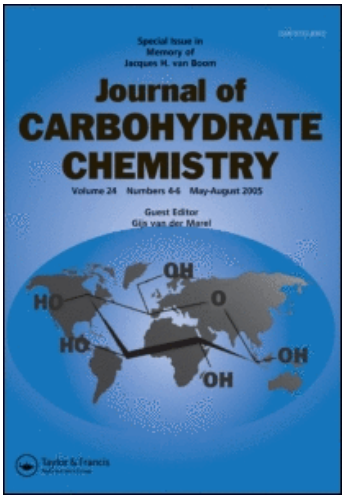

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REVIEW

POLYSACCHARIDE BASED VACCINES FOR THE PREVENTION OF PNEUMOCOCCAL INFECTIONS

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Table of Contents

1. INTRODUCTION

Streptococcus pneumoniae remains an important cause of infections in this end of the 20th century and is responsible for invasive diseases - pneumonia, meningitis, bacteremia, septicemia - as well as for noninvasive diseases such as pharyngitis, conjunctivitis, otitis media.[1,2]

2. POLYSACCHARIDE VACCINES

Streptococcus pneumoniae, a diplococcus gram positive bacterium was independently described in 1881 by Pasteur and Sternberg. The first pneumococcal vaccine proposed by Wright in 1911[3] was a whole-cell killed bacteria vaccine and this vaccinal approach was pursued by Lister.[4]

Meanwhile Dochez, Avery and Heidelberger[5,6] described the carbohydrate composition of the capsule and its antigenic properties. They established the principle of capsular based serotyping; today 90 different serotypes have been identified[7] and the capsular polysaccharide is the main virulence factor, due to its antiphagocytic properties. Francis[8] and then Finland[9] demonstrated the immunogenicity of the purified polysaccharides in animals and humans. Those findings led scientists to use purified pneumococcal capsular polysaccharides as a vaccine: this concept was validated with a hexavalent vaccine which was released on the market in 1946.

The hope brought by antibiotics stopped the development of the preventive approach and further research on vaccines for more than twenty years, but pneumococcal infections still persisted with an increasing attack rate due to the emergence of antibiotic-resistant strains.

Under the auspices of Austrian,[10] a multivalent vaccine, containing 14 valences (1, 2, 3, 4, 6A, 7F, 8, 9N, 12F, 14, 18C, 19F, 23F, 25) with a theoretical coverage of 70-80 % of adult pneumococcal infections, was released in the US in 1977. In 1983, the vaccine was improved with the launch of a 23 valent vaccine (changing 6A to 6B, and adding serotypes 5, 9V, 11A, 15B, 17F, 19A, 20, 22F, 35) with a coverage of more than 85% of the cases.

It is well known that polysaccharides activate B-cells without the participation of T-cells; they are T-independent and induce mainly IgM.[11] The overall efficacy of the 23 valent vaccine is approximately 60 % in adults,[2] but it depends on the age group: antibody response to the vaccine polysaccharides is high for young adults, but the most important drawback of this vaccine is that polysaccharides induce very few, if any, anti-polysaccharide antibodies in infants; this is due to the fact that infants have an immature immune system and that B-cells fail to generate specific anti-polysaccharide antibodies against these T-independent antigens.

3. NEED FOR AN IMPROVED VACCINE AND POTENTIAL PNEUMOCOCCAL VACCINE CANDIDATES

Epidemiological studies show that incidence of pneumococcal infections is higher in infants and young children than in other age groups.[12] Since the existing polysaccharide vaccine is ineffective in this age group, T-dependent antigens, either proteins or polysaccharides conjugated to proteins, are required for the prevention of pneumococcal diseases in infants.

3.1 Pneumococcal proteins[13]

Among potential protein vaccine candidates, pneumolysoid and Pneumococcal surface protein A (PspA) have been under investigation for some years.

3.1.1 Pneumolysin is a highly conserved protein through the different serotypes; it is a cytoplasmic, cytotoxic thiol activated toxin, contributing to the virulence of the bacteria. Anti-pneumolysin antibodies give some protection in animals. When genetically modified,[14] this protein looses 99.5 % of its hemolytic properties but keeps its protective properties. Because of a non-complete clearance of the bacteria by anti-pneumolysin antibodies, pneumolysin is no longer considered as a vaccine by itself, but it could contribute to protection in association with polysaccharide conjugates.[15]

3.1.2 Pneumococcal surface protein A elicits protective antibodies,[16] but several different proteins have been identified; to cover most of pneumococcal diseases it is expected that several PspA's would be necessary. PspA protective efficacy has not yet been established in humans.

3.2 Conjugate vaccine

In 1929, Avery showed that covalent coupling of polysaccharide to a protein increased the immunogenicity of the polysaccharide in animals.[17] Robbins in 1980 introduced the idea of polysaccharide conjugate vaccines for infants.[18,19] If mechanisms for

the processing of conjugate antigens have not yet been clearly established,[21] the efficacy of the first conjugate vaccine against *Haemophilus influenzae* type b has been extensively demonstrated in infants.[20,22] The covalent linkage of polysaccharides to proteins converts these T-independent antigens into T-dependent antigens able to induce polysaccharide specific antibodies.

4. PNEUMOCOCCAL CONJUGATE VACCINES

4.1 Composition of the pneumococcal conjugate vaccine

Since the prevalence of individual pneumococcal serotypes in pneumococcal diseases differs according to geographic areas, the composition of a vaccine will depend on the targeted populations.[23] The vaccine should contain the most frequent serotypes isolated from pneumococcal invasive and noninvasive diseases: serotypes 6B, 14, 19F, 23F are responsible globally for about 60 % of the cases; with additional serotypes like 4, 9V, 18C, the theoretical coverage of child pneumococcal diseases should be more than 75 % in developed countries. But it would be necessary to add serotypes 1, 5 for developing countries, 7F for European countries and type 3 for China and Latin America. Therefore, to develop a vaccine for worldwide use, 11 serotypes would be necessary.

4.2 Carrier protein

The carrier is the constituent of the conjugate molecules which allows the T-dependent type of response against the carbohydrate part of the conjugates; it may be a protein or a peptide. If in theory, one T-epitope motif is sufficient to induce a T-dependent response, it is more likely that several T-epitopes should be present on the carrier. Commercialized conjugates have been prepared from non toxic toxin (CRM 197), detoxified toxin (diphtheria or tetanus) or with an outer membrane protein complex from *Neisseria meningitidis*; molecular weights vary from 38 to several hundred kDa.

Epitopic suppression being suspected to occur by using a large amount of a given carrier protein,[38] new carrier proteins or multiple carrier proteins, some of them with vaccinal interest, may be used in order to control the amount of carrier when combination

vaccines are prepared. This implies extensive developments both for processes and in the clinic to generate safety data. Therefore, the first pneumococcal conjugate vaccines have been prepared using the Hib conjugate carriers with efforts towards decreased carrier protein load in the vaccine doses. New carriers are currently under development to allow the expansion of conjugate vaccines; some have already been used in clinical studies, protein D from nontypeable *Haemophilus influenzae,*[39] exotoxin A from *pseudomonas aeruginosa.*[40]

4.3 Preparation of the conjugate vaccine

Pneumococcal polysaccharides (PS) have a large variety of chemical structures; all have been established[24] and some are presented in Figure 1; therefore, conjugation processes should be defined in relation with the functional groups present on the polysaccharides: aldonyl group, carboxyl group or amino group, etc.. Functional groups can be introduced to achieve a specific coupling technology, e.g., aldehydes via periodic oxidation, amino groups via reductive amination or via cyanogen OH activation, but whatever the chemical reaction chosen, it should not degrade the antigen, since very little is known on immunogenic determinants.[25-28]

The main rule in preparing conjugate vaccines is to maintain the chemical structure and more specifically the immunogenic epitopes of the polysaccharides; therefore it is crucial to know the polysaccharide's structure, or at least its composition, and the involvement of chemical functions in immunogenicity in order to adapt the chemical reactions for cleavage (if desired) or coupling.

The ideal size of the polysaccharide to couple is not yet established since it is impossible to draw general rules from the published data obtained either in animals, or in infants, with *Haemophilus infuenzae* type b conjugate vaccines or other conjugates. Many studies have been done comparing different sizes of polysaccharide in conjugates; they often present contradictory results most likely because conjugates were prepared with different capsular polysaccharides of different lengths, different carrier proteins, with different coupling processes and resulting conjugates were tested in different animal models, with or without adjuvant.[29-31] In addition, if *Haemophilus influenzae* type b conjugates prepared with small oligosaccharides and polysaccharides display similar

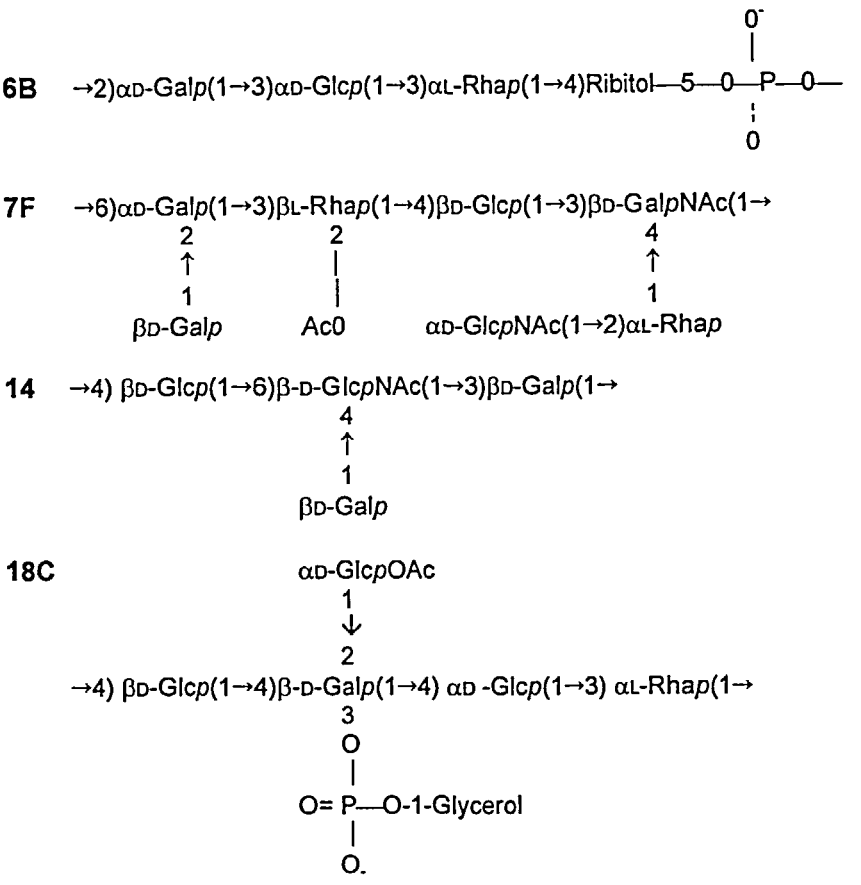

Figure 1. Some structures of *Streptococcus pneumoniae* capsular polysaccharides.

immunogenicity in human infants,[32] then studies done on type 6B, 14, 19F and 23F pneumococcal polysaccharides showed that polysaccharide based vaccines are better immunogens than oligosaccharide based vaccines for a given carrier protein and a given coupling process.[33] Influence of the size of polysaccharide in a conjugate can be related to the chemical structure of this polysaccharide and/or the molecular characteristics of the conjugate obtained (carrier protein , coupling process, etc..).

We have chosen to use depolymerized polysaccharides, and coupling was done after introduction of amino groups using a reductive amination reaction.

4.3.1 Pneumococcal polysaccharide depolymerization

For the coupling of oligosaccharides to protein, depolymerization is a major problem since the cleavage process has to be adapted to the chemical characteristics of the polysaccharides to be included in the vaccine.

In addition, if some information indicates that the *O*-acetyl group on serotype 1 polysaccharide is an antigenic determinant,[28] recent data show that de *O*-acetylated type 9V polysaccharide is immunogenic, no indication being given for other *O*-acetylated polysaccharides. Very little is known on the part played by other chemical residues or carbohydrates for most of the pneumococcal polysaccharides.

Therefore, it appears that because of this gap in knowledge, the depolymerization process should preserve all the labile groups, either phosphodiester groups or *O*-acetyl groups as well as side-chain substituents. Soft depolymerization reactions, mild acid hydrolysis, controlled periodic oxidation, enzymatic hydrolysis can be applied , but if mild acid or alkaline hydrolysis is possible for serotype 3, 4, 5, 6B, 19F because of their linear structure with no labile side-chain, degradation is suspected during the depolymerization for serotypes 1, 7F, 9V, 18C, these polysaccharides having labile side-chains or branched sugars. In addition, enzymatic hydrolysis is only possible with serotype 14 polysaccharide and mild periodic oxidation used to depolymerize Hib polysaccharide, with (→1 Ribitol 5→ PO_4) in its structure, is not applicable to any of those selected pneumococcal polysaccharides. Therefore the oxidative-reductive depolymerization technique (ORD) has been developed and shown to maintain all the labile groups.

This mode of depolymerization is well known but was described as a degradative depolymerisation.[34,35] Nevertheless, by controlling the reaction we have found that depolymerization can occur without removing labile groups such as *O*-acetyl or phosphodiester groups.

The random depolymerization being initiated by hydroxyl radicals, the kinetics of the sizing of the molecule was monitored by the determination of the molecular weight (KDa) of the depolymerized polysaccharide by exclusion gel chromatography, as shown in Figure 2, and the targeted size may be obtained by acting on the parameters of the reaction.

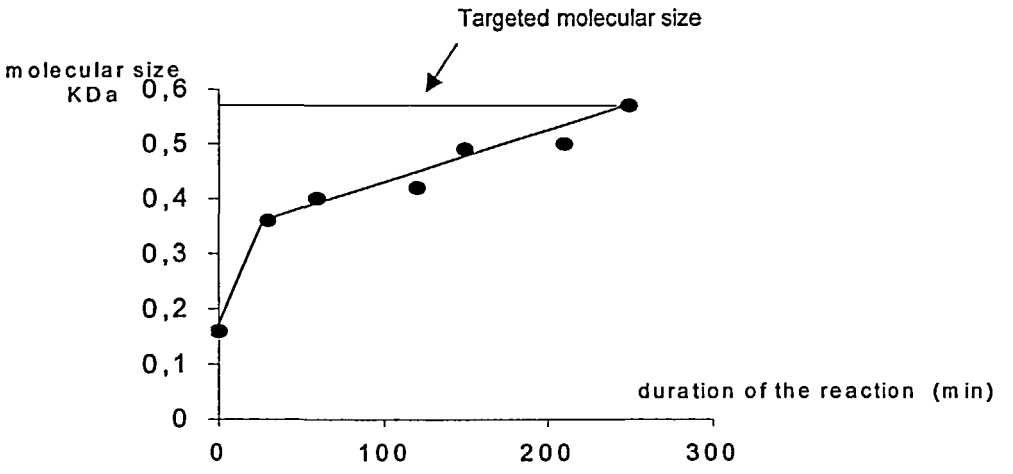

Figure 2. Oxidative-reductive-depolymerisation (ORD) of the serotype 9V pneumococcal polysaccharide: Kinetics of the depolymerisation (exclusion gel chromatography on TSK4000).

The fragments obtained with a molecular weight of 50 kDa, had narrow dispersity (ratio Mw/Mn : 1.5) and targeted size polysaccharides accounted for about 90 % of the starting material. Whatever the PS serotype, when analyzed for their composition, native and depolymerized polysaccharides showed identical sugar, *O*-acetyl and phosphate contents; 18C pneumococcal polysaccharide with a structure presenting (Fig. 1) an *O*-acetyl group , a phosphoglycerol and a sugar branched on the backbone carbohydrate chain was the best example; ^{1}H NMR spectroscopy (Fig. 3) confirmed the identity between native and depolymerized polysaccharides. Increase of reducing functions was followed during the depolymerization, and those functions were used for the activation via reductive amination.

4.3.2 Coupling technologies

The choice of the coupling technology depends on the chemical functions available on the polysaccharide (amino, carbonyl, carboxyl and hydroxyl groups) and on the protein (amino, sulfhydryl, carboxyl groups).

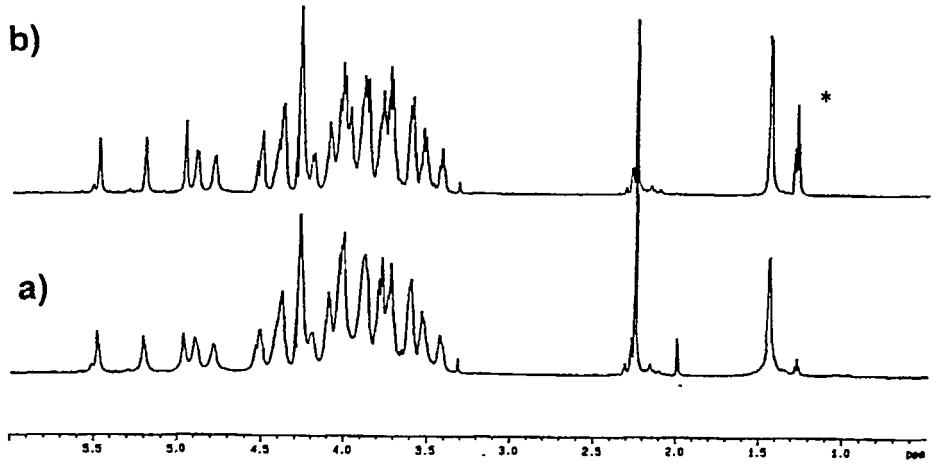

Figure 3. 600 MHz ^{1}H NMR Spectra of serotype 18C pneumococcal polysaccharide: a) native b) depolymerized. * Signal of ethanol used at the purification step.

Table 1. Coupling technologies: chemical reactions/reagents

Functional groups	Reagents / reactions
CHO	Reductive amination
OH	Carbonyldiimidazole
CHOH-CHOH	Periodate oxidation
	Cyanogen bromide
COOH	Carbodiimide
NH_2	Carbodiimide
	N-OH succinimidyl
SH	Maleimide
	Haloacetyl

A panel of chemical reactions can be used but they have to be selected in order to avoid degradation, therefore gentle chemistry is required: examples of the reagents or reactions associated with the functional groups are shown in table 1.

To overcome any steric hindrance problem at the coupling step or to obtain a better presentation of the antigen, spacer molecules may be synthesized during the conjugation reaction or voluntarily introduced.

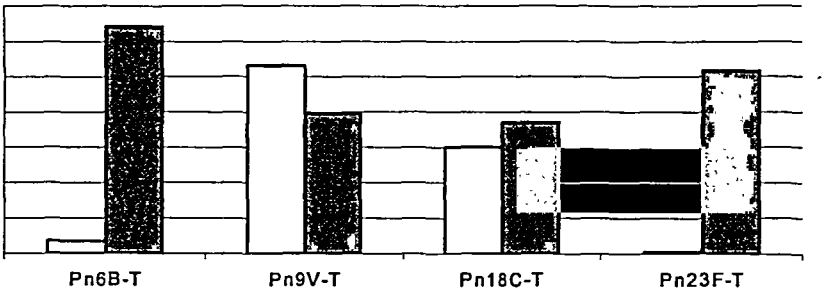

Figure 4. Relative anti-polysaccharide IgG response induced by serotype 6B, 9V, 18C, 23F polysaccharide-tetanus toxoid conjugates.
☐ Response in mice after 2 injections (IgG titers expressed as ELISA arbitrary units/mL of serum).
☑ Response in infants after 3 injections (IgG titers expressed as μg/mL of serum).

Spacers are also a way of improving the yield of the conjugation step. The chemistry and the length of the spacer will depend on the choice of the conjugation process.

4.4 Quality controls

4.4.1 Unavailability of a predictive animal model

Previous experience with the first conjugate vaccines has evidenced the unavailability of an animal potency test which would correlate with immunogenicity in human infants: in Figure 4 is presented the relative antibody response of four tetanus toxoid conjugates when injected in mice and in infants: pneumococcal 6B and 23F conjugates which, in the absence of adjuvant, induce very few antipolysaccharide antibodies in mice after two injections (no further antibody increase after the third injection), induce anti-polysaccharide antibodies in infants. New animal models need to be developed.

4.4.2 Quality control of the conjugates

As no animal test has been shown to correlate with immunological properties of

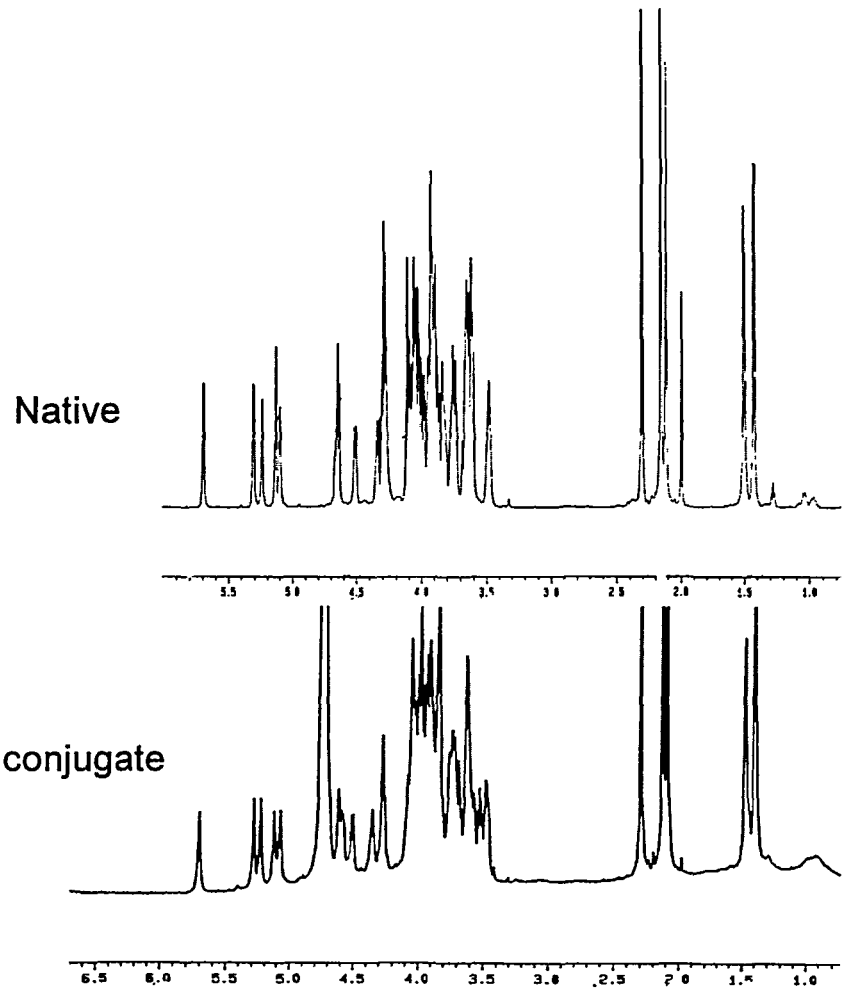

Figure 5. 600 MHz ^{1}H NMR spectra of native and conjugated serotype 7F pneumococcal polysaccharide.

conjugate vaccines in human infants, an extensive physicochemical characterization is necessary:

- Proton NMR analysis showed that the polysaccharide structure was preserved throughout the coupling process from the native polysaccharide to the conjugate as presented in Figure 5.
- Polysaccharide and protein contents were determined: the polysaccharide content was used for the quantification of the final vaccine.

• The conjugate preparation was also characterized by the molecular weight of the conjugate molecule and the protein/polysaccharide ratio.

In addition :

• Immunogenicity of the conjugates was tested in mice to demonstrate their T-dependent properties as indicated by the booster effect observed on polysaccharide specific antibodies after the second injection of conjugates.

• As T-independant antigens, unconjugated polysaccharides are not desired and had to be quantified

• Removal of chemical reagents is controlled to insure safety

5. CLINICAL STUDIES

Different pneumococcal conjugate vaccines are currently in clinical trials. Published data indicate that all the candidate vaccines, whatever the coupling process and carrier protein, are safe and immunogenic in adults and toddlers as well as in infants, showing in young children a booster effect on the anti-polysaccharide specific antibodies after the second or the third injection. As an example, data obtained in infants with PMC octavalent conjugate vaccine are presented in table 2.

It has been noticed that anti-polysaccharide titers can be very different in amplitude from one polysaccharide serotype to another, and furthermore different patterns in the response kinetics were observed: type 3 and 4 already gave a significant antibody increase after the first injection which was further increased after the second and the third injection. For other types (type 14 and 19F) antibody increase was observed after the second injection with further increase after the third shot. For serotypes 6B, 9V, 18C and 23F the antibody increase was observed after the third injection only.

What are the reasons for such discrepancies? Are they related to the structures of polysaccharides? Can some specific coupling technology improve the amplitude of the response to conjugated polysaccharides? Is aspecific coupling needed for each specific polysaccharide structure? From published data,[41,42] it seems that whatever the carrier protein and the coupling technology, 6B conjugates are always among the weakest immunogens giving generally increase in IgG response after the third injection in infants;

Table 2. Immunogenicity of PMC octavalent pneumococcal polysaccharide – Tetanus toxoid conjugate vaccine in infants, (25 infants; 3 injections at 2, 4, 6 months of age; dose: 1 μg; IgG: geometric means titers in μg/mL).

PS serotypes	antipolysaccharide IgG titer			
	Pre	post 1	post 2	post 3
3	**0.6**	2.2	4.0	**6.9**
4	**0.1**	0.8	2.1	**3.0**
6B	**0.2**	0.1	0.3	**1.2**
9V	**0.2**	0.1	0.2	**0.8**
14	**0.3**	0.3	1.1	**2.6**
18C	**0.1**	0.1	0.2	**0.7**
19F	**0.5**	0.5	1.5	**4.2**
23F	**0.2**	0.2	0.3	**1.0**

in this particular cases the kinetics of the response seems linked to the structure of the polysaccharide.

All those data put the emphasis on the fact that the knowledge in conjugate processing by the immune system is very poor. If different mechanisms have been suggested for the processing of conjugate antigens,[17,18] none of them has been experimentally confirmed even if the efficacy of the first conjugate vaccines against *Haemophilus influenzae* type b has been extensively demonstrated in infants. Multi-center programs are now under development in order to understand the mechanism of the conjugate vaccine and to find a correlation between physico-chemical characteristics and immune potency of conjugate molecules.

The booster effect of antipolysaccharide specific IgG upon re-injection of conjugates indicated the T-dependent property of the immune response and that T-cell memory has been induced. This was further confirmed in infants vaccinated at 2, 4 and 6 months of age with a tetravalent conjugate vaccine then boosted one year after with one injection of Pneumo 23® vaccine which contains 6B, 14, 19F, 23F native polysaccharides.[36]

Table 3. Evidence of T-cell memory: booster effect observed after immunization with purified polysaccharide vaccine (Pneumo 23®) in the second year of life of infants previously vaccinated with tetravalent pneumococcal conjugate vaccine.

	Anti PS IgG titer (μg/mL serum)		
PS serotypes	Post 3 conj.[a]	Pre PS[b]	Post PS[c]
6B	0.9	0.7	5.2
14	2.8	1.0	9.6
19F	3.7	1.4	17.2
23F	0.8	0.3	2.2

a. IgG: geometric mean titers measured 1month after the third injection of conjugate.
b. IgG: geometric mean titers measured 8 months after the third injection of conjugate vaccine and before polysaccharide vaccine injection.
c. IgG: geometric mean titers measured 1 month after the polysaccharide vaccine injection.

6. CONCLUSION

Pneumococcal conjugate vaccine efficacy data have already been published by one of the manufacturers developing such a vaccinal approach; it shows that a heptavalent formulation provides 100% protection, in infants and young children, against pneumococcal invasive diseases in the US.[37] Results from efficacy studies for otitis are pending.

It is, therefore, expected that pneumococcal conjugate vaccines for infant use will be introduced to the market in the coming years and that prevention of these threatening and high cost infections will be achieved.

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