Review Article

Meningococcal vaccines: past, present, and future perspectives

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Abstract

Meningococcal disease remains a significant global public health issue and is unique among causes of bacterial meningitis and sepsis where not only does it cause sporadic disease but also outbreaks. The prevention of meningococcal disease also presents a serious challenge. Although polysaccharide vaccines have been available for serogroup A, C, Y, and W135 for many years, serogroup C polysaccharide-protein conjugate vaccine has only recently been licensed in many countries. More recently, a conjugate vaccine for a combination of serogroup A, C, Y, and W135 has been made available. The major hurdle in achieving the goal of eradication is the development of a safe and immunogenic vaccine against serogroup B infections. Outer membrane vesicle vaccines are already used in some countries, and will likely be used more widely in the next few years, but efficacy for endemic disease in children has so far been disappointing. Through the recent availability of the meningococcal genome sequence, many new vaccine candidates are being identified and there is increasing optimism that a solution to the problem can be found. However, none of these has yet been presented as the “universal” protective antigen and work in this field continues to be held back by our limited knowledge concerning the mechanisms of natural protection against serogroup B.

Key Words: Neisseria meningitidis, Meningococcal vaccines, Reverse vaccinology, Developing countries.

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1. Microbiological features and pathogenesis of Neisseria meningitidis

*Neisseria meningitidis* is a gram-negative diplococci with aerobic metabolism which can be isolated on chocolate agar [1]. *N. meningitidis* is an encapsulated bacterium and classified into 13 serogroups on the basis of the chemical composition and immunologic properties of the capsular polysaccharides. Only five main serogroups (A, B, C, Y and W135) cause disease. The main meningococcal capsular polysaccharides associated with invasive disease, with the exception of serogroup A, are composed of sialic acid and its derivatives. The capsular polysaccharide of serogroups A and C are composed of N-acetyl-mannosamine-1-phosphate residues and repeating units of N-acetyl-5-acetyl-neuraminic acid respectively. The capsular polysaccharide of serogroup B has some different properties in comparison with the other serogroups where it protects the organism against the complement mediated lysis, inducing immunological tolerance which may lead to autoimmune disease. Capsular polysaccharides of serogroup A, C, W135 and Y of meningococci are the basis for currently employed meningococcal
vaccines. The outer membrane proteins of *N. meningitidis* are classified according to their molecular weights and can be considered as a basis for subserotyping (class 1) and serotyping [2,3]. Most meningococci strains possess 1, 2 and 3 classes of outer membrane proteins which are similar to Por proteins of *Neisseria gonorrhoeae*. These outer membrane proteins are associated with the structure of pores. The basis of immunotyping is lipo-oligosaccharides. *N. meningitidis* also could be classified by molecular subtyping using multi loci enzyme electrophoresis, pulsed-field gel electrophoresis, or DNA sequencing in order to identify closely related strains [2]. The B and C serogroups are able to cross-switch by exchanging their genetic material responsible for capsule production. The exchanges in capsular composition may be a virulence factor of *N. meningitides*, an important consideration in serogroup specific vaccination [3,4].

Humans are the sole natural reservoir of *N. meningitidis* and the nasopharynx is the site of entry of this organism. The organism can asymptomatically become a part of normal flora at this site (in five to ten percent of adults) and can also penetrate into the bloodstream and cause bacteremia in a small group of individuals. However, in the majority of people exposed to *N. meningitidis*, carriage is an immunization process leading to a systemic antibody response. During the colonization, the organism gets attached to non-ciliated columnar cells of the nasopharynx, mediated by long filamentous surface type IV pili [5]. The pilus has been suggested to bind to the membrane cofactor protein (MCP or CD46) which is expressed in all human cells except erythrocytes. Subsequently, the opacity associated proteins, opa and apc, bind to CD66 and heparin sulfate proteoglycan receptors, respectively [6, 7, 9]. These bindings lead to the engulfment of the organism by epithelial cells [10]. Following the engulfment, the organism can survive in the epithelial cells through IgA1 protease and PorB [4,11].

2. Epidemiology aspects of meningococcal infection

Meningococcal infection, in both endemic and epidemic forms, is an important cause of morbidity and mortality in developing countries as well as in the industrialized world. In the absence of epidemics, approximately 1.2 million cases of invasive meningococcal disease occur annually, resulting in more than 135,000 deaths.

Serogroups A, B and C account for most cases of meningococcal diseases throughout the world, with serogroups B and C responsible for the majority of cases in Europe, America and serogroups A and C predominating throughout Asia and Africa [4]. Half of these cases occur in sub-Saharan countries (Africa), the so-called meningitis belt, along Ethiopia in the east to Senegal in the west, with a population of about 300 million [12]. However, hyper-endemic disease caused by serogroup A was observed in Finland and Russia and New Zealand in the late 1970s and 80s, and several pandemics have been described that originated in China. A recent study warns of the possible emergence of serogroup A in Greece [13,14].

In the beginning of 2006 during the meningitis season, outbreaks occurred at 32 districts in 7 countries of the African Meningitis Belt. In these affected countries, a total of 5,719 suspected cases, including 580 deaths, were reported to the World Health Organization (WHO). These occurred in two foci, one in West Africa, affecting Burkina Faso, Côte d'Ivoire, Mali and Niger, and characterized by the predominance of *N. meningitidis* serogroup A. Outbreaks in the second epidemic foci, in eastern Africa, concern Kenya, Sudan and Uganda, and were mainly caused by *N. meningitidis* serogroup W135 [15].

During January to April of 2007, the Ministry of Health of Burkina Faso reported 22,255 suspected cases including 1,490 deaths. Similar reports were received by the WHO from Uganda (from 1 to 21 January 2007 with 241 suspected cases and 16 deaths); the Sudan (from 1 January to 11 March 2007 with 6,946 suspected cases and 430 deaths); and the Democratic Republic of Congo (from 1 to 31 January 2007 with 53 suspected cases and 6 deaths). Cerebrospinal fluid specimens from all affected areas tested positive for *N. meningitidis* serogroup A by latex agglutination test and/or culture [15].

In 2000, serogroup W135 meningococci caused sporadic cases of disease, which was associated with large outbreaks among Hajj pilgrimages in Saudi Arabia and was also responsible for a severe epidemic in Burkina Faso.
in 2002. Serogroup W135 was observed in families of pilgrims returning to London, Singapore, and the USA from the Mecca in Saudi Arabia. Although carriage rates in family members of returning pilgrims are increased, these strains do not appear to have caused significant outbreaks more widely [13,16-18].

The importance of serogroup Y-caused disease has been rising in recent years, currently accounting for over 30% of cases in the USA [13]. The remaining capsular serogroups are rarely associated with disease, although outbreaks caused by meningococci of serogroup X have been recently described in Africa [18].

In industrialized countries, the incidence rate of such endemic disease is usually around 1-2 cases per 100,000 individuals, although explosive epidemics periodically occur in the Meningitis Belt with incidence rates that may exceed 500 per 100,000 [12,18].

Meningococcal disease occurs year-round, but the majority of cases occur during the winter and early spring. The rates of disease are highest among infants whose protective antibodies have not yet developed. The rates, however, decline after infancy and then increase during adolescence and early adulthood. Although the rates of meningococcal disease once again drop after early adulthood, more cases occur in individuals of 23 to 64 years old than in any other age groups (unpublished data). This change has important implications for preventive strategies [4,12,19].

3. Risk factors for infection

Since the carriage of *N. meningitidis* is widespread and the disease rate in most of the populations is relatively low, it has been suggested that individual susceptibility and the competency of the immune system may play an important role in determining the result of organism colonization. Some deficiencies of the immune system such as complement deficiency dispose individuals to recurrent episodes of disease, which are mostly caused by less common serogroups [20,21]. Deficiencies of the innate immunity such as the deficiency of mannose binding proteins (MBP) are known to be risk factors. Hypogammaglobulinemia is another risk factor which reflects the importance of the antibodies in complement-mediated bacteriolysis and opsonophagocytosis [22-24]. Although there are limited data and reports, hypo-splenism also may increase the susceptibility of meningococcal disease. Active or passive exposure to tobacco smoke, living and working in crowded families and factories, poor socioeconomic situation, and current viral infection in the upper respiratory tract are the other risk-modifying factors. The relevance of genetic polymorphism that leads to protein polymorphism related to meningococcal disease susceptibility and severity has been the subject of many detailed studies [25-29].

4. Correlations between vaccine efficacy and laboratory assays

Antibody dependent functions, the complement cascade, and inflammatory mediators are the most important parts of the immune system in protection against meningococcal disease. Serum bactericidal activity (SBA) and opsonophagocytosis are two main mechanisms that reflect the role of antibody and complement-mediated defense against *N. meningitidis*. SBA correlates with protection against meningococcal infection as shown in several clinical trials [30-33]. Also, patients with deficiencies in the terminal steps of complement-mediated lysis, such as membrane attack complex (MAC) formation are highly susceptible to meningococcal disease, especially to uncommon serogroups [34]. In addition to antibody and complement-mediated bacteriolysis, the role of opsonins is significant in the *in vivo* defense against the organism. The most important opsonins that facilitate phagocytic killing are immunoglobulins (especially IgG class) and complement mediators such as C3b and C4b. Ross et al. [35] have shown that serogroup B meningococci is more resistant to bactericidal killing than serogroup A and C but is highly susceptible to polymorphonuclear (PMN) killing mechanisms after opsonization [36].

Furthermore, a human monoclonal antibody against meningococcal group B polysaccharide that is highly opsonic but not bactericidal conferring high protection in animal models has been produced. There is relevance between serum bactericidal activity and the opsonophagocytosis mechanism. In fact, the antibodies acting in
bactericidal activity (especially IgG antibodies) are potent opsonins as well. A broad range of immune cells possess receptors specific for the Fc segment of IgG molecules (FcγR).

Also, components such as C3b and C4b that deposit on the bacterial surfaces following the complement cascade activation are other important opsonins and there are specific receptors for these mediators at the surface of PMNs (CD35) that mediate phagocytosis. Indeed, IgM and IgG triggering of complement activation and C3b and C4b (opsonins) deposition comprise effective SBA. An immune response in which the most efficient function is based on phagocytosis rather than bacteriolysis will be beneficial to the host, since intracellular destruction will minimize intravascular release of endotoxin and reduce the risk of septic shock [37-39].

4.1. Serum bactericidal activity

The complement-dependent bactericidal antibodies are the main immune response against meningococcal infection both in the natural disease and following vaccination. Thus, the presence of these antibodies has been widely accepted as evidence of the potential efficacy of meningococcal vaccines [40,41]. Serum bactericidal activity (SBA) was first demonstrated by Goldschneider and colleagues in 1969. From 1976 until now, the SBA assay requirement was recommended by the WHO Expert Committee on Biological Standardization as the gold standard for meningococcal polysaccharide vaccine licensure [41]. The accepted standard is that more than 90% of vaccinated adult persons should have at least a four-fold rise in SBA titer by the specified SBA assay for target strains of N. meningitidis. Meningococci are more susceptible to complement mediated lysis in the presence of exogenous rabbit complement in comparison with human complement. Thus, the accepted titer of ≥ 4 as a protective titer for human complement SBA (h SBA) and the rabbit SBA (r SBA) titer of ≥ 8 are currently established as a protective titer for meningococcal disease. In addition to r SBA titers of ≥ 8, additional evidence such as a seroconversion of ≥ 4 fold rise in SBA titer from pre- to post-vaccination is indicative of protection against meningococcal disease [40,42,43].

4.2. Opsonophagocytosis

N. meningitidis is prone to phagocytosis by peripheral blood polymorphonuclear (PMN) leukocytes, and an antibody raised against such a bacterium bears bactericidal properties in the presence of PMN and complement. Consequently, deficiency of a terminal complement pathway leads to susceptibility to meningococcal disease, particularly meningococci of uncommon serogroups [39,44]. However, the presence of serum opsonins facilitates phagocytic killing of meningococci which is of prime importance in in vivo defense mechanisms. Previous studies have revealed that serogroup B meningococci are more resistant to killing by antibodycidal properties of bacteria viz serogroup A and C meningococci, while, serogroup B meningococci are highly susceptible to destruction by PMN after being opsonized. Determination of opsonophagocytic activity of antibodies to meningococci is a measure of their in vivo functional activity. This method is based on semi-automated, noninfectious targets, providing useful laboratory correlates of protection, and can be multiplexed [44].

Therefore, the in vitro opsonophagocytic activity (OPA) of antibodies to meningococcal components may approximate their functional activity in vivo. The measurement of OPA can be performed by flow cytometry, chemiluminescence, or outdated oxidative burst activity of PMN. [35,44,45].

4.3. Antigen binding assays

OPA and SBA are related effector functions. Both represent a good surrogate of protective immunity, but the antibody levels (ELISA data) for each component in the vaccine is an essential part of, and more convenient for, evaluating vaccine immunogenicity. Furthermore, an ELISA may be particularly helpful for comparing antibody responses to vaccination in different age groups or populations. Therefore, it is useful to have an ELISA to measure total or isotype-specific serum antibody responses in a larger number of vaccinated subjects. An accurate assessment of vaccine immunogenicity and information on the type of antibody response, that helps to distinguish native polysaccharide from conjugated polysaccharide antigens, has been provided by the ELISA method [46].

High serum antibody responses to meningococcal polysaccharide by ELISA in the presence of low or undetectable bactericidal antibody have been detected, especially in infants, toddlers, and even in some adults who receive
meningococcal polysaccharide vaccines. The most likely explanation is that the polysaccharide vaccine, as a T-cell independent antigen, does not give rise to antibody affinity maturation so that the immune response consists principally of low-avidity anti-capsular antibodies. These antibodies are detected by ELISA but appear to be either less functional or nonfunctional compared to detectable bactericidal antibody in the SBA. These results are problematic if the IgG ELISA antibody responses are being compared after one or two doses of plain polysaccharide or after one or two doses of protein conjugate vaccine; a single dose of either vaccine can elicit low-avidity antibodies, particularly in infants and toddlers [40, 41].

5. Meningococcal polysaccharide vaccine

Encapsulated bacteria such as Streptococcus pneumoniae, N. meningitidis, and Haemophilus influenzae type B (Hib) are major causes of diseases all over the world. The development of vaccines against these organisms has targeted their capsular polysaccharides (CPS), as anti-capsular antibodies often protect against these diseases [47]. The capsular polysaccharide vaccines that have been available against these organisms are neither immunogenic nor protective in young children and certain immune compromised individuals. Immune responses against carbohydrate antigens have been categorized as T cell-independent (TI) in nature because they do not require cognate interactions between antigen specific B and T cells. These antigens are known to stimulate an antibody response in athymic mice. In general, polysaccharide (PS) antigens elicit a T cell-independent immune response, characterized by lack of memory, and poor immunogenicity at the extremes of life [47, 48].

Further division of TI antigens into TI-1 and TI-2 types was proposed by Mosier et al. in 1977. TI-1 antigens are defined as B cell mitogens, or antigens capable of inducing proliferation and differentiation of both naive and mature B cells. Conversely, TI-2 antigens possess large molecular weights and have highly repetitive structures that exhibit no intrinsic B cell stimulating activity. These antigens are also characterized by their poor in vivo degradability and inability to stimulate MHC class II restricted T cell help. TI-2 antigens will activate only mature B cells and are hypothesized to act by cross-linking the cell surface immunoglobulin (Ig) of specific mature B cells. Capsular PS from S. pneumoniae and N. meningitidis exemplify TI-2 antigens [47, 48].

5.1. Polysaccharide vaccines against group A, C, Y, and W135 meningococcal disease

Gotschlich et al. produced the first successful purified CPS vaccines against serogroup A and C N. meningitidis. Large-scale field trials were conducted in the 1970s among different age groups in many parts of the world including Europe, Africa and Latin America. These trials showed that the CPS A vaccines were effective in controlling epidemics of serogroup A disease in almost all age groups [49]. It soon became clear that antibody responses among children to serogroup A and C CPS vaccines depended on a number of factors including the age of the child, the number of doses of antigen, and the prior experience of the child with naturally occurring antigens cross reactive with the meningococcal CPS. Furthermore, the molecular weight of CPSA had been too small (less than 20,000 instead of 80,000 or greater) to induce good responses. Inadequate molecular weight, however, was not the only problem in the development of meningococcal polysaccharide vaccines [50].

The serogroup A and C CPS vaccines have major short-comings. First, they are serogroup-specific and therefore not protective against serogroup B. Second, they offer only short-lived protection which lasts not more than 3 years. Third, they offer little, if any, protection to the most vulnerable age group, namely children under 2 years old. Children under 2 respond, particularly to CPS A, but with only small and temporary increases in specific antibodies [50].

It is interesting to note that the serogroup A capsular polysaccharide dose not appear to be a traditional T cell independent antigen as is the group C capsular polysaccharide [51]. First, group A polysaccharide vaccine has been shown to be immunogenic in children under 2 years. Second, the CPS A appears to stimulate affinity maturation, as indicated by an increase in avidity indices following vaccination [52, 53].

As mentioned above, CPS A is more immunogenic than C in infants aged less than 2 years old, but protective immunity after one dose lasts only a short time, and even after repeated injections, booster responses are low. Although
there is continuing debate about this subject, 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 not optimum properties for a vaccine intended for routine immunization. As result, polysaccharide vaccines against serogroup A and C meningococci have been widely used in epidemics, for outbreak control, or as secondary prophylaxis for close contacts of index cases with a good safety records [54]. Some countries, primarily in the Eastern Mediterranean Region, have adopted preventive use of polysaccharide vaccine in some form. It will be useful to evaluate the long-term effectiveness of these approaches. In the developed world, use of meningococcal polysaccharides is largely reserved for the control of outbreaks caused by strains with a capsular group represented in the vaccine. It is also recommended for the prevention of disease in high-risk groups, such as those with complement deficiencies or hyposplenia, travellers to highly endemic areas, military recruits, and possibly for college students [13,55,56].

Meningococcal tetravalent polysaccharide vaccine against meningococci of serogroup A, C, Y, and W135 is commercially available, as are bivalent products against serogroup A and C only.

MPSV4 is a tetravalent meningococcal polysaccharide vaccine (Menomune-A,C,Y, W135; manufactured by Sanofi Pasteur Inc., Swiftwater, Pennsylvania) available in the United States. Each dose consists of the four (A,C,Y,W135) purified bacterial capsular polysaccharides. The immunogenicity and clinical efficacy of the serogroups A and C meningococcal vaccines have been well established. Vaccine efficacy rates of 80-90% against group C disease have been observed in school-aged children and adults, whereas the serogroup A vaccine has demonstrated estimated clinical efficacies of 95% [57]. Thus serogroup A polysaccharide induces antibody responses among certain children as young as age 3 months, although a response comparable to that occurring in adults is not achieved until 4-5 years of age [58,59]; the serogroup C component is poorly immunogenic among recipients aged < 18-24 months. Serogroup Y and W135 polysaccharides are safe and immunogenic among adults and children aged > 2 years; however, clinical protection has not been demonstrated [60-62].

Vaccination with these polysaccharides induces production of bactericidal antibodies. The antibody responses to each of the four polysaccharides in the tetravalent vaccine are serogroup-specific and independent. As mentioned above, the poor immune responses to these and other polysaccharide antigens are attributed to the T cell independent nature of the immune responses they induce and the poor adaptation of the immature immune system to the mounting of such responses. A characteristic feature of T cell independent responses is the lack of induction of immunologic memory.

Reduced clinical efficacy has not been demonstrated among persons who have received multiple doses of vaccine. However, recent serological studies have reported that multiple doses of serogroup A and C polysaccharide vaccine might cause immunologic hyporesponsiveness (i.e., a reduced antibody response after subsequent challenge with the same polysaccharide antigen) to serogroup A and C polysaccharide. The clinical relevance of such hyporesponsiveness is unclear [63-66].

Meningococcal polysaccharide vaccines have been used extensively in mass vaccination programs as well as in the military and among international travelers. Adverse reactions to polysaccharide meningococcal vaccines are usually mild; the most frequent reaction is pain and redness at the injection site, lasting for 1-2 days. Estimates of the incidence of such local reactions have varied (range: 4-56%) [67,68]. In certain studies, transient fever occurred among ≤ 5% of persons vaccinated, more commonly among infants. Several reactions to polysaccharide meningococcal vaccine are uncommon [69].

5.2. Polysaccharide vaccines against group B meningococcal disease

When purified serogroup B polysaccharide was used to vaccinate adult volunteers, no measurable increase in anti-capsular antibody was noted [70]. 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 [71,72]. 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 [3,74]. Expression of this cross-reactive polysialic acid diminishes in most but not all adult tissues. Jennings and co-workers first proposed an innovative strategy for overcoming immunological tolerance to the group B meningococcal polysaccharide [75,76]. 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 [77]. 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 PorB 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, sponsored by Baxter Health Care (formerly, North American Vaccines) [78]. One safety concern with N-Pr group B meningococcal polysaccharide conjugate vaccines is that a subset of the anticapsular antibodies elicited has autoreactivity with host polysialic acid. Whether or not the autoreactive antibodies cause 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 diseases or interfere with neural migration in the developing fetal brain [12,78,79].

6. Non-capsular vaccine candidates for prevention of group B disease

Non-capsular antigens potentially offer protection that is independent of the capsular polysaccharide group, but with the rapid development of effective conjugate vaccines against groups A, C, Y, and W135, vaccines based on noncapsular antigens are often inaccurately described as group B vaccines [80,81].

Thus, alternative vaccine candidates have been sought since the early 1970s and research has been focused on major outer membrane proteins (OMPs) and lipopolysaccharide (LPS).

Outer membrane vesicle (OMV) vaccines have been developed by using detergent, mostly deoxycholate (DOC), to extract LPS in order to reduce the LPS content and hence the local and systemic reactogenicity [82,83]. Because of its central role in the pathophysiology of meningococcal disease, lipooligosaccharide has received some attention as a vaccine candidate with the potential to offer cross-protective immunity against diverse meningococci [84]. Isolates of N. meningitidis produce one of several different glycoforms of lipooligosaccharide, one of which (immunotype L3,7,9) is generally associated with isolates causing invasive disease [85]. Animal studies suggest that vaccines based on detoxified lipo-oligosaccharide or L3,7,9-toxoid conjugates elicit opsonic antibody responses that seem immunotype specific [84]. Plested et al. showed that conserved inner core epitopes of lipo-oligosaccharide can also elicit protective immunity in animals, indicating that the inner core structure may be potential as a cross-protective vaccine component. However, clinical trial data assessing the effectiveness of these vaccines is not yet available [86].

7. Meningococcal glyconjugate vaccines

Bacterial polysaccharides, including those comprising the capsule of N. meningitidis, are T cell-independent antigens. T cell-independent antigens do not elicit a memory response; they stimulate mature B lymphocytes but not T lymphocytes, thus inducing a response that is neither long-lasting nor characterized by anamnestic response after subsequent challenges with the same polysaccharide vaccines having inherent limitations [47]. Efforts to overcome the obstacles associated with the PS antigens have focused on converting the TI-2 antigens to T cell-dependent (TD) antigens. The major strategy used to achieve a TD immune response to PS antigens is through conjugation of the PS to a carrier protein [48]. Most of the studies characterizing the cellular interactions responsible for TD immunity to conjugate vaccines have been performed with the
H. influenzae type b (Hib) vaccine preparations [87]. The proposed mechanism of action for conjugate vaccines combines aspects of TI-2 and TD B cell activation. B cells recognize the PS component through specific interactions with Ig surface receptors. The PS specific B cells can then internalize the PS-protein conjugate. Intracellularly, the protein is processed; peptide fragments are presented by MHC class II molecules and activation of peptide-specific T cells results. PS-specific B cells are thought to receive stimulatory cytokines from activated T cells and become antibody-secreting B cells. Furthermore, it is postulated that the function of a conjugate is to ensure localization of protein antigens near the site of PS recognition [47,48,88].

Several highly immunogenic proteins have been proposed as the protein component, but mainly four have been used: diphtheria (DT) or tetanus (TT) toxoids, CRM197 (a non-toxic variant of diphtheria toxin), and a complex outer-membrane protein (OMP) mixture from N. meningitidis. The polysaccharides or an oligosaccharide are linked to the carrier, either directly or with carbon spacers. The toxoids were chosen as the carrier proteins because, apart from their inherent immunogenicity, if the recipient had been earlier immunized with the toxoid, a booster effect was expected. However, under certain circumstances, suppressive effects can also occur [88,89].

7.1. Serogroup C conjugate vaccines
A thymus-dependent immune response is achieved through conjugation of group C-specific meningococcal polysaccharide to a protein carrier. In November 1999, monovalent serogroup C conjugate vaccines were introduced in the United Kingdom. The national vaccination campaign introduced a 3-dose infant vaccination series and implemented a mass catch-up campaign during 1999 to 2000, targeting all persons aged 1 to 17 years [90].

Presently, three groups of C meningococcal conjugate (MCC) vaccines are currently licensed internationally. The three serogroups C conjugate vaccines used in the United Kingdom are Meningtec™ (Wyeth Lederle Vaccines and Pediatrics, Pearl River, New York); Menjugate™ (Chiron Vaccines, Siena, Italy); and NeisVac™ (Baxter Hyland Immuno, Beltsville, Maryland). All contain the same amount of meningococcal polysaccharide antigen (10µg), which is less than that contained in most polysaccharide vaccines (50µg). Two vaccines (Meningtec and Menjugate) contain short-chain oligosaccharide (O-acetylated) derived from serogroup C capsular polysaccharide, conjugated to CRM197, a nontoxic mutant diphtheria toxin. The third vaccine (NeisVac) contains a serogroup C polysaccharide (de-O-acetylated) conjugated to tetanus toxoid [91,92].

Both types of conjugate vaccines induce enhanced levels of IgG anti-capsular antibodies and memory B-cells. In late 1999, immunization against group C meningococcal disease using MCC vaccines became part of the national immunization program in the United Kingdom, where at that time the incidence of meningococcal serogroup C disease was approximately 2 per 100,000 population. Infants were vaccinated at 2, 3, and 4 months of age and children aged 4 to 13 years and teenagers were offered catch-up vaccination [12]. Large-scale serological studies in the United Kingdom showed that 16 months following vaccination with a single dose of the MCC vaccine, 88% of children aged 1.2 years still had protective antibody levels, whereas among adolescents aged 15 to 17 years, 96% had protective levels. Preliminary data based on a serum bactericidal assay suggested that three doses of MCC vaccine at intervals of 2 months provided high levels of protection in infants. Following the introduction of MCC vaccination into the routine infant schedule and extensive catch-up vaccination of young children and teenagers, there was a rapid decline in group C meningococcal disease. Moreover, careful surveillance has shown no evidence of changes in the prevalent serogroups and serotypes among invasive meningococcal isolates since the MCC programme was launched in the United Kingdom. The United Kingdom experience confirms that the safety profile of the current MCC vaccines is excellent. Fortunately, previous immunization with unconjugated group C polysaccharide does not compromise the immune response to MCC vaccines and none of the MCC vaccines interfere with the response to co-administered vaccines of the national immunization programme (United Kingdom). Although clearly inducing immunological memory, the period of observation is too short to allow conclusions on the duration of
protection in the various age groups. However, based on experiences gained with the successful conjugate vaccine against \textit{H. influenzae} type b, the MCC vaccines may be expected to provide high levels of protection for at least 10 years following completion of the 3-dose course in infants or a single dose in adolescents [12,56]. Furthermore, the observed relatively high post-immunization concentrations of mucosal anti-group-C antibodies are likely to counteract colonization of group C meningococci and hence result in a herd immunity effect [93,94].

7.2. Serogroup A conjugate vaccines

A more complex picture evolves regarding the immunogenicity of serogroup A conjugates. Owing to the high immunogenicity of the group A polysaccharide, IgG responses to group A antigen after two doses of the conjugate vaccine in infants were not significantly higher than after two doses of the plain polysaccharide vaccine [95,96]. The different quality of the response to the conjugate vaccine is nonetheless illustrated by the fact that the SBA results were 20-fold higher in conjugate recipients [95]. Regarding the induction of memory, data for serogroups A and C differ again. Memory has conventionally been assessed by the administration of a plain polysaccharide booster to children who had been “primed” with conjugate vaccines. Most studies administered the booster to individuals at around 1 year of age, when polysaccharide responses are usually minimal and memory responses for serogroup A conjugates have been less consistent. A study in the Gambia failed to demonstrate immunologic memory in individuals 2 years of age following a three-dose schedule in infancy. Further follow-up of this cohort confirmed that at 5 years of age, only those individuals who had received an additional group A conjugate vaccine dose at 2 years of age had meaningful immunologic memory [13,97,98]. In those who had not received a conjugate booster, IgG levels rose but not SBA titers. In contrast, a study in Niger, in which three conjugate doses in monthly intervals were also given, showed vigorous responses to a polysaccharide booster administered at 1 year of age. The reasons for this discrepancy are unclear. The two vaccines were based on different carrier proteins: the Niger study tested a diphtheria-conjugate, whereas the Gambian study used the mutant diphtheria toxoid CRM197 as a carrier. Possibly more importantly, it has been suggested that the size of the oligosaccharide molecules in the Gambian study vaccine may have been suboptimal. No efficacy data are available for group A conjugate vaccines so far [13,99].

7.3. Meningococcal tetravalent conjugate vaccine

In 1981, MPSV4 (Menomune) was licensed in the United States on the basis of data on safety and immunogenicity. Immunogenicity of this vaccine was compared with that of the vaccine then licensed for use in the United States, A/C meningococcal polysaccharide vaccine, which had demonstrated 97% efficacy against serogroup A and 90% efficacy against serogroup C [100]. The immunologic criterion used for licensing was a fourfold or greater rise in SBA among 90% of adults at 3 to 4 weeks after vaccination. As a result, in 2005, MCV4 (Menactra) was licensed on the basis of findings indicating that it was not inferior to MPSV4 in terms of immunogenicity and safety. A primary criterion in determining immunogenic non inferiority of the new vaccine was the percentage of vaccines having a fourfold or greater increase in bactericidal antibody for MCV4 compared with MPSV4 [56]. Vaccine composition MCV4 is a tetravalent meningococcal conjugate vaccine (Menactra, manufactured by Sanofi Pasteur Inc., Swiftwater, Pennsylvania). A single dose (0.5 mL) of this vaccine contains 4 µg each of capsular polysaccharide from serogroups A, C, Y, and W-135 conjugated to 48 µg of diphtheria toxoid. MCV4 is available only in single-dose vials [56].

7.4. Alternative carrier proteins for meningococcal vaccines

The increasing use of glycoconjugate vaccines employing toxoids and CRM197 as carriers has led to concerns about carrier-induced immune suppression, particularly with multivalent conjugate vaccines in which the dose of carrier protein can be high. A recent clinical study showed that the immune response to MCC with CRM197 vaccine was significantly reduced when it was coadministered with a 9-valent pneumococcal CRM197 vaccine. These concerns have stimulated interest in new carrier proteins that may augment the immune response or provide additional protection against meningococcal or other diseases. Carriers for group C polysaccharide tested in preclinical studies include a recombinant
membrane-associated P64 protein from *N. meningitidis* N19, a string of human universal CD4 T cell epitopes from various pathogen-derived antigens, and *Bordetella pertussis* filamentia. A recombinant porin protein (rProB) from *N. meningitidis* was used as a group B carrier and the immunogenicity and potency of a tetravalent meningococcal conjugate vaccine developed for infants has been reported to be enhanced when diphtheria toxoid was substituted with a surface protein from pneumococci, PspA. In addition, the potential of outer membrane protein D of *H. influenzae* as a carrier has been demonstrated in preclinical studies of Hib PRP conjugates [18].

8. Outer membrane protein vesicle-based vaccines

Outer membrane vesicles (OMV) can be prepared by detergent extraction from meningococcal bacterial cells. The composition of OMV is complex. The main constituents are PorA, phospholipids, LPS, residual DNA, RmpM, and residual detergent. The interaction of these compounds with each other through various non-covalent forces determines the physicochemical properties and stability of OMV [101].

The first OMV-based vaccines were developed by the Finlay Institute, Cuba, and the National Institute of Public Health, Norway, in response to group B meningococcal outbreaks in these countries [102]. The vaccine produced by the Finlay Institute has already been registered in 19 countries, mainly in Latin America. Recently GlaxoSmithKline and Chiron Vaccines have signed commercial agreements with the Finlay Institute and the Norway National Institute of Public Health, respectively, for further development and commercialization of first generation of OMV-based meningococcal vaccines for parts of Europe and the USA. The use of these vaccines has not shown any notable serious adverse effects on many individuals, except minor local reactions at the injection site, transient malaise, and fever [103].

While OMV based vaccines have been efficacious, these vaccines have multiple limitations. First, the major components show sequence and antigenic variability and, consequently, the induced protection is strictly serotype-serosubtype specific. In addition, the complex multiple-protein-based composition of these vaccines makes them difficult to manage from standardization and quality control points of view [18].

As described below, these vaccines elicited strain-specific bactericidal antibodies, which undoubtedly contributed to the level of protection. There was also some evidence from the efficacy trials that protection was not restricted to the homologous strains from which the vaccines were prepared. Although further data are needed to formulate sound conclusions, this finding suggests that immune mechanisms, in addition to complement-mediated bactericidal antibody, may contribute to protection [104].

In 1998, the WHO approached vaccine manufacturers to develop a vaccine designed specifically against the lineage III, P1-7, 4 strain which is responsible for nearly a decade of epidemic group B disease in New Zealand. In response, Chiron Vaccines and the National Institute of Public Health (Norway) have recently signed an agreement with the New Zealand Ministry of Health, and the vaccine is expected to be available in 3 to 4 years [12].

Currently, to provide protection against additional strains, a hexavalent recombinant PorA vaccine is being developed in the Netherlands at Rijksinstituut voor Volksgezondheid en Milieu with OMV prepared from two strains that each express three different PorA proteins [105]. Wyeth Vaccines and the institute have an agreement to advance the commercialization of such a vaccine. The hexavalent PorA OMV vaccine has been assessed clinically in adults, children, and infants [106-108]. In infants, the bactericidal antibody responses after a primary series of two or three injections were only modest. However, a fourth booster dose given in the second year of life elicited higher titers [106]. Thus, one unresolved issue is whether the modest responses after primary immunization will be sufficient to confer protection before the booster injection, especially since infants under a year of age are at greatest risk of group B disease. A second issue is the large number of PorA strains that would need to be included to confer broad protection—e.g., in the USA, a hexavalent vaccine would have the potential to prevent less than 50% of endemic group B meningococcal disease. Thus, the clinical data from OMV-based vaccines provide the clearest proof that certain membrane protein
antigens can elicit protective antibodies in humans to encapsulated group B meningococci [109]. In addition to OMV-based vaccine development, activities on subunit development have been initiated, which include OMPs. A few new OMP candidates were recently discovered via the availability of full genomic sequences for vaccine design [12,18].

9. Future meningococcal vaccines

9.1. Room to be developed

To improve immunogenicity, conjugates of CPS A have been synthesized. One of the CPS A conjugates is composed of a partially hydrolyzed, size-fractionated oligosaccharide, being less immunologic than CPS A. Reports of the other CPS A conjugates provide only limited information on their syntheses and composition [110]. The serogroup A conjugate vaccines have proven, in multiple studies, to be safe and to induce anti-polysaccharide antibodies at least at the same levels and with similar or higher bactericidal titers compared to the polysaccharide vaccine. Although previous studies did not demonstrate induction of immunological memory in infants, as measured by the response to a booster dose of serogroup A polysaccharide vaccine, this is contrasted by recent studies. Immunological memory to a serogroup A conjugate vaccine in infants and children has also been demonstrated as indicated by a rise in IgG avidity maturation [111].

Jin et al. (2003) showed that CPS A conjugates are more immunogenic than the CPS A purified vaccine [110]. In 2005, the planned meningitis vaccine project employed serogroup A conjugate for preclinical studies and clinical trials. Later on, a Phase 1 study of this vaccine (in India) showed that the product is safe and immunogenic. Phase 2 studies have begun in Africa and will be ended by 2008-9 [111].

An alternative approach for the development of a T-cell dependent meningococcal vaccine is the use of protein-based OMV vaccines. Serogroup B meningococcal vaccines based on OMVs have been developed, and in clinical trials these vaccines have been shown to be safe and efficacious. Significant increases in bactericidal antibody response against the homologous strain were observed in 96–98% of the vaccines in all age groups studied [111]. Previous studies have demonstrated memory induction and a prolonged duration of antibody response following three doses [112,113]. Serogroup B OMV-based vaccines might therefore be effective to control epidemic meningococcal disease where most cases are caused by homologous organisms. This strategy is now being tried in New Zealand and results from phase I/II clinical trials are promising. Epidemics caused by serogroup A meningococci have basically been clonal; thus there could be a rationale for preventing meningococcal meningitis epidemics in Africa with vaccines targeting subcapsular antigens [111]. It was shown (2005) that an OMV vaccine towards serogroup A meningococcal disease was very immunogenic and induced bactericidal antibodies in mice. Their studies also showed that the antibodies were mainly directed towards the PorA outer membrane protein. The production process of OMV vaccines is simpler than the production of conjugate vaccines, and the researchers therefore believe that this could be a realistic and less expensive alternative to a conjugate vaccine [111].

In the previous study, Fukasawa et al. (1999) showed that serogroup C polysaccharide conjugated with OMV can be used as a serogroup B/C bivalent antigen. Therefore, the development of B/C bivalent anti-meningococcal vaccine would be desirable and this glycoconjugates vaccine could be a good candidate [46].

In 2006-7 Siadat et al. reported that conjugates of CPS A with OMV of serogroup B can induce highly protective antibody with bactericidal and opsonophagocytic activity against serogroup A N. meningitidis in animal models [32,33,114].

9.2. Genomic and reverse vaccinology

As described previously [12,13,56], very effective vaccines based on the capsular polysaccharides against meningococcus C are already on the market and anti-meningococci A, C, Y, and W135 polyvalent vaccines are expected to be launched in the near future. However, there are no vaccines available for the prevention of group B meningococcal disease responsible for a large proportion (from 32% to 80%) of all meningococcal infections. Because the CPS B is a poor immunogen in humans, the use of this polysaccharide in a vaccine may elicit autoantibodies [12,56]. An alternative approach to vaccine development is based on the use of surface-exposed proteins, which in some instances have been shown to elicit protective
bactericidal antibodies. In this context, every protein synthesized by the bacteria can be tested as a vaccine candidate without any prior selection based on their in vitro expression or their role in virulence and immunogenicity [115,116]. This method based on the identification of vaccine candidates from a whole genome sequence rather than from live microorganisms has been termed reverse vaccinology [117]. Thus the first potential vaccine against N. meningitidis serogroup B was developed with the reverse vaccinology approach; this pioneer project led to the definition of a new paradigm of a more general use in developing the next generation of vaccines [18].

N. meningitidis serogroup B represents the first example to which reverse vaccinology has been applied [18].

The genome sequences of MC58 (a serogroup B meningococcus) and of Z2491 (a serogroup A strain) were elucidated by the random shotgun strategy and published in 2000 [118,119]. While the MC58 genome sequencing was in progress, Pizz et al. began identifying the open reading frames (ORFs) that were predicted to encode either membrane bound, surface exposed or exported proteins [120]. Among the 2,158 putative open reading frames annotated, 650 ORFs were selected on the basis of these criteria. These putative antigens included different classes of proteins, according to their predicted localization on the bacterial cell structures: outer membrane or secreted proteins; lipoproteins; inner membrane proteins; periplasmic proteins; and also other proteins homologous to bacterial factors involved in virulence and pathogenesis.

The selected 650 ORFs were amplified from meningococcus by PCR, and cloned into Escherichia coli in order to express each gene as either His-tagged or glutathione S-transferase (GST) fusion protein. Out of these 650 putative ORFs, 50% were successfully expressed, purified, and used to immunize mice. Screening of immune sera was performed by Western blot on meningococcus total cell lysates and outer membrane vesicles to verify whether the protein was really expressed in meningococcus and to determine its subcellular localization. The surface-exposure of each antigen was then confirmed by fluorescence-activated cell sorter (FACS) analysis and ELISA on whole cell bacteria. Finally, sera were tested in a bactericidal assay, an assay which is known to correlate with protection in humans. Ninety-one proteins were found to be surface-exposed; 29 of them were able to induce bactericidal antibodies [30,120].

Most of the antigens identified by the conventional approach showed strain variability or were expressed only in some strains, resulting in protection against the homologous but not the heterologous strains in humans. Therefore, the candidate antigens selected by genome-analysis, were evaluated for gene presence and sequence conservation in a panel of 31 N. meningitidis serogroup B strains isolated in different parts of the world, and representative of the major serogroups. Sequence alignment revealed that the majority of the antigens analyzed are well conserved while only a few of them present highly variable regions and carry multiple epitopes conserved in most strains. Finally, most of these antigens, tested in bactericidal assays, were able to induce cross-protection against heterologous strains. Surprisingly, the antigens identified by the genomic approach are quite different from those identified using conventional vaccinology. In fact, in addition to classical outer membrane proteins with variable loops, many of the newly selected antigens were lipoproteins or surface-exposed proteins with a globular structure and without membrane crossing domains. Furthermore, some of these antigens were not abundant on the bacterial surface [116,118]. In conclusion, in only a few years, reverse vaccinology has resulted in the identification of more vaccine candidates as compared to those discovered during the previous 40 years [117]. Even if it is too early to speculate when a vaccine against meningococcus will be available, the investigators found over 28 novel proteins that elicited group B meningococcal antibodies which bound to the bacterial surface or had bactericidal activity [120]. The use of genomic technologies allows discovering previously unknown and undescribed proteins. It is crucial to further characterize these novel molecules as vaccine candidates and also to understand their role and function [118]. Many of the novel outer membrane or surface-exposed proteins identified in N. meningitidis serogroup B share interesting homologies to known virulence factors. Among these the newly-identified antigens GNA33, NadA, GNA992 and GNA1870 have been further characterized from the biochemical and functional
point of view. GNA33 (genome derived Neisseria antigen) is a lipoprotein highly conserved among meningocococcus serogroup B strains, other meningococcal serogroups, and gonococcus [121,122]. Biochemical analysis confirmed that the molecule is a murein hydrolase of the lytic transglycosylase class as it is capable of degrading both insoluble murein sacculi and unsubstituted glycan strands. It has been shown that the recombinant GNA33 elicits antisera that are bactericidal and confer passive protection against bacteraemia in infant rats by mimicking a surface-exposed epitope on loop 4 of Porin A in strains with serosubtype P1.2. NadA (Neisseria adhesin A, NMB1994) induces strong bactericidal antibodies against both homologous and heterologous strains, suggesting that this protein could be a good candidate for a vaccine. This protein has, in fact, a carboxyl terminal membrane anchor domain and an internal region with high coiled-coil probability. Interestingly, NadA forms very stable high molecular weight oligomers, and such oligomers are anchored to the outer membrane of meningococcus. The gene is present in three out of four hypervirulent lineages and its sequence is highly conserved among different strains. Furthermore, NadA is able to bind to human cells in vitro. GNA992 has been postulated to promote adherence of meningococcus to host cells by mimicking the cell–cell recognition phenomena that occur at the neural level. By FACS analysis and Western blot on outer membrane vesicles, GNA992 has been shown to be surface-exposed. Antibodies elicited by GNA992 are bactericidal against a subgroup of N. meningitidis serogroup B strains and therefore this antigen is regarded as a possible component of a multi-component protein-based vaccine [18,122-124]. Masignani et al. (2003) discovered GNA1870, a new surface-exposed lipoprotein of N. meningitidis that induced high levels of bactericidal antibodies. This antigen is expressed by all strains of N. meningitidis. Sequencing of this gene showed that the protein can be divided into three variants. The level of expression varies among strains, which can be classified as high, intermediate and low in the level of gene expression. Bactericidal titers are highest against those strains expressing high yields of the protein, and even very low levels of gene expression can be efficacious. This novel antigen is a top candidate for development of new vaccine against meningococcus [125].

9.3. Live vaccines

Mucosal colonization with a complex microbial ecosystem is often necessary for the development and maturation of natural immunity against infectious agents. The development of natural immunity elicited during childhood through exposure to cross-reactive and cross-protective antigens of nonpathogenic commensal Neisseria spp in the nasopharynx could be modified and applied as live vaccine [126]. Epidemiological evidence suggests that the nonpathogenic N. lactamica, a commensal species, has common antigenic structure/s with N. meningitidis, may be involved in natural immunity. It is likely that the use of antigens from this species in vaccines would be effective against N. meningitidis. The immunization with N. lactamica can mimic infection and enhance natural immunity to the meningococcal disease by detectable SBA against three important serogroups of N. meningitidis. This hypothesis could be supported through studying the response of cross-reactive antibody elicited by antigens of N. lactamica to the meningococcus and the potential exploitation of such common antigens for the development of a comprehensive vaccine against meningococcal diseases [127].

The most critical point to consider in the application of such live vaccines is safety, particularly the possibility and the risk of appearance of wild type strains through taking up exogenous DNA; but it is unlikely that this reversion would occur if the vaccine strain was derived from an organism never associated with the pathogenicity, such as N. lactamica. The molecular understanding and genetics of pathogenic/nonpathogenic strains could be very informative in the analysis and assessment of these strains so as to choose the virulence factors for designing and development of meningococcal live vaccines [81].

Recently, it has been shown that immunization of mice with OMVs derived from N. lactamica induced protective immunity in the absence of detectable SBA [128].

An alternative approach for combating bacterial infection is the development of live attenuated vaccines. Notable examples of live attenuated bacterial vaccines are Salmonella
enterica serovar Typhi vaccine Ty21a and Mycobacterium bovis BCG; thus these two recombinant typhoid strains are in development. Multivalent live attenuated vaccines can also be used to circumvent limitations imposed by antigenic variation. Li et al. (2004) reported that the selection and construction of attenuated strains of serogroup B N. meningitidis must be based on analysis of such genes involved in systemic infection. They experimentally showed that the attenuated strains were used in murine models, both to examine the basis of protection and to define the specific epitope(s) responsible for the development of immunity [129].

There is still a long way to go in developing live vaccines but the availability of modern techniques and instruments makes the goal attainable.

References

59. Peltola H, Kayhty H, Kuronen T, et al. (1978) Meningococcal group A vaccine in children three months to five years of age: adverse reactions and immunogenicity related to endotoxin content and


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