Avidity of specific IgG antibodies elicited by immunisation against *Haemophilus influenzae* type b

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**Abstract**

**Aim**—To investigate the avidity of specific IgG polyribosyl ribitol phosphate (PRP) antibodies induced by three *Haemophilus influenzae* type b (Hib) conjugate vaccines: PRP—meningococcal outer membrane protein complex (PRP-OMP), PRP—nontoxic mutant diphtheria toxin, CRM₄₅ (HbOC) and PRP—tetanus toxoid (PRP-T).

**Methods**—One hundred and ten infants were immunised with one of the vaccines at two, three and four months of age. Blood samples were taken after each vaccination and serum stored at −20°C. Serum samples collected after the third course (that is, at five months of age) were submitted to antibody avidity assessment, using a urea enzyme linked immuno-sorbent assay (ELISA).

**Results**—All three conjugate vaccines elicited IgG PRP antibodies of high median avidity. The resultant antibody populations were heterogeneous with regard to avidity, which in turn was independent of PRP antibody concentration.

**Conclusions**—With the recent findings of a correlation between bactericidal killing and affinity, our data highlight the need for a protective level to be expressed qualitatively as well as quantitatively.


Keywords: Avidity, *Haemophilus influenzae*, IgG antibodies.

*Haemophilus influenzae* type b (Hib) infection is a major cause of childhood bacterial infections such as meningitis, epiglottitis, and septic arthritis. Before routine immunisation, one in 600 children each year were at risk of acquiring an infection in the United Kingdom by their fifth birthday. Hib infection proved to be an age related disease because of the immaturity of the immune system which is unable to respond to polyaccharide antigens in the first two years of life. Recently, conjugate Hib vaccines have been developed to overcome this problem. These vaccines contain the relatively immunogenic region of Hib—that is, polyribosyl ribitol phosphate (PRP) conjugated to different protein carriers. Carriers include the outer membrane protein of *Neisseria meningitidis* (PRP-OMP), diphtheria toxoid (PRP-D), a mutant diphtheria protein (HbOC), and tetanus toxoid (PRP-T). An ideal vaccine elicits both protective immunity and memory so that subsequent exposure to the pathogen will result in its elimination and a boost in immunity. Vaccine trials have shown the ability of these conjugates to activate and prime previously immature, non-responsive infants. When compared with unconjugated vaccines, conjugate vaccines have not only been able to induce an immune response in young infants but, more importantly, they were able to prime the immune system (memory induction) and therefore promote secondary responses on subsequent boosts with either conjugated or unconjugated vaccines.

Although conjugated vaccines have been reported to elicit different levels of Hib PRP antibodies, depending on the conjugate used, the levels of specific antibodies reached provide protection against disease. However, the level of Hib antibodies required for protection remains controversial. It has been suggested that a PRP antibody level of 0.15 μg/ml confers short term protection from Hib infection, but that a level of 1.0 μg/ml is required to confer long term protection. Antibody affinity is thought to play an important role in vaccine efficacy and this has recently been supported by the correlation between antibody affinity and bactericidal killing. Whereas intrinsic affinity measures the interaction between a univalent binding site and a single antigenic determinant, one usually refers to the binding affinity (functional affinity) as a measure of the strength of interaction between a bivalent (or multivalent) antibody and a polynvalent antigen. We have investigated the avidity of specific IgG antibodies produced in a comparative study of three different conjugate Hib vaccines.

**Methods**

Two hundred and fifty infants participating in an immunogenicity trial (Begg et al, unpublished data) were immunised at two, three and four months of age with one of three conjugate vaccines: PRP-OMP (15 μg), PRP-T (10 μg), or HbOC (10 μg). Infants received Hib vaccine via intramuscular injection in the right thigh while receiving routine triple (diphtheria-pertussis-tetanus) vaccine in the left thigh. Blood samples were collected before the first immunisation and one month after each subsequent immunisation. Serum was separated on the day of venepuncture and stored at −20°C until required.

Serum samples were collected from 110 infants (43 received PRP-T; 25 PRP-OMP and
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Figure 1  The effect of urea on a single serum. End point titres are determined at 35% of maximum absorbance. 2.5 M urea was selected as it gave a reproducible and measurable shift in curve. For this particular sample, the end point titre without urea is 600 (x) and with urea it is 360 (x), avidity = 60%. Any sample which dropped below this 35% cut off in the presence of urea was assigned an arbitrary value of 15%.

42 HbOC) at two months (before immunisation) and at five months (that is, after the third immunisation). Serum samples were selected on the basis of PRP antibody concentrations—that is, samples were collected only from infants who had responded to immunisation with at least 0.5 μg/ml of PRP antibody. This is the minimum concentration required to assess antibody avidity by this method.

IgG antibodies to PRP were measured by enzyme linked immunosorbent assay (ELISA). The method used to measure avidity was adapted from the technique described by Hedman et al. Polyribosyl ribitol phosphate (provided by Pasteur Merieux Serum et Vaccins, France) was covalently bound to poly-L-lysine and diluted in 0.05 M carbonate buffer (pH 9.6) to approximately 1 μg/ml. This solution was added to flat bottomed microtitre plates (Immulon 1, Dynatech) using 100 μl per well, sealed with selfseal tape and incubated overnight at 4°C. Patient serum samples were diluted serially, by doubling dilution, in phosphate buffered saline (PBS) plus 0.05% Tween 20 and 100 μl was applied in quadruplicate rows to the microtitre plate. After one hour of incubation, plates were washed four times with PBS/Tween 20; 100 μl of 2.5 M urea (in PBS/Tween 20) was added to two wells and 100 μl of PBS/Tween 20 added to the corresponding two wells. After 10 minutes of incubation plates were washed with PBS/Tween 20. The residual antigen bound IgG was detected with antibody to human IgG conjugated to alkaline phosphatase (1 in 4000 dilution in PBS/Tween 20 (Sigma)) as before. The conjugate was applied for one hour, washed with PBS/Tween 20 and developed with p-nitrophenyl phosphate (5 μg/ml; Sigma) in diethanolamine buffer for one hour in the dark. The reaction was stopped by the addition of 50 μl of 3 M sodium hydroxide. The absorbance was measured at 405 nm (A405) by a Multiscan ELISA plate reader (Lab-systems). Two dilution curves (A405 v serum dilution) were plotted for each patient, one with and the other without urea. The presence of urea caused a leftward shift of the curve proportional to antibody avidity. End point titres (EPR) for each dilution curve were calculated at 35% of maximum absorbance and the ratio of titres expressed as a percentage: (titre (urea+)/titre (urea−))×100. All incubations and washes were carried out at room temperature.

For comparative purposes samples were obtained from a small group of adult volunteers (n = 7) previously immunised with PRP-T. The avidity of the United States Food and Drug Administration (FDA) standard for H influenzae type b capsular polysaccharide antibodies (serum) (lot 1983, provided by Dr Carl Frasch) was also assessed.

Standardisation of the assay involved using varying concentrations of urea which shifted the dilution curve in a linear, dose dependent manner (fig 1). The close proximity of the parallel curves demonstrates that the same degree of shift is obtained at either 35 or 50% (as recommended by Joyson et al) of the maximum absorbance.

Statistical analyses were performed using Epi Info version 5. Analysis of variance was used to compare median avidity data obtained for each conjugate vaccine. The Kruskal–Wallis test was used for comparison of non-parametric data. Data correlation was carried out using linear regression analysis on Macintosh Cricket Graph.

Results

REPRODUCIBILITY
Two samples of noticeably different avidity were used as quality control serum samples
throughout this study. Intraplate variance was assessed by testing each sample several times (n=11) in a single assay. The coefficient of variance (CV) was calculated and found to be 4% for both samples. Interplate variances of 12% (n=13) and 14% (n=13) were obtained.

### COMPARISON OF ANTIBODY PROVOKED BY DIFFERENT VACCINES

There was no correlation between PRP concentration and avidity for any of the vaccines; correlation values (r) were 0.005 for HbOC vaccine, 0.025 for PRP-OMP vaccine and 0.004 for PRP-T vaccine. Nor were there significant differences between the median avidities or ranges obtained for each conjugate vaccine (fig 2). All three vaccines elicited PRP antibodies of heterogeneous avidity. However, the avidity distribution pattern differed for each vaccine (fig 3). The biphasic avidity distribution pattern elicited by HbOC (fig 3A) suggests the presence of two antibody populations, one distributed about a mean of 43 (of relatively low avidity) and the other distributed about a mean of 73 (of relatively high avidity). A biphasic distribution is also seen in fig 3B but it is not as pronounced as in fig 3A. The low avidity population is distributed about a mean of 33 and the high avidity about a mean of 70. PRP-T elicits PRP antibodies which are distributed normally about a mean of 62. Adult serum samples showed a median high avidity of 80% (fig 2). The FDA serum had an avidity of 65% as measured by this ELISA method.

### Discussion

These findings suggest that, after an accelerated series of conjugate Hib vaccine immunisation with either PRP-T, HbOC or PRP-OMP, the Hib specific IgG antibodies induced in five month old infants are heterogeneous with respect to avidity. No correlation was demonstrated between the type of vaccine preparation and median avidity though the range of avidities was much wider with two of the three vaccines. Avidity was also independent of antibody concentration. The latter finding confirming that the selection of serum samples with PRP IgG antibody levels greater than 0.5 μg/ml was acceptable.

The ability of a vaccine to protect an individual from disease has been attributed to an increase in antibody concentration.8 The inconsistencies seen in the literature regarding the “protective level”9,18 and the existence of vaccine failures19 indicates that other factors, possibly antibody class20 and idotype21 distribution, are involved in protection besides antibody concentration. There have been very few publications which have investigated antibody avidity in relation to vaccines. However, its biological importance in protection must not be underestimated.12,13

To our knowledge Schlessinger and Granoff22 are the only other group to have studied PRP antibodies elicited by these three Hib conjugate vaccines. They demonstrated that PRP-CRM (HbOC) and PRP-T conjugate vaccines elicited higher avidity antibodies than PRP-OMP in infants immunised at two, four and six months of age. However, their method of assessing avidity differs fundamentally in

![Avidity distribution of PRP antibodies. EPR = end point titre.](image-url)
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principle from our ELISA. We chose an elution ELISA which utilised urea as the chaotropic agent. It is a simple, cost-effective and rapid procedure which is reproducible and effective in antibody avidity determination. Radioimmunoassays are unable to detect low affinity antibodies, whereby ELISAs can detect both low and high avidity antibodies. Radioimmunoassays are also unable to distinguish between immunoglobulin types (IgG, IgM, etc.) unlike the ELISA. Solid phase reactions are non-homogeneous and consequently the antibody is not free to react with all of the antigenic determinants exposed in solution. In addition, immobilisation of antigen to a solid phase increases the avidity of the interaction by several orders of magnitude. This may improve the sensitivity of the assay to some extent as well as detect antibody that binds epitopes with the lowest avidity. It is not surprising our results differed when these factors are taken into account.

Even though all three conjugate vaccines elicit IgG PRP antibodies of a relatively high median avidity, each differs in the extent to which low avidity antibodies are induced. Low avidity antibodies are less efficient in their biological function than high avidity antibodies. We found no correlation between antibody avidity and concentration, confirming that avidity is totally independent of PRP concentration.

The presence of high avidity PRP antibodies in the FDA standard correlates well with data presented recently by Hetherington and Rutkowski. Adult serum samples also possess high avidity PRP antibodies. It is possible therefore that the presence of small concentrations of maternal antibodies in the infants serum samples at two months of age may have contributed to the overall avidity. However, this is unlikely to be relevant in the post-immunisation samples taken at five months of age. We have demonstrated that avidity of PRP antibodies varies greatly from one infant’s serum to another and is independent of vaccine preparation and antibody concentration. Our findings draw attention to antibody avidity which is an important factor in protection and needs to be considered when evaluating vaccine efficacy as the presence of a high concentration of PRP antibodies does not necessarily guarantee protection. We are grateful for the assistance of the nursing staff involved in the Gloucester trial, for the cooperation of all the parents and children involved in that trial and to Dr R. Booy for helpful discussions.

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