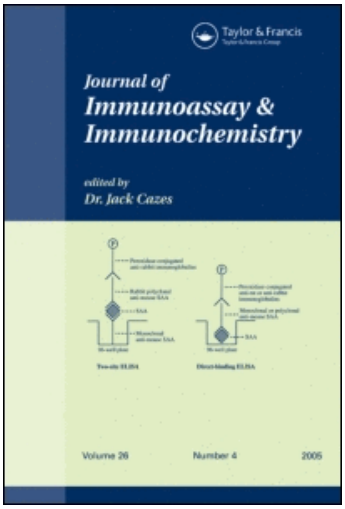

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An Enzyme-Linked Immunosorbent Assay for Quantitation of Haemophilus Influenzae Type b Polysaccharide-Specific IgG1 And IgG2 in Human and Infant Rhesus Monkey Sera

Kathleen T. Dolan[a]; Joan M. Staub[a]; Timothy L. Schofield[b]; Vincent I. Ahonkhai[cd]; Ronald W. Ellis[a]; Philip P. Vella[a]
[a] Departments of Cellular and Molecular Biology, Merck Sharp and Dohme Research Laboratories West Point, PA [b] Biometric Research, Merck Sharp and Dohme Research Laboratories West Point, PA [c] Clinical Research Merck Sharp and Dohme Research Laboratories West Point, PA [d] R. W. Johnson Pharmaceutical Research Institute Rarriton, NJ

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AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR QUANTITATION OF HAEMOPHILUS INFLUENZAE TYPE b POLYSACCHARIDE-SPECIFIC IgG1 AND IgG2 IN HUMAN AND INFANT RHESUS MONKEY SERA

Kathleen T. Dolan[1], Joan M. Staub[1], Timothy L. Schofield[2], Vincent I. Ahonkhai[3], Ronald W. Ellis[1] and Philip P. Vella[1]
Departments of Cellular and Molecular Biology,[1] Biometric Research,[2] and Clinical Research[3]
Merck Sharp and Dohme Research Laboratories
West Point, PA 19486

ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) has been developed and validated to quantitate IgG1 and IgG2 antibody to polyribosyl- ribitol phosphate (PRP), the capsular polysaccharide of Haemophilus influenzae type b (Hib). The sera of children and infant Rhesus monkeys immunized with an Hib conjugate vaccine composed of Hib PRP covalently linked to an outer membrane protein complex (OMPC) from Neisseria meningitidis serogroup B (PedvaxHIB®, PRP-OMPC, Merck, Sharp and Dohme Research Laboratories). The solid-phase antigen employed in the ELISA is a conjugate of PRP to human serum albumin. The enzyme-labeled antibody is alkaline phosphatase-conjugated mouse monoclonal (mAb) anti-human IgG1 or IgG2. A human serum standard was calibrated using parallel titrations with a known antibody standard. The geometric mean titer (GMT) of the anti-PRP IgG1 response to one dose of PedvaxHIB® was 3.87 µg/ml (n=82), 11.80 µg/ml (n=62) and 14.57 µg/ml (n=74) in infants and children 12 to 17 months, 18 to 23 months and $\geq$ 24 months old, respecitvely. Infants 2 to 11 months old responded with an IgG1 anti-PRP response of 7.10 µg/ml while infant monkeys responded with a GMT of 150.65 (n=9) after

[3]Current address: R.W. Johnson Pharmaceutical Research Institute
Rarriton, NJ 08869

Requests to:

Philip P. Vella
Department of Cellular and Molecular Biology
Merck Sharp and Dohme Research Laboratories
West Point, Pennsylvania 19486

two doses of vaccine. The anti-PRP IgG2 GMT responses in all groups were <0.25 µg/ml, except for humans greater than or equal to 18-months old who exhibited a GMT of ≥0.40 µg/ml (n=75). PedvaxHIB®, immunization of human infants and children and infant Rhesus monkeys elicits primarily an IgG1 response to PRP. The monkey model appears to be a reliable indicator of the human immune response.

KEY WORDS: ELISA, Hemophilus influenzae, Antibodies.

INTRODUCTION

Haemophilus influenzae type b (Hib) is a major cause of bacterial meningitis, epiglottitis, sepsis and pneumonia in infants and young children, primarily affecting those less than two years old (1,2). The major contributor to the increased susceptibility of those in this age group is their inherent inability to respond immunologically to T-independent antigens, including bacterial polysaccharides such as the Hib capsular polysaccharide (PRP) (3,4,5). Licensed Hib PRP vaccines are ineffective in this age group and, until recently, no protective immunization was available.

New vaccines have been developed which offer protection against Hib disease through an alternative immunological mechanism than that provided by the PRP vaccines. Conjugation of PRP to proteins, thus far of bacterial origin (6,7,8,9,10,11), converts the PRP to a T-dependent antigen and thereby enables the elicitation of an active immune response in this susceptible infant population. PedvaxHIB® is such a conjugate vaccine, consisting of PRP covalently conjugated to an outer membrane protein complex (OMPC) from Neisseria meningitidis serogroup B (NMB) (12). PedvaxHIB® has been shown to be highly immunogenic in

children as young as 2 months of age (10) as well as in an infant Rhesus monkey preclinical model which appears to predict the immune response of infant children (11). Furthermore, PedvaxHIB® has induced protection against invasive Hib disease after one dose administered at two months of age (13). Other Hib conjugate vaccines have been developed (ProHIBit®, Connaught Laboratories; HibTITER®, Praxis Biologics) using diphtheria toxoid or CRM197 non-toxic mutant protein of diphtheria toxin as the carrier. Comparable immunogenicity in two month old infants after one dose has not been demonstrated for these other two vaccines or for conjugates of PRP to tetanus toxoid (14). However, HibTITER® has been shown to protect against invasive Hib disease beginning after the third dose at six months of age (15).

The antibody produced in response to natural infection or vaccination with Hib conjugate vaccines consists of IgM, IgG, and IgA, with IgG predominating (7,16,17). Anti-PRP also has been shown to mediate the functional activities of serum bactericidal, opsonization, and passive protection of infant rats from Hib challenge (18). The IgG subclasses involved in antibody responses to PRP-OMPC vaccination are of interest in order to learn more about the immunological mode of action of this new Hib conjugate vaccine. In some studies, bacterial polysaccharides including Hib PRP have been shown to induce relatively equal concentrations of IgG1 and IgG2 anti-PRP in children and adults (16,19,20,21). PRP-OMPC-vaccinated children who receive booster doses of either PRP-OMPC or PRP vaccine at >24-months old likewise produce similar

concentrations of anti-PRP IgG1 and IgG2 (8). However, the primary immune response to PRP-OMPC is predominantly of the IgG1 subclass in 2- to 17-month old children. This suggests that a relationship may exist between the inability to produce antibody in infants against PRP vaccines (T-independent antigens) and the elicitation of primarily anti-PRP IgG1, typical of a T-dependent immune response, by Hib conjugate vaccines (3,4,5).

In order to confirm and extend these observations, we have developed and validated an ELISA to measure anti-PRP IgG1 and IgG2 in PRP-OMPC-vaccinated children of four different age groups and in the preclinical infant Rhesus monkey immunogenicity model. The assays have been standardized and validated to permit easier comparison of such data in the field, as was done recently by the U.S. Food and Drug Administration Center for Biologicals Evaluation and Research (CBER) for the Farr-type radioimmunoassay (RIA) for anti-PRP.

MATERIALS AND METHODS

Vaccine

Three clinical lots of PRP-OMPC (PedvaxHIB® Lot 1072/C-P298, 1080/C-P749, and 1085/C-R132) were used to conduct clinical studies. The vaccine was a lyophilized product and was resuspended with aluminum hydroxide (0.225 mg Al^{+++}/dose) diluent prior to use and contained 15 μg PRP/0.5 ml. The vaccine was administered intramuscularly into the thigh.

Human Subjects

Children aged 2 months to 5 years were vaccinated with either one dose (≥12 mo.) or two doses (<12 mo.) of PRP-OMPC at a two month interval. Serum samples were obtained just prior to each injection and one month after the last injection. All sera were stored at -70°C and were assayed in serial two-fold dilutions beginning at 1:20 (as described under IgG subclass ELISA).

Infant Rhesus Monkeys (Macaca mulatta)

The infant Rhesus monkeys were maintained with their mothers at the New Iberia Research Center Primate Facility, New Iberia, Louisiana. Two- to three-month old Rhesus monkeys were given two doses of PRP-OMPC (0.25 ml in the muscle of each thigh) 28 days apart. Bleedings were obtained just prior to injection.

Antigen

Since PRP will not coat polystyrene microtiter plates consistently, a PRP-human serum albumin (HSA) conjugate was made as described previously (12) to eliminate the possibility of non-PRP-specific binding of antibody. The optimal concentration for use in the ELISA was determined through checkerboard titrations to be 5.0 μg conjugated PRP/ml.

Test Serum Standard

A Human Serum Standard (A144) (obtained from D. M. Granoff, Washington University School of Medicine, St. Louis, MO) was prepared as a pool from the serum of adults vaccinated with PRP.

This pool was titrated at least four times in the subclass ELISAs in parallel with the PRP Standard Serum (OOB Lot 1983) obtained from CBER, Bethesda, Maryland. The anti-PRP IgG1 and IgG2 values for the PRP Standard Serum as determined by RIA of purified anti-PRP IgG subclass preparations were published previously (22), and at least four relative potency calculations were performed as described in Results in order to obtain a value for A144. The quantities for A144 were determined to be 9.64 μg anti-PRP IgG1/ml and 7.8 μg anti-PRP IgG2/ml.

Alkaline Phosphatase-Conjugated Anti-Human Subclass Reagents

Alkaline phosphatase-conjugated murine MAbs to human IgG1 (clone #JDC-1) and IgG2 (clone #AB4-DH4) were obtained from Southern Biotechnology Associates through Fisher Scientific (Pittsburgh, PA). The specificity of each enzyme-conjugated reagent was tested by direct ELISA with homologous and heterologous IgG subclass myeloma proteins. The 99% purity of IgG subclass isolates of human antibodies used to produce the MAbs also was verified by the manufacturer with an isotype-specific competitive RIA.

IgG Subclass ELISA

All incubations were performed in covered plates at ambient temperature (20-25°C) on a rocker platform (Bellco Biotechnology, Vineland, NJ; frequency 6.5). Polystyrene optically-certified microtiter plates (Corning #25801) were coated in duplicate wells with 0.1 ml of PRP-HSA conjugate at 5.0 μg PRP/ml diluted in 0.05

M $NaHCO_3$, pH 9.5. Duplicate wells were coated with 0.1 ml of the antigen dilution buffer as a control for non-specific binding to the plate. After an overnight incubation, the plates were washed three times with phosphate-buffered saline containing 0.05% Tween 20 (PBST) using a Cetus/Perkin Elmer (Norwalk, CT) ProWash system (0.5 ml/wash, 5 second soak/wash). Serial two-fold dilutions were made of the standard, test controls, and test sera in PBST + 1.0% (w/v) BSA, and 0.1 ml was loaded into each of four wells in the plate (two replicates with diluted antigen, two with antigen dilution buffer alone). After a two-hour incubation, plates were washed three times, and 0.1 ml of the previously- determined optimal dilution of the alkaline phosphatase-conjugated murine MAb anti-human IgG1 or IgG2 was added and incubated for two hours. Plates were washed and 0.1 ml of pre-warmed 1.0 mg p-nitrophenyl phosphate (Sigma, St. Louis, MO)/ml in 1.0 M diethanolamine, pH 9.8 was added. Color development at ambient temperature took place until the antigen-coated wells containing the first dilution of the standard curve attained an OD_{405} equal to 1.0-1.5. The enzyme-substrate reaction then was stopped by the addition of 0.05 ml 3.0 M NaOH/well. Plates then were read at OD_{405} on a BioTek EL310 platereader. Data was collected on a portable NEC 8300 and transferred into a four column format for processing as described in the statistical methods section.

Specificity Of Subclass ELISAs

The specificity of the anti-PRP IgG1 and IgG2 ELISAs for detection of anti-PRP over that of other bacterial polysaccharides was tested by incubating serial two-fold dilutions of the Human

Serum Standard with 5 or 100 μg/ml of 1) the Hib PRP-HSA conjugate used as the coating antigen in the ELISA, 2) unconjugated Hib PRP, 3) pneumococcal type 6B polysaccharide, or 4) pneumococcal type 14 polysaccharide. The serum/adsorbent solutions were incubated overnight at 4°C and tested directly in the ELISA. The reactivity of the adsorbed serum dilutions was compared to a similarly-incubated unadsorbed control.

Statistical Methods

Net OD was calculated for each sample as the ratio of average OD from PRP-coated wells and average OD from antigen buffer-coated wells. Dilutions of the standard were fit by a four-parameter logistic regression, using a program provided by the National Institutes of Health. The concentration of the standard was determined from a reference sample provided by CBER using standard parallel-line calibration methods, and μg anti-PRP/ml for unknown samples were interpolated from the standard curve in each run.

The sensitivity limit of each assay was calculated as described previously (23). The reproducibility was determined from multiple runs on a panel of heterogenous samples and is expressed as percent variability. Parallelism between test and standard samples was judged through dilution studies of several unknown samples.

RESULTS

Validation of IgG subclass ELISAs

To validate the anti-PRP IgG1 and IgG2 ELISAs, it was necessary to establish reproducibility, specificity, sensitivity and parallelism.

Reproducibility was determined by assaying the same panel of sera from four to eight times within an assay over the course of at least four separate assays. The average interassay percent variability of a sample was 21% in the anti-PRP IgG1 ELISA and 11% in the IgG2 assay. Control serum samples were included on every plate within every assay.

Specificity of the ELISAs was tested using passive adsorption with PRP and pneumococcal bacterial polysaccharides. Both ELISAs were found to be specific for the detection of anti-PRP (Figures 1 and 2).

The sensitivity of each subclass ELISA was determined from results using the quantitated serum standard A144 over fourteen assays performed on 4 to 5 different days. The sensitivity limit was calculated to be 0.020 μg anti-PRP IgG1/ml and 0.011 μg anti-PRP IgG2/ml in their respective assays.

The issue of parallelism of human and monkey sera to a human serum standard was addressed by analyzing results of several sera over serial two-fold dilutions, looking for concommitant two-fold differences in uncorrected titers as read from the human serum standard curve. The average fold difference in titer for a pair of serial two-fold dilutions was 2.01 for anti-PRP IgG1 and 2.04 for anti-PRP IgG2.

Quantitation Of Standard A144

The PRP Standard Serum has been assigned an RIA titer of 70 μg anti-PRP/ml by CBER. The assigning of subclass-specific anti-PRP titers to this standard using purified and RIA-quantitated anti-PRP subclass preparations was obtained

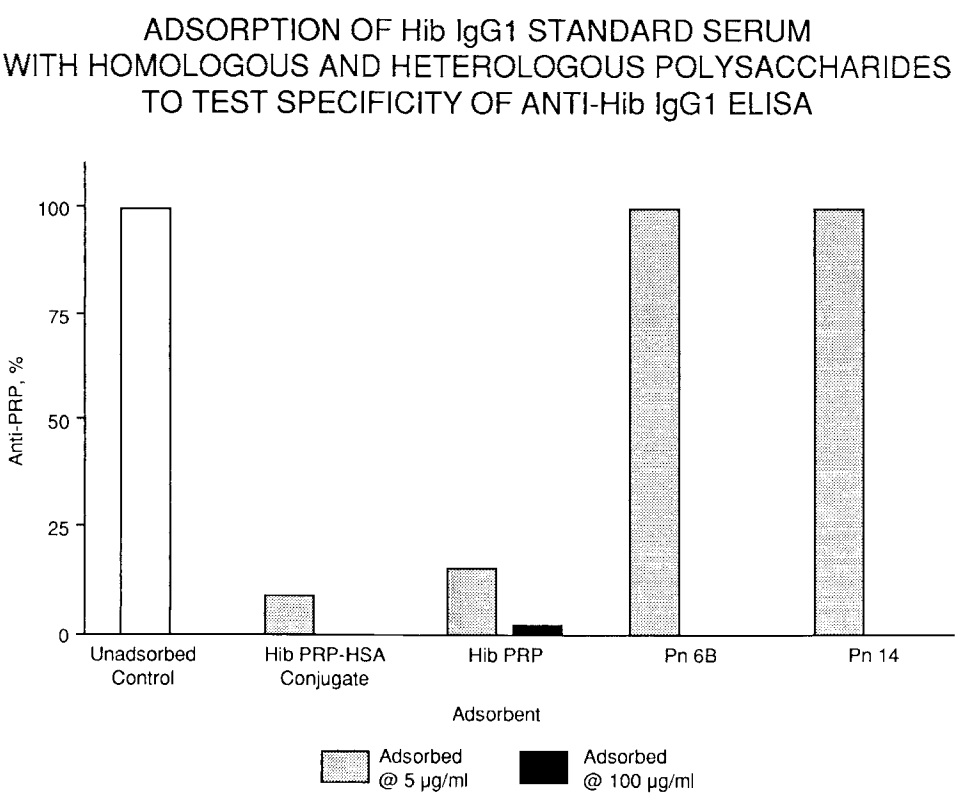

Fig. 1

Figures 1 and 2 Legend:

Competitive inhibition ELISAs were performed using homologous (Hib PRP-HSA conjugate, Hib PRP) and heterologous (pneumococcal types 6B and 14) bacterial polysaccharide preparations as described in Materials and Methods for anti-PRP IgG1 (Fig. 1) and anti-PRP IgG2 (Fig. 2). Adsorbed serum titers were compared to unadsorbed serum control values in each assay to obtain a % reactivity value.

through affinity chromatography (22); anti-PRP IgG subclass ELISAs were performed to assign relative IgG subclass values to the PRP Standard Serum. The values for this PRP Standard Serum were 27.0 µg anti-PRP IgG1/ml and 13.4 µg anti-PRP IgG2/ml and were used to

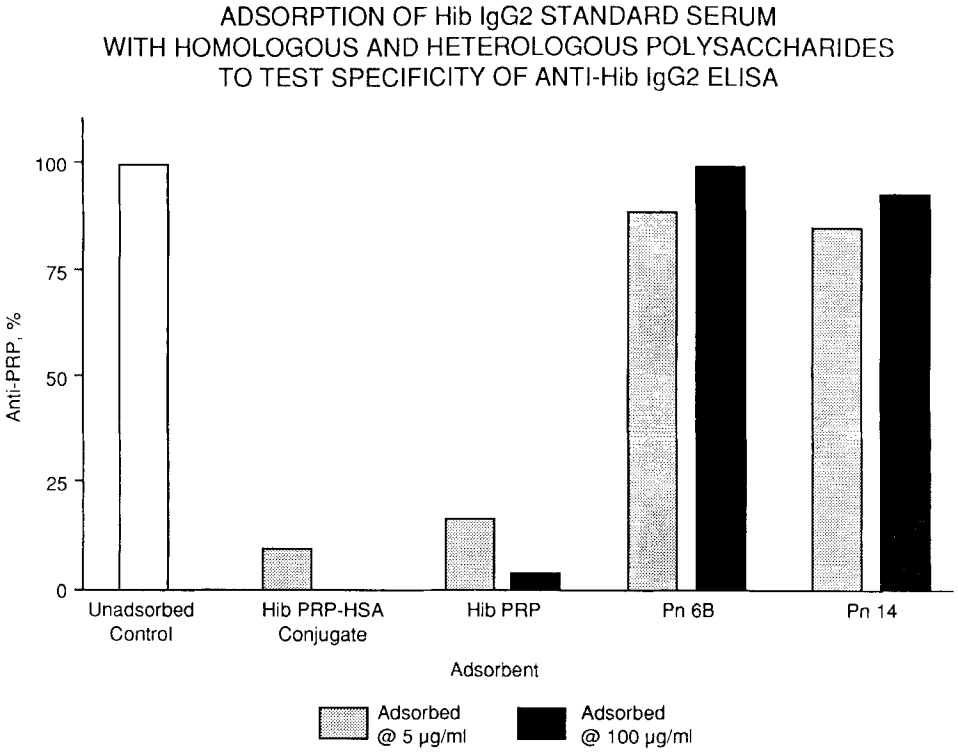

Fig. 2

assign comparable titers to the Human Serum Standard (A144). The relative potency of the PRP Standard Serum to the Human Serum Standard in the IgG1 assay was 2.80, resulting in a titer of 9.64 µg anti-PRP IgG1/ml in A144, and the relative potency in the IgG2 assay was 1.72, resulting in a titer of 7.80 µg anti-PRP IgG2/ml in A144.

Human Clinical Trials

The geometric mean anti-PRP RIA titers for PedvaxHIB®-immunized children and anti-PRP IgG subclass ELISA responses are

TABLE 1

Hib PRP-specific Antibody Responses in PedvaxHIB®-vaccinated Children

Age range, months	Doses, no.	GMT, μg anti-PRP/ml*								
		No. of subjects	RIA (95% CI)**	% >1.0 μg/ml	No. of subjects	ELISA IgG1 (95% CI)	% ≥1.0 μg/ml	No. of subjects	ELISA IgG2 (95% CI)	% ≥1.0 μg/ml
<12	2	271	5.42 (4.50, 6.03)	90	94	7.10 (5.66, 8.91)	94	39	<0.25 (<0.25, <0.25)	0
12-17	1	182	3.76 (3.11, 4.55)	84	82	3.87 (2.82, 5.30)	87	43	<0.25 (<0.25, <0.25)	0
18-23	1	110	8.88 (6.83, 11.53)	96	62	11.80 (7.92, 17.58)	94	35	0.44 (0.32, 0.59)	17
≥24	1	145	16.10 (13.03, 21.15)	96	74	14.57 (9.86, 21.51)	95	40	0.40 (0.28, 0.57)	15

* post 1 dose for children ≥12 months old, post 2 doses for children <12 months old

** 95% confidence interval

shown in Table 1. Ninety percent of children less than 12-months old attained anti-PRP levels in the RIA test of greater than 1.0 μg/ml after two doses of PRP-OMPC as did 84% of children 12- to 18-months old after one dose and 96% of children greater than 18-months old after one dose. IgG1 responders (≥1.0 μg of anti-PRP/ml) were comparable in all four age groups listed in Table 1. Response rates of 98%, 86%, 94% and 96% were observed for the <12 month, 12-17 month, 18-23 month and ≥24-month-old age groups, respectively; GMT of 7.14 μg, 4.18 μg, 11.26 μg and 14.40 μg anti-PRP/ml, respectively, were observed for each of the groups. IgG2 responders (≥1.0 μg of anti-PRP/ml) were not observed in the <12 month and 12-17 month-old age groups while 21% and 16% responder rates were observed for the 18-23 month and ≥24 month-old age groups, respectively. The geometric mean titer of the IgG2 anti-PRP response in children less than 18-months old was <0.25 ug/ml and for all age groups was <0.5 μg/ml.

Infant Rhesus monkey studies

The infant Rhesus monkey was developed as a preclinical immunogenicity model for PRP-OMPC and has proven to be an excellent predictor of the ability of different vaccines to induce protective immune responses in the pediatric population (11). The IgG subclass responses of the infant Rhesus monkey were similar to those of children in demonstrating IgG1-restricted anti-PRP IgG responses; no anti-PRP IgG2 was detectable in any of the monkey sera tested (Table 2).

TABLE 2

Anti-PRP Responses in PedvaxHIB®-Immunized Infant Rhesus Monkeys

No. of subjects	Bleeding	GMT, μg anti-PRP/mL		
			ELISA	
		RIA	IgG1	IgG2
9	pre	<0.13	<0.60	<0.25
9	post	112.0	150.56	<0.25

DISCUSSION

The primary objective in developing a bacterial vaccine is to use an immunogen which will stimulate antibodies (serum bactericidal and opsonins) capable of interfering with an essential functional bacterial activity. The development of serological assays to delineate the IgG subclass specificity of vaccine-induced immune responses is necessary to help further an understanding of the mechanisms involved in such responses. There have been reports of functional activity for anti-PRP IgG1 and IgG2 in serum bactericidal and opsonin assays, but varying potencies have been observed (18,24). Until the biological or genetic regulation of IgG subclass antibody induction is better understood and the roles that the separate IgG subclasses play in

protection from natural bacterial challenge are defined more clearly, the strategies for the design of bacterial vaccines will be restricted to manipulations which enhance the overall immune response, or total antibody production, while still promoting functional activity (25). The pediatric population of less than 18 months of age is incapable of mounting immune responses to purified bacterial polysaccharides. Manipulations of PRP have included the conjugation of the PRP to proteins such as diphtheria toxoid, ProHIBit® (9) or its variant, HibTITER® (16), tetanus toxoid (7), or an OMPC of NMB in the case of PedvaxHIB® (12). Covalent attachment of these proteins to PRP confers T-dependent properties to the PRP. The anti-PRP IgG subclass ratio shifts, from equal amounts of IgG1 and IgG2 as seen in adults vaccinated with PRP or other polysaccharides (16,19,20,21) to heavily favor IgG1 as seen in this and other studies of conjugate vaccines in children (7,8,9,20,26) due to the T-dependent nature of the protein component (9,17,18). The fact that bacterial proteins such as tetanus toxoid have been shown to induce primarily IgG1 in adults and children (27,28) supports this view.

The ELISA currently is employed by many other investigators for analysis of anti-PRP levels in serum (7,17,29,30,31). Our subclass assays have been shown to be reproducible, sensitive, and specific for the detection of anti-PRP. The use of an alkaline phosphatase-conjugated murine MAb to human IgG1 or IgG2 enhanced the specificity of the ELISA when compared to the use of a polyclonal alkaline phosphatase-conjugated anti-human IgG in an

ELISA for anti-PRP IgG (data not shown). The sensitivity of the subclass ELISAs was only slightly lessened as determined by the aforementioned comparison and may be explained by recognizing that MAbs are known to have generally less avidity than polyclonal preparations (32).

As more laboratories become involved in the analysis of the immune responses to this relatively new family of bacterial conjugate vaccines, the need for interlaboratory comparison of results is evident. Uniform and precise quantitation of the anti-PRP IgG1 and IgG2 levels is therefore very important in order to avoid variation in results as reported in OD units or reciprocal dilutions. Use of the ELISA for analysis of IgG subclass content in serum is widespread, but antigen-specific subclass quantitation is not obtained so easily. Therefore few investigators report their results in terms of concentration. However, interlaboratory variation in technique can lead to wide discrepancies in interpretation of results, and the subclass ELISA ultimately may require standardization by a central laboratory which would distribute quantitated standards to investigators interested in studying subclass immune responses to Hib conjugate vaccines. In the meantime, indirect quantitation methods such as those used by this laboratory allow a closer comparison of results between laboratories than those methods involving endpoint or reciprocal dilution titers.

As our technique is run currently, the results are obtained in as few as 24 hours post-antigen coating; this time can be

reduced further by employing an automated platewasher that washes the entire microtiter plate at once. The use of alkaline phosphatase-conjugated murine MAb anti-human IgG subclass reagents allows direct detection of bound antibody. However, since these reagents are not widely available, the investigator is reliant on the manufacturer for consistent production of enzymatically-active materials. The ELISA is adaptable to automation at every step, from loading plates to washing to reading optical densities to data analysis. Unlike the RIA, the reagents are not radioactive and involve no toxic or carcinogenic materials.

The development of the IgG subclass ELISAs also allows for independent verification of the results obtained for total antibody response using the RIA. The anti-PRP IgG1 and IgG2 responses in PRP-OMPC-immunized children as young as two months old serve as confirmation of the anti-PRP RIA titers in proving that this vaccine is highly immunogenic, even after one dose. Functional activity in the form of serum bactericidal antibody and passive protection of infant rats is evident in the majority of children with an anti-PRP RIA titer of >1.0 μg/ml (10). An efficacy study with PRP-OMPC has demonstrated that it is highly efficacious at preventing invasive Hib diseases in young infants who are most at risk (32). The magnitude of the responses is worthy of note, especially considering the previously documented nonimmunogenicity of unconjugated PRP in children less than 18 months old (33,34,35,36).

The infant Rhesus monkeys mounted an equally impressive antibody response to two doses of PedvaxHIB® but were unresponsive

to PRP alone (11). In further support of the use of infant Rhesus monkeys as a preclinical immunogenicity model, the IgG subclass ELISAs showed a response comparable to that of the vaccinated children with respect to IgG subclass composition, being restricted to anti-PRP IgG1.

The development and standardization of the anti-PRP IgG subclass ELISAs enables a further definition of the components of the T-dependent immune response elicited in young infants and children who are immunized with PedvaxHIB®. Identification of IgG1 as the dominant subclass induced during the primary immunization series suggests that the PRP acts in conjunction with the protein carrier as a T-dependent immunogen. Studies of the functional capability of IgG1 versus IgG2 show similar potencies for the two subclasses and the anti-PRP IgG1 elicited in these studies clearly has such activity. Preliminary data obtained in this laboratory show that a booster dose of PRP-OMPC given to 17 to twenty-four month olds one year or more after a primary immunization results in a shift in the anti-PRP IgG1 to IgG2 ratio from 36:1 to 8:1; however the significance of this in terms of functional activity remains to be defined. All the parameters examined so far, i.e., safety profile, tolerability, immunogenicity in the target population, and functional activity, have been favorable regarding the development of this new Hib conjugate vaccine, PedvaxHIB®.

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