low antibody titers, between those who are primed for memory and those who are not.

New-generation bacterial conjugate vaccines are currently in accelerated development following the dramatic success of the *Haemophilus influenzae* type b (Hib) conjugate vaccines in reducing the incidence of invasive Hib disease [1]. The success of such vaccines is due to the chemical conjugation of the capsular polysaccharide to a carrier protein, thereby rendering the carbohydrate component immunogenic, even in very young children. In addition to their immunogenicity, the vaccines also appear to prime for memory responses, which is crucial if the vaccine is to provide long-lasting immunity. Evaluation of Hib conjugate vaccine immunogenicity relies on the measurement of serum antibody titers to Hib capsular polysaccharide (polyribosylribitol phosphate [PRP]) and the interpretation of the titer in the light of the accepted levels for short-term (>0.15 μg/mL) or long-term (>1.0 μg/mL) protection [2]. These levels, however, were derived by passive immunization or immunization with pure polysaccharides; thus, their relevance for protection in children who have been primed for memory responses remains unclear. Ideally, therefore, the evaluation of vaccine immunogenicity should incorporate a surrogate marker of priming.

Antibody avidity, the strength with which an antibody binds to an antigen, increases over time following encounter with an antigen. Memory responses are characterized by the production of high-avidity antibody; thus, avidity could be considered a surrogate of successful priming. Recent advances in the measurement of antibody avidity permit the application of such measurements to large numbers of sera, such as those obtained during a vaccine trial [3].

We have developed an assay for the measurement of anti-PRP antibody avidity and have evaluated sera obtained from infants who have undergone primary immunization with Hib conjugate vaccines and received a booster dose at 1 year of age. We have measured both titer and avidity of Hib-specific IgG after primary immunization, before booster, and after booster immunization to assess whether antibody avidity may provide a surrogate marker of successful priming following primary immunization.

**Methods**

This study is a follow-up of a previously reported study in which we assessed the interchangeability of two different Hib conjugate vaccines given according to the United Kingdom accelerated immunization schedule [4]. We enrolled infants aged 8–12 weeks of age and randomly allocated them to receive one of six different sequences of the two Hib conjugate vaccines, which were administered at the same time as the routine immunization with diphtheria-tetanus toxoids–pertussis (DTP) and oral poliovirus vaccine at 2, 3, and 4 months of age. A booster dose of either of the two conjugate vaccines was given at 1 year of age. This was an open randomized study. The two Hib conjugate vaccines studied were PRP-T (ActHIB; Pasteur-Mérieux-MSD, Lyon, France) and HbOC (HibTITER; Cyanamid-Lederle-Praxis Biologicals, Pearl River, NY). All infants also received the Wellcome DTP whole cell vaccine in 0.6 mg of Al(OH)₃ (Biotech, Beckenham, UK) and oral poliovirus vaccine. DTP was mixed with PRP-T or HbOC in the...
same syringe [5] and administered intramuscularly in the anterolateral aspect of the buttock, thigh, or arm.

Serum was obtained by venipuncture or heel prick from each infant before the first dose, 4 weeks after the third dose, and before and 4 weeks after the booster dose and was stored at −70°C until analyzed. IgG antibody to PRP was assayed by ELISA as previously described [6] by investigators blind to the randomization groups. Antibody avidity was measured by an elution ELISA, previously described by us [3, 7] and modified for the Hib assay. Briefly, sera were diluted in PBS–0.05% Tween 20–1% bovine serum albumin (PBS-T–BSA) to a final concentration of ~0.5 μg/mL (minimum dilution, 1:4) and were then allowed to bind for 2 h at room temperature to an antigen-coated plate. After washing, ammonium thiocyanate diluted in PBS-T–BSA was added in duplicate at concentrations of 0–1 M. After 15 min at room temperature, the wells were washed, and antibody binding was then detected by the addition of a mouse anti-human IgG (R10; Recognition Sciences, Birmingham, UK) followed by a sheep antimouse–horseradish peroxidase conjugate. The assay was then developed by use of the chromogenic substrate o-phenylenediamine in citric acid–phosphate buffer with hydrogen peroxide. The reaction was stopped with 4 N H2SO4 and the absorbance at 490 nm was read by use of an automatic microtiter plate reader (Dynatech, Chantilly, VA). Results were then expressed as the log percentage of reduction in absorbance in the presence of ammonium thiocyanate plotted against the concentration of ammonium thiocyanate. Antibody avidity was displayed as an avidity index corresponding to the molar concentration of ammonium thiocyanate required to produce a 50% reduction in absorbance [3].

Statistical analyses were done by use of the statistical software Minitab (Minitab, State College, PA). Anti-PRP titers and avidity were positively skewed and thus log-transformed before analysis. Geometric mean titers were compared between the groups by two-sample Student’s t tests.

Results

As previously reported, 512 infants were analyzed before and after primary immunization. The geometric mean titer (GMT) of anti-PRP IgG for the entire cohort 1 month following primary immunization was 6.23 μg/mL (95% confidence interval [CI], 6.15–6.30), with 92.4% achieving an antibody titer >1.0 μg/mL [4]. Paired sera from 376 infants were available for analysis before and after boosting. At 1 year of age, the GMT of anti-PRP IgG had fallen to 0.40 (95% CI, 0.34–0.47) for all vaccinees; 38.8% had antibody titers below the short-term protective titer of 0.15 μg/mL and only 31.9% had antibody titers >1.0 μg/mL. After booster immunization at 1 year of age, antibody titer increased significantly for all vaccinees (GMT, 113.92; 95% CI, 97.4–133.2).

The geometric mean avidity index (GMAI) for all vaccinees at 5 months of age following three doses of Hib conjugate vaccine was 0.28 (95% CI, 0.27–0.3). After boosting, avidity increased significantly compared with post–primary immunization avidity to a GMAI of 0.52 (95% CI, 0.49–0.56; P < .001). In a small subgroup of infants (n = 40) in whom antibody titers were high enough for antibody avidity to be analyzed before boosting at 1 year of age, the GMAI had increased from 0.28 (95% CI, 0.24–0.31) after primary immunization to 0.55 (95% CI, 0.46–0.64) before boosting. After boosting, the GMAI increased further to 0.60 (95% CI, 0.5–0.73) (figure 1). Postbooster avidity correlated with the prebooster avidity for subjects in this group (r = .71).

When the postbooster avidity indices of individual vaccinees in whom data on antibody titer were available at all time points were stratified according to their antibody titer after primary immunization, differences were noted. Subjects with an antibody titer <1.0 μg/mL at 5 months of age had significantly lower GMAIs after boosting than did vaccinees who mounted a long-term protective response after primary immunization (table 1). In addition, the antibody response of this group after boosting was significantly lower than that of the subjects who mounted an IgG response >1.0 μg/mL after primary immunization. Interestingly, the GMAI for this group after boosting (0.28 [95% CI, 0.23–0.35]) was the same as that seen for all vaccinees after the primary immunization series. For all of the vaccinees, however, the correlation between either post–primary immunization avidity or titer and postbooster avidity or titer was relatively weak (0.3 and 0.4, respectively). Thus, the effect seen when the subjects are stratified according to their initial response is a threshold effect.

Responses to the two conjugate formulations used for boosting were compared. The GMT of anti-PRP IgG for the group receiving PRP-T as a booster was 139.86 (95% CI, 109.2–179.1) compared with a GMT of 85.70 (95% CI, 68.9–106.5; P = .004) for those boosted with HbOC. Despite the difference

Figure 1. Geometric mean titer (GMT) of anti-PRP (Hib capsular polysaccharide) IgG before immunization and GMT and geometric mean avidity index after primary immunization and before and after boosting with Hib conjugate vaccine in subgroup of the main cohort (n = 40). Bars indicate 95% confidence intervals.
in antibody titer for recipients of the two conjugates, no difference was noted in the GMAI after boosting. Recipients of a PRP-T booster had a GMAI of 0.52 (95% CI, 0.46–0.57), compared with a GMAI of 0.53 (95% CI, 0.49–0.58) for the recipients of an HbOC booster ($P = .60$).

Discussion

Surrogate markers of priming are relevant to an understanding of the immunologic basis of protection afforded by the new-generation bacterial conjugate vaccines. Theoretically, persons with low or undetectable specific IgG titers will be protected from infection if they are primed for memory responses, as they are able to rapidly produce specific IgG of relatively high avidity on encounter with the relevant antigen. Thus, in persons with low titers of antibody, measures of antibody avidity may be able to discriminate between those successfully primed in infancy, who may be considered to be protected, and those with low-avidity antibody, who may technically be at risk of invasive Hib disease. Evidence of the superiority of high-compared with low-avidity antibody in its ability to kill Haemophilus influenzae type b in vitro has been shown [8].

The strict requirements for the measurement of “true” antibody affinity (monoclonal sources of antibody, highly purified haptenic antigens) have precluded the widespread use of such assays in the assessment of vaccine responses. Our approach has been to adapt solid-phase assays developed for the measurement of antibody titer to measure antibody avidity. Avidity, defined as the bivalent interaction of antibody with complex antigens, is, we believe, more closely analogous to the complex interaction encountered in vivo and thus a more relevant biologic measurement than is affinity. We, and others, have validated the thiocyanate elution assays by comparison with equilibrium dialysis as well as with biospecific interaction, analysis [9, 10].

In this study, we have shown that antibody avidity is relatively low following primary immunization and significantly higher following boosting. The majority of the increase in avidity was seen in the months after primary immunization, and boosting only slightly increased the avidity further. It is possible that further increases in avidity may take place in the months after the booster dose.

In a small subgroup of subjects with suboptimal primary responses, the avidity after boosting was low and the same as that seen after primary immunization. The suggestion that these subjects had not been adequately primed and thus were mounting a primary response at 1 year of age was reinforced by the lower GMT of anti-PRP IgG achieved after boosting, a titer comparable to that achieved by older children after a single dose of Hib conjugate [11]. It will be interesting to see whether these subjects show increase in antibody avidity over time after this booster dose. With increased sensitivity of the assay, it will be possible to measure the antibody avidity of low-titer serum. This will permit studies of naturally occurring anti-PRP IgG antibodies and an analysis of changes in antibody avidity with increasing age as well as the study of vaccine failure.

Previous reports of higher antibodies being induced by booster doses of PRP-T compared with other Hib conjugate formulations [12, 13] were confirmed in our study. Despite the higher anti-PRP IgG titers after boosting with PRP-T, the GMAI was similar for the two vaccines. These data support those of Agbarakwe et al. [14], who analyzed antibody avidity by means of a urea elution assay and found no difference in the avidity of antibodies induced by primary immunization of infants with PRP-T, HbOC, or PRP conjugated to outer membrane proteins (PRP-OMP). In contrast, in a study using a radioantigen-binding technique for the measurement of avidity, antibodies induced by primary immunization with HbOC were shown to be of higher avidity than those induced by PRP-T and PRP-OMP [15].

Currently, antibody titer alone is the sole measure of vaccine immunogenicity, and while such measurements may adequately reflect the immediate response to a vaccine, they cannot be relied on to reflect immune status months or years after the immunization with a vaccine that induces memory. Current concern surrounding the reduced immunogenicity of combination vaccines containing acellular pertussis and Hib conjugate in the same formulation [16] highlight the urgent need for an improved understanding of the relationship between primary antibody responses and long-term protection. Reduced anti-

<table>
<thead>
<tr>
<th>Post–primary immunization response</th>
<th>Prebooster GMT</th>
<th>Postbooster GMT</th>
<th>Postbooster GM avidity index</th>
</tr>
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<tbody>
<tr>
<td>&lt;$1 \mu g/mL$ ($n = 26$)</td>
<td>0.10 (0.07–0.13)</td>
<td>62.6 (39.72–98.8)</td>
<td>0.28 (0.23–0.35)</td>
</tr>
<tr>
<td>$\geq 1 \mu g/mL$ ($n = 290$)</td>
<td>0.43 (0.36–0.51)</td>
<td>144.8 (84.71–201.5)</td>
<td>0.55 (0.51–0.59)</td>
</tr>
<tr>
<td>$P$</td>
<td>&lt;.001</td>
<td>.05</td>
<td>&lt;.001</td>
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NOTE. Data are $\mu g/mL$ for GMTs. Data in parentheses are 95% confidence interval. PRP, Hib capsular polysaccharide. Suboptimal responses were defined as anti-PRP IgG titers <$1.0 \mu g/mL$ 1 month after third dose of Hib conjugate vaccine. Differences in antibody levels were compared by 2-sample Student’s $t$ test.
body titers immediately after immunization in the face of successful priming may not necessarily compromise postimmunization protection from disease. In the light of our demonstration of an increase in avidity in the months after vaccination, immunogenicity studies incorporating avidity measurements would optimally require the analysis of serum at time point(s) additional to 4 weeks after vaccination. Furthermore, validation of avidity as a surrogate for priming will require studies in which antibody titers induced by a booster dose of pure PRP months or years after primary immunization are compared with avidity. A high proportion (38.8%) of subjects in our study had antibody titers below the minimum protective titer at 1 year of age, yet, despite this, the effectiveness of the Hib conjugate vaccine in the United Kingdom, where no booster dose of Hib conjugate vaccines is given, remains high [17]. This reinforces our view that antibody titers below the minimum protective level in primed persons do not necessarily reflect a susceptibility to infection, and the search for adequate markers of such protection continue. Antibody avidity may play a part in this analysis.

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References