Vaccine Design

The Subunit and Adjuvant Approach

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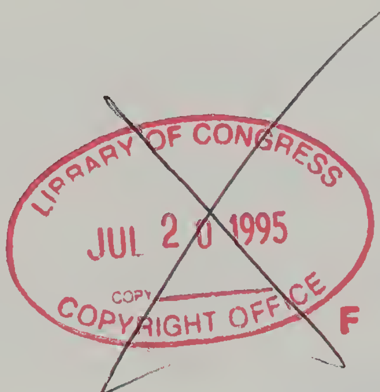

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Chapter 30

Haemophilus influenzae Type b Conjugate Vaccines

Peter J. Kniskern, Stephen Marburg, and Ronald W. Ellis

1. INTRODUCTION

Haemophilus influenzae, a gram-negative rod-shaped bacterium, is a major human pathogen that causes a range of diseases in infants, children, and adults. There are six antigenic types of *H. influenzae*, termed a–f, whose antigenic specificity is provided by the capsular polysaccharide (Ps). There also are nontypeable strains which lack a capsular Ps. *H. influenzae* type b (Hib) is the most pathogenic *H. influenzae* strain for infants and young children and a major cause of invasive bacterial infections. Until the 1990s, Hib caused an estimated 10,000 cases of meningitis per year in children 2 months to 5 years of age in the United States (Vadheim and Ward, 1994). The mortality rate is up to 5% in developed countries, and up to 35% of survivors may develop permanent neurological sequelae. Other invasive Hib infections include cellulitis, empyema, endocarditis, endophthalmitis, epididymitis, epiglottitis, osteomyelitis, pericarditis, pneumonia, septic arthritis, and tracheitis (Kaplan, 1994). While the total incidence of such Hib infections was approximately equal to that of Hib meningitis in the United States, these diseases vary significantly in relative incidence elsewhere in the world. As a result of the development and widespread use of Hib conjugate vaccines in several developed countries, the incidence of invasive Hib diseases has decreased by about 95% in the United States (M.M.W.R., 1994) and 60–80% in other countries (Vadheim and Ward, 1994).

The most significant epidemiological feature of invasive Hib diseases in unvaccinated populations is its age-related incidence, where the risk is generally highest at approximately 6 to 12 months of age. In many areas the risk is lower below 6 months of age because of transplacental acquisition of maternal antibodies. The risk decreases with age above 2

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years, presumably as a result of immune maturation as well as exposure to other pathogens serologically cross-reactive with Hib. While the incidence of invasive Hib disease is lower in developed than in developing countries, there are selected populations with significantly higher incidence of disease. For example, Australian aborigines, Eskimos, and native Indian populations of North America have a higher incidence than their respective country averages, perhaps because they live under conditions similar to those in developing areas (Vadheim and Ward, 1994).

A series of classical experiments established the Hib capsular Ps as a candidate vaccine antigen. The basis for antibodies mediating in vitro protection against Hib first was shown in bactericidal assays with normal human sera (Fothergill *et al.*, 1933). Hyperimmune rabbit antisera to Hib then were employed successfully for passive immunotherapy of Hib infections (Alexander *et al.*, 1943); the activity of such sera could be absorbed by purified Hib capsular Ps (Alexander *et al.*, 1944). The Ps was purified and its structure shown to be polyribosylribitol phosphate (PRP) (Crisel *et al.*, 1975). PRP was used to develop a Farr-type radioimmunoassay (RIA) to quantify serum antibodies to PRP (anti-PRP) (Robbins *et al.*, 1973). Levels of such antibodies were shown to correlate with protection from invasive Hib disease (Peltola *et al.*, 1977).

On the basis of these studies, purified PRP was developed into a vaccine. It was shown to be immunogenic in older children and adults, but not in children less than 2 years of age (Robbins *et al.*, 1973; Anderson *et al.*, 1977). When PRP vaccine was tested in a double-blind controlled efficacy trial in 50,000 infants and children in Finland, the rate of efficacy in children 18–71 months old was 90%, but no protection was observed in children less than 18 months old (Peltola *et al.*, 1984). On this basis, the vaccine was licensed in the United States in 1985 for routine use in children at least 2 years old and in children 18–23 months of age at high risk of Hib infection. Long-term protection was shown to correlate with anti-PRP levels ≥ 1.0 μg/mL and short-term protection with levels ≥ 0.15 μg/mL, as quantified by RIA (Käyhty *et al.*, 1983). Nevertheless, since most invasive Hib disease occurs in children less than 18 months old, a second-generation Hib vaccine was needed for optimal disease control.

The strategy for developing a second-generation Hib vaccine can be rationalized from immunological considerations. As is the case for most types of Ps, PRP is a T-independent (TI) antigen, which stimulates B lymphocytes directly to produce anti-PRP without the participation of T lymphocytes such that immunological memory is not established. TI antigens are not immunogenic in infants and young children. On the other hand, T-dependent (TD) antigens, such as proteins, stimulate helper T lymphocytes to interact with B lymphocytes, causing the latter to produce anti-PRP. In the course of the TD immune response, immunological memory is established, antibody class-switching occurs, and antibody affinity increases as a result of somatic mutations in anti-PRP immunoglobulin genes. Since TD antigens are immunogenic in infants and young children, the conversion of PRP from a TI to a TD antigen would be expected to make it immunogenic in infants.

The principle of coupling Ps to protein to increase its immunogenicity had long been established (Goebel and Avery, 1931). Thus, the covalent coupling of PRP to a carrier protein would be expected to convert PRP to a TD antigen that would elicit anti-PRP in infants. This is the immunological basis for Hib conjugate vaccines, the second-generation

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Hib vaccines that have been shown to protect infants from invasive Hib diseases as described below.

2. RATIONAL CONJUGATE DESIGN, CHEMISTRY, AND PROCESS CONTROL

2.1. Outline of the Processes

Fundamentally, the processes developed for the four licensed vaccines (Table I) differ from one another in the following attributes: (1) the choice of protein carrier, (2) the size of the starting PRP, (3) the linking or bridging molecule, (4) the architecture of the chemical process steps, i.e., whether the processes flow in a single, sequential stream (PRP-TT and HbOC) or in two parallel derivatization streams which join at the point of chemical conjugation (PRP-OMPC and PRP-DT), and (5) the process steps used to remove unconjugated PRP and other reactants. These differences and other attributes of the four processes are discussed in more detail in the following sections. The quantitative aspects of covalency and removal of unconjugated PRP also are discussed in Sections 2.2 and 2.4, respectively.

2.1.1. PRP-D

This process (Gordon, 1986) begins by hydrolyzing the PRP to reduce its molecular weight, after which it is activated with cyanogen bromide to an electrophilic species (considered to be a cyanate ester). The protein (Pr) carrier for PRP-DT is diphtheria toxoid (DT), which is prepared by formalin treatment of diphtheria toxin secreted by *Corynebacterium diphtheria*. In a second process stream, the carboxyl groups of DT are functionalized with adipic acid dihydrazide, using the water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) as the condensing agent, creating a nucleophilic DT derivative. The electrophilic and nucleophilic intermediates then react to form the conjugate product (depicted below and as a schematic model in Fig. 1A) which is purified by ammonium sulfate precipitation:

$$\mathit{DT}\text{–HNNHCO}(\text{CH}_2)_4\text{CONHNHC}(=\text{N})\text{O–}\mathit{PRP}$$

Analytical size-exclusion chromatography (SEC) was used to provide indirect proof of covalency (see Section 2.2.1).

2.1.2. PRP-OMPC

This process also proceeds in two streams (Marburg *et al.*, 1986, 1987, 1989). The first, which involves derivatization of native PRP, differs from the equivalent steps used in the other three conjugation processes in that it takes place in a nonaqueous system, eliminating the nucleophilic competition between the hydroxyl groups on PRP and the hydroxyls of water. To accomplish this, PRP is converted from the calcium salt to the dimethylformamide (DMF)-soluble tetrabutyl ammonium salt by titration with tetrabutylammonium hydroxide. This hydrophobic PRP salt is reacted in DMF with carbonyl diimidazole (CDI), which activates some of the PRP hydroxyls to the electrophilic

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Table I
Characteristics[a] of the Carrier Proteins and Polysaccharides

Conjugate name[a]	Trade name	Manufacturer	Carrier protein (Pr)	Mass (kDa) of Pr	PRP sizing method	Mass (Da) of PRP[b]
HbOC	HibTITER®[c]	Lederle–Praxis	Mutant diphtheria toxin[d]	58	$NaIO_4$ treatment	3400–11,000
PRP-DT	ProHIBit®	Connaught	Diphtheria toxoid[e]	58	Hydrolysis @ 100°C	2×10^5–2×10^7
PRP-OMPC	PedvaxHIB®	Merck	Outer membrane protein complex[f]	50,000	Oxalic acid treatment[g]	~8×10^4
PRP-TT	ActHIB® OmniHIB®[h]	Pasteur–Merieux	Tetanus toxoid[e]	150	None	~1×10^5

[a]See Section 2 for relevant reference citations and abbreviations for conjugate names.
[b]Values as reported in the literature or estimated from K_d data reported in the literature.
[c]Also available in combination with DTP as Tetramune®.
[d]Cross-reactive material (CRM_{197}).
[e]Characteristics are given for the toxin prior to toxoiding.
[f]Isolated from *Neisseria meningitidis* serogroup B.
[g]This is not a sizing operation *per se*, but rather is the result of a chemical event that occurs during the process.
[h]Distributed by SmithKline Beecham in the United States only.

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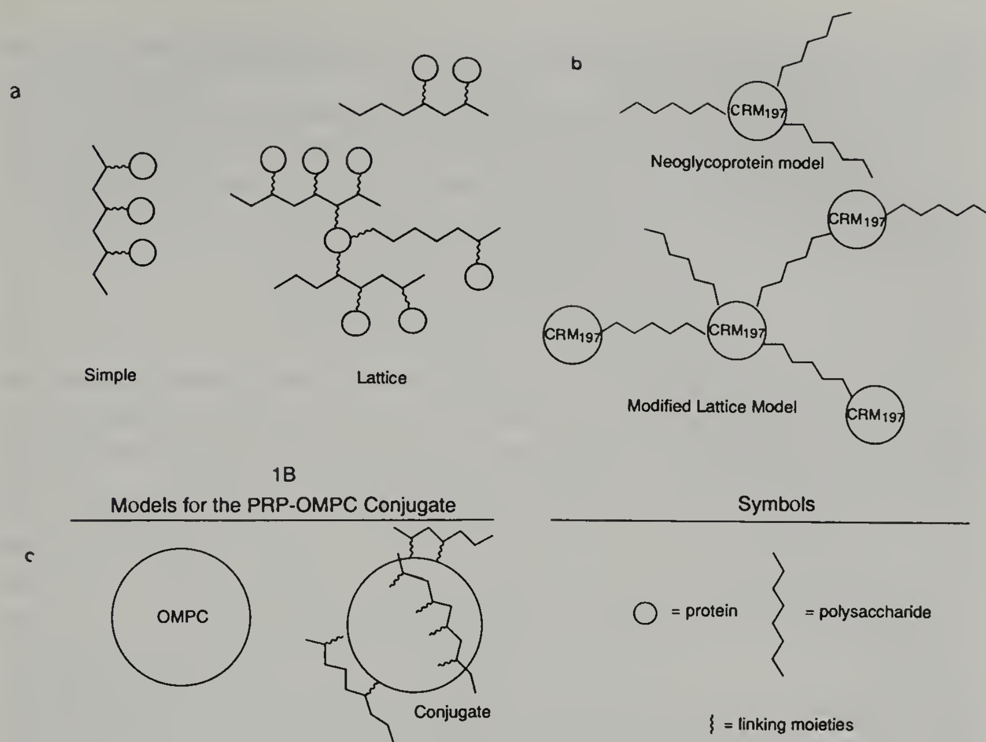


Figure 1. (A) Diagrammatic representation of PRP-DT and PRP-TT. Because of the polyfunctionality of both Pr and Ps, the products are a mixture of geometries which are simple lattice types.

(B) Diagrammatic representation of the PRP-OMPC conjugate. In this case, there are a number of Ps molecules of varying chain length linked through bridging moieties to each large OMPC particle. The term "ladder-type" conjugate seems appropriate in describing the OMPC conjugates. This type of structure differs from the lattice type associated with the PRP-DT and PRP-TT vaccines in that each Ps molecule is linked to only one Pr moiety.

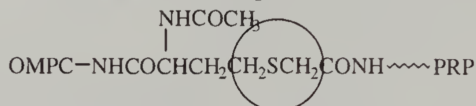
(C) Diagrammatic representation of HbOC. This conjugate can be described as a mixture of a neoglycoprotein model (Dick and Bevrett, 1989) and a lattice (the latter being a modified lattice because the PRP fragment is only bi- and not polyfunctional).

imidazolyl urethane function. The activated PRP then is reacted directly with an aqueous solution of 1,4-butane diamine (BuA_2); the NMR spectrum also indicates that little if any cross-linking has occurred. The final step in the PRP derivatization stream involves the bromoacetylation of the derivatized PRP, which is monitored by NMR for degree of bromoacetylation and derivatization.

The second, parallel process stream functionalizes the Pr carrier which is the outer membrane protein complex (OMPC) from *Neisseria meningitidis* serogroup B. This carrier is a particle (~ 50,000 kDa) that forms a stable colloidal suspension in aqueous systems. This high mass allows OMPC and its conjugates to be ultracentrifuged to a pellet for separation from the soluble nonconjugated PRP. The conjugate also can be retained by ultrafiltration membranes through which unconjugated PRP can permeate. In order to form its thiolated derivative, the OMPC is reacted with *N*-acetylhomocysteine thiolactone; the

number of sulfhydryl groups per gram of Pr is determined by Ellman (Ellman, 1959) and Lowry (Lowry *et al.*, 1951) assays.

At this point, the bromoacetylated PRP and thiolated OMPC are reacted to form a thioether bond by bromide displacement from the activated PRP with the thiol functionality on the OMPC. This creates the conjugate, which is shown schematically in Fig. 1B. The thioether (the encircled atoms in the structure depicted below) is the relevant covalent bond:



It is unlikely that a significant percentage of Pr is cross-linked, since the final conjugation reaction takes place in the presence of a large excess of activated PRP and light scattering analysis and scanning electron microscopy indicate little change in particle size distribution (J. Hennessey, personal communication).

Excess bromoacetyl groups in the conjugate (those that did not react with the OMPC thiols) next are reacted with *N*-acetylcysteamine to form a "capped" conjugate (~ 50,000 kDa) which then is separated from unconjugated PRP (~ 80 kDa) by diafiltration. The final capped conjugate is characterized as described in Section 3, and an aliquot is hydrolyzed with HCl to cleave all of the peptide bonds in the Pr and the amide bonds. In addition to all of the component amino acids of OMPC, two novel amino acids, *S*-carboxymethylhomocysteine (SCMHC) and *S*-carboxymethylcysteamine (SCMC), are released and are detectable in the amino acid analysis spectrum. This approach for defining covalency has been termed the "bigeneric spacer" method (Marburg *et al.*, 1986, 1987, 1989).

2.1.3. PRP-TT

This vaccine (Clemens *et al.*, 1992) is based on the method of Chu *et al.* (1983) and Schneerson *et al.* (1986). In a single process stream, the native PRP is activated with cyanogen bromide and reacted in situ with adipic acid dihydrazide to form a nucleophilic derivative. As discussed further in Section 2.3, the degree of derivatization (the number of adipic hydrazide units per 100 repeat units of PRP) for this intermediate corresponds to about a 2% weight loading, which is approximately 4 adipic hydrazide units per 100 repeat units of PRP. The carrier protein in this case is tetanus toxoid (TT) which is obtained by formalin treatment of tetanus toxin secreted by *Clostridium tetani*. The toxin has a mass of approximately 150 kDa prior to toxoiding (Mangalo *et al.*, 1968). To form the conjugate (depicted below and as a schematic model in Fig. 1A), the carboxyl groups of TT are condensed with the hydrazide group of activated PRP with EDAC:

Low-mass by-products are removed by SEC. The high-mass material contains the immunogen, which is characterized as described in Section 3.

2.1.4. HbOC

This methodology differs significantly from the other three processes in that the first step in the PRP derivatization process requires generation of aldehyde functionalities in

the PRP. This is accomplished by using sodium metaperiodate to cleave the vicinal diols in PRP (Anderson and Eby, 1990), resulting in concomitant size reduction of the PRP polymer to small oligosaccharide fragments. The products of the reaction are fractionated by ultrafiltration. The degree of polymerization, defined as the number of repeat units in a single molecule, is 15–30 and the weight-average is approximately 20.

The Pr carrier used for HbOC is a nontoxic, genetic mutant of diphtheria toxin designated CRM_{197} (58 kDa) (Pappenheimer *et al.,* 1972; Colombatti *et al.,* 1989) which is antigenically equivalent to DT. The use of CRM_{197} offers a potential advantage as carrier over a chemically-created toxoid, because more nucleophilic nitrogens are available to form bonds with electrophilic partners such as aldehyde groups since none of the available lysines in CRM_{197} were reacted with formaldehyde in a toxoiding process.

The bond-forming chemistry used for this process is termed reductive amination and involves the reaction of an amine (from the Pr) with an aldehyde (from the periodate-treated PRP). The resultant Schiff base is reduced in situ by sodium cyanoborohydride, resulting in an alkylated amine. Thus, the activated PRP and CRM_{197} are reacted to form the conjugate:

$$CRM_{197}\text{–NHCH}_2\text{CH}_2\text{OPO}_2\text{–}(PRP)_{15\text{–}30}\text{–OCH}_2\text{CH}_2\text{NH–}CRM_{197}$$

which also is depicted as a schematic model in Fig. 1C.

The unreacted aldehydes then are reduced with sodium borohydride, and the conjugate is ultrafiltered to remove small reagents and unconjugated PRP. The retentate contains the conjugate, which is characterized as described in Section 3. Since the activated PRP previously was fractionated by ultrafiltration, the copresence of Ps and Pr in the retentate is indirect support for covalency (see Section 2.2 for discussion on direct proof for covalency).

2.2. Process Design and Quantitative Aspects of Covalency

As discussed in Section 1, the immunological basis for the Hib conjugate vaccines is the covalent coupling of PRP to a carrier protein, thereby converting PRP to a TD antigen. If PRP and carrier protein are linked by noncovalent forces, their separation in vivo becomes possible and the efficacy of the conjugate potentially is compromised. Therefore, a covalent bond probably is required, and all of the processes for the licensed Hib conjugates have been designed to use chemical reactions that predictably will achieve covalent linkage. However, the products of these conjugation reactions are of high mass, and the conventional physicochemical analyses of structure are not applicable for demonstrating that covalent bonds have been formed. Therefore, classical approaches to defining the structural nature of the product(s) are not practical for conjugates, and the determination of the covalency of the bond is addressed in two ways.

2.2.1. INDIRECT PROOF OF COVALENCY

This determination relies on separation of products and starting materials on the basis of size or physiochemical property. If Pr and Ps appear, after such a separation, in the same "compartment" (for example, a chromatographic fraction), and such a compartment is

different from the "compartments" into which the reactants would separate, it can be concluded that a bond between the two must have been formed. However, these observations do not specify what type of bond (noncovalent versus covalent) exists. All of the conjugates provide methodologies for indirect confirmation of covalency.

2.2.2. DIRECT ASSAYS FOR PROOF AND QUANTITATION OF COVALENCY

Assays that detect a new amino acid that was created during conjugation can provide further proof that a covalent bond has been formed. Since the constituent amino acids of the carrier Pr and the new amino acid formed during conjugation are released by hydrolysis and determined in the same assay, a constituent amino acid can be used as an internal standard. Consequently a ratio can be calculated to define the degree of covalency, a value that is useful as a process control parameter. Because of the fundamental differences among the respective bond-forming chemistries, the optimal degree of covalency must be determined empirically for each conjugate type. Within each vaccine type, such assays, which verify that this ratio is within the specifications, provide another critical process control parameter, along with the Ps/Pr ratio, to verify consistency of manufacture.

As discussed in Section 2.1.2 for PRP-OMPC, SCMHC and SCMC are determined and these values, when referenced internally to a constituent of the conjugate, can be used to calculate the number of covalent bonds formed and the number of unreacted side chains capped after the conjugation reaction. The importance of such analytical control was illustrated clearly when certain postlicensure lots of PRP-OMPC were shown to have less-than-expected immunogenicity in the field. A thorough investigation of this issue by the manufacturer revealed that overderivatization of a subpopulation of PRP molecules with BuA_2 had occurred for some lots. This overderivatized population then preferentially reacted with the thiolated OMPC; this was responsible for the reduced immunogenicity. This phenomenon has been referred to as "hyperconjugation," which has been controlled by a process modification. In addition, levels of SCMHC and SCMC now have been employed to establish release specifications for the ratios of SCMHC/PRP and (SCMHC + SCMC)/PRP, the limits of which provide a check of all lots to ensure that the implemented changes continue to control the process and eliminate possibly hyperconjugated lots.

For HbOC, two types of analysis were developed to provide additional evidence for covalency (Smith *et al.*, 1989; Seid *et al.*, 1989). The first employs demonstration of the coincidence of antigenic reactivity to PRP and to CRM_{197} after SDS-PAGE and immunoblotting. The second approach follows the strategy developed for PRP-OMPC (*vide supra*) and involves the detection of the new amino acid formed when the lysines on the CRM_{197} are reductively aminated with the activated PRP. When the resultant conjugate is hydrolyzed in acid, N_{ε}-(2-hydroxyethyl)lysine is formed and detected by amino acid analysis.

2.3. Characterization of Starting Materials and Process Intermediates

Heretofore, vaccine materials were natural products, characterized by their isolation processes and the characteristics of the final immunogen. In the case of conjugate vaccines, these natural products are transformed into a series of chemical intermediates that require

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definition just as do the intermediates in a multistep organic chemical synthesis. This staged analytical system not only provides control of the overall process but also affords an understanding of the final product in structural terms.

The characteristics of the protein carriers (Table I) have been important factors in the development of these vaccines in that they have dictated: (1) the processes used for conjugation and reactant removal; (2) the properties of the final conjugates that bear on vaccine formulation and the immune response to it; and (3) the analytical assays required for characterization of process intermediates and of the final conjugate immunogens. In addition for PRP-TT and PRP-DT, the reaction of the epsilon-amino groups of lysine by formalin treatment in the toxoiding step has implications for subsequent process steps for PRP-DT and PRP-TT. Therefore, this toxoiding step should be consistent for different lots of DT and TT and for any formalin-treated proteins that might be used for other future conjugates.

Numerous publications and patents exist describing the purification and characterization of PRP (Anderson *et al.*, 1976; Kniskern *et al.*, 1981), which has a repeating unit composed of ribose, ribitol, and phosphate in equimolar ratios. Specifications (WHO Technical Report, 1991) require that the PRP preparations must contain > 32% ribose, 6.8–9.0% phosphorus, <1% protein, and <1% nucleic acid content. PRP is a family of polymers that vary in the number of the repeating units (Crisel *et al.*, 1975), hence molecular weight (Table I). In some cases an index of the molecular weight heterogeneity (sometimes termed polydispersity) also is determined (Hennessey *et al.*, 1993). For PRP-DT, PRP-OMPC, and PRP-TT, the size of the PRP that enters the conjugation reaction is only slightly smaller than Ps shed by the Hib bacterium growing in liquid culture. These PRP molecules initially still have several hundred repeating units, but may suffer some size degradation in the process of chemical modification. For the case of HbOC, the chemistry employed results in the partial depolymerization of PRP. By controlling the amount of periodate used for the oxidative depolymerization reaction, oligosaccharide fragments are obtained that are within the range of sizes that result in an immunogenic conjugate (Anderson *et al.*, 1989).

For all of the Hib conjugates, using either oligo- or polysaccharides results in an efficacious vaccine. This indicates that some or all of the protective epitopes are displayed on the final conjugate molecules. It should be noted, however, that some Ps, notably those of *N. meningitidis* B (Lifely *et al.*, 1987), *Streptococcus pneumoniae* type 14 (Wessels and Kasper, 1989) and Group B Streptococcus type III (Jennings *et al.*, 1981), display conformational epitopes that elicit neutralizing antibodies. It is not known whether PRP contains conformational epitopes, but it must be kept in mind that such epitopes may be required for a neutralizing antibody response. Therefore, in the development of new Ps-Pr conjugates, it may be critical that synthesis of a particular conjugate start with Ps of sufficiently high molecular weight to ensure that such size-associated conformers are present in the final conjugate. These data usually are determined immunologically by RIA, ELISA, or nephelose methods which are used to verify that antigenicity has been preserved.

For all of the processes, the derivatized PRP is characterized to ensure consistent functionalization. For the conjugates that proceed in two process streams (PRP-DT and PRP-OMPC), the derivatized Pr carrier also is subject to analytical control. Manufacturers also conduct additional analyses, such as verification of antigenic integrity.

2.4. Separation of Unconjugated Polysaccharide

It is unclear whether rigorous removal of unconjugated Pr from the final products is necessary. However, the effective dosages of the vaccines are based on *conjugated* PRP, and the release assays cannot discriminate between conjugated and unconjugated PRP. Furthermore, there is some evidence from animal immunogenicity studies that the presence of free Ps in a conjugate preparation can reduce the desired immune response (Peeters *et al.*, 1992). Therefore, processes must be employed to reduce the free Ps to appropriate low levels. Processes generally rely on biochemical separations based on the size differences of the conjugates and the free Ps; these size-based separations are most efficient if the starting Ps and Pr have molecular weights that differ by approximately 10-fold (Kniskern and Marburg, 1994). For the licensed vaccines, the specifications for unconjugated PRP (expressed as percent free Ps) are in the range of 10–37% (Table III).

3. CHARACTERIZATION OF BULK CONJUGATES AND FINAL CONTAINER VACCINES

Table II summarizes the assays and methodologies used to release and follow the stability of the Hib conjugate vaccines. Table III provides a compilation of the characteristics of the different immunogens and their corresponding vaccine formulations. Clearly, the inherent differences in the vaccine designs and processes have resulted in as many differences as common features among these products.

The vaccine content of the final container is calculated from the PRP assay. The PRP/Pr ratios vary considerably among the licensed conjugates. However, within a vaccine type, this determination will give an important indication of process consistency. The PRP-OMPC and HbOC conjugates also would be expected to have unique specifications related to the newly synthesized amino acids which relate to degree of conjugation. These parameters, along with the immunochemical and immunobiological assays discussed below, form the basis for releasing consistent and stable vaccine with predictably assured immunogenicity and efficacy in the target population.

The conjugated PRP must maintain its antigenic epitopes which are displayed as structural components on the bacterial capsule in order to elicit antibodies that bind to the bacterium and initiate complement-dependent bacteriolysis and phagocytic destruction of the Hib organism. Chemical assays for PRP do not measure antigenicity, which is determined by binding experiments using antibodies raised to native PRP as displayed on the bacterial capsule (van Dam *et al.*, 1990; Kniskern and Marburg, 1994). Some or all of this antigenicity can be lost during the processing steps. Therefore, such quality control is important not only in the final product but at each phase of a multistep process.

Because antigenicity *a priori* does not predict immunogenicity, the determination of the ability of the final vaccine to elicit functional antibodies in an appropriate animal model is critical. These tests, which will be discussed here only in terms of process control, rejection criteria, and release of vaccine, have been reviewed in detail (Vella and Ellis, 1992). Such in vivo assays, which measure the ability of conjugate vaccines to induce anti-PRP antibodies in experimental animals, have been developed in mice (Chu *et al.*, 1983; Vella *et al.*, 1990) and in infant rabbits (Anderson and Smith, 1977); nonconjugated

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Table II
Characterization Assays

Chemical and physical assays	Method[a]	Used for conjugate: HbOC	PRP-DT	PRP-OMPC	PRP-TT
Assays on Ps and intermediates					
Molecular weight	SEC or UF	Yes	Yes	Yes	Yes
Degree of derivatization or functionalization	NMR	Yes	No	Yes	No
Antigenicity	Nephelose or RIA	Yes	Yes	Yes	Yes
Assays on Pr and intermediates					
Degree of derivatization or functionalization	Ellman	No	No	Yes	No
Assays on conjugate					
Concentration (dosage) Ps	Orcinol	Yes	Yes	Yes	Yes
Concentration Pr	Lowry	Yes	Yes	Yes	Yes
Ps/Pr ratio	Calculation				
Percent unconjugated Ps	SEC or UF	Yes	Yes	Yes	Yes
Direct assay of covalent linkage	AA analysis	Yes	No	Yes	No
Degree of cross-linking	AA analysis	Yes	No	Yes	No
Antigenicity					

(*continued*)

Table II
(continued)

Chemical and physical assays	Method[a]	Used for conjugate			
		HbOC	PRP-DT	PRP-OMPC	PRP-TT
Preclinical Immunogenicity					
In vivo potency (ED_{50})	Adult mice or rabbits	Yes	Yes	Yes	Yes
T-cell dependency	Athymic (nude) mice	Yes	Yes	Yes	Yes
Infant primate immunogenicity	Infant Rhesus monkeys	No	No	Yes	No
Safety					
Pyrogen	Rabbit thermal induction	Yes	Yes	Yes	Yes
LPS	LAL	Yes	Yes	No	Yes
General safety	Mice and guinea pigs	Yes	Yes	Yes	Yes
Stability-indicating assays					
Antigenicity (in vitro potency)	Nephelose or RIA	Yes	Yes	Yes	Yes
In vivo potency	Adult mice or rabbits	Yes	Yes	Yes	Yes
% unconjugated PRP	SEC or UF or UC	Yes	Yes	Yes	Yes
pH		NS	NS	Yes	NS
Completeness of adsorption[b]	Nephelose or RIA	No	No	Yes	No
Covalency	AA analysis	NS	NS	Yes	NS

[a]Abbreviations: SEC = size-exclusion chromatography; UF = ultrafiltration; NMR = nuclear magnetic resonance; RIA = radioimmunoassay; UC = ultracentrifugation (the size of OMPC allows UC separation for PRP-OMPC); AA = amino acid; LAL = *Limulus* amoebocyte lysate; NS = not specific.
[b]At present, only PRP-OMPC is adsorbed to aluminum as an adjuvant. Other Hib conjugate vaccines in future combination vaccines also will be adsorbed to some extent.

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Table III
Characteristics of the Conjugate Vaccines

Vaccine	PRP K_d (CL-4B)[a]	Proof of covalency	Unconjugated PRP removal	% free PRP	PRP dose	Protein dose	PRP/protein ratio	Form	Adjuvant	Preservative	References
HbOC	0.3–0.6[b]	Direct and indirect	Ultrafiltration	< 10 %	10 μg	ca. 25 μg	ca. 0.4	Liquid	None	Thimerosal[e]	Anderson and Eby (1990), WHO Technical Report (1991), USP DI (1992), *European Pharmacopoeia* (in preparation), *PDR* (1994)
PRP-DT	95% < 0.75	Indirect	$(NH_4)_2SO_4$ precipitation	< 37%	25 μg	ca. 18 μg	ca. 1.4[f]	Liquid	None	Thimerosal[e]	Schneerson *et al.* (1986), Gordon (1986), WHO Technical Report (1991), USP DI (1992) *European Pharmacopoeia* (in preparation), *PDR* (1994)
PRP-OMPC	85% < 0.25	Direct and indirect	Ultrafiltration	< 15%	15 μg[c]	ca. 250 μg[d]	ca. 0.06	Lyo[h]	Al^{3+}	Thimerosal	Marburg *et al.* (1986, 1987, 1989), WHO Technical Report (1991), USP DI (1992), *European Pharmacopoeia* (in preparation), *PDR* (1994)
PRP-TT	60% < 0.20	Indirect	NA[g]	< 20%	10 μg	ca. 24 μg	ca. 0.4	Lyo[h]	None	None	Schneerson *et al.* (1986), WHO Technical Report (1991), *European Pharmacopoeia* (in preparation)

[a]Percentage of starting PRP whose distribution constant (K_d) is below the value specified as analyzed by CL-4B SEC.
[b]K_d (CL-2 B) for the starting PRP before it is reduced to an oligosaccharide by periodate oxidation for conjugation (see text for detailed description of the process).
[c]Developer is in the process of changing dose to 7.5 μg PRP and ca. 125 μg protein based on immunological equivalence.
[d]Reconstituted with diluent supplied by the manufacturer at time of use; manufacturer is in process of developing liquid formulation.
[e]Only in multidose vials.
[f]The release specification is a range from 1.2 to 1.7 (P. H. McVerry, personal communication).
[g]NA = not available.
[h]Manufacturer is developing a liquid formulation.

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PRP is not immunogenic in either of these species. It also is possible to verify the functional activity of the induced anti-PRP by examining its ability: (1) to kill Hib organisms in a complement-dependent manner (Musher *et al.*, 1986); (2) to passively protect infant rats from challenge with Hib (Smith *et al.*, 1973); and (3) to enhance the ability of leukocytes to phagocytose and kill Hib (opsonophagocytosis) (Gray, 1990).

The TD nature of the immune response to the conjugates, which provides biological confirmation of covalent linkage, can be determined by comparing the anti-PRP responses of athymic nude mice, which are congenitally devoid of T cells, with those of their heterozygous littermates, which have a normal complement of T-helper cells (Baker, 1975). An infant primate immunogenicity assay also has been developed; this provides a predictor of vaccine immunogenicity in the target human infant population (Vella *et al.*, 1990). The inconsistent seasonal availability of infant Rhesus monkeys makes such an assay impractical for routine testing of every commercial vaccine lot, leaving the mouse and infant rabbit tests as the only practical in vivo release assays.

Table II summarizes the stability-indicating assays that are used to establish vaccine shelf life. Currently all of the licensed vaccines are stable for at least 2 years, which is their recommended shelf life. For the same reasons that it is important as a release specification, the measurement of free Ps also stands out as a critical parameter in determination of vaccine stability. Moreover, the same antigenicity and immunogenicity assays that were used to release the vaccine can be followed over time as an aid in predicting stability of the formulations. Phosphodiester linkages in PRP are susceptible to hydrolysis at elevated temperatures and at extremes of pH. This chemical event causes a pH change; therefore, pH drift is monitored for some of the conjugates as an early indication that such hydrolysis might be occurring. Future combination of the Hib conjugate vaccines with DTP, which is formulated on aluminum-based adjuvants, may necessitate that measurement of Al adsorption be applied to all conjugate vaccines.

The conjugate vaccines are tested for safety in mice and guinea pigs in which they may not cause loss of weight during a specified observation period (*Code of Federal Regulations*, 21CFR 610.11, 1991). Additionally, because PRP is isolated from Hib organisms which contain the pyrogenic lipopolysaccharide (OMPC also contains LPS), the conjugates and the formulated vaccines are tested for pyrogenicity in rabbits (*Code of Federal Regulations*, 21CFR 610.13, 1991). Since these conjugates are produced by reactions involving reactive chemical intermediates, a unique safety concern is raised (WHO Technical Report, 1991), viz., the reactive functional groups that were created for forming the requisite covalent bonds might also modify host Pr and other host macromolecules if they were present in reactive form in the final vaccine preparation. These safety considerations are addressed by incorporating special capping reactions into the processes, as described for PRP-OMPC and HbOC, or by validation of the ability of the process to reduce levels of potential reactive intermediates to insignificant levels. In addition, the question of whether an immune response is elicited to the linker moiety was addressed for PRP-OMPC. In these experiments, no antibody responses to SCMHC were detected in the sera of a group of vaccinated human infants (2 to 16 months of age), even after three doses of vaccine (J. Staub and P. P. Vella, unpublished data). All of the Hib conjugate vaccines have an excellent clinical safety record. Furthermore, Hib conjugate vaccines (and future Ps conjugates for which the PRP conjugates will provide the prototypes) are being

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developed into combination vaccine formulations with other pediatric vaccine components. In fact, one such combination of HbOC with DTP (Tetramune™, Lederle–Praxis Biologicals) already has been licensed.

4. IMMUNOGENICITY AND EFFICACY

The four Hib conjugate vaccines elicit anti-PRP in infants and young children but show different immunogenicity profiles. Sufficient studies of the immunogenicity and efficacy of these vaccines in infants were performed in the 1980s to early 1990s such that anti-PRP became accepted as an immunological surrogate for an effective Hib conjugate vaccine, as described below. Evaluations of the immunogenicity of these vaccines in infants typically were performed by immunizations at 2, 4, 6 and 15 months of age to coincide with the ages for administration of diphtheria–tetanus–pertussis (DTP) vaccine in the United States (other ages in other countries) and with regularly scheduled visits to pediatricians. Since there are variations in the quantitation of anti-PRP among different laboratories, the most reliable comparative immunogenicity data have come from head-to-head studies of different vaccines in the same clinical study with all sera assayed in blinded fashion in the same laboratory.

HbOC elicits no detectable anti-PRP post-dose one, low levels (approximately 0.5 μg/mL) post-dose two, but substantial anti-PRP post-dose three (approximately 5 μg/mL). The anti-PRP is of relatively higher avidity than that elicited by PRP-T or PRP-OMPC, is active in bactericidal and opsonophagocytic assays, is predominantly IgG, and primes for a memory response to PRP even after only two doses (Daum and Granoff, 1994). Hence, HbOC shows the properties of a TD antigen. The immune response to HbOC is augmented by carrier priming, in that infants receiving diphtheria toxoid at 1 month of age followed by HbOC (and DTP) at 2 and 4 months of age achieve higher anti-PRP responses than infants not receiving the 1-month dose (Granoff *et al.*, 1993). HbOC was tested in an efficacy trial in north California (Black *et al.*, 1991). Efficacy in 20,800 fully vaccinated children (2, 4, 6 months) compared with unvaccinated controls was 100% post-dose three [95% confidence interval (C.I.) 68–100%], 100% post-dose two (95% C.I. 47–100%), and 26% post-dose one (95% C.I. –166 to +80%). These results mirrored the immunogenicity data in that there was no invasive Hib disease in children receiving two doses of HbOC who are known to be fully primed to a robust post-dose-three or booster response. In a parallel uncontrolled efficacy study in Finland where 55,000 infants were vaccinated at 2, 4, and 14–18 months of age, there were only two cases of invasive Hib disease (Eskola *et al.*, 1990a,b). HbOC was licensed in 1990 in the United States for use in infants at 2, 4, 6, and 15 months of age.

PRP-OMPC is unique among Hib conjugate vaccines in eliciting a significant post-dose-one anti-PRP response (approximately 1–2 μg/mL) which boosts moderately post-dose two (2–5 μg/mL) but does not boost further post-dose three. The anti-PRP is of relatively lower avidity than that elicited by PRP-TT or HbOC, is active in bactericidal and opsonophagocytic assays, is predominantly IgG, and primes for a memory response to PRP (Daum and Granoff, 1994). Thus, PRP-OMPC shows characteristics of both TI antigens (strong post-dose-one response, less efficient boosting) and TD antigens. PRP-OMPC was tested in a double-blind placebo-controlled efficacy study in Navajo Indians

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in the southwestern United States (Santosham *et al.,* 1991), a population with about ten times the incidence of invasive Hib disease as the general population and an earlier peak incidence of disease. The 5200 infants in the study were randomized to receive either vaccine or placebo at 2, 4, and 15 months of age. Efficacy was 93% post-dose two (95% C.I. 53–98%) and 100% post-dose one (95% C.I. 15–100%). These results were consistent with the immunogenicity data which show a strong post-dose-one anti-PRP response. PRP-OMPC was licensed in 1990 in the United States for use in infants at 2, 4, and 12–15 months of age.

PRP-TT elicits low levels of anti-PRP post-dose one, moderate levels post-dose two (approximately 1 μg/mL), and substantial levels post-dose three (approximately 5 μg/mL). The anti-PRP is of somewhat lower avidity than that elicited by HbOC but higher than that by PRP-OMPC, is active in bactericidal assays, is predominantly IgG, and primes for a memory response to PRP (Daum and Granoff, 1994). The immune response to PRP-TT also is augmented after carrier priming as for HbOC (Granoff *et al.,* 1992). Two double-blind randomized efficacy trials for PRP-TT were begun in the United States (south California and North Carolina) in 1989, but both trials were aborted early because of the licensure of HbOC for infants. While the trials did not proceed long enough for the results to have statistical significance, there were no cases of invasive Hib disease among approximately 6000 vaccinated infants compared to five in controls (Parke *et al.,* 1991; Fritzell and Plotkin, 1992). PRP-TT was used routinely between about 1990 and 1992 to vaccinate Finnish infants at 4 and 6 months of age; there were no cases of invasive Hib disease in 1991 (Peltola *et al.,* 1992). Since it was not possible ethically to conduct another efficacy trial in the United States, the FDA evaluated PRP-TT on the basis of anti-PRP as a surrogate marker of efficacy, where PRP-TT was compared to licensed Hib conjugate vaccines (HbOC, PRP-OMPC) in terms of anti-PRP levels post-dose three and their persistence out to the 15-month booster dose, priming for boost by PRP, bactericidal or opsonophagocytic activity, and class and subclass of anti-PRP. On the basis of this evaluation, PRP-TT was licensed in the United States in 1993 for use in infants at 2, 4, 6, and 15 months of age (Frasch, 1994).

PRP-DT is much less immunogenic in infants than the other Hib conjugate vaccines, eliciting only approximately 0.3 μg anti-PRP/mL post-dose three (Decker *et al.,* 1992). PRP-D was tested in an efficacy trial in Finland from 1985 to 1987 (Frasch, 1994). Efficacy in 58,000 vaccinated children, given doses at 3, 4, 6, and 14–18 months of age, was compared with an equal number of unvaccinated controls and was 90% (95% C.I. 70–96%) post-dose three and 100% post-dose four. PRP-DT also was tested in a double-blind placebo-controlled trial in Native Alaskans from 1984 to 1988 with vaccinations at 2, 4, and 6 months of age (Ward *et al.,* 1990). Efficacy post-dose three was only 43% (95% C.I. –43 to 78%), which was not statistically significant. Since PRP-DT has similar immunogenicity profiles in both efficacy studies, the disparity in outcomes is likely related to differences in epidemiology and study populations. The incidence of disease in Native Alaskans was much higher than in Finnish infants (1700 versus 57/100,000 per year, respectively) and peaks at a much earlier age (6 versus 15 months, respectively). Moreover, Native Alaskan families have a greater average number of children, more crowding, less breast-feeding, far inferior economic conditions, and different racial background compared to Finnish families. Nevertheless, given the difference in outcomes between the trials

which have not yet been fully understood coupled with the availability of the more highly immunogenic Hib conjugate vaccines described above, PRP-DT has not been licensed in the United States for use in infants but is available as a booster dose at 15 months of age. In addition, PRP-DT was licensed for infants in both Germany and Iceland on the basis of the Finnish efficacy study, given that these countries have similar epidemiologies of Hib infection as that in Finland.

Thus, three Hib conjugate vaccines are licensed for infants in the United States and in many other developed countries. It is expected that additional Hib conjugate vaccines will be licensed based on immunological comparability to existing vaccines with anti-PRP as a surrogate marker of efficacy.

5. LESSONS FOR THE FUTURE

The vaccines described above are highly immunogenic and efficacious in the at-risk infant population for which they are licensed, and it is unlikely that they will be the subject of further development *per se*. However, they assuredly serve as the prototypes for future Ps conjugate vaccines, and therefore it will be useful to review lessons learned from their development.

5.1. The Implications of Carrier Choice and of Size of Starting Ps

5.1.1. CARRIER CHOICE

The kinetics of response to PRP-OMPC differ from the other three conjugates in that a significant immune response, which usually results in a protective level of antibody, occurs after the primary immunization; there is a moderate boost post-dose two, but no further boost with the third dose. The other three vaccines require a second vaccine administration to achieve similar anti-PRP titers, and this level is boosted by a third immunization. The carrier-specific need for priming with DT and TT is discussed in Section 4, and the processes developed for free Ps removal, which are dictated in part by the physicochemical attributes of the carriers, are discussed in Section 2.4.

5.1.2. SIZE OF STARTING PS

All bacterial capsular Ps are polymers composed of repeating subunits. For PRP, which has a linear structure, this has allowed development of immunogenic conjugates from both oligo- and polysaccharide starting materials. However, for development of other Ps conjugates (such as pneumococcal), the size of the starting Ps may be more constrained, and the immunogenicity of the resultant conjugate will continue to be verified on a case-by-case basis. With this caveat, the size of starting Ps for future conjugates can be chosen to facilitate free Ps removal in the process (see Section 2.4).

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5.2. The Importance of Rigorous Process Control and Analytical Testing of the Process Intermediates, Final Products, and Formulated Vaccines

The Hib conjugates are already beginning to serve as models for the development of other Ps conjugate vaccines (see Chapter 31 for a review of the progress for pneumococcal Ps conjugates). To ensure the successful development of such future conjugates, the lessons regarding process control and analytical testing learned during the development of the Hib conjugates will be critical. In this regard, the importance of and proof of covalent conjugation is discussed in Sections 2.2 and 2.3; the importance of process control and analytical qualification for process intermediates is discussed in Section 2.3; the importance of low levels of free Ps is discussed in Section 2.4; and the analytical and immunological release testing of final products and formulated vaccines is discussed in Section 3.

In addition to serving as prototypes for other Ps conjugates, such as those for the Group B Streptococcus, *Staphylococcus aureus* and *N. meningitidis*, as well as for *S. pneumoniae* (as mentioned above), further development of these Hib conjugate vaccines will be necessary as they are incorporated into combined formulations with other licensed vaccines for routine pediatric administration. In addition to Tetramune®, other combinations including DTP with Hib, hepatitis B, and inactivated polio (IPV) vaccines are in advanced stages of development, and incorporation of other Ps conjugates such as those mentioned above (such as pneumococcal) undoubtedly will result in licensing of combinations with perhaps as many as a dozen components. Such combination formulations will make analytical release testing based on physicochemical criteria very challenging, and in vitro and in vivo immunological tests will evolve as the key methods to ensure compatibility and lack of interference among the components.

6. SUMMARY

In summary, all of the Hib conjugate vaccines are highly immunogenic and efficacious in children older than 12–15 months of age, and HbOC, PRP-OMPC, and PRP-T are highly immunogenic and demonstrated to be efficacious in infants as young as 2 months old. HbOC, PRP-OMPC, and PRP-T have been licensed in numerous countries for infants and are recommended for infant immunization. However, perhaps the greatest tribute one can pay to all four Hib vaccines described in this review is to note the dramatic decrease in the incidence of Hib disease that has occurred since their introduction. In fact, according to the *Morbidity and Mortality Weekly Report* (March 4, 1994), the incidence of Hib disease in children less than 5 years old has declined by 95% from 41 cases per 100,000 in 1987 to 2 cases per 100,000 in 1993, timing that coincides with the availability and use of the Hib conjugate vaccines (Anderson, 1994). As universal administration is achieved and the apparent vaccine-induced reduction in carriage of Hib by the population continues, Hib vaccines may follow the lead of past vaccines (such as smallpox, measles, mumps, rubella, and polio) toward eradication of disease or at least a high degree of medical control, thereby virtually eliminating the mortality and insidious morbidity associated with invasive Hib diseases.

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