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Review

Meningococcal polysaccharide vaccines: A review

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ABSTRACT

Polysaccharides produced by *Neisseria meningitidis* are pharmaceutically important molecules, and are the active components of vaccines against *N. meningitidis* serogroups A, C, W135 and Y. Effective vaccines based on capsular polysaccharide, polysaccharide conjugates and outer membrane vesicles have been developed for strains expressing capsular polysaccharides that define the sero groups A, C, Y and W135. However, conventional approaches to develop a vaccine for group B strains have been largely unsuccessful. This review focuses on the various aspects of fermentative production of meningococcal polysaccharide from *N. meningitidis*, methods of conjugation for improving the immunogenicity of polysaccharide vaccine, and efficient and cost effective methods for the purification of *N. meningitidis* capsular polysaccharide and outer membrane vesicles. In addition, different analytical techniques for the quantitative determination of polysaccharide vaccine and evaluation of structural integrity of conjugate vaccine have been described.

1. Introduction

Neisseria meningitidis is a common cause of serious bacterial infection around the world. Infants, immunocompromised individuals and young adults living in close quarters are at higher risk for invasive meningococcal disease than the general population (Campbell et al., 2002). Even with antimicrobial therapy and the availability of advanced intensive care, case fatality rates are 5–10% in industrialized countries, and are even higher in the developing world (Jodar, Feavers, Salisbury, & Granoff, 2002). Three bacterial species, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Neisseria meningitidis*, are responsible for most cases of meningitis occurring beyond the neonatal period. The incidence of *S. pneumoniae* is greatest in infants and children below 2 years old, that of *H. influenzae* in children from 6 months to 2 years of age, and that of *N. meningitidis* in children, adolescents and young adults from 1 to 29 years of age. *N. meningitidis* moreover is the only bacterium capable of generating epidemics of meningitis. Epidemics have been described as early as 1805 in Europe and recognized for more than 100 years in sub-Saharan Africa.

Neisseria meningitidis are Gram negative, oval cocci, 0.6–0.8 μm, occurring typically in pairs, with adjacent sides flattened or concave (Chakraborty, 1996). The *N. meningitidis* capsule is composed of high-molecular-weight anionic polysaccharides (Romero & Outschoorn, 1994). Polysaccharides produced by *N. meningitidis* are medically important molecules, and are the active components of vaccines against *N. meningitidis* serogroups A, C, W135 and Y. Polysaccharide vaccines for prevention of these diseases have been available for many years. However, vaccines based on plain polysaccharides have important drawbacks, as their immunogenicity is age-related, and they fail to elicit booster response upon reinjection (Cadoz, 1998). Polysaccharide–protein conjugate vaccines are much more effective than unconjugated polysaccharide vaccines in young children (Wenger, DiFabio, Landaverde, Levine, & Gaafar, 1999). Effective conjugate vaccines for the prevention of meningococcal disease caused by group C strains have been licensed in UK and in other European countries (Jodar et al., 2002). Lipopolysaccharide released by *N. meningitidis* in spent media can also be used as antigenic components for production of outer membrane vesicle (OMV) vaccine. OMV vaccines have shown good immunogenicity and safety profiles as they induce immunological memory and bactericidal antibodies against homologous strains in all age groups (Gu & Tsai, 1991). Table 1 shows a summary of studies of meningococcal outer membrane protein vaccine.

2. Meningococcal meningitis

2.1. Epidemiology of meningococcal disease

Meningococcal disease is an endemic as well as worldwide epidemic illness. *N. meningitidis* is a common inhabitant of the mucosal membranes of the human nasopharynx, where it usually lies as a harmless commensal. Up to 5–10% of the population may be asymptomatic carriers in non-epidemic settings. Most cases are acquired by person-to-person contact through aerosol droplets or contacts with respiratory secretions from asymptomatic carriers. A small minority of those who become infected eventually will develop an acute inflammation of the meninges.

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Table 1
Summary of studies of meningococcal outer membrane protein vaccines.

Year of vaccination	Vaccine strain and formulation	Location (population vaccinated)	Age	Percent efficacy (95% CI)
1987–1989	B:4:P1.15 C PS/alum	Cuba (106 000)	10–14 years	83 (42–95)
1989–1990	B:4:P1.15 C PS/alum	Sao Paolo, Brazil (2.4 million)	3–23 months 24–47 months 4–7 years	−37 (−100 to 73) 47 (−72 to 84) 74 (16–92)
1990	B:4:P1.15 C PS/alum	Rio de Janerio, Brazil (1.6 million)	6–23 months 24–47 months 4–9 years	41 (−96 to 82) 14 (−165 to 72) 71 (34–87)
1987–1989	B:4:P1.3 C PS/alum	Iquique, Chile (40 800)	1–4 years 5–21 years	−39 (−100 to 77) 70 (3–93)
1988–1991	B:4:P1.7,16 alum	Norway (171 800)	14–16 years	57 (28–NR)

In industrialized countries, annual attack rates of meningococcal disease average 1–3 per 1, 00,000 of population. The highest incidence is in children under the age of 5 years, with a secondary peak in teenagers and young adults. Case fatality rates in children under 5 years are about 5%, whereas rates of up to 25% are seen in teenagers and adults (Harrison, Pass, & Mendelsohn, 2001). Some groups in general populations, such as university students living in dormitories, or those using catered dining facilities are at several fold higher risk than others of similar age. Additional risk factors include alcohol consumption, smoking and viral respiratory infections such as influenza. Individuals with complement deficiencies, hyposplenism or other forms of immunosuppression are also at increased risk of developing meningococcal disease.

Neisseria meningitidis can be divided into 12 groups based on chemically and antigenically distinct polysaccharide capsules. Meningococci are further classified into serogroups and subtypes on the basis of the immunologic reactivity of their PorA and PorB outer membrane proteins, respectively. Five groups, designated A, B, C, Y, W135, account for virtually all disease-causing isolates. Table 2 illustrates the occurrence of different serogroups with respect to countries (Jodar et al., 2002).

2.2. Clinical manifestations of meningococcal meningitis

Fever and chills, intense headache, stiff neck, vomiting, lethargy or drowsiness or irritability characterizes acute meningitis. Systemic meningococcal disease carries a significant risk of death. Despite antimicrobial therapy, 10% of patients die, typically within 24–48 h of onset of symptoms. Another 10–20% of survivors are left with permanent sequelae such as neurological disorders or sensorineural deafness. When meningitis follows hematogenous dissemination of *N. meningitidis,* the disease usually presents as an acute illness of 1–3 days following a short latency period dominated by cold like symptoms. Frequency and severity of the main clinical manifestations varies with serogroups and clones of *N. meningitidis* and is also influenced by age and immunocompetence of patients (Rang, Dale, & Ritter, 1995).

2.3. Therapeutic treatment and preventive measures of meningococcal meningitis

Due to rapidity and severity of the disease urgent measures must be taken for any patient displaying signs of meningococcal infection. Treatment should be initiated immediately, before diagnostic conformation. Isolation of the patient is not necessary. Treatment of meningococcal disease has two facets: antibiotic therapy and supportive care to handle systemic problems and avoid further complications.

Presently, a range of antibiotics may be used for the treatment including penicillin, ampicillin, chloramphenicol and ceftriaxone. Under epidemic conditions in Africa, oily chloramphenicol is the drug of choice but has adverse reactions like aplastic anemia, hypoplastic anemia, agranulocytosis, thrombocytopenia, anaphylaxis, gray baby syndrome, pseudomembranous colitis, optic neuritis and blurred vision. Supportive care is usually complex and demanding, and must be specifically adapted to the case. Close contacts of all persons with invasive disease, whether sporadic or in an outbreak, are at high risk and should receive chemoprophylaxis with in 24 h of diagnosis of the primary case. Prophylaxis is best ensured through administration of a few specific antiinfective drugs, which eliminate nasopharyngeal carriage. Rifampicin is the drug of choice (Satoskar & Bhandarkar, 1993). Rifampicin has adverse reactions such as nausea, vomiting, abdominal pain, diarrhea, skin rash, headache and pain at injection site (Simmons, Jones, & Calder, 2000). Vaccination is generally recommended for the following groups who are at increased risk for meningococcal disease (Jodar et al., 2002).

- People involved in mass concentrations, movements or schools (military, refugees, *etc.*)
- College students
- Travellers to hyper endemic and epidemic areas
- Individuals with health risks (anatomic or functional asplenia, *etc.*)
- Pathological laboratory staff concerned with blood or serum sample testing

2.4. Cell wall structure of N. meningitidis

The inside or cytoplasm of the meningococcus is surrounded by a cell envelope consisting of three layers, an inner layer called the cell membrane, a middle layer composed of peptidoglycans, and an outer layer called the cell wall (Fig. 1). The cell wall is made up of sugar-like molecules (lipopolysaccharides or LPS) and outer membrane proteins (OMPs), which are linked together to form a mesh or sheet, giving the bacterium its shape. The OMPs enable the organism to interact with and adhere to host cells and also act as

Table 2
Occurrence of different meningococcal serogroups with respect to countries (Jodar et al., 2002).

Group	Predominance in countries	Other remarks
A	Sub-saharan African countries, China	Incidence rates up to 30 per 100,000 in countries such as Niger
B	Norway, Netherlands, Germany and Denmark	50–90% Cases in Europe. Highest in infants less than a year old in all countries
C	Czeck republic, Slovakia, Greece, Republic of Ireland, Spain and UK	Common in teenagers and young adults
Y	USA	Capsular group accounting for 1/3rd of cases
W_{135}	–	Common in Hajj pilgrims

transport proteins allowing control of the intracellular environment. These OMPs, together with the capsular polysaccharide form the principal surface antigens of the organism. Meningococci that cause disease also have a polysaccharide capsule, which is attached to the outer cell wall. This meningococcal capsule forms an additional layer around the bacterium, protecting it from phagocytosis. The lipopolysaccharide (endotoxin) from the outer membrane confers serum resistance and is involved in the pathogenesis of meningococcal disease. A number of functionally important lipoproteins (e.g. LbpB and TbpB) are loosely tethered to the external surface of the outer membrane (Morley & Pollard, 2001).

2.5. Structure of meningococcal polysaccharides

The polysaccharides are heteropolymers of sialic acid alternating with either glucose (serogroup Y) or galactose (serogroup W135) (Bhattacharjee, Jennings, Kenny, Martin, & Smith, 1975; Romero & Outschoorn, 1994). Purified polysaccharides are large, unbranched structures made up of *O*-acetylated residues in the 3 position of α-D-mannosamine-6-phosphate linked (1–6). These are homopolymers of mannosamine phosphate in case of serogroup A or in case of serogroup C these are linear homopolymers made up of *O*-acetylated residues in position 7 and/or 8 (in *O*-acetyl-positive form) or made up of non-acetylated (*O*-acetyl-negative) *N*-acetyl neuraminic acid linked α (2–9).

3. Vaccines against groups A, C, Y and W135 meningococcal disease

3.1. Polysaccharide vaccine

The polysaccharide capsules of *N. meningitidis* are important determinants of virulence. Mutants without capsular expression are serum sensitive i.e. killed by complement, and non-pathogenic. Serum antibody to capsule polysaccharide protects against disease by activating complement-mediated bacteriolysis or opsonization, or both. Polysaccharide vaccines against various groups are licensed and available worldwide (Jodar et al., 2002).

The first successful capsular polysaccharide vaccines against groups A and C were developed in response to epidemics of meningitis among US military recruits (Liu, Gotschlich, Jonssen, & Wysocki, 1971). However, it was found that most polysaccharide vaccines are poor immunogens in infants and fail to induce immunological memory in people of any age. Additionally, immune hyporesponsiveness is recorded after repeated vaccination with group C polysaccharide vaccine, which may cause difficulties for individual who need long-term protection (Ley, Biezen, & Poolman, 1995).

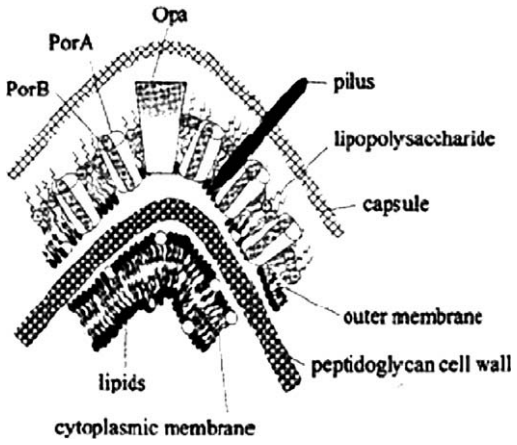

Fig. 1. Cell wall structure of *Neisseria meningitidis* (Pollard & Frasch, 2001).

Finally, there is only transient or no effect on colonization or transmission of the organism. The requirement for multiple doses of vaccine throughout childhood, the short duration of protective immunity after one dose, and the absence of an effect on colonization or transmission of the organism are some unfavorable properties for a vaccine intended for routine immunization (Jodar et al., 2002).

There are however, several disadvantages of this vaccine:

- It produces a diminished response in young children and for that reason is not licensed for use in children under the age of 2 years
- It does not provide long-term protection. At best, immunity lasts for 5 years and at worst this might be as little as 1 year particularly in younger children
- Response to this vaccine is diminished after the second or third dose. Hyporesponsiveness is a real concern in vaccines being used for public health protection (Ravenscroft et al., 1999)

3.1.1. Polysaccharide production

Polysaccharides produced by different serogroups of *N. meningitidis* constitutes the antigen for the vaccine against meningitis. Little information is available in the literature about production of these polysaccharides on industrial scale. The majority of the reports mention only the cultivation of this organism at small scale in solid or liquid Muller–Hinton medium in shake flasks (De Voe & Gilchrist, 1973; Paz et al., 2003). The relationship between biomass and polysaccharide production is extremely important in large-scale production. The cost of polysaccharide production and purification can be reduced by attaining maximum polysaccharide concentration at the end of the cultivation in the bioreactor (Pf) and simultaneously attaining the maximum cell/polysaccharide yield factor (Yp/x), because in purification process the rest of cell structure is a contaminant.

The production of polysaccharide is reported to be growth-associated in the bacterial population up to the 12th-h of cultivation. After this period, in the stationary phase where there is no bacterial growth, polysaccharide production continues; in other words, there is non-growth-associated production (Paz et al., 2003).

The major factors affecting the yields and cellular growth of polysaccharide during fermentation are type of medium, pH, aeration, temperature and growth time.

3.1.1.1. Growth medium. *Neisseria meningitidis* grows on the relatively simple agar medium containing mineral salt, lactate and few amino acids like glutamine, arginine, glycine, serine and cysteine (Catlin, 1973). Usually, large-scale cultivation of *N. meningitidis* is carried out using one of the following three media:

1. Frantz medium (Frantz, 1942)
2. Modified Frantz medium (with replacement of glucose by glycerol)
3. Catlin 6 (a synthetic medium with glucose)

Compositions of the above three media are compared in Table 3. Paz et al. (2003) compared these three media on the basis of the final polysaccharide concentrations (Pf), the yield coefficient cell/polysaccharide (Yp/x), substrate consumption and cell growth. It was found that Pf and Yp/x were highest with Frantz medium with an average of 0.134 g/l and 0.121 g/l, respectively. The same analysis showed that the poorest yields (an average of 0.030 g/l and 0.028 g/l, respectively) were obtained with modified Frantz medium. Modified Frantz medium supported production of a large biomass of *N. meningitidis* with low/no polysaccharide production.

Table 3
Composition of Frantz, modified Frantz and Catlin media used for the production of meningococcal polysaccharide (Paz et al., 2003).

Compounds	Frantz	Modified Frantz	Catlin
L-Glutamic acid	1.60	1.60	3.90
L-Cysteine·HCl	0.02	0.02	0.10
L-Arginine·HCl	–	–	0.15
L-Serine	–	–	0.50
Glycine	–	–	0.25
$CaCl_2 \cdot 2H_2O$	–	–	0.03
NaCl	6.00	6.00	5.80
$Na_2HPO_4 \cdot 12H_2O$	6.24	6.24	–
K_2HPO_4	–	–	4.00
K_2SO_4	–	–	1.00
KCl	0.09	0.09	–
NH_4Cl	1.25	1.25	1.00
$MgCl_2 \cdot 6H_2O$	–	–	0.40
$MgSO_4 \cdot 7H_2O$	1.23	1.23	–
Glucose	5.00	–	10.0
Glycerol	–	5.00	–
Fe (III) citrate	–	–	0.04
Yeast extract	2.00	2.00	–

Results obtained with Catlin 6 medium were 0.095 g/l and 0.067 g/l, respectively.

In Frantz and Catlin 6 medium, where glucose was used as the carbon source, residual value at the end of cultivation was greater than 1 g/l and between 3 and 5 g/l, respectively. With modified Frantz medium, where glycerol was used as a source of carbon, there was also a residue of substrate at the end of cultivations. To avoid the excess of carbon source at end of cultivation, the concentration of this component can be adjusted in initial formulation.

In media with glucose as carbon source (Frantz and Catlin 6), the pH fell to around 5.0, indicating production of acidic metabolites. With modified Frantz medium, where glycerol was used as a source of carbon, there was a slight rise in pH values to around 7.6 at the end of cultivation. This indicates that acidic metabolites were not formed, and that there was sequential consumption of amino acids as a source of carbon together with consumption of glycerol (Paz et al., 2003). Masson and Holbein (1985) reported that low pH supports polysaccharide production; this may be the reason for greater yields with Frantz and Catlin 6 medium as compared to modified Frantz medium.

Baruque-Ramos et al. (2001) reported that optimization of polysaccharide production can be done by limiting an essential nutrient in the medium such as nitrogen source in the presence of excess glucose. Similar results were reported for the production of other bacterial polysaccharide such as gellan gum from *Sphingomonas paucimobilis* ATCC 31461 (Ioannis, Linda, & Brian, 2000). Thus a greater production of meningococcal capsular polysaccharide can be obtained in the medium with high C:N ratio.

Catlin 6 medium is a better medium for *N. meningitidis* polysaccharide production, as it not only gives reasonably good yields, but it is also a chemically defined medium. The other two media used in this study were chemically undefined and may contain undesirable contaminants right up to the end of cultivation process, which may not happen using Catlin 6 medium. Another advantage of the Catlin 6 medium (when compared to the other two cultivation media which use dialyzed yeast extract) is that the components need not be dialyzed. This results in operational saving. In addition, considering that the final product, a vaccine against the disease caused by *N. meningitidis* serogroup C is an immunobiological injection, the use of the Catlin 6 medium satisfies the cGMP and FDA specifications.

3.1.1.2. Temperature. Not many studies have been carried out for optimization of cultivation temperature for production of capsular polysaccharide from *N. meningitidis*. As *N. meningitidis* is a pathogenic organism, the original cultivation temperature is 36–37 °C. However, it is suggested that cultivation at 35 °C could increase the capsular polysaccharide yield, possibly due to slower bacterial growth. In most cases, fermentations were carried out at around 35 °C (Gu & Tsai, 1991; Paz et al., 2003).

3.1.1.3. Aeration and agitation. Little information is available in the literature about the effect of aeration and agitation on the production of capsular polysaccharide. It is recommended that the oxygen volumetric transfer coefficient before incubation be 0.07/min; airflow rate of 5 L/min and agitation frequency of 120 rpm should be kept constant for better polysaccharide yield. Four baffles should be used for enhanced mixing during fermentation (Paz et al., 2003).

3.1.2. Analytical methods

1. *Cell concentration: it* can be expressed as dry biomass and is determined by centrifugation of a sample at 10,000*g* followed by pellet drying at 60 °C for 48 h.
2. *Glucose concentration:* D-*glucose* oxidase method (colorimetric) (Spiro, 1966).
3. *Glycerol concentration: it* can be determined by a glycerol oxidation method in which glycerol reacts with sodium periodate in acid solution generating aldehyde and formic acid. Formic acid is then titrated with 0.125 M NaOH, and the spent volume corresponds to glycerol concentration. Phenolphthalein was used as an indicator.
4. *Polysaccharide concentration: cells* can be disrupted and precipitated by the addition of Cetavlon. After the centrifugation, the supernatant is eliminated and the precipitated biomass resuspended in a 1 M $CaCl_2$. The suspension is centrifuged again and the supernatant collected for the polysaccharide determination by the resorcinol–HCl colorimetric method which detects the polysaccharide monomers, formed after acid hydrolysis (Svennerholm, 1951).

3.1.3. Purification of the polysaccharide

Gotschlich, Liu, and Artenstein (1969) first described the method for purification of *N. meningitidis* capsular polysaccharide, which is the French technology currently used in Brazil. This method includes the following steps:

- The negatively charged polysaccharide is precipitated along with the cells by the addition of cetavlon to the bacterial culture to a final concentration of 0.1% (w/v),
- The cetavlon precipitate is recovered by centrifugation and the paste is resuspended in 1 M $CaCl_2$. Nucleic acid, LPS and some proteins dissolve, while the cell debris is removed by centrifugation,
- The nucleic acid is then removed by fractional precipitation using ethanol at a final alcohol concentration of 25% (v/v) and the precipitate is removed by centrifugation,
- The polysaccharide is precipitated by adjusting the ethanol concentration to 80% (v/v),
- The polysaccharide is recovered by centrifugation and the residual cetavlon and $CaCl_2$ are removed by washing the pellet thrice with absolute ethanol; at this stage the polysaccharide contains large amounts of proteins and LPS,
- The contaminating protein is removed from crude polysaccharide by cold phenol extraction. One volume of 45% w/v [The concentration of phenol is reported as 45% for LPS digestion and 90% for protein digestion by Wu, Tsai, and Frasch (1987) and Gu and Tsai (1993).] phenol solution is added to a 10 mg/ml of polysaccharide in 10% sodium acetate and is shaken vigorously. The

aqueous phase is recovered and re-extracted twice more with phenol. The pooled aqueous phases are dialyzed against distilled water to remove the phenol, and
- The LPS is separated from the solution by ultracentrifugation at 100,000*g*.

The last two steps in this process are inconvenient for large-scale production. Phenol is a very corrosive reagent, and ultracentrifugation is expensive for large-scale production (Gotschlich et al., 1969). Therefore, an alternative process to substitute the phenol extraction and ultracentrifugation was developed by Tanizaki et al. (1996). In this method, a mixture of proteinase, nagarase and trypsin was used for the digestion of protein contaminants instead of phenol. The treatment with these three enzymes followed by tangential ultrafiltration was found to eliminate about 97% of total protein contaminants. Elimination of LPS and low molecular weight proteins is generally achieved by ultrafiltration in 100 kDa cut-off hollow fiber in the presence of 0.5% deoxycholate (DOC) (Tanizaki et al., 1996).

The yields of polysaccharide reported after $CaCl_2$ treatment, before ultrafiltration and of final product are shown in Table 4 (Tanizaki et al., 1996). The main favorable point for the use of this process in large-scale production is the economic reason as the cost of a hollow fiber is around one hundred times more economical than an ultracentrifuge. Another advantage in using hollow fiber is the fact that larger volumes for instance a scale of 40–400 l, of fermentation broth can be processed at the same time and by in the same manner, instead of having the necessity of many ultracentrifuges (Tanizaki et al., 1996).

Pato, De Pádua, Barbosa, and da Silva (2006) have described a new purification methodology employing liquid chromatography that resulted in a polysaccharide showing the characteristics recommended by the WHO for vaccine purposes. In this method, steps of the traditional procedure that yield low recovery and use toxic materials were modified. The present process consists of the following steps:

- Continuous flow centrifugation of the culture for removal of the cells,
- Supernatant concentration by tangential filtration (100 kDa cut-off),
- Addition of 0.5% DOC, heating to 55 °C during 30 min and tangential filtration (100 kDa cut-off),
- Anion exchange chromatography on chromatographic medium Source 15Q (Amersham, Pharmacia Biotech, Sweden),
- Size exclusion chromatography on medium Sepharose CL-4B (Amersham, Pharmacia Biotech, Sweden), and
- The polysaccharide C fraction so obtained was dialyzed and freeze-dried. The structural identity of the polysaccharide was demonstrated by ^{1}H NMR spectrometry.

3.2. Conjugate vaccines

In the past two decades, the development of conjugate vaccines, consisting of bacterial capsular polysaccharides conjugated to protein carriers, has been successful. Experience with the *H. influenzae* type b (Hib) conjugate vaccine has reduced the incidence of the invasive disease due to this pathogen by >95% (Peltola, 2000). Pneumococcal conjugate vaccines showed that the immunogenicity of polysaccharides could be improved by chemical conjugation to a protein carrier, thereby eliciting a T-cell dependent antisaccharide antibody response (Wuorimaa & Kayhty, 2002). The polysaccharide–protein conjugate vaccines are safe, immunogenic in young infants and induce long-term protection (Lesinski & Westerink, 2001). Table 5 discusses comparisons of polysaccharide vaccine with conjugate polysaccharide vaccine.

3.2.1. Production of meningococcal groups A and C polysaccharide–protein conjugate vaccine

Meningococcal oligosaccharides and polysaccharide–protein conjugate vaccines have been prepared for prevention of diseases caused by serogroups A, B and C organisms (Beuvery, Miedema, Delft, & Haverkamp, 1983). Till date, data from humans are limited to serogroups A and C meningococcal oligosaccharides protein conjugate vaccines (Zimmer & Stephens, 2004).

A first-generation prototype vaccine was prepared using meningococcal A and C oligosaccharides that were independently coupled to CRM 9, carrier protein (a cross-reactive non-toxic mutant diphtheria toxin) (Costantino et al., 1992). The conjugation method is based on selective end-reducing group activation of oligosaccharides and subsequent coupling to the protein through a 6-carbon "spacer" molecule, adipic acid (Fig. 2). In adults, the immunogenicity of the first-generation meningococcal A and C conjugate vaccine appeared to be similar to that of an unconjugated control (Anderson et al., 1994).

Table 4
Purification of *Neisseria meningitidis* group C polysaccharide (Tanizaki et al., 1996).

Sample	Volume (ml)	P(total) (g)	LPS(KDO) (total) (mg)	Protein (total) (mg)	Nucleic acid (total) (mg)	PS Recovery (%)
After $CaCl_2$	600	2.62	134.3	861.5	ND	100
Before ultrafiltration	250	1.52	61.7	614.6	ND	58.0
Final product	250	1.38	3.5	30	19.7	52.6

Table 5
Comparison of meningococcal polysaccharide vaccine with conjugate polysaccharide vaccine.

	Polysaccharide vaccine	Conjugate vaccine
Type of vaccine	Purified bacterial capsular polysaccharide (AC, AC/W135, Y)	Purified bacterial capsular polysaccharide conjugated to a protein (only serogroup C available)
Number of doses	One	Three doses for infants, one dose for older children
Booster	Every 3–5 years	None
Contraindication	Severe adverse reaction to previous dose	Severe adverse reaction to previous dose
Adverse reactions	Occasional mild local reaction, mild fever	Occasional mild local reaction, mild fever
Special precautions	Children aged under 2 years of age are not protected by the vaccine	–

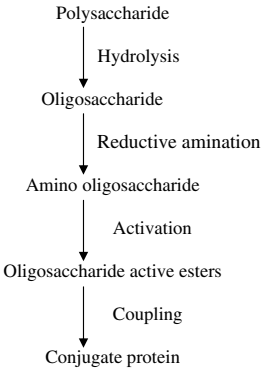

Fig. 2. Strategy of conjugation of meningococcal oligosaccharides to CRM diphtheria toxoid, used in the first-generation meningococcal A and C conjugate vaccine.

A "second-generation" meningococcal C vaccine was prepared using similar chemistry, except that very small oligomers were removed prior to conjugation. In phase I and II clinical trials in humans, both vaccines have been shown to be safe in infants, toddlers and adults (Reingold et al., 1985) Expanded clinical trials with the second-generation meningococcal C conjugate vaccine are currently in progress.

3.2.1.1. The polysaccharide. Group C polysaccharide is a linear homopolymer of α-(2→9) linked *N*-acetyl neuraminic acid (sialic acid). Conjugate vaccine manufacturers frequently elect to reduce the size of the polysaccharide generating oligosaccharides of a consistent size for vaccine production. The degree of size reduction of the polysaccharide will depend upon the manufacturing process. The average size distribution (degree of polymerization) of the processed polysaccharide should be measured by a suitable method.

The size should be specified for each type of conjugate vaccine with appropriate limits for consistency, as the size may affect the reproducibility of the conjugation process.

3.2.1.2. Carrier protein. Proteins that have been used as carriers in meningococcal conjugate vaccines are as follows:

- Tetanus toxoid (Richmond et al., 2000),
- The non-toxic mutant diphtheria toxin (CRM197) (Ho, Bolgiano, & Corbel, 2000), and
- Membrane associated protein from *N. meningitidis* that is obtained by recombinant DNA technology (P64k protein) (Carmenate et al., 2004),
- *Bordetella pertussi* fimbriae (Crowley-Luke, Reddin, Gorringe, Hudson, & Robinson, 2001) and diptheria toxoid (Burrage et al., 2002).

The manufacturer has a choice of possible carrier proteins, provided that the resulting conjugate vaccine is safe and efficacious in infants and young children. Test methods are used to characterize such proteins, to ensure that they are non-toxic, and that the national control authority should approve their purity and concentration. Proteins and purification methods that could be used are as follows:

1. *Tetanus or diphtheria toxoid*: this should satisfy the relevant requirements published by WHO (WHO Technical Report Series, No. 800, Annex 2, 1990). The recommendations suggest the purity to be a minimum of 1500 Lf/mg (Lf = limit of flocculation) protein (non-dialyzable) nitrogen and

2. *Diphtheria CRM197 protein:* this a non-toxic mutant of diphtheria toxin, isolated from cultures of *Corynebacterium diptheriae* C7/γ197. When produced in the same facility as diphtheria toxin, methods must be in place to distinguish the CRM197 protein from the active toxin. The carrier protein should be characterized for its purity, concentration and non-toxicity. The identity may be determined serologically. Physico-chemical methods that may be used to characterize the protein include SDS–PAGE, isoelectric focusing, HPLC, amino acid analysis, amino acid sequencing, circular dichroism, fluorescence spectroscopy, peptide mapping and mass spectrometry as appropriate (Jodar, Griffiths, & Feavers, 2004).

A major drawback of the use of carrier proteins is the observation that the antibody response to an antigen coupled to a carrier protein could decrease in individuals previously immunized with the carrier. This carrier-induced epitope-specific suppression was first described as a general regulatory process found among different hapten carrier systems and mouse strains. Pre-immunization with a carrier can impair the antibody response, mostly for the IgG2a isotype and the hapten coupled to the same carrier. The same phenomenon was observed with TT as a carrier, with a strong suppression of the IgG1 response against two different coupled antigens. The effect has been further found in humans (Rauly et al., 1999).

3.2.1.3. Conjugation methods. Table 6 illustrates essential properties of licensed monovalent group C meningococcal conjugate vaccines. Several methods for the chemical modification of polysaccharides prior to conjugation have been found to be satisfactory. The national control authority should approve the chosen method (Jodar et al., 2004). Suitable methods include the following:

In the first method, Jin, Chu, Robbins, and Schneerson (2003) carried out the production of size-reduced polysaccharide by controlled acid hydrolysis and size fractionation. The resulting oligosaccharide fraction is reductively aminated and activated by coupling through the amine group to a functional linker, *bis-N*-hydroxysuccinamide ester of adipic acid. The reaction between the activated oligosaccharide and the protein generates the conjugate vaccine. This method is also used for the preparation of group A meningococcal capsular polysaccharide conjugates.

Jodar et al. (2004) produced size-reduced polysaccharides by periodate oxidation generating aldehyde groups. On mixing with the carrier protein, Schiff's bases forms between the aldehyde groups of the oligosaccharide and the amino groups of the protein, which can be reduced to form stable covalent bonds by treatment with sodium cyanoborohydride.

Cuello et al. (2007) obtained the conjugate vaccine by neoglycoconjugate intended to immunize against *N. meningitidis* serogroup C. Amine groups generated by basic hydrolysis in the Meningococcal group C polysaccharide were successfully conjugated to carboxyl groups of tetanus toxoid (TT), using carbodiimide-mediated coupling. The results indicated that the meningococcal group C polysaccharide and TT maintained its chemical and antigenic structure after the conjugation process.

As part of in-process controls, the processed polysaccharide to be used in the conjugation reaction may be assessed for the number of functional groups introduced (WHO Technical Report Series, No. 924, Annex 2, 2004). All conjugation methods currently in use involve multi-step processes. Both the method and the control pro-

Table 6
Essential properties of licensed monovalent group C meningococcal conjugate vaccines (Richmond et al., 2000).

Manufacturer	Wyeth	Baxter	Novartis
Polysaccharide	Oac-dp	De-OAc	Oac-dp
(10 μg)	20–47	"Fragment"	14–19
Carrier protein	CRM197	Tetanus toxoid	CRM197
Spacer	No	Adipic acid	No
Adjuvant	$AlPO_4$	$Al(OH)_3$	$Al(OH)_3$

cedures used to ensure the reproducibility, stability and safety of the conjugate should be established once the immunogenicity of the meningococcal conjugate vaccine has been demonstrated. The derivatization and conjugation process should be monitored by analysis of unique reaction products or by other suitable means. Residual unreacted functional groups potentially capable of reacting *in vivo* may be present following the conjugation process. The manufacturing process should be validated to show that activated functional groups do not remain at the end of the manufacturing process.

After the conjugate has been purified, tests to assess consistency must be performed. NMR spectroscopy may be used to confirm the identity and integrity of the saccharide in the conjugate (Jones, Lemercinier, Crane, Gee, & Austin, 2000; Ravenscroft, D'Ascenzi, Proietti, Norelli, & Costantino, 2000; Turula et al., 2004). The determination of the saccharide–protein ratio provides an indirect measurement of the extent of conjugation reaction (Jodar et al., 2004).

3.2.1.4. Adjuvants. Three group C meningococcal conjugate vaccines licensed to date have included aluminum salts as an adjuvant. If an adjuvant has been added to the vaccine, its content should be determined by a method approved by the national control authority. If aluminum compounds are used as adjuvants, the amount should not exceed 1.25 mg per single human dose. The adsorption of the antigen to the adjuvant should be controlled. Consistency is important and the adsorption of production lots should be demonstrated to be within the range of values found for vaccine lots shown to be clinically effective (Ho et al., 2000).

3.2.1.5. Amount of free polysaccharide. Only the meningococcal polysaccharide that is covalently bound to the carrier protein is immunologically important for clinical protection. Excessive levels of unbound polysaccharide could potentially result in immunological hyporesponsiveness to group C polysaccharide (Gold, Lepow, Goldschneider, Draper, & Gotschlich, 1975; Granoff, Gupta, Belshe, & Anderson, 1998; Leach et al., 1997; MacDonald et al., 1998). The clinical importance of vaccine-induced hyporesponsiveness to group C polysaccharide is unknown.

For quality control purposes, each batch of conjugate should be tested for unbound or free polysaccharide in order to ensure that the amount present in the purified bulk is within the limits agreed by the national control authority based on lots shown to be clinically safe and efficacious. Methods that have been used to assay unbound polysaccharide include gel filtration, ultrafiltration and hydrophobic chromatography or ultracentrifugation with high performance ion exchange chromatography with either pulsed amperimetric or colorimetric detection (Ho et al., 2000).

Various modern physico-chemical methods for characterization of polysaccharide–protein conjugate vaccine with a degree of precision have been developed. These techniques are based on the structure of the active ingredient and are thus complementary to traditional bioassays used in the industry. The techniques for analysis of conjugate vaccines include optical spectroscopy (circular dichromism and fluorescence spectroscopy), size exclusion chromatography (especially when combined with light scattering detection techniques) and mass spectrometry, whereas the saccharide component of glycoconjugates (comprised of protein–saccharide) can be characterized by NMR spectroscopy and composition analysis performed using the sensitive technique of high performance anion exchange chromatography (Egan, Frasch, & Anthony, 1995; Holliday & Jones, 1999). Immune responses may be affected by different properties of conjugate vaccines including the structure of the polysaccharide, choice of carrier protein, conjugation chemistry, the degree of cross-linking, the presence of aluminum salts and the perhaps amount of free polysaccharide (Jodar et al., 2004).

3.2.2. Meningococcal group B polysaccharide–protein conjugate vaccine

Efforts to develop a group B polysaccharide–protein conjugate vaccine have been hindered by the dangers of induction of autoantibodies that cross-react with glycosylated host antigens. There are now many promising novel vaccine candidates and the prospects have never been greater for achieving a group B meningococcal vaccine that is both safe and capable of eliciting broad based protection. (Jodar et al., 2002) When purified serogroup B polysaccharide was used to vaccinate adult volunteers, no measurable increase in anticapsular antibody was noted (Wyle et al., 1972). The use of serogroup B polysaccharide non-covalently complexed to outer membrane proteins as a human vaccine generates only short-lived IgM responses (Zollinger, Mandrell, Griffiss, Altieri, & Berman, 1979). Furthermore, antimeningococcal serogroup B antibodies generated by such vaccinations or by natural infection are reported to be of low avidity (Mandrell & Zollinger, 1982).

3.2.2.1. Capsular polysaccharide-based group B meningococcal vaccines. The group B polysaccharide capsule is composed of a homolinear polymer of α-(2→8) linked *N*-acetyl neuraminic acid (polysialic acid). Efforts to use the capsular polysaccharide as a group B meningococcal vaccine have been hampered by its poor immunogenicity, even when conjugated to a carrier protein (Romero & Outschoorn, 1994). This poor immunogenicity is attributed to immunological tolerance induced by developmental exposure of the fetus to cross-reactive polysialated glycoproteins, which are expressed in various host tissues. Cross-reactive, long chain polysialated glycoproteins are especially abundant in the fetal brain, where they are seen on the neural cell adhesion molecule. Expression of this cross-reactive polysialic acid diminishes in most but not all adult tissues (Jodar et al., 2002).

Jennings and co-workers first proposed a strategy for overcoming immunological tolerance to the group B meningococcal polysaccharide. These investigators replaced the native *N*-acetyl (*N*-Ac) groups on the polysaccharide with *N*-propionyl (*N*-Pr) groups, and conjugated the *N*-Pr group B meningococcal polysaccharide to a protein carrier. The resulting conjugate vaccine was highly immunogenic in laboratory animals; elicited IgG anticapsular antibodies that activated complement-mediated bacteriolysis *in vitro*, and passively protected laboratory animals that were infected with group B meningococci. A second-generation version of this vaccine was prepared that used a chemically modified *N*-propionylated polysialic acid from *Escherichia coli* K1 polysaccharide capsule (which is structurally identical to the meningococcal group B polysaccharide), coupled to purified recombinant Por B outer membrane protein as a carrier. This vaccine is highly immunogenic in non-human primates, and is being investigated in a phase 1 clinical trial in Europe (Jennings, Gamian, & Ashton, 1987; Jennings, Roy, & Gamian, 1986).

One safety concern with *N*-Pr group B meningococcal polysaccharide vaccines is that a subset of the anticapsular antibodies elicited has autoreactivity with host polysialic acid. Whether or not the autoreactive antibodies cause the disease is unknown, but the antibodies are predominantly IgG, and can cross the placenta. Large clinical studies with extensive follow-up will be needed to prove that such a vaccine does not evoke autoimmune disease or interfere with neural migration in the developing fetal brain (Jodar et al., 2002). To investigate the molecular basis for antigen recognition, Moe, Dave, and Granoff (2006) have cloned and sequenced the variable region (*V*) genes of five bactericidal anti-*N*-Pr MBPS murine mAbs and produced computer models of the combining sites.

The results were compared to those reported in the literature for two autoreactive anti-MBPS.

A number of experimental approaches have been used to develop vaccines for the prevention of meningococcal B disease. These include vaccines based on eliciting antibody to the group B capsule (Fusco, Michon, Tai, & Blake, 1997; Jennings et al., 1987) or to non-capsular antigens.

3.2.2.2. Non-capsular vaccine candidates for prevention of group B disease. Non-capsular antigens potentially offer protection that is independent of the capsular polysaccharide group (Jodar et al., 2002). The non-capsular approaches include outer membrane vesicle (OMV) vaccines (Bjune, Gronnesby, Hoiby, Closs, & Nokleby, 1991; Boslego et al., 1995; Norheim et al., 2005; Perkins et al., 1998), specific OMPs such as PorA (Borrow et al., 1998; Christodoulides et al., 1998; Idanpaan-Heikkila et al., 1995; Muttilainen et al., 1995; Peeters et al., 1996; van der Voort et al., 1996; van der Voort et al., 1997), iron-regulated proteins (Banerjee-Bhatnagar & Frasch, 1990; Lissolo et al., 1995; Pettersson et al., 1990), class 4 (Rmp) proteins (Rosenqvist et al., 1999), Opc (Musacchio, Carmenate, Delgado, & Gonzalez, 1997), neisserial surface protein A (Martin, Cadieux, Hamel, & Brodeur, 1997; Martin, Roiux, Villeneuve, Hamel, & Brodeur, 1998) and detoxified lipopolysaccharide (Gu & Tsai, 1993).

3.2.2.3. Outer membrane protein (OMV) vesicle-based vaccines. The production process of OMV vaccines is simpler than that of conjugate vaccines. Hence, this could be a realistic and less expensive alternative to a conjugate vaccine (Norheim et al., 2005). The outer membrane protein complex carrier is derived by detergent extraction of whole cells. OMV can be prepared from meningococcal bacterial cells. The resulting OMVs contain the major porin proteins, lesser amounts of other proteins including those with high molecular weights of 60–100 kDa, and lipopolysaccharide. These vaccines have been used in many people without notable serious safety concerns, although local reactions at the injection site and transient malaise and fever are common. The vaccine has showed efficacy in the range of 50–80%. However, no important protection was recorded in children below 4 years of age, the age group at greatest risk of acquiring meningococcal disease (Jodar et al., 2002). These vaccines elicited strain-specific bactericidal antibodies, which undoubtedly contributed to the level of protection (Tappero et al., 1999). There was also some evidence from the efficacy trials that protection was not restricted to the homologous strains from which the vaccines were prepared (Holst et al., 2003). This finding suggests that immune mechanisms, in addition to complement-mediated bactericidal antibody, may contribute to protection. The bactericidal antibody responses to OMV vaccines appear to be directed predominantly at the class 1 porin protein (PorA). The predicted structure of PorA contains eight hydrophilic exposed loops that are created as the porin traverses the outer membrane (OM) (Van der Ley, Heckels, Virji, Hoogerhout, & Poolman, 1991). To expand protection to a greater number of strains, a recombinant hexavalent PorA vesicle vaccine has been developed. In summary, OMV vaccines can clearly induce protective antibody in humans and for this reason, they have been regarded as important vaccine candidates. However, this approach has some important limitations. These include restricted bactericidal antibody response to hypervariable regions of PorA, the propensity for disease during non-epidemic periods to be caused by multiple strain variants, and the potential for antigenic drift that may be accelerated by selective pressures resulting from the immune responses induced by vaccination (Anderson, Donnelly, & Gupta, 1997). However, due to variability of PorA, these vaccines may be less useful in control of endemic disease. Genome-based vaccine discovery was evaluated in an attempt to produce a candidate capable of conferring a broadly protective vaccine against a diversity of meningococcal B strains (Danzig, 2006).

3.2.2.4. Transferrin binding protein B (TbpB). Iron is important in the metabolism of *N. meningitidis* and it has developed elaborate means to scavenge iron from the environment (Masson & Holbein, 1985). TbpB is a subunit of transferrin binding protein, a surface-exposed *N. meningitidis* group B lipoprotein whose expression is up-regulated when the organism is grown in iron restricted media. Thus, TbpB is an attractive vaccine candidate and is currently being developed by a commercial organization (Moe, Siqi, & Granoff, 1999). In a recent phase I study in adult humans, a recombinant TbpB vaccine was reported to elicit high IgG antibody titres as measured by an enzyme-linked immunosorbent assay (ELISA), but the resulting antibodies showed minimal complement-mediated bacteriolytic activity. Given the importance of bactericidal antibody in protection, its absence in most of the post-immunization sera raises concerns about the potential efficacy of this TbpB vaccine in humans. Future vaccine work on TbpB is likely to focus on investigating improved formulations and use of adjuvants (Danve et al., 1998).

3.2.2.5. Neisserial surface protein A (NspA). Neisserial surface protein A (NspA) is a highly conserved, surface-exposed outer membrane protein of *N. meningitidis* that has been shown to induce a bactericidal immune response in animals against all pathogenic neisserial serogroups (Halperin et al., 2007). This protein is a first of the novel group of promising vaccine candidates that are characterized by highly conserved membrane proteins being present in low copy number in the outer membrane, and being weak immunogens during natural infections. Immunization of mice, rabbits and non-human primates with recombinant NspA elicits serum bactericidal antibodies that confer passive protection in animal models of group B meningococcal bacteraemia. Two major challenges to develop an effective NspA vaccine are (i) to understand the mechanism that makes some strains susceptible to anti-NspA complement-mediated bacteriolysis, and others resistant and (ii) to develop vaccine formulations that are more immunogenic than natural infections (Martin et al., 1997).

Martin et al. (1997) showed that NspA mixed with the adjuvant, QuilA, elicited bactericidal antibody responses against serogroups A, B and C. Halperin et al. (2007) found that the unfolded rNspA meningococcal vaccine was well tolerated and immunogenic in healthy adult volunteers but did not elicit bactericidal antibodies.

4. Availability and storage of meningococcal polysaccharide vaccines

A meningococcal polysaccharide vaccine is a freeze-dried preparation of the group-specific polysaccharide antigens from *N. meningitidis*, groups A, C, Y and W135 for subcutaneous injection. The diluent is sterile pyrogen-free distilled water to which thimerosal [mercury((*o*-carboxyphenyl)thio)ethyl sodium salt or sodium ethylmercuric thiosalicylate] 1:10,000 is added as a preservative. After reconstitution with diluent as indicated on the label, each 0.5 ml dose contains 50 μg of "isolated product" from each groups A, C, Y and W135 in isotonic sodium chloride solution preserved with thimerosal. Each dose of vaccine also contains 2.5–5 mg of lactose added as a stabilizer. The vaccine when reconstituted is a clear colorless liquid. Any unused reconstituted vaccine remaining in a 50-dose vial should be discarded and not retained for later use. The single dose vial should be used within 24 h of reconstitution.

Storage conditions should be based on stability studies and approved by the national regulatory authority. Storage of both liquid and freeze-dried vaccines at a temperature of 2–8 °C has been

found to be satisfactory. Group C conjugate vaccines have generally proved to be stable over a wide range of storage temperatures, although some formulations are affected by repeated freeze-thawing. The expiry date should be approved by the national control authority, and based on the stability of the final product as well as the results of the stability tests.

5. Dosage and administration

Vaccines should be visually inspected for extraneous particulate matter and discoloration prior to administration and reconstituted using only the diluent supplied for this purpose. Vials should be shaken until the vaccine is dissolved and administered subcutaneously.

The immunizing dose is a single injection of 0.5 ml. The vaccine can be given at the same time as other immunizations, if needed. There are no data on the incidence and degree of reactions following booster doses of quadrivalent meningococcal vaccine.

Vaccines should be administered only with automatic hypodermic jet apparatus. Special care should be taken by using the deltoid area to avoid injecting the vaccine intradermally or intravenously since clinical studies have not been done to establish safety and efficacy of the vaccine using these routes of administration.

6. Safety and immunochemical characterization of serogroup C-tetanus toxoid conjugate vaccine

Biochemical assay, ELISA and isopycnic CsCl gradient ultracentrifugation can be used to characterize conjugate vaccines. The safety and immunogenicity of a single dose of a new serogroup C *O*-deacylated meningococcal polysaccharide–tetanus toxoid conjugate vaccine have been tested in 30 adult volunteers. As stated above, the poor immunogenicity of polysaccharide can be overcome by conjugation to protein carrier molecules such as diphtheria or tetanus toxoids. Conjugate vaccines induce a T-cell-dependent antibody response even in infants, which leads to affinity, maturation and induction of immunological memory.

The serogroup C meningococcal polysaccharide-tetanus toxoid conjugate technology has proved to be immunogenic and safe in healthy adult volunteers. In addition to measuring the anticapsular IgG and serogroup C specific bactericidal antibody, the change in IgG avidity over time is an important parameter and should be evaluated, as this can provide evidence of a T-cell dependent antibody response and may be useful as a surrogate marker of priming for immunological memory (Richmond et al., 2000).

6.1. Serology

Meningococcal serogroup C specific IgG antibody concentrations can be measured using a standardized enzyme-linked immunosorbent assay (ELISA) with a standard reference serum (CDC1992) (Richmond et al., 2000). ELISA can also analyze tetanus antitoxoid antibodies and the results are expressed as ELISA units per milliliter (EU/ml). Antibody avidity has been measured by an elution ELISA using the chaotrope, thiocyanate and the method was modified for meningococcal IgG assay by Richmond et al. (2000).

6.2. Adverse reactions

Thirty healthy adults were enrolled by Richmond et al. (2000) and received a single dose of MCC-TT vaccine. The vaccine was well tolerated and there were no serious adverse events. There was no significant change in general health status, hematological, biochemical or immunological parameters post-immunization. The local and systemic reactions are shown in Table 7. The majority of reactions were mild. The only significant (>3 cm) local erythema and swelling was related to an injection site hematoma, which resolved rapidly. No subjects had a fever >38 °C within 48 h of vaccination. In another example, a conjugate vaccine against meningococcus A and C was prepared by Costantino et al. (1992), using the non-toxic mutant of diphtheria toxin CRM 197 as a carrier protein. After the preclinical studies, which showed that the vaccine was safe and immunogenic in animal models, a pilot phase 1 clinical trial, blind versus placebo, was performed on adult volunteers. The difference between the incidence of adverse reactions associated with vaccine and placebo administration was not statistically significant. All the volunteers who received the vaccine had a significant increase in antibodies to groups A and C meningococcal capsular polysaccharides after the first dose.

Other adverse reactions reported after receipt of meningococcal vaccine includes injection site reactions, allergic reactions, Guillain–Barré syndrome as serious events. Fever, headache, dizziness, injection site hypersensitivity, urticaria and paresthesia are among the most common adverse reactions reported (Ball, Braun, & Mootrey, 2001).

6.3. Immunogenicity

According to a study done by Richmond et al. (2000), subjects showed a significant increase in meningococcal C specific SBA (serum bactericidal antibody) titre and IgG following immunization. Out of 30, 17 subjects had SBA titres <8 μg/ml and 24 had IgG levels <2 μg/ml. All subjects developed a 4-fold or greater increase in SBA and IgG levels. SBA geometric mean titre (GMT) increased from 11 to 3649 with a mean 323-fold rise. Post-immunization, the geometric mean avidity index (GMAI) for all subjects was 0.21. It was also found that there was an increase in the avidity of meningococcal C-antibodies 6 months following MCC-TT vaccination indicating successful induction of a T-cell dependent antibody response.

6.4. Thermostability of the conjugate

CPS/TT was incubated in fluid and at lyophilized conditions at 4, 37 and 56 °C for 1 month. The antigenic activities of both components were then tested in the homologous ELISA system. It was concluded by Beuvery et al. (1983) that storage of the conjugate in fluid conditions at 4 and 37 °C and in lyophilized condition did not alter the antigenic activities of CPS and TT components. Storage in fluid conditions at 56 °C, however, resulted in a considerable loss of the ability of both components to bind their corresponding antibodies.

Table 7
Frequency of adverse events within 7 days of meningococcal C-tetanus toxoid conjugate vaccine in healthy adults (Richmond et al., 2000).

Symptoms	Number of subjects
Local (injection site)[a] tenderness	15/30 (50%)
Erythema	15/30 (50%)
Pain	13/30 (43%)
Induration	8/30 (27%)
Systemic fever ($\geqslant$38 °C)[b]	1/30 (3%)
Headache	2/30 (7%)
Myalgias	1/30 (3%)
Arthralgia	0/30 (0%)

[a] 85–100% of reported injection site complaints were mild in intensity.
[b] There were no fevers within 72 h of vaccination.

7. An assay for meningococcal conjugate vaccine

The principal component of the vaccine is the *N*-propionylated group B meningococcal polysaccharide (*N*-Pr-GBMP) covalently attached to a carrier protein. It is important to determine whether any whole polysaccharide, oligosaccharide or monosaccharide, from minute to moderate levels, become detached from the conjugate. The ability of the conjugate vaccine to elicit an immune response in the form of specific and protective antibodies depends upon the maintenance of the carrier protein and the maintenance of the polysaccharide epitope. Degradation of the polysaccharide, or loss of wholeness, of the vaccine can compromise its efficacy. The level of free saccharide, any poly, oligo or monosaccharide detached, must therefore be monitored to demonstrate that conjugate vaccine remains principally intact.

Formulations of conjugate vaccines vary, but many are adsorbed onto or dispersed into adjuvants, which serve as excellent vehicles for delivery. Aluminum-based adjuvants have been extensively used in vaccine formulation for this purpose. Adjuvant and buffer systems must be formulated to ensure conjugate structural integrity for the specified shelf life of the vaccine. Analysis of the final formulation for the evolution of free saccharides provides a good way to determine structural wholeness or integrity. Analysis of formulated vaccine for free saccharide requires:

(i) A high degree of selectivity because the saccharide must be isolated from all other vaccine components,
(ii) A high degree of sensitivity because vaccine dose concentrations are usually low and the detection of even a low percentage of free saccharide is necessary, and
(iii) An accuracy that provides the true concentration. For the analysis of low levels of free saccharide integrated pulsed amperometric LC detection (iPAD)[3] has proven most useful. However, to gain the sensitivity advantage of the HPAEC-iPAD system, the free saccharide must be first isolated from the adjuvant and then depolymerized into its basic repeat unit, typically mono or disaccharide. Depolymerization for many bacterial polysaccharides can be accomplished by acid hydrolysis. Conditions such as temperature, acid strength and incubation time are optimized to maximize the release of the monomeric monosaccharides, while minimizing the degradation to the liberated monosaccharides.

The meningococcal C polysaccharide is a linear homopolymer of polysialic acid, α-(2→9) linked. Due to the linkage, the polysaccharide is readily hydrolyzed under mild conditions. The HPAEC-iPAD has been used successfully for the analysis of sialic acid and polysialic acids (Manzi, Diaz, & Varski, 1990). The HPAEC-iPAD analysis of commercial meningitidis type C has been successfully employed to monitor conjugate wholeness. High resolution-magic angle spinning (HRMAS) NMR spectroscopy has been applied to serogroup A *N. meningitidis* (NMA) to determine precise structures of capsular polysaccharide (CPS) expressed on the meningococcal surface. The ^{1}H NMR HRMAS spectral patterns correlated with the purified CPS ^{1}H NMR profiles. HRMAS NMR can distinguish detailed complex carbohydrate structures expressed on bacteria. NMA express both *O*-3- and *O*-4-acetylated polymers but not in equimolar ratio amounts *in vivo* (Gudlavalleti, Szymanski, Jarrell, & Stephens, 2006).

In contrast, the meningococcal B polysaccharide, also a linear homopolymer of polysialic acid but α-(2→8) linked, does not depolymerize completely with either acid or base catalyzed hydrolysis. This is due to the proximity of the C_9 hydroxy group, which under acidic conditions lactonizes with the α-carboxylic acid group on C_1 of the adjacent monomer, sustaining the linkage from dissociation. Also, complicating the ability to depolymerize the GBMP (group B meningococcal polysaccharide) is the difference in labilities of the internal glycosidic bonds. Thus, to analyze for structural wholeness of the meningococcal B conjugate vaccine formulation for low levels of detached saccharide, it is necessary to develop an approach that improved the efficiency of depolymerization. The use of anhydrous methanolysis is generally employed for the preparation of polysaccharides gas chromatography and also the means by which depolymerization can be accomplished (Fig. 3). Conditions can be optimized such that the methanolysis produce a common methyl glycoside of sialic acid over a broad range of levels of free saccharide as detected with HPAEC-iPAD. Sufficient sensitivity and linearity has been demonstrated for the application

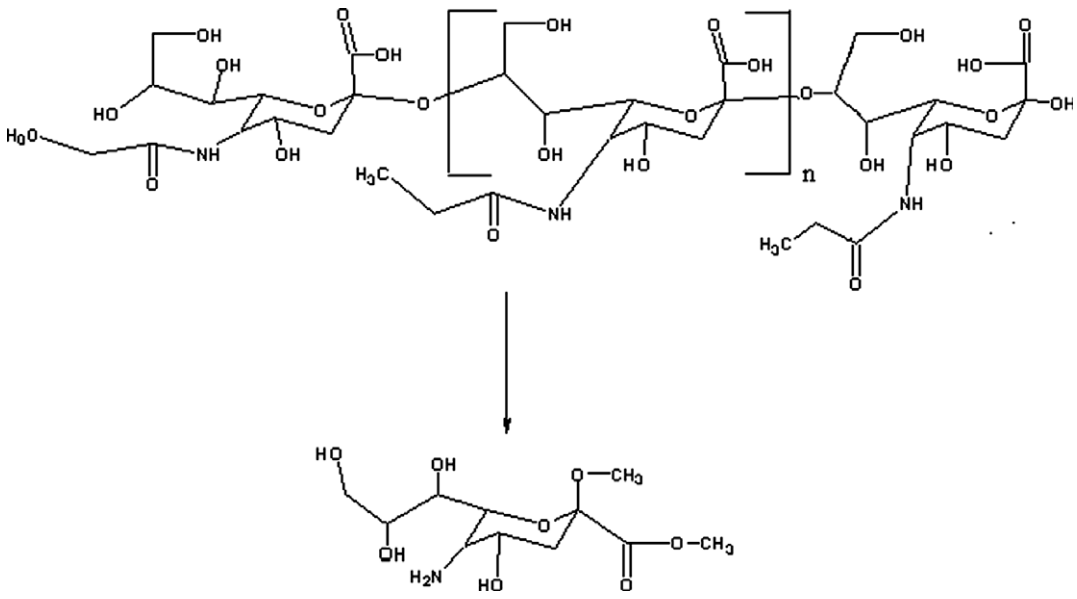

Fig. 3. Formation of the β form of deacetylated sialic acid methyl glycoside.

with vaccine dose formulations with low total saccharide concentrations (Turula et al., 2004).

8. Precautions for meningococcal polysaccharide vaccine

Care must be taken by health-care providers for the safe and effective use of meningococcal polysaccharide vaccine. Prior to an injection of any vaccine, all known precautions should be taken to prevent adverse reactions. This includes a review of the patient's history with respect to possible sensitivity to the vaccine or similar vaccines and to possible sensitivity to dry natural latex rubber. Epinephrine injection (1:1000) must be immediately available to combat unexpected anaphylactic or other allergic reactions.

Health-care providers should also obtain the previous immunization history of the vaccine, and inquire about the current health status of the vaccine. A separate, sterile syringe and needle or a sterile disposable unit should be used for each patient to prevent transmission of hepatitis and other infectious agents from person-to-person. Needles should not be recapped and should be disposed of according to biohazard waste guidelines. Persons with a history of anaphylaxis to thimerosal should only receive vaccine from a single dose vial preparation, which does not contain thimerosal. Persons with a history of anaphylaxis to a vaccine component, but who are at risk for meningococcal disease, should be referred to a health-care provider for evaluation and possible administration of meningococcal vaccine. Persons whose spleens have been removed because of trauma or lymphoid tumors and persons who have inherited complement deficiencies have acceptable antibody responses to meningococcal vaccine; however, clinical efficacy of vaccine has not been documented in these patients, and they may not be protected by vaccination. Meningococcal polysaccharide vaccines should be considered for pregnant women at increased risk for meningococcal disease.

Special care should be taken to avoid injecting the vaccine intradermally, intramuscularly or intravenously since clinical studies have not been done to establish safety and efficacy of the vaccine using these routes of administration (Massachusetts Department of Public Health, Massachusetts Immunization Program, model standing orders, April 2004).

9. Conclusions

The meningococcal polysaccharide–protein conjugate are safe, immunogenic in young infants and induce long-term protection than polysaccharide vaccines. The introduction of conjugated vaccines into pediatric vaccination schedules has led to a drastic decrease of the incidence of invasive diseases caused by Hib, MenC or Pneumococcus, a major public health success. Surveillance of asymptomatic carriage has furthermore, demonstrated that the conjugate vaccines provide herd protection through their impact on nasopharyngeal colonization among vaccinated children. The generalized use of a quadrivalent MenA/C/Y/W135 conjugate vaccine would obviously be ideal in the future, but this remains as an elusive goal at this time in view of the costs involved. The outer membrane vesicle LPS can be used efficiently for the production of vaccines for children above 4 years of age, as no important protection is recorded in children less than that. The outer membrane vesicle yield is more than the capsular polysaccharide yield, making it more cost effective than conjugate vaccines. In outer membrane vesicle vaccines, there are evidences that the protection is not restricted to the homologous strains from which the vaccines were prepared. Thus, outer membrane vesicle vaccines have good future prospects. Thus, meningococcal polysaccharide vaccines have proved effective and have decreased the fatality associated with bacterial meningitis. At this time, the problem of vaccines to fight meningococcal disease due to group B meningococcus still remains an unsolved challenge, although several candidate outer membrane proteins are being thoroughly studied. Group B meningococcal vaccine, which is both safe and capable of eliciting broad based protection, has not been achieved.

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