Development and characterization of the immune response induced by peptides and DNA constructs that mimic the capsular polysaccharide of Neisseria meningitidis serogroup C

Deborah Marie Prinz
Medical College of Ohio

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Development and Characterization of the Immune Response Induced by Peptides and DNA Constructs that Mimic the Capsular Polysaccharide of Neisseria Meningitidis Serogroup C

Submitted by

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In partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Medical Sciences

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Development and Characterization of the Immune Response Induced by Peptides and DNA Constructs that Mimic the Capsular Polysaccharide of *Neisseria meningitidis* Serogroup C

Deborah Marie Prinz

Medical College of Ohio

2005
DEDICATION

This work is dedicated to my family and friends, who without their continued love and support I probably would not have been able to accomplish this step in my life. To mention every one of those individuals who have influenced my life and made the past 4 years a pleasurable journey would take an infinite amount of time. But to everyone who has been there for me, I thank you for touching my life and making this a wonderful experience.

To my mother who is my dearest and closest friend, I thank you for everything you have done for me. Thank you for always being there no matter what the circumstance and for helping to keep my drive going in order to achieve my goals. You are the most amazing woman I know, and if I can be half the woman you are, I know I can succeed at anything I try to do. You have taught me so much and have instilled in me so many qualities that I need in order to preserve and excel. No words can be said to thank you for all the sacrifices you have made for me to succeed.

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I would like to dedicate this work to all those who have passed away in my family, especially my grandmother Margaret Mulhern, my grandfather, Paul Prinz (Pop-pop), my grandfather, James Mulhern, and my dear great uncle John Minnich. You will never be forgotten.
Finally, my biggest supporter on my journey passed away a year before I finished. I told her that I would dedicate this work in her memory. Well I finally finished Gram, so here’s to you! I love you more than you’ll ever know and thank you for being my grandma.
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Two different methods result in the selection of peptides that induce a protective antibody response to *N. meningitidis* serogroup C

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INTRODUCTION

*Neisseria meningitidis* (*N. meningitidis*), an encapsulated gram-negative bacterium, is one of the leading causes of bacterial meningitis worldwide. The polysaccharide capsule surrounding the organism is the major virulence factor and is essential for pathogenicity. It has been demonstrated that antibodies directed against the capsular polysaccharide protect individuals from infection by inducing complement-dependent bactericidal activity (Goldschneider, et al. 1969). Consequently, one of the first vaccines targeted against *N. meningitidis* was composed of purified capsular polysaccharide (Gotschlich, et al. 1969). The major disadvantage of this vaccine is its inability to induce protective immunity in children less than 2 yrs of age. The poor immunogenicity of the capsular polysaccharide is associated with its T-independent nature. Efforts to convert this T-independent antigen to a T-dependent antigen have resulted in the polysaccharide conjugate meningococcal serogroup C vaccine (Jennings and Lugowski 1981).

An alternate method of converting a T-independent antigen to a T-dependent antigen is through the use of molecular mimicry. Molecular mimicry is defined as the ability of structurally unrelated molecules to exert the same biological effect. The concept of mimicry is based on Jerne’s idiotypic network hypothesis that theorizes mimicry as the function of reproducing the binding interactions between an antibody and antigen (Jerne, 1974). Recently, this concept has been extended through the development of phage display libraries that are capable of selecting peptides that mimic capsular polysaccharides. Both techniques of anti-idiotypic antibodies and phage display libraries have been used to identify peptides that mimic carbohydrate
antigens and are capable of eliciting functional anti-carbohydrate antibody responses (Westerink, et al. 1995; Pincus, et al. 1998; Lesinski, et al. 2001). In principle, the major advantage of using peptides as vaccine candidates targeted against encapsulated bacteria is the inherent ability of a peptide to be recognized and processed by MHC molecules, thus inducing a desired T-dependent immune response, including memory.

A natural extension of the observation that peptides can mimic polysaccharides is the development of DNA constructs that encode peptide mimics of polysaccharides. The DNA constructs have the potential to induce long lasting humoral and cellular immune responses. The DNA constructs are easily altered to manipulate the magnitude and orientation of the desired immune responses. Recently, studies (Velders, et al. 2001; Whitton, et al. 1993) have demonstrated the success of encoding multiple B and T cell epitopes in a single DNA construct. This is important in vaccine development for an encapsulated organism like *N. meningitidis* where multiple epitopes representing carbohydrate as well as protein antigens can be included, directing the response to specific meningococcal polysaccharide as well as conserved protein epitopes.

The following experiments will describe the evaluation of a peptide mimic of the capsular polysaccharide of *N. meningitidis* serogroup C (MCPS) selected through biopanning phage libraries compared with a peptide mimic of MCPS selected using anti-idiotypic technology. These studies also will describe the evaluation of the immune response to vaccination with a multi-epitope DNA construct encoding the peptide mimic of MCPS. The hypotheses of these studies are as follows:
1. Peptides that mimic the capsular polysaccharide of *N. meningitidis* serogroup C (MCPS) can be selected using various methods.

2. Both anti-idiotypic antibody and phage display selected peptide mimics of MCPS can elicit a protective anti-MCPS antibody response in mice.

3. A multi-epitope DNA construct encoding a peptide mimic of MCPS can induce a functional anti-MCPS antibody response that can protect mice against lethal meningococcal challenge.

4. A multi-epitope DNA construct encoding a peptide mimic of MCPS can induce a T-dependent immune response with predominantly Th1 characteristics.
LITERATURE REVIEW

Neisseria meningitidis

History

Cerebrospinal meningitis was first described in Geneva, Switzerland in 1805 by Vieusseux as a “malignant purpuric fever” suggestive of meningococcal disease (Vieusseux, 1805). Marchiafava and Celli (1884) first documented the organism Neisseria meningitidis in 1884 as the causative agent of cerebrospinal meningitis when they discovered micrococcii in phagocytes from cerebrospinal fluid. Three years later in 1887, Anton Weichselbaum (1887) isolated the meningococcus from patients with meningitis and described the organism as an intracellular diplococcus. Weischelbaum named the organism Diplococcus intracellularis meningitidis, but was later renamed N. meningitidis after the German scientist and clinician Albert Neisser (Neisser, 1879). Although these were the first discoveries of the meningococcus, clinical descriptions and epidemics have been recorded as early as the 16th century (Willis 1684; Skinner 1970). Since then epidemic meningitis has been documented and reported in several countries due to the high morbidity and mortality associated with meningococcal disease.

N. meningitidis Structure

N. meningitidis is an encapsulated gram-negative, spherical bean-shaped bacilli that is seen naturally growing in pairs (van Deuren, et al. 2000; Pollard and Frasch 2001). The meningococcus cell envelope is built up with both an outer membrane and an inner (cytoplasmic) membrane, which are divided by a
peptidylglycan cell wall (DeVoe and Gilchrist 1975; Morley and Pollard 2001) (Figure # 1).

Figure 1. The Structure of *Neisseria meningitidis*

The outer membrane is composed of lipids, lipopolysaccharides (LPS or endotoxin), numerous outer membrane proteins (OMPs), and surface appendages, called pili or fimbriae (Poolman, et al. 1982; Pollard and Levin 2000). Attached to the outer membrane is the most external layer, the polysaccharide capsule. The polysaccharide capsule is the main virulence factor of the organism and is essential for pathogenicity.
It protects the meningococcus from its external environment and helps confer resistance to phagocytosis and complement-mediated lysis (Morley and Pollard 2001). Together the outer membrane proteins and the capsular polysaccharide form the principle surface of the meningococcus.

*Neisseria meningitidis* is classified into serogroups, serotypes, subtypes, and immunotypes. Thirteen different serogroups exist (A, B, C, X, Y, Z, W135, 29E, (Z’), H, I, K, and L) defined on the basis of the chemical composition of the polysaccharide capsule (Frasch 1990). Some meningococcal capsular polysaccharides are composed entirely of polysialic or sialic acid [N-acetylneuraminic acid (NANA)] linked to alpha 2-8/alpha 2-9 or glucose/galactose (i.e., B, C, Y, and W135), whereas others are not composed of sialic acid [N-acetylemannosamine 1-phosphate] (i.e., A) (Liu, et al. 1971; Bhattacharjee, et al. 1976). Capsule switching of the meningococcus has been known to occur and have been documented with serogroups B and C (Swartley, et al. 1997; Jodar, et al. 2002). It is suggested this occurs by gene conversion or genetic exchange of the capsule polymerase. The ability to switch capsules enhances the invasiveness of the organism by helping it to evade opsonization and neutralization due to pre-existing antipolysaccharide antibodies (Swartley, et al. 1997).

Twenty different serotypes exist based on the antigenic differences in the serotype antigens which are class 2 and 3 OMPs (Menzel and Rake 1942; Frasch and Chapman 1973), and further divided into 10 subtypes based on differences in class 1 OMPs (Frasch, et al. 1985; Abdillahi and Poolman 1988). Thirteen different immunotypes exist based on the heterogeneity in the LPS of the cell membrane and
other chemically undefined antigens unrelated to the serotype protein determinants (Mandrell and Zollinger 1977; Riedo, et al. 1995). Lastly, further typing may be established based on the antigenic properties of the immunoglobulin A1 (IgA1) proteases and pili/fimbriae characteristics (Stephens, et al. 1985).

**Surface Antigens and Virulence Factors**

The survival and proliferation of *N. meningitidis* in the host is by the virtue of several bacterial virulence factors, summarized in Table I. The main virulence factor is the polysaccharide capsule that protects the organism from environmental insults and is anti-phagocytic. Numerous surface antigens and OMPs have been described and shown to attribute to the virulence of the meningococcus. Many of the surface antigens are not conserved between serogroups and are highly variable. These proteins enable the organism to adhere to host cells, confer serum resistance, act as transporter proteins, and aid in pathogenesis of disease (Prinz, et al. 1999; Vogel and Frosch, 1999). Meningococci also demonstrate a high degree of “blebbing” (release of blebs) that consist of surface antigens such as LPS, periplasmic proteins, OMPs and phospholipid (Devoe and Gilchrist 1973; Andersen, et al. 1979; Zhou, et al. 1998). It has been suggested that blebbing may be caused by peptidoglycan turnover or cell autolysis (Pollard and Levin 2000). Blebbing contributes to the pathogenicity of the meningococcus by drawing attention away from intact organisms.
### Table I. Virulence Factors of *Neisseria meningitidis*

<table>
<thead>
<tr>
<th>Meningococcal Antigen</th>
<th>Function</th>
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<tbody>
<tr>
<td>Polysaccharide capsule</td>
<td>Protects against complement mediated bacteriolysis and phagocytosis (Klein, et al. 1996)</td>
</tr>
<tr>
<td>Lipopolysaccharide (LPS)</td>
<td>Confers serum resistance, down regulates complement-mediated lysis (Vogel and Frosch 1999; Pollard and Frasch 2001)</td>
</tr>
<tr>
<td>Pili/Fimbriae</td>
<td>Attachment to mucosal cells (Virji, et al. 1992)</td>
</tr>
<tr>
<td>Class 2 and 3 OMPs (Por B)</td>
<td>Cationic porin – transport solutes and macromolecules across cell membrane; Protects from apoptosis (Bjerknes, et al. 1995; Massari, et al. 2000)</td>
</tr>
<tr>
<td>LbpB</td>
<td>Iron acquisition from lactoferrin; further investigations of function are currently studied (Pettersson, et al. 1998; Prinz, et al. 1999)</td>
</tr>
<tr>
<td>Ferric binding protein (Fbps)</td>
<td>Binds iron from human transferrin (Banerjee-Bhatnagar and Frasch 1990; Pettersson, et al. 1990)</td>
</tr>
<tr>
<td>IgA1 proteases</td>
<td>Prevents IgM and IgG binding to the bacterial surface; inhibits complement-mediated bactericidal activity</td>
</tr>
</tbody>
</table>
Epidemiology of Meningococcal Disease

Neisseria meningitidis is a major cause of morbidity and mortality worldwide. It is the leading cause of bacterial meningitis and septicemia in children under the age of 2 yrs, with a secondary peak incidence in adolescent years (Moore 1992; Kaczmarski 1997; Rosenstein, et al. 1999). The incidence of meningococcal disease varies from 1-3 cases per 100,000 individuals in developed countries to 10-25 cases per 100,000 in developing countries (Schlech, et al. 1987; Harrison and Broome 1987; Wenger, et al. 1990), with over 350,000 cases occurring worldwide annually (Jackson, et al. 1995; Raymond, et al. 1997). Recent studies have shown that in the United States alone, 2400-3600 cases of meningococcal disease occur each year with an average 0.8-1.3 cases per 100,000 individuals. This is associated with a case fatality rate of 10-13%, 46% of which occur in children <2 yrs of age (Jackson and Wenger 1993; 2000, Lingappa, et al. 2001).

Although 13 serogroups have been described, serogroups A, B, C, Y and W-135 account for more than 90% of all cases of meningococcal disease (Apicella 2000). All serogroups cause epidemics and sporadic disease (epidemic defined as $\geq 100/100,000$ individuals in a 1-year period (Riedo, et al. 1995). The majority of epidemics are the result of serogroup A, whereas sporadic disease is usually caused by serogroups B, C, Y and W-135 (Peltola 1998). Serogroup A is responsible for recurrent epidemic disease in Sub-Saharan Africa known as the “meningitis belt.” The “meningitis belt” consists of 15 countries in Central Africa that extends from the Sudan in the east to Mali and Guinea in the west (Lapeyssonnie 1962; Peltola 1983). The epidemics start in the dry season (December-June) and end in the rainy season,
and occur in 8-12 year cycles (Greenwood, et al. 1987; 1997). The incidence of meningococcal disease in the Sudan is a 100 times greater than the incidence in developed countries. Attack rates have approached nearly 1000/100,000 individuals, affecting up to 10% of the population (Moore 1992; Pinner, et al. 1992). A recent outbreak of serogroup A in the Sudan resulted in 22,000 cases of meningococcal disease and over 1600 deaths (Cartwright, et al. 2001).


Pathogenesis of Meningococcal Disease

*Neisseria meningitidis* is a pathogen that inhabits the upper respiratory tract in humans. Meningococci are spread by intimate contact with the oral secretions or respiratory droplets of an infected individual (Peltola 1983). *N. meningitidis* causes meningitis, meningococcemia, and septicemia, but more commonly results in asymptomatic nasopharyngeal carriage. Approximately, 10% of adults and up to
30% of adolescents carry the meningococcus in their nasopharynx at any one time (Riedo, et al. 1995; Morley and Pollard 2001). There are four stages that are required for the pathogenesis and establishment of meningococcal infection (van Deuren, et al. 2000). First, exposure to a pathogenic strain must occur via direct contact with an infected patient/individual. Second, the bacteria must colonize the nasopharyngeal mucosa. Pili/fimbriae from the bacteria’s outer membrane bind to the surface of the mucosal cell and contribute to the colonization (Virji, et al. 1992). This occurs at the exterior surface of the host mucosal cell layer as well as the intra- or sub-epithelial layer (Stephens and McGee 1981; Stephens and Farley 1991). Next, after attachment of the pili, the class 5 OMPs, Opa and Opc, stimulate the engulfment of the meningococci by the mucosal epithelial cells and transport of the meningococci through the mucosa (Dehio, et al. 1998). As a result of endocytosis the bacteria invade and pass through the mucosal epithelium via phagocytic vacuoles (McGee, et al. 1983; Read, et al. 1995). Lastly, the meningococci survive and proliferate in the infected individuals bloodstream by the assistance of numerous virulence factors, such as the capsular polysaccharide and OMPs (Tbp, PorA and B), see Table I.

*Meningococcal Disease*

Diagnosis of meningococcal infection is determined by blood and cerebrospinal fluid cultures and specimens of skin lesions and other infected areas (Lepow, et al. 1999). In 1968 clinical classifications of meningococcal disease were established by Wolfe and Birbara (1968) (Niklasson, et al. 1971). The first classification is “bacteremia without sepsis” which is defined as the presence of bacteria in the blood regardless of clinical symptoms. The second classification is
“meningococcemia without sepsis” which is associated with leukocytosis, headache, muscle aches, rash, and seizures that occur in approximately 20% of infected individuals (Lepow, et al. 1999; van Deuren, et al. 2000). The third classification of meningococcal disease is “meningitis with or without meningococcemia.” Meningeal signs in this classification are associated with <100 white blood cells/mm$^3$ of cerebrospinal fluid. The infected individual presents with headache, fever, with either a full alert or depressed mental status. The last classification of meningococcal infection is “meningoencephalitic manifestation” defined as ≥100 white blood cells/mm$^3$ of cerebrospinal fluid, with an increased concentration of endotoxin and cytokines in the plasma (van Deuren, et al. 2000). This is the most severe form of disease resulting in shock, multiple organ failure, skin and limb necrosis and cerebral edema. Other symptomatic problems as a result to meningococcal infection include myocarditis, endocarditis, pericarditis, and purulent arthritis (Mason, et al. 1979). Although there are 4 outlined clinical classifications of meningococcal disease, individuals who are infected with the bacteria present with various manifestations that may not fall into one specific category.

The progression from nasopharyngeal carriage to invasive disease remains unclear but is influenced by a variety of factors associated with both the host and the infecting bacteria. These predisposing factors include rate of bacterial propagation, climatological and social conditions, and the immune status of the infected individual. Influences by climatic conditions have been documented in the Sudan where the incidence of disease varies seasonally (Greenwood and McGuire 1957; Peltola 1983). Numerous cases of disease occur in the dry season when the harmattan (dessert wind)
is stronger then during the rainy seasons. Overcrowding and poor living conditions are predisposing conditions to infection. This has been shown in the crowded living conditions associated with military camps and more recently with an increase of incidence in university dormitory settings (Peltola 1983; Neal, et al. 1999; Brundage, et al. 2002; Williams, et al. 2003). Close contact in living conditions and households with an infected individual increase the risk of colonization from 500 to 800- fold up to 3000 to 4000- fold (Community Report 1976; Olcen, et al. 1981).

Smoking is another risk factor associated with meningococcal disease due to the physical damage sustained to the nasopharyngeal mucosa resulting in increased risk of carriage (Stanwell-Smith, et al. 1994). Current infections, such as acute respiratory disease or those caused by immunosuppressive viruses, alter the integrity of the mucosal surface and increase the risk of disease by transiently suppressing the immune system (Haneberg, et al. 1983; Rouse and Horohov 1986; Cartwright, et al. 1991). Likewise, immunodeficient individuals, such as asplenic persons or those with complement or IgM/IgG2 deficiencies, have a greater risk of succumbing to invasive meningococcal disease (Francke and Neu 1981; Bass, et al. 1983; Ross and Densen 1984; Hobbs 1986; Figueroa and Densen 1991). Additional risk factors also include chronic alcoholism, low socioeconomic status, and an excess of anti-polysaccharide IgA antibodies that may inhibit the binding of the bacteria with anti-polysaccharide IgM or IgG antibodies (Hedrich 1952; Wenzel, et al. 1973; Griffiss 1975, 1982; Salmi, et al. 1976).
Immune Response to N. meningitidis

In the early 1900s studies by scientists such as Matsunami et al. (1918) and Heist et al. (1922), suggested immunity to the meningococci was dependent on specific blood-borne immunologic factors. The definitive role of antibodies in protection against meningococcal infection was later demonstrated by Goldschneider et al. (1969) by the relation between functional anti-meningococcal antibodies and level of susceptibility to infection. Subsequently, serum complement-dependent bactericidal activity was determined critical for a host defense against invasive disease (Nicholson and Lepow 1979).

The immunological mechanisms responsible for defense against meningococcal disease include antibody-dependent complement lysis, antibody-mediated phagocytosis, and antibody-dependent cellular toxicity (Goldschneider, et al. 1969; Lehmann, et al. 1999). Although the precise nature of these defense mechanisms is not certain, it is suggested there are four possible routes of complement activation that leads to phagocytosis of the bacteria (Pollard and Frasch 2001). These include (1) lysis by anti-meningococcal antibodies followed by activation of the classical pathway of complement, (2) lysis and activation of complement via the alternative complement pathway, (3) opsonization by complement or phagocytosis by other opsonins such as mannose binding lectin via its receptor, and (4) opsonization by anti-meningococcal antibodies and/or complement followed by phagocytosis of the bacteria. In immune individuals the classical pathway of complement activation occurs followed by binding of specific anti-meningococcal antibodies to the capsular polysaccharide and surface OMPs (Pollard
and Frasch 2001). In non-immune individuals the alternative pathway of complement activation is favored. The final result of either pathway of complement activation is the formation of the membrane attack complex that “punches” holes throughout the outer membrane and phagocytosis caused by deposition of complement on the cell surface (Carlone, et al. 1997).

Treatment of Meningococcal Disease

The first successful treatment of meningococcal disease before the use of antibiotics was introduced in the 1900s by Dopter et al. (Delville 1973) in France and later by Jochmann (1906) in the United States. This treatment consisted of introducing horse serum or an antiserum targeted against the meningococci to the infected patient in order to lyse the bacteria and reduce toxicity. This technique was defined as serotherapy and supported the role of antibody as a requirement for protection from disease. In addition to serotherapy, patients were treated with lumbar punctures, ice bags, and hexamethylenetetramine (Seleste 1941; Peltola 1983). In 1930 sulfonamides were discovered and antibiotics became the primary treatment of meningococcal infection (Peltola 1983). Shortly after sulfa became the common antibiotic of choice. The first reports of reduced sensitivity to the antibiotic were documented in the United States during World War II (Schoenbach and Phair 1948; Love and Finland 1954; Leedom, et al. 1966). Additional resistance to antibiotic treatment was documented to serogroups A, B, and C not only in the United States but also in Europe (Millar, et al. 1963; Alexander, et al. 1968; Kaiser, et al. 1974). Due to meningococcal resistance to sulfonamides, alternative antibiotics such as penicillin and chloramphenicol were used in the 1950s and 1960s (Riedo, et al. 1995).
However, penicillin resistant strains were described shortly after its introduction in Spain, England, South Africa and North America (Riedo, et al. 1995).

Currently, antibiotics such as ciprofloxacin, and third-generation cephalosporins are being used as treatments. Rifampin has been shown to eradicate nasopharyngeal carriage of meningococci and likewise ciprofloxacin has been proven to be greater than 90% effective at eliminating the carrier state (Broome 1986). With the continuing discovery of antibiotic resistant strains, continual surveillance is required in order to determine the choice of antimicrobial treatment used for infection.

*Encapsulated Bacteria – Carbohydrate Antigens*

*T-Independent Antigens – TI-1 and TI-2*

Carbohydrate antigens such as the capsular polysaccharides of *N. meningitidis* and *Streptococcus pneumoniae* are considered T-independent (TI) in nature. The original concept of an antigen as TI was characterized by the antigens ability to stimulate an immune response in neonatally thymectomized mice (Mond, et al. 1995). Studies (Mosier, et al. 1977b) with X-linked B cell deficient (CBA/N) and immunologically mature mice led to the definition of the two classes of TI antigens, TI type 1 (TI-1) and TI type 2 (TI-2). TI-1 antigens are known as polyclonal B cell mitogens. These antigens are capable of inducing the proliferation of both naïve and mature B cells (Janeway, et al. 1999). TI-1 antigens are able to stimulate anti-carbohydrate immune responses in mature and neonatal mice as well as CBA/N or xid mice (Mosier, et al. 1977a; Mond, et al. 1995). Examples of TI-1 antigens
include bacterial cell wall components such as lipopolysaccharides, and antigens like *Brucella abortus*.

Conversely, TI-2 antigens do not have intrinsic B cell stimulation activity as TI-1 antigens do, and are only able to activate mature B cells (Janeway, et al. 1999). TI-2 antigens are unable to induce anti-carbohydrate responses in neonates of CBA/N mice (Mosier, et al. 1977a; Endres, et al. 1983). TI-2 antigens are usually high molecular weight polysaccharides or polypeptides characterized by their high density of repeating epitopes, poor in vivo degradability, leading to their prolonged persistence, and inability to stimulate MHC class II dependent T cell help (Stein 1985; Haneberg, et al. 1998). Examples of TI-2 antigens include carbohydrates such as Dextran B512 and trinitrophenylated-ficoll (TNP-Ficoll), *S. pneumoniae* capsular polysaccharide, *Haemophilus influenzae* capsular polysaccharide, and *N. meningitidis* capsular polysaccharide.

**TI-2 Immune Responses**

T-independent type 2 antigens are believed to elicit humoral immunity by extensively crosslinking the cell surface immunoglobulin of specific mature B cells. Multiple crosslinking of the B cell receptor by the polysaccharide antigen can eventually lead to the production of anti-polysaccharide IgM antibodies, yet extensive crosslinking has been shown to render the B cell unresponsive (Snapper and Mond 1996). For complete B cell activation, a second signal is required in order to secrete immunoglobulin. This signal can be provided by the bacterial constituents of the antigen itself or by non-thymus accessory cells such as natural killer (NK) cells or macrophages (Snapper and Mond 1996).
One proposed mechanism of secondary signaling required for B cell activation by a TI-2 antigen depends on the alternative pathway of complement (Janeway, et al. 1999; Lesinski and Westerink 2001). It is suggested that the C3 convertase of the alternative pathway cleaves molecules of C3 into several split products (iC3b and C3dg) that bind to the TI-2 antigen. The new TI-2 antigen bound complex is subsequently attached to mature B cells by the complement receptor CR2 (also known as CD21) that is an important part of the B cell co-receptor complex on the mature resting B cell (Iida, et al. 1983; Tedder, et al. 1984). Co-ligation of the CD-19 co-stimulatory molecule and CR2 provides the secondary signal necessary for B cell activation (Janeway, et al. 1999).

B cells that respond to TI-2 antigens have unique properties that are distinct from those that respond to protein antigens (Stein 1985). First, TI-2 B cells are compartmentalized within the splenic marginal zone and develop late in ontogeny (MacLennan, et al. 1982; Lane, et al. 1986), whereas protein responsive B cells reside in the follicular zones and arise early in ontogeny (Liu, et al. 1992). It has been suggested that specific marginal zone antigen presenting cells (APCs) are involved in the presentation of TI-2 antigens to B cells (Stein 1985). The half-life of polysaccharide specific B cells is relatively long, whereas protein specific B cells become long-lived memory B cells within a short period of time. TI-2 specific B cells are biased to the production of low affinity IgM and IgG3 antibodies. Protein specific B cells, on the other hand, produce antibodies that undergo affinity maturation and isotype switching (Stein 1992).
Neonatal Immune Responses to TI-2 Antigens

TI-2 antigens are poorly immunogenic in the neonatal population (< 2 yrs of age). Early life immunodeficiencies in this population to TI-2 antigens are caused by the immaturity of B and T cell functions and the rapid decline of circulating maternal antibodies (Goldschneider, et al. 1969). Neonates have the ability to produce specific IgM antibodies, but much lower titers of IgM and IgG antibodies are detected. Studies (Gathings, et al. 1981) have shown B cells that produce fetal antibodies are immature in nature and do not undergo complete immunoglobulin class switching as seen with adult B cells. This is evident in the observation that the majority of neonatal B cells express surface IgM and IgD compared to adult B cells (Gathings, et al. 1981).

Since the initiation of TI-2 antigen immune responses has been suggested to take place in the splenic marginal zone, investigators have reported differences in infant marginal zone B cells compared to adult B cells (Timens, et al. 1989). The presence of CD21 (CR2) on mature B cells is a marker of the differentiation from immature B cells. The glycoprotein CD21 is an important part of the B cell co-receptor complex and provides the necessary secondary signals to specific B cells for activation. Infant marginal zone B cells lack or have reduced expression of CD21 and thus are not able to form the B cell co-receptor complex in order to activate polysaccharide-specific B cells (Rijkers, et al. 1998). When neonates approach approximately 2 yrs of age, the expression of CD21 can be detected on mature B cells. This correlates with the ability to respond to TI-2 antigens.
Another possible reason for the poor immunogenicity of TI-2 antigens in ontogeny is the dependence on a specific subset of B cells, Lyb-5+ B cells (Stein, et al. 1983). Studies (Boswell, et al. 1980; Stein, et al. 1983) using the TI-2 antigens TNP-Ficoll and Dextran B512 have demonstrated Lyb-5+ B cells are required for the induction of anti-carbohydrate antibody responses to these two antigens. Lyb-5+ B cells have been found in low frequencies in neonates. The failure to respond to TI-2 antigens may correlate with low expression of this particular B cell subset. Lastly, immature immune responses to TI-2 antigens in neonates may be contributed to low secretion of particular cytokines. This includes cytokines such as IFN-\(\gamma\) and IL-4 that are required for the activation of B cells (Rijkers, et al. 1998; Pertmer, et al. 2001).

**T Cell Dependent Characteristics of TI Immune Responses**

It has been suggested that responses to antigens recognized as TI in nature are actually dependent on some form of T cell or T cell derived help (Mond and Brunswick 1987). This observation has been supported by studies demonstrating that TI antigens promote class switching from IgM to IgG3 in vivo. Mongini et al. (1981) reported both IgM and IgG antibodies could be detected to TI antigens in athymic mice. Likewise, Snapper et al. (1994) demonstrated when T cell depleted spleen cells were transferred into SCID mice, the production of TNP-Ficoll specific IgM and IgG antibodies could be induced by a challenge with the TI-2 antigen TNP-Ficoll. The ability to switch antibody isotypes suggests a T cell role in the response to TI antigens. Investigators have reported the T cell help may be provided by non-T cells such as NK cells or by \(\gamma\delta\) T cells (Snapper, et al. 1992; Chien, et al. 1996). NK cells
have the ability to secrete cytokines, specifically Interferon-gamma (IFN-\(\gamma\)), that may enhance B cell differentiation and thus isotype switching from IgM to IgG.

**T-dependent antigens**

Antibody responses to soluble proteins, viruses and parasites are considered T-dependent (TD) in nature. These antigens require the presence of mature T cells in order to elicit an immune response. T-dependent antigens have the ability to associate with MHC class I and II molecules at the surface of APCs which interact with peptide specific T cells. These immune responses are characterized by immunologic memory, affinity maturation, and isotype switching.

**Cytotoxic T cells**

T-dependent immune responses involve two different types of T cell subsets, CD8 T cells and CD4 T cells (Janeway, et al. 1999). These T cells recognize peptide fragments of the antigen in the form of peptide:MHC molecule complexes on the surface of APCs. CD8 T cells, or cytotoxic T cells, are involved in host defenses against pathogens such as viruses or intracellular bacteria that are found in the cytosol. During infection, the cytosolic antigens are degraded into small peptide fragments by large multicatalytic proteases (proteosomes) and transported to the endoplasmic reticulum where they bind to the MHC class I molecules (MHC-1) (Janeway, et al. 1999). The peptide:MHC-1 complex is presented at the surface of antigen presenting cells (APC) to CD8 T cells. The effector CD8 T cells are activated by binding of the peptide:MHC-1 complex on the APC to a receptor on the T cell, combined with the appropriate secondary signals. Specific co-stimulatory molecules, such as glycoprotein B7 on the APC and the CD28 and CTLA-4 receptor on the T
cell, provide these secondary signals (Janeway, et al. 1999). Activated CD8 T cells are functionally cytotoxic that kill their targets by lysing the cell with lytic granules and programming them to undergo apoptosis.

**Helper T Cells**

Immune responses to extracellular pathogens and some parasites involve the second subset of T cells, CD4 T cells, or helper T cells. Helper T cells fall into two functional classes, (1) Th1 cells that stimulate phagocytosis and the intracellular killing of microbes, and (2) Th2 cells that stimulate high titers of antibody production from B cells (Spellberg and Edwards 2001). During infection, antigens presented to CD4 T cells are either internalized via endocytosis into intracellular vesicles with B cells or macrophages or directly invade phagocytic cells during their replication. Endosomal or lysosomal proteases within the macrophage are activated by the ingestion of the organism and degrade the antigen into peptide fragments. The small peptide fragments bind to MHC class II molecules (MHC-II) and are transported to the cell surface as a peptide:MHC-II complexes where they are presented to CD4 T cells. Similar to CD8 T cells, CD4 T cells require appropriate secondary signals in order to become activated effector T cells. The secondary signals are provided co-stimulatory molecules such as glycoprotein B7 and the CD28 receptor. Membrane-bound effector molecules also include the CD40 ligand and/or Fas ligand on the T cell which attach to the CD40 receptor or Fas receptor which trigger target cell activation (macrophages or B cells) (Janeway, et al. 1999).
Differentiation of T Cell Subsets

Naïve CD4 T cells (Th0 cells) are differentiated into either Th1 or Th2 cells. This occurs by polarization of the already activated naïve CD4 T cell. This depends on (1) the cytokine environment provided by the antigen [specifically Interleukin-12 (IL-12) and Interleukin-4 (IL-4)], (2) the presence of immunologically active hormones, (3) antigen dose and route of administration, (4) the type of APC presenting the peptide:MHC complex, and (5) the strength of the co-stimulatory signals (Spellberg and Edwards 2001). The differentiation into Th1 cells is mainly polarized by the cytokine IL-12. IFN-γ produced by newly differentiated Th1 cells suppresses any IL-4 secretion. This leads to the inhibition of Th0 differentiation into Th2 subsets resulting in the increase of Th1 cells (Janeway, et al. 1999). Th2 subsets are polarized by the cytokine IL-4 and secrete excess IL-4 and Interleukin-10 (IL-10) to inhibit the production of IL-12. This inhibits the differentiation of Th1 cells and results in an increase in Th2 subsets. When both IL-12 and IL-4 are present at the time of polarization, Th2 differentiation is favored due to the fact that IL-4 takes precedence (Hsieh, et al. 1993).

Th1 and Th2 cells are not only characterized by differences in the cytokine milieu they secrete but also in their specific effector functions. Th1 responses are characterized by strong cell-mediated immune responses that lead to the activation of macrophages and the stimulation of phagocytosis. Th1 cells mainly produce IL-2, IL-12, IFN-γ, and TNF-β cytokines that assist in the activation of macrophages (Janeway, et al. 1999). Conversely, Th2 responses are characterized by strong humoral responses leading to B cell proliferation, antibody production and class
switching. Th2 cells mainly secrete IL-4, Interleukin-5 (IL-5), Interleukin-6 (IL-6), IL-10, and Interleukin-13 (IL-13) that increase the activation and differentiation of B cells (Spellberg and Edwards 2001).

Vaccine Development for Neisseria meningitidis

History

Vaccine development for *N. meningitidis* has ranged from killed whole cell and autolysate vaccines to the presently available capsular polysaccharide and polysaccharide-protein conjugate vaccines. The earliest efforts to develop vaccines against *N. meningitidis* were in the early 1900s and consisted of killed whole organisms or crude extracts of broth cultures (Lapeyssonnie 1962; Triau 1974). These early vaccines demonstrated little evidence of protection and were plagued by severe adverse reactions due to the presence of high endotoxin content (Ferry and Steele 1935; Kuhns, et al. 1938). In the mid 1940s Scherp and Rake (1945) first demonstrated that antibodies to capsular polysaccharide antigens could passively protect mice against lethal challenge. Shortly afterward, in the 1960s, Gotschlich et al. (1969) introduced the meningococcal serogroup A and C capsular polysaccharide vaccines. The capsular polysaccharide vaccines have proven effective in adults but are poorly immunogenic in children < 2 yr of age due to the TI nature of the capsular polysaccharide. In the 1970s, the development of a meningococcal polysaccharide-protein conjugate vaccine was introduced by Jennings and Lugowski (1981) as a method to overcome the TI response associated with the polysaccharide vaccine.
Capsular Polysaccharide Vaccines

The capsular polysaccharide of \textit{N. meningitidis} is an important determinant of the organism for virulence. As early as the 1960s it was evident that purified capsular polysaccharide from encapsulated bacteria could elicit type-specific immune responses in adults and that type-specific antibodies were the effector molecules that could confer protection and decrease naso-pharyngeal carriage of the organism (Goldschneider, et al. 1969; Gotschlich, et al. 1969). The first group specific meningococcal polysaccharides were isolated by Rake and Scherp in 1933 (1933). These isolated polysaccharides were large in molecular weight and were further chemically defined by Gotschlich (1969) and Liu et al. (1971). These early studies (Goldschneider, et al. 1969; Gotschlich, et al. 1969) with the capsular polysaccharide first suggested the correlation between susceptibility to meningococcal infection with the lack of serogroup specific anti-polysaccharide antibodies. It was further demonstrated that serogroup specific serum antibodies to the capsular polysaccharide protected against disease by activating complement-mediated bacteriolysis or opsonization.

The first successful capsular polysaccharide vaccines were against serogroups A and C in response to meningitis epidemics among United States military recruits. The vaccines were safe and tolerable, and were shown to induce significant bactericidal antibody responses in adult recruits (Artenstein, et al. 1970; Devine, et al. 1970). The polysaccharide vaccines resulted in an 87\% reduction in serogroup A and C disease and demonstrated that army recruits vaccinated with polysaccharide had reduced acquisition rates of meningococci as compared to unvaccinated recruits.
Polysaccharide vaccines for serogroup A, C, Y and W-135 have been developed since these first studies in military recruits. Two polysaccharide vaccines are routinely used containing serogroups A and C, or A, C, Y, and W-135 (Menomune A/C/Y/W-135). A polysaccharide vaccine for serogroup B has not been successfully developed due to its similarity to the N-acetyl neuraminic adhesion molecule in the brain (Cadoz 1998). The majority of clinical trials in adults have proven the effectiveness of the polysaccharide vaccine in inducing bactericidal anti-polysaccharide antibodies. These antibodies have shown to remain elevated for at least 5 yr post-immunization in normal immune competent adults (Brandt and Artenstein 1975). Thus re-vaccination is recommended in adults every 5 yr. Although the vaccine is effective in adults, two important factors have limited the usefulness of the polysaccharide vaccine in controlling meningococcal disease, (1) the polysaccharides’ poor immunogenicity in infants and young children, and (2) the induction of serological hyporesponsiveness after repeated immunization.

Immunization studies with the capsular polysaccharide vaccine in young children have shown that plain meningococcal polysaccharide is poorly immunogenic and induces short-lived responses. Early studies (Goldschneider, et al. 1972) demonstrated serogroup A and C polysaccharide vaccines elicited average antibody responses approximately 60% that of adult levels 2 wk post-immunization. Goldschneider et al. (1973) demonstrated that immunization in young children with several doses of polysaccharide resulted in low antibody titers with no apparent booster effect. In infants < 2 yr of age, decreasing antibodies are actually detected
after each subsequent boost, with the resulting anti-MCPS response 10% of that seen in adults, and an anti-MAPS response 30% of that seen in adults (Gold, et al. 1977).

A difference in the antibody response elicited to serogroup A polysaccharide and serogroup C polysaccharide in infants has been observed. Studies (Gold, et al. 1979) have shown serogroup C polysaccharide vaccines induce relatively low antibody levels that do not persist in young children. Anti-MCPS antibodies usually decline rapidly to baseline levels 3-5 mo post immunization (Cadoz 1998). On the other hand, the serogroup A polysaccharide has been shown to elicit anti-MAPS antibodies that are persistent in children as young as 3-9 mo of age (Kayhty, et al. 1980; Reingold, et al. 1985). The levels of anti-MAPS antibodies are usually short-lived though, and are not comparable to adult antibody levels until 4-5 yr of age.

Immune responses to the capsular polysaccharide vaccines in toddlers have been found to be dependent on age of the vaccinee, the presence of pre-existing antibodies due to previous exposure, and whether the vaccinee had been previously immunized with the polysaccharide vaccine. Overall vaccine efficacy in this age group has been shown to decrease from 87% 1 yr post-immunization to 70% and 54% at 2 and 3 yr, respectively (Reingold, et al. 1985). Similar to that seen in infants, persistence of anti-MAPS antibodies is greater than that of anti-MCPS antibodies. In toddlers, anti-MCPS antibodies may decline as much as 75% 1 yr post-immunization (Lepow, et al. 1977). The immune response to the polysaccharide vaccine is strongly dependent on pre-existing antibodies from prior vaccination with the polysaccharide vaccine. Studies (Leach, et al. 1997; MacLennan, et al. 1999) that examined toddlers who had been previously immunized with the polysaccharide vaccine, demonstrated
serum bactericidal responses 10 fold lower following booster immunization compared to toddlers immunized for the first time. When a second dose of polysaccharide was administered 2 yr following primary immunization, anti-PS antibodies progressively decreased until they returned to baseline levels 5 yr after immunization (Ceesay, et al. 1993). These studies demonstrated that previous immunization in childhood with the polysaccharide vaccine was capable of inhibiting the immune response to booster doses several years later and that a hyporesponsive state could be induced.

In addition to the hyporesponsive state detected in previously immunized toddlers, there also has been increasing evidence of the induction of hyporesponsiveness in adults as well. Numerous studies (MacDonald, et al. 1998; Richmond, et al. 1999b) have detected impaired bactericidal antibody responses following a second dose of the polysaccharide vaccine administered several months after primary immunization. These secondary responses are comparable to antibody levels found in non-immunized adults (Fairley, et al. 1996; Leach, et al. 1997). In addition, when the booster dose was as low as 1/50 of the primary polysaccharide dose, a refractory state was still induced (Granoff, et al. 1998). The ability to induce hyporesponsiveness in all age groups has raised concern that previous vaccination with the polysaccharide vaccine could increase the risk of developing meningococcal infection. The exact mechanism for hyporesponsiveness after repeated doses is not fully known. It has been suggested that the TI environment of the immune response induced by polysaccharide immunization may possibly deplete polysaccharide-specific memory B cell, although this remains a controversial issue (MacLennan, et al. 2001).
Purified meningococcal polysaccharides are not good immunogens in young children and infants due to the poor immune responses and short-lived immunity induced by the vaccine. Therefore, the meningococcal polysaccharide vaccine has been recommended only for the control of disease outbreaks caused by serogroups represented in the vaccine and use among certain high risk groups (Centers for Disease Control and Prevention, 1997). These high risk groups include individuals with complement deficiencies or hyposplenia, travelers to highly endemic areas, laboratory personnel who are exposed to the bacteria, military recruits, and collegiate students (Centers for Disease Control and Prevention, 2000).

Polysaccharide Conjugate Vaccines

Thymus cell dependent immune responses to polysaccharide antigens have been successfully induced following conjugation of the polysaccharide to a protein. It has been demonstrated by the efficacy of the *Haemophilus influenzae* type b vaccine that chemical conjugation of the polysaccharide to a protein carrier, such as tetanus or diphtheria toxoid, converts the immune response from that of a T-independent to a T-dependent response characterized by immunological memory, even in infants and young children.

Glycoconjugate vaccines consist of partially hydrolyzed polysaccharide or fractions of oligosaccharide derived from the polysaccharide capsule covalently coupled to a large immunogenic protein carrier (Morley and Pollard 2001). Currently licensed vaccines and conjugates in clinical trials contain either tetanus toxoid (TT), diphtheria toxoid (DT), a non-toxic synthetic form of diphtheria toxoid, Diphtheria Cross Reactive Material 197 (CRM197), or *N. meningitidis* group B OMP complex as
the carrier protein (Peeters, et al. 1991; Morley and Pollard 2001). These proteins are commonly used because they are safe, have equivalent immunogenicity, and have previously been used in human vaccination without adverse side effects (Decker, et al. 1992).

Chemically reactive amino or carboxyl groups on the capsular polysaccharide are not readily available for covalent conjugation to a protein, therefore, two methods have been predominantly used to couple the polysaccharide to a selected protein (Lindberg 1999). The first method is reductive amination in which carbohydrate groups previously oxidized from the polysaccharide capsule are directly coupled to the protein carrier (Anderson, et al. 1985). The second method involves the addition of a bifunctional nucleophilic spacer, such as adipic acid dihydrazide (ADH), that creates a polysaccharide-ADH derivative which can then be coupled to a protein carrier by carbodiimide-mediated condensation (Schneerson, et al. 1980; 1986). The resulting glyconconjugate produced by these methods is a vaccine with multiple attachments between the polysaccharide and the carrier protein. This technology has been successfully used in the creation of conjugate vaccines against encapsulated bacteria such as *H. influenzae* type b, *S. pneumoniae*, and *N. meningitidis*.

The proposed mechanism by which a conjugate vaccine imparts increased T-dependent immunogenicity to a carbohydrate antigen is a combination of T-independent and T-dependent B cell activation (Beuvery, et al. 1982). It has been suggested the function of a conjugate vaccine is to localize the protein antigens near the site of polysaccharide recognition. (Mosier, et al. 1987). After immunization, the conjugated polysaccharide protein complex is first transported to the lymphoid tissue
adjacent to the injection site. Polysaccharide specific B cells recognize the polysaccharide-protein complex through specific interactions with immunoglobulin surface receptors, and initiate a TI immune response (Lesinski and Westerink 2001). Concurrently, the polysaccharide-protein complex is internalized by receptor-mediated endocytosis, processed, and carrier derived peptide fragments are presented via MHC class II molecules to CD4\(^+\) T cells (Insel and Anderson 1986; Breukels, et al. 1999). Activated T helper cells provide polysaccharide specific B cells essential costimulatory signals and specific cytokines. As a result, these B cells can mature into antibody producing plasma cells involving isotype switching or develop into polysaccharide specific memory B cells (Guttormsen, et al. 1999; Kamboj, et al. 2001).

The first conjugate vaccine was licensed in 1987 directed against \textit{H. Influenzae} type b. The majority of \textit{H. Influenzae} strains is not encapsulated and rarely causes invasive disease. However, a small number are encapsulated and do pose a threat of infection (types a-f). Of these six, type b is by far the most virulent, and like many encapsulated organisms the capsular polysaccharide is T-independent in nature and induces a poor immune response in children under the age of two.

Four different Hib conjugate vaccines are currently licensed; PRP-D (DT), PRP-T (TT), HbOC (CRM\(_{197}\)), and PRP-OMP (\textit{N. meningitidis} OMP complex). The conjugates differ in carrier proteins used, linkage type between polysaccharide and protein (with or without spacer), polysaccharide size, and whether they are linked at the ends or in chains. Immunizations with the Hib conjugates have demonstrated to be safe, immunogenic in infants, able to induce immunologic memory, and protect

The success of the Hib conjugate vaccine has led to the use of this technology in the development of conjugate vaccines targeted against the meningococcal capsular polysaccharide. Before the licensing of the Hib conjugate in 1987, attempts to develop a conjugate vaccine for *N. meningitidis* had been ongoing. In the early 1980s, Jennings and Lugowski (1981) and Beuvery et al. (1983), prepared some of the first meningococcal conjugate vaccines. The vaccines were water-soluble conjugates composed of the capsular polysaccharides from serogroups A, B, and C conjugated to TT. The conjugates induced high levels of functional anti-MAPS and anti-MCPS antibodies in rabbits and mice that were boostable with polysaccharide, although anti-MBPS antibodies were not detected. By the end of the 1980s, conjugate vaccines for serogroups A and C were developed and tested in phase I clinical trials in infants and toddlers. These were proven to be safe, immunogenic and able to induce a memory response in children (Rappuoli 2001). Due to the advancements of these studies, monovalent (C), bivalent (A and C), and even quadrivalent (A, C, Y, W-135) formulations have been created and are being tested in clinical studies. This has resulted in the licensing of the first serogroup C
meningococcal conjugate vaccine (MnCC: C-CRM$_{197}$) in November of 1999 in the United Kingdom (Jodar, et al. 2002).

Several studies with meningococcal conjugate vaccines in infants, children and adults have demonstrated that the vaccines are safe, well tolerated, and immunogenic. Early immunizations in Gambian infants with an A/C-CRM$_{197}$ conjugate resulted in high levels of anti-MCPs and MAPS antibodies compared to titers induced by the polysaccharide vaccine alone (Twumasi, et al. 1995). The immune response induced by the conjugate was boostable with polysaccharide in the majority of previously immunized children, demonstrating the ability to prime for immunologic memory, although some demonstrated a state of hyporesponsiveness similar to that seen with the polysaccharide vaccine (Twumasi, et al. 1995; Leach, et al. 1997; MacLennan, et al. 2001). Several studies in infants in the United Kingdom immunized with a C-CRM$_{197}$ conjugate or the A/C-CRM$_{197}$ showed significantly greater bactericidal antibodies compared with those induced by the polysaccharide vaccine. The immune response was boostable 1 yr later resulting in an anamestic response (Fairley, et al. 1996; MacDonald, et al. 1998; Richmond, et al. 1999a; Borrow, et al. 2002). Similar immunizations with meningococcal conjugates like those in the Gambian and UK have resulted in increased anti-polysaccharide titers compared to the polysaccharide vaccine. The antibody responses typically declined rapidly within 12 mo after the last immunization. It is proposed, that despite low titers of antibodies, the conjugate will provide long term protection via immunologic memory as shown for the Hib conjugate. Immunization studies with meningococcal conjugate vaccines in adults has been limited, but current studies have demonstrated a
slight increase in levels of anti-polysaccharide antibodies induced by the conjugate, but not significantly different from those induced by the polysaccharide vaccine (Anderson, et al. 1994; Richmond, et al. 1999b; Campbell, et al. 2002).

In addition with the development of conjugate vaccines to induce T-dependent immunity against encapsulated organisms, several concerns about their overall effectiveness have been raised. First, as demonstrated in the Gambian immunization trials, a partial state of hyporesponsiveness was detected after several doses with a bivalent A/C meningococcal conjugate vaccine (Twumasi, et al. 1995). This state was similar to that seen in individuals previously immunized and boosted with plain polysaccharide. In some individuals this was overcome with another does of the vaccine, although when re-vaccinated 1 yr later, those previously demonstrating hyporesponsiveness continued to do so, and actually demonstrated antibody titers lower than controls immunized with the polysaccharide vaccine (Leach, et al. 1997). In addition, other studies have shown the immune response to the polysaccharide, elicited by the conjugate vaccine, continued to possess characteristics of a T-independent response, despite the ability to elicit T cell help and a memory response (Seppala, et al. 1988).

A second concern with meningococcal polysaccharide-protein conjugate vaccines is that the choice of protein carrier may be important for the vaccines immunogenicity. Several investigators (Campagne, et al. 2000; Richmond, et al. 2001) using DT, TT, or CRM\textsubscript{197} conjugated to MCPS have demonstrated select carriers were superior to others by inducing different levels of anti-MCPS antibodies after immunizations, with some having antibody titers lower than that induced by
polysaccharide alone. In addition to choice of carrier, others (Ahman, et al. 1998; Dagan, et al. 1998) have shown the antibody response to the conjugate can be suppressed by increasing doses of the carrier protein. This is known as “carrier induced epitope suppression” in which the selected carrier protein may interfere with immunologic priming with other conjugate vaccines using the same carrier protein (Herzenberg and Tokuhisa 1982). Carrier induced suppression has been described in individuals previously immunized with unmodified TT- or DT-protein and subsequently immunized with a polysaccharide coupled to TT or DT (Gaur, et al. 1990; Peeters, et al. 1991). Likewise epitope suppression also has been observed when multivalent polysaccharide formulations have been conjugated to the same carrier protein (Cross, et al. 1994; Fattom, et al. 1999).

Another possible concern with the advancements of meningococcal conjugate vaccines, such as MnCC licensed in the UK, is the increased colonization with serogroups not included in the conjugate vaccine such as B, Y, and W-135. Horizontal genetic exchange within the meningococcal population continually takes place and has been documented to occur between serogroups B and C (Swartley, et al. 1997). An exerted pressure to reduce serogroup C meningococci in the population, may increase the occurrence of non-serogroup C clones. This has been demonstrated in previously immunized infants and toddlers with the multivalent pneumococcal conjugate vaccine and possibly may occur with the meningococcal vaccine (Dagan, et al. 1997).

Lastly, questions have been raised about the overall immunogenicity of the meningococcal conjugate in neonates and adults. First, the conjugate vaccine
requires 2-3 doses at least 1 mo apart which complicates an already crowded infant immunization schedule (Riedo, et al. 1995). Second, conjugate immunization switches antibody isotypes to other isotypes that are less protective against bacterial infection (Lesinski and Westerink 2001). Third, a potential loss of somatic mutation of specific germline-encoded anti-polysaccharide antibodies may occur (Lesinski and Westerink 2001). These antibodies have evolved to be optimally protective against bacterial infection, and B cells expressing the original germline are diminished in a T-dependent response. Last, similar levels of bactericidal antibodies are stimulated in adults immunized with either the conjugate or polysaccharide vaccine. This questions the efficacy of the vaccine in the adult population.

With these concerns in mind, it is, therefore, debatable whether the conjugate-polysaccharide technology, used to virtually eradicate Hib disease, is directly applicable to meningococcal disease. Therefore, additional methods to induce a T-dependent immune response to a T-independent antigen should remain a priority.

*Outer Membrane Protein Vaccines*

Alternative meningococcal vaccine candidates being investigated consist of non-capsular surface antigens such as serogroup/serotype specific epitopes on outer membrane proteins (OMPs). These vaccine candidates are targeted predominantly for serogroup B meningococci. This is due to the fact that the capsular polysaccharide of serogroup B structurally mimics polysialated glycoproteins that are expressed in various human tissues, especially in the fetal brain on the neural cell adhesion molecule (Finne, et al. 1987). Efforts to develop a polysaccharide vaccine for serogroup B have been hindered due to dangers of inducing autoantibodies that
cross-react with glycosylated host antigens. Attempts to overcome this with a protein-polysaccharide conjugate vaccine have not been successful, and currently there is no vaccine licensed for serogroup B. Therefore, efforts to develop a vaccine for group B focus on sub-capsular components such as OMPs and lipopolysaccharides, and have extended to studies with other serogroups.

Outer membrane protein vaccine candidates are based on the fact that specific OMPs administered alone induce specific anti-meningococcal antibodies. Two variations of OMP vaccines exist, aggregate OMP vaccines and more recently, outer membrane vesicle vaccines (OMV). Aggregate OMP vaccines consist of outer membrane preparations depleted of LPS (endotoxin) by detergent and subsequent precipitation of the OMP to produce protein aggregates (Griffiss, et al. 1987). The latter, OMV vaccines, are based on the ability of meningococci to excrete outer membrane blebs. These blebs or vesicles are depleted of LPS and contain the unmodified OMP of interest in its native conformation and natural lipid environment (Poolman 1996; Katial, et al. 2002).

Several meningococcal proteins that include class 1 and 2/3 porin proteins (PorA and PorB), neisserial surface protein (NspA), transferring binding protein (Tbps) and class 5 protein (Opa and Opc) have been used in OMV constructs and are currently in human clinical trials (van der Ley, et al. 1991; Martin, et al. 1997; Cadieux, et al. 1999; West, et al. 2001). These trials have yielded limited immune responses and protection in adults to the vaccines, including no protection in children under the age of two. Studies (Bjune, et al. 1991; Rosenqvist, et al. 1995; Sacchi, et al. 2001) in Norway, Cuba, and Brazil using OMV vaccines demonstrated an overall
efficacy of 50-80% with rapid declining antibodies post-immunization and little to no detectable bactericidal antibodies in several immunized individuals. An additional study (Tappero, et al. 1999) in Chile using the same OMV vaccine administered in Cuba and Norway, demonstrated an efficacy of antibodies against heterologous meningococcal strains of 31-35% in children, 37-60% in adults, and no response in infants. On the other hand, when antibodies were tested against homologous strains, the efficacy of the OMV vaccine was 67% in adults and children and 90% effective in infants.

Outer membrane proteins and outer membrane vesicles as vaccine candidates against *N. meningitidis* have been of interest since the 1970s, but due to the antigenic variation of the organism, studies have demonstrated low efficacy in targeted populations. Several explanations for OMP/OMVs poor immunogenicity have been suggested. First, multiple OMPs are restricted to specific homologous meningococcal serotypes and thus will not induce cross-reactive antibodies to heterologous strains (Martin, et al. 1997). This suggests that OMP/OMV vaccines will not confer protection against heterologous strains of the meningococcus. This would make them useful only during epidemics when antigenic variation of the meningococcus causing disease is low, unless they are combined with other constituents in a multivalent construct (Tappero, et al. 1999). Second, the significance of specific OMP antibody in a protective immune response remains to be elucidated. Several conserved proteins, such as Lip (H.8) and Rmp, once considered as potential vaccine candidates, were later demonstrated to induce non-bactericidal antibodies that blocked the bactericidal activity of antibodies directed at other proteins (Cannon, et al. 1984;
Bhattacharjee, et al. 1990; Munkley, et al. 1991). Third, some studies (Sanchez, et al. 2001) have shown administration of OMP/OMV vaccines lead to several problems including diminishing carrier rates or hampering of colonization by related commensal neisseria species, such as *Neisseria lactima*. It has been demonstrated that common OMPs and several cross-reactive antigens exist between these organisms, thus the use of similar OMPs may hinder the natural mechanism for acquisition of immunity (Troncoso, et al. 2000).

*Alternative Strategies for Vaccine Development Against Encapsulated Bacteria*

*Anti-Idiotypic Antibodies*

An alternative vaccine strategy to develop a T-dependent immune response to a carbohydrate antigen is through the development of anti-idiotypic antibodies. Anti-idiotypic vaccine technology is based on the immune network hypothesis of Niels Jerne (1974) and Jan Lindenmann (1973) that proposes in principle that anti-idiotypic antibodies (Ab2) directed at anti-carbohydrate antibodies can act as “internal images” of the carbohydrate antigen. The anti-idiotypic antibodies, used as immunogens, can induce anti-carbohydrate antibodies directed toward the nominal antigen. Several studies (Kohler, et al. 1989; Schreiber, et al. 1991; Westerink, et al. 1995; Gulati, et al. 1996) have demonstrated the use of anti-id technology in producing mimics of microbial and viral antigens, and in the induction of immune responses against tumor antigens and various biological active ligands.

The immune network hypothesis of Jerne and Lindenmann defines the immune system as a vast web of interacting antibodies and lymphocytes. Idiotopes are antigenic determinants in or near the paratope of an antibody, the portion that
defines immunoglobulin specificity (Greenspan and Bona 1993). The combination of all idiotopes on an antibody molecule defines its “idiotype.” In the normal immune system network, antibodies are directed at one another’s idiotypies, thus a particular antibody (Ab1) will produce a corresponding anti-idiotypic antibody (Ab2) (Westerink, et al. 1988). In this respect, some Ab2s will have “internal images” or structural analogs of the external antigen and theoretically will be able to mimic the antigen. Thus, Ab2 when used as an immunogen will induce specific antibodies (Ab3) directed at the nominal antigen. A summary of the idiotypic network hypothesis is illustrated in Figure 2.
Figure 2. Schematic representation of Jerne’s idiotypic network hypothesis (1974). The nominal antigen elicits an immune response and triggers a specific antibody response referred to as Ab1. Idiotypes on the Ab1 molecule act as immunogens and thus induce anti-idiotypic antibodies (Ab2) directed at the various idiotypes. Three different anti-id antibodies may be induced, Ab2α, Ab2β, or Ab2γ. Of these Ab2β antibodies contain surface characteristics that are internal images of the Ab1 paratope. Therefore, Ab2β antibodies have the ability to mimic the nominal antigen in binding to Ab1 and can induce anti-anti-idiotypic antibodies (Ab3) that cross-react with the nominal antigen.
As depicted in figure 2, immunization with Ab1 induces three different subsets of anti-id antibodies, Ab2α, Ab2β, or Ab2γ. These categories were originally assigned by Jerne et al. (1982) and later expanded by Bona and Kohler (1984), and were defined on the ability to map the location of the target idiotope. This was accomplished by using the relevant antigen as an inhibitor in the binding of Ab2 to the idiotope. When the target idiotope was distant from the antigen binding site and no inhibition of antigen occurred, the anti-idiotypic antibody was designated Ab2α. Alternatively, when inhibition of the antigen was observed and the idiotope was near the antigen-binding site, the anti-idiotypic antibody was called Ab2β. Last, when the antigen was inhibited, but only due to steric interference, the anti-idiotypic antibody was classified as Ab2γ. Because Ab2β antibodies are the only to bind in or near the antigen binding sites, they are the only subset with the ability to mimic the nominal antigen (Jerne 1974).

The first experimental evidence supporting the concept that anti-id antibodies mimic the biological properties of an antigen was provided in 1978 by Sege and Peterson (1978). They demonstrated that Ab2β antibodies raised against insulin specific antibodies could bind to the insulin receptor and mimic the functions of the hormone by regulating blood glucose levels. These observations were further supported by Shechter et al. (1984) who showed mice immunized with insulin developed anti-insulin antibodies as well as Ab2β antibodies to specific mouse insulin antibodies binding to the insulin receptor. This suggested anti-insulin receptor antibodies were induced as part of the idiotypic network in which Ab2β antibodies could mimic the structure of the insulin receptor. These data showed for the first time
the ability of anti-idiotypic antibodies to mimic the natural functions of a ligand and
the interactions with its specific receptor. The idea of anti-idiotypes as a new vaccine
strategy against infectious disease was suggested in 1981 by Nisonoff and Lamoyi
(1981) and others (Roitt, et al. 1981) after several successful preparations and
characterizations of anti-idiotypic antibodies produced against many diverse antigens.
Since then Ab2β antibodies have been shown to mimic different antigens derived
from viral, parasitic, and bacterial pathogens (Schreiber, et al. 1991; Westerink, et al.

Several anti-idiotypic antibodies directed at V domains of anti-carbohydrate
antigens have been shown to elicit carbohydrate binding antibody responses when
used as immunogens themselves (Harris, et al. 1997). Protective anti-polysaccharide
antibody responses have been induced by Ab2β antibodies produced against
Streptococcus pyogenes, group B Streptococcus, Streptococcus pneumoniae,
Pseudomonas aeruginosa, and N. meningitidis (McNamara, et al. 1984; Monafo, et al.
al. (1988) have previously described the production of a monoclonal antibody Ab2,
designated 6F9, that mimics the polysaccharide of N. meningitidis serogroup C.
Immunization with mAb 6F9 induced a T-dependent anti-MCPS antibody response
that was functional in mice and subsequently protected mice from lethal challenge
(Westerink, et al. 1990; Westerink and Giardina 1992). In addition, the immunogenic
site of 6F9 responsible for the induction of the anti-MCPS antibody response was
determined using computer assisted molecular modeling. A synthetic peptide that
represented the immunogenic complementarity determining region 3 (CDR3) of mAb
6F9 was synthesized and immunization studies were performed (Westerink, et al. 1995). These experiments showed that immunization with a peptide mimic of the immunogenic CDR3 region of mAb 6F9 resulted in complete protection against a lethal challenge with serogroup C (Westerink, et al. 1995), and further demonstrated that peptides could mimic carbohydrate antigens.

Anti-idiotypic technology as a vaccine strategy offers several advantages over conventional vaccines. First, anti-id vaccines do not contain nominal antigen or its fragments. This reduces side affects associated with the vaccine and eliminates the possibility of reversion to a virulent pathogen. In addition, anti-id vaccines are proteins and can be easily modified or coupled to carrier proteins to enhance their immunogenicity. Last, the technology has been shown to be useful at identifying potential vaccine candidates in organisms where it is difficult to detect immunogenic portions. However, there are also several disadvantages associated with anti-id vaccines. First, the production of an anti-id antibody is costly and time consuming. Development of an anti-idiotypic antibody includes the generation of monoclonal antibodies (Ab1) from single B cell hybridomas, immunization with Ab1 to elicit Ab2s, and time needed to culture large quantities of Ab2 for immunizations and in vitro assays. Second, vaccination with monoclonal Ab2 may induce undesirable immune responses to non-immunogenic portions of the immunoglobulin molecule. Third, studies (Elias, et al. 1984; Suciu-Foca, et al. 1984) have demonstrated that idiotype-specific immunosuppression could be induced in neonates when anti-id concentrations were high. Due to these limitations, anti-id derived products are not
frequently used in vaccine clinical trials, but the technology is very useful in identifying peptides that can be used as vaccine candidates.

Phage Display Libraries

Recent studies have indicated the potential of phage display libraries in selecting peptide mimics that are capable of inducing anti-carbohydrate immune responses. Several investigators (Pincus, et al. 1998; Valadon, et al. 1998; Grothaus, et al. 2000; Lesinski, et al. 2001) have identified peptide mimics of polysaccharides from a diverse group of organisms including Cryptococcus neoformans, Streptococcus group B, Streptococcus pneumoniae, and N. meningitidis serogroup A. The concept of peptide mimicry is an extension of the idiotypic network that suggests mimicry is the function of reproducing the binding interactions between the antibody and antigen (Fields, et al. 1995). Peptide mimics allow the immune response to focus on specific epitopes of a monoclonal antibody or ligand that will induce protection against an antigen, while concurrently avoiding non-immunogenic epitopes that are not needed or may interfere with the response (Nussbaum, et al. 1997).

Phage libraries are constructed by incorporating an oligonucleotide encoding a random peptide sequence into the tailcoat protein gene of filamentous phage (Smith and Scott 1993). Investigators (Grant, et al. 1981; Felici, et al. 1991) have shown foreign DNA fragments can be inserted into either the pIII, pVI, or pVIII genes of the phage to create an infective “fusion phage” that displays the encoded peptide on its surface (Parmley and Smith 1988). Phage display libraries encompass between 1x10^7 and 1x10^10 clones, each displaying a unique peptide sequence (Valadon and Scharff 1996). These libraries are screened with various selectors such as monoclonal
antibodies, natural ligands of receptors, cultured cells, serum samples and even whole animal (Cwirla, et al. 1990; Devlin, et al. 1990; O'Neil, et al. 1992; Cortese, et al. 1996; Amersdorfer, et al. 2002) in a process called “biopanning” (Valadon, et al. 1996). During the biopanning process, if a particular phage displays a peptide that is a strong ligand for the selector, it can be readily affinity purified out of the library.

Phage display libraries offer several benefits in selecting vaccine candidates for infectious disease. First, large numbers of phage can be screened at one time. Tens of millions of short peptides can be surveyed to identify several different peptide mimics (Scott and Smith 1990). Second, screening of a phage display library is a time efficient and simple method for identifying reactive peptides. Peptide mimics of antigens are easily defined through several rounds of biopanning. This is in contrast to the method for producing an anti-id antibody, which would take several months. Next, if a particular phage that expresses a peptide of interest is recovered in a low yield, it can be amplified by repeating several rounds of biopanning (Devlin, et al. 1990). Third, a peptides’ immunogenicity can be easily enhanced by coupling it to a carrier molecule or through the use of an adjuvant (Lowell, et al. 1988b).

Several investigators have demonstrated the ability to select peptides, using anti-polysaccharide antibodies, that functionally mimic the native carbohydrate antigen. Recent studies by Fleuridor et al. (2001) demonstrated that carbohydrate peptide mimics of Cryptococcus neoformans could be selected using the mAb 2E9 specific for the capsular polysaccharide. Immunization with a peptide mimic, designated P13, resulted in an anti-polysaccharide immune response that protected mice against infection in vivo. Similarly, Phalipon et al. (1997) using a mAb specific
for the O-antigen of *Shigella flexneri* and Pincus et al. (1998) using a mAb specific for the Group B streptococcal type III capsular polysaccharide have demonstrated the ability of carbohydrate peptide mimics to elicit significant anti-polysaccharide immune responses. Our laboratory has likewise used the technique of phage display to select carbohydrate peptide mimics of the *S. pneumoniae* serotype 4 capsular polysaccharide (Lesinski, et al. 2001) and the capsular polysaccharide of *N. meningitidis* serogroup A (Grothaus, et al. 2000). Both selected peptides when complexed to meningococcal serogroup B outer membrane proteins (proteosomes) were shown to elicit specific anti-polysaccharide antibody responses when administered in a mouse model.

The mechanisms responsible for enabling a peptide to mimic a carbohydrate antigen remain to be elucidated. Several studies (Evans, et al. 1994; Luo, et al. 1998) have postulated that structural similarity to the corresponding carbohydrate epitope is the basis for antigenic mimicry, whereas others (Harris, et al. 1997) have proposed antigenic mimicry is based on the antigen-antibody binding site of the anti-carbohydrate antibodies. Peptides that mimic capsular polysaccharides or bind to anti-carbohydrate antibodies have been shown to have similar peptide structures and common amino acid sequences. Sequences of carbohydrate mimics have demonstrated a preference for aromatic, acidic, hydrophobic, and hydrogen-bonding amino acid residues (Pincus, et al. 1998; Glee, et al. 1999; Phalipon and Sansonetti 1999). Hoess et al. (1993) identified peptide mimics of the Lewis Y antigen, and suggested the preference for certain amino acid residues may be attributed to their resemblance to sugar moieties of the carbohydrate epitopes along with a structural
role similar to the branched nature of carbohydrates. Pincus et al. (1998) selected a peptide mimic of group B streptococcal type III capsular polysaccharide (GBS) that contained aromatic amino acid residues, and suggested the presence of these residues to reflect sialic acid present in carbohydrates. Using x-ray crystallography Luo et al. (2000) proposed that the predominant forces involved include hydrogen bonding, stacking of aromatic side chains and van der Waals forces. Young et al. (1997) attribute the affinity of binding between an anti-carbohydrate antibody and a peptide mimic to the presence of these hydrogen bonds. Although the nature of peptide mimicry of polysaccharide antigens remains to be elucidated, the phage display libraries have been demonstrated to be effective means for identifying peptide mimics of carbohydrate antigens.

Phage display libraries select for carbohydrate peptides with a great deal of diversity, one restriction of this method is that it does not discriminate between peptides that bind to the monoclonal antibody with high and low affinity. Phages that are selected may not necessarily make an effective peptide mimic of the carbohydrate antigen solely on the basis of their affinity for the selecting mAb. The selection of particular phage during the biopanning process may be due to multivalent interactions between the phage and the selecting mAb (Goldsmith and Konigsberg 1977). The multivalent binding leads to strong adherences between the phage and the monoclonal antibody, regardless of affinity, thus allowing it to be propagated to the next round (Cwirla, et al. 1990). Some peptides displayed on the phage may be intrinsic to components used in biopanning such as plastic, streptavidin, or the blocking agents used (Adey, et al. 1995; Caparon, et al. 1996; Davies, et al. 1999). Last, multiple
cycles of selection may create a bias to certain peptides that do not interfere with the propagation rate and therefore multiply faster than the rest, despite their affinity to the monoclonal antibody (Cabilly 1997; Stern and Gershoni 1997). It is for these reasons that peptides selected must be evaluated with respect to their biological effects or the ability to mimic the carbohydrate antigen, and not how strongly they bind to the selecting monoclonal antibody.

Several investigators have documented that isolated peptide mimics demonstrating high affinity to the selecting carbohydrate monoclonal antibody did not prove immunogenic in vivo. Conversely, peptide mimics demonstrating low affinity to the selecting monoclonal antibody, have been shown to be highly immunogenic in eliciting anti-carbohydrate immune responses. Studies by Valadon et al. (1998) using the glucoronoxylanmannan (GSM) portion of \textit{C. Neoformans} were able to isolate high affinity binding peptides that inhibited interactions between the antigen and selecting monoclonal antibody. Upon immunization with the peptide mimic, a significant anti-carbohydrate antibody response to the \textit{C. Neoformans} capsular polysaccharide was not detected. Likewise, studies by Phalipon et al. (1997) also demonstrated that high affinity binding to the isolating agent did not correlate with its ability to be a good immunogen. Among nineteen peptides that were selected to mimic the O-antigen of \textit{Shigella flexneri}, only two were capable of eliciting anti-carbohydrate antibody responses. On the other hand, studies by Monafo et al. ((1987) and Raychardhuri et al. (1990) imply that peptides with low affinity could still induce anti-carbohydrate immune responses, and that the immune response to a peptide mimic has little to do with affinity for the selecting monoclonal antibody.
Other restrictions inherent to phage display libraries include the inability to display proteins that are toxic to bacterial cells. Alternative vector systems such as the use of bacteriophage γ, bacteriophage T4, or the psu protein on the P4 capsid have been considered for the presentation of toxic proteins (Maruyama, et al. 1994; Lindqvist and Naderi 1995). The use of these vectors represses the synthesis of the toxic protein during the lysogenic state, while allowing the induction of the protein shortly before lysis. Thus, the use of these new vector systems allows the screening of toxic proteins without damaging the bacteria. Phage display libraries also may not provide all possible peptides capable of binding to the selecting monoclonal antibody. For this reason, several different libraries varying in size (i.e., 7-mer, 10-mer, and 15-mer) have been constructed to allow the selection of peptides with diverse lengths (Cwirla, et al. 1990). Recently, conformationally constrained libraries have been increasingly used due to their ability of presenting peptides in a conformation similar to that in the natural protein (O'Neil, et al. 1992). Constrained libraries are constructed by flanking the random peptide sequence with a specific amino acid residue, such as cysteine, in the hope of limiting the number of possible conformations of the resulting peptide cyclic loop (Hoess, et al. 1994). Studies (Zhong, et al. 1994) have demonstrated by displaying peptides in a specific conformation, such as a β-loop or α-helix, monoclonal antibodies could select for constrained peptides with a specific three-dimensional structure that were generally more antigenic and immunogenic mimics than selected unconstrained peptide counterparts.
The primary advantage of using peptide mimics as a vaccine strategy for carbohydrate antigens is the ability to overcome the T-cell independency associated with the polysaccharide antigen and to increase the efficiency of the immune response to the carbohydrate. Peptides are intrinsically T cell dependent antigens, and are able to interact with both MHC I and II molecules. Therefore, peptides are displayed on the surface of antigen presenting cells which leads to affinity maturation directed toward the carbohydrate antigen as well as the facilitation of carbohydrate-specific cellular responses. Unfortunately, linear peptides are usually poor immunogens and are degraded and eliminated very rapidly in vivo when administered alone (Partidos, et al. 2001). Therefore, peptides must be coupled to a carrier protein, such as tetanus toxoid or hepatitis B core antigen (Vreden, et al. 1991), or given in formulation with an adjuvant. Several delivery systems have been considered which include the use of virosomes where the peptide is maintained in virus-like particles (Gluck, et al. 2000), lipid carriers such as immune stimulating complexes (ISCOMs) (Hsu, et al. 1996), microspheres (Men, et al. 1997), multiple antigen peptides (MAPS) that attach several branched peptides to a lysine core (Tsuji and Zavala 2001), and last DNA vectors that contain an oligonucleotide sequence encoding the peptide. These systems have been demonstrated to increase the immunogenicity of the peptide antigen, usually by protecting them from host protease degradation. This allows for the induction of specific anti-carbohydrate humoral and cellular immune responses.
DNA Vaccination

A natural extension of the observation that peptides/proteins can induce anti-carbohydrate responses is the development of DNA vectors that encode peptide mimics. DNA immunization is based on the concept that an encoded protein will be processed similarly to an antigen synthesized by the infectious organism, resulting in a humoral and cellular immune response specific for that pathogen (Donnelly and Liu 1998; Babiuk 1999). This approach has been applied to a wide range of infectious agents, and has more recently been demonstrated by Lesinski et al. (2001) and others (Kieber-Emmons, et al. 2000) to induce anti-carbohydrate immune responses to encoded epitopes of encapsulated organisms. Moreover, the ability to modulate the immune response to specific protein and carbohydrate epitopes makes DNA vaccines ideal for use against encapsulated organisms, where antibodies can be directed at both the polysaccharide and other relevant proteins such as conserved cell wall components.

History

DNA vaccine technology was first reported by Wolff et al. in 1990 (1990) when it was demonstrated that direct intramuscular immunization of plasmid DNA encoding multiple reporter genes could induce protein expression within muscle cells. This observation was further expanded several years later by Tang et al. (1992) who demonstrated antigen-specific antibody responses to human growth hormone could be induced in mice injected with gene-gun delivered plasmid DNA encoding hGH. Subsequently, the first documented protective immune response by DNA immunization was reported 1 yr later when Ulmer et al. (1993) and Robinson et al.
(1993) showed protection against influenza challenge occurred in DNA immunized subjects. It was further demonstrated from these challenge experiments, that both humoral and cellular immunity could be stimulated by the DNA construct. These early studies showed several important features about DNA vaccines; (1) DNA vaccines could be used against a variety of infectious agents including bacterial, viral and parasitic, (2) humoral and cell-mediated immune responses could be generated after administration of DNA, (3) a response could be generated using different routes of DNA immunization, and (4) a protective immune response could be induced by DNA vaccination against a specific pathogen.

Advantages of DNA vaccines

There are several advantages to DNA immunization, making it a good vaccine candidate to treat infectious disease (Robinson 1997). First, DNA vaccines are safe and pose no risk of reverting to virulent forms unlike live attenuated or killed whole vaccines. Second, DNA vaccines are easily constructed using recombinant DNA technology. Epitopes inserted into the plasmid can be modified readily allowing the removal/insertion of sequences that could affect the processing of the encoded protein. Sequences also can be modified to enhance the antigenic potency of the individual epitopes or removed if the epitopes trigger unwanted immune responses. Next, DNA vaccines are thermally stable at high and low temperatures, and stored in solution or dry, thus eliminating the need for “cold chain” transportation (series of refrigeration required to maintain the viability of a vaccine). This is essential for delivering the vaccine to areas of endemic disease. Last, DNA vaccines are very malleable and can be easily altered to modulate the magnitude and orientation of the
immune response. This can be achieved through the use of multiple adjuvants, methods/sites of administration, or the addition of epitopes that encode for cytokines, costimulatory molecules, or conserved proteins among different strains of the pathogen.

**Requirements for a DNA Vaccine Vector**

DNA constructs consist of a foreign gene of interest cloned into a bacterial plasmid vector. The plasmid vector is divided into a mitogenic unit, present in the DNA backbone and necessary for bacterial replication, and a transcriptional unit that directs antigen synthesis (Donnelly and Liu 1998; Tighe, et al. 1998; Hasan, et al. 1999). The control elements of the bacterial plasmid necessary for propagation of plasmid DNA in bacteria include an origin of replication that allows for the production of large copy numbers of plasmids in bacteria, and a selectable marker, such as a bacterial antibiotic resistance gene (i.e., ampicillin), used in growth selection for screening transformed bacterial cells. Elements important for transcription and regulation of gene expression in the host consist of a strong viral promoter for optimal expression in mammalian cells (i.e., cytomegalovirus) and a polyadenylation signal sequence for stabilization of mRNA transcripts (i.e., bovine growth hormone) (Liu 1995; Donnelly, et al. 1997). Plasmid DNA used for immunization is purified from transformed bacterial cells, and is injected into the animal as a DNA vaccine. Transformation occurs in host cells with the plasmid directing the synthesis and secretion of the vaccine antigen (Hilleman 1995; Ellis 1999), (Figure 4).
A gene of interest for a specific antigen is cloned into an expression plasmid. Bacteria are transformed and amplified in culture. Plasmid DNA used for immunizations is purified and animals are immunized with the purified antigen-expressing plasmid.
Means of DNA Administration

The various routes of DNA administration include intramuscular, intradermal, intranasal, oral, vaginal, and intraperitoneal delivery. Immunization by needle injection into the skeletal muscle is the immunization route predominantly used in studies. The plasmid DNA is injected using distilled water or saline as the delivery vehicle. Several investigators have used agents such as cardiotoxin, bupivacaine, or hypertonic solutions to improve responses to intramuscular injection. The use of these agents has been shown to increase the numbers of inflammatory cells, such as macrophages, to the inoculation site which results in better uptake and presentation of the DNA (Davis, et al. 1993). The optimal dose of DNA for intramuscular immunization depends on the particular antigen and model system used, but successful vaccination usually requires 10 to 100µg of plasmid DNA to elicit a response.

The second most frequent route of DNA administration is intradermally using the method of gene-gun delivery. This technique using a gas-driven biolistic bombardment devise propels DNA-coated gold particles directly through the plasma membrane into the cytosol of epidermal cells (Bennett, et al. 1999). Any cells in the path of the beads are transfected with the DNA vector, thus eliminating the need for cellular internalization of the antigen. Immunization with DNA by gene-gun often requires smaller amounts of DNA, 0.1-1.0µg, to induce antibody and cellular responses. This characteristic enables gene-gun delivery to be a more efficient mode of delivery compared to intramuscular injection, even though both have been shown to elicit humoral and cell-mediated responses (Lodmell, et al. 1998).
Mucosal DNA immunizations have used intranasal, genital-tract, aerosolized, or intratracheal routes of administration (Cohen, et al. 1998). More recently, mucosal DNA delivery has involved the use of microparticles containing the plasmid DNA delivered orally. Delivery at these sites induces immune responses necessary for limiting the original infection at the site of entry of the pathogen. The main advantage of a mucosal vaccine is the potential to stimulate both mucosal and systemic immunity (Babiuk 1999).

The specific type of immune response generated by DNA vaccination is dependent on the method of administration, and can be skewed to either specific cell-mediated or specific antibody responses. As previously stated, intramuscular needle immunization injects plasmid DNA into the interstitial space, where uptake occurs into the myocytes. This involves specific intracellular signals that lead to an increase in a cell mediated Th1 immune response. This response is characterized by elevated IgG2a to IgG1 antibody ratios with increased levels of IFN-gamma and IL-2, and little to no IL-4 secretion (Donnelly and Liu 1998; Webster 1999). On the other hand, gene-gun administration directly transfects target cells, bypassing any uptake mechanisms. The gene-gun method skews the immune response to a humoral Th2 response characterized by elevated IgG1, less IFN-gamma, and predominantly IL-4 and IL-10 cytokine production (Feltquate, et al. 1997; Robinson 1997). Therefore, by using different routes of administration, immune responses can be modulated in a specific way, by either cytokine production or antibody responses, that are needed to optimize the response against the targeted pathogen.
**Mechanisms of DNA Vaccination**

The precise mechanism involved in the generation of immune responses following DNA immunization remains to be elucidated. Several mechanisms have been postulated to explain how DNA vaccines exert their effect; (1) antigen presentation mediated directly by transfected myocytes, (2) presentation by transfected dendritic cells (DCs) or macrophages, and (3) cross-priming of antigen from transfected myocytes to professional antigen presenting cells (APCs).

It was first theorized that after intramuscular immunization myocytes are transfected directly with plasmid DNA. Several studies (Wolff, et al. 1992) had demonstrated that expression of the DNA encoded proteins were present in myoblasts and myotubules suggesting myocytes were responsible for presenting antigen and stimulating the primary immune response. However, due to the lack of MHC class II molecules, as well as costimulatory molecules, B71 and B72, myocytes are poor antigen presenting cells and are not likely to induce primary antibody and cellular immune responses. In this respect, it was hypothesized that transfected myocytes act as antigen secreting factories supplying infiltrating APCs, such as macrophages and dendritic cells, with soluble proteins. The professional APCs are recruited to the muscle as a part of local inflammatory responses caused by the immunization procedure. The released antigens are taken up by the APCs in the interstitial spaces and carried to the draining lymph nodes where they are presented to T and B lymphocytes (Davis, et al. 1997; Leitner, et al. 1999). This process of antigen secretion by the myocyte is known as cross-priming and is based on the idea that antigens synthesized by somatic cells, myocytes or keratinocytes, can be transfected
to professional APCs such as DCs or macrophages (Corr, et al. 1999). It is suggested that in gene-gun delivery cross-priming occurs by directly transfected keratinocytes, Langherhans cells, or dermal fibroblasts that are found in relatively larger proportions in the skin compared to muscle (Klinman, et al. 1998; Corr, et al. 1999; Hasan, et al. 1999). In both intramuscular and gene-gun immunizations, DCs and macrophages migrating to the site of immunization induce the primary immune response, whereas nonmigratory cells such as myocytes and keratinocytes influence the magnitude of the response through soluble antigen production (Fynan, et al. 1993; Klinman, et al. 1998; Corr, et al. 1999; Hasan, et al. 1999).

The second theory suggests direct transfection of bone-derived professional APCs are responsible for the primary response following DNA immunization. Dendritic cells and monocyte/macrophages express high levels of MHC class I and II molecules, as well as necessary costimulatory molecules, and have been shown to be efficient at uptake and presentation of antigen. It is suggested that these APCs are directly transfected, migrate to the lymph node, and stimulate CD8 T cells, CD4 T cells, and B cells (Maecker, et al. 1998).

Several investigators have demonstrated that both direct transfection of professional APCs and cross-priming by myocytes occur after DNA immunization. Corr et al. (1996) and others (Doe, et al. 1996) have shown, using myocyte and bone-marrow derived chimeras, that professional APCs were responsible for priming the immune response after DNA immunization and that myocytes were not converted into APCs after transfection with DNA. Likewise, studies by Casares et al. (1997) and Chattergoon et al. (1998) demonstrated that plasmid DNA could be isolated from
lymph-node derived and skin-derived DCs after both intramuscular and intradermal immunization. Both of these studies support the theory of direct transfection of APCs distant and near the site of inoculation. On the other hand, Ulmer et al. (1996, 1997) have demonstrated support for the cross-priming theory, where transplantation of myoblasts transfected with influenza NP DNA induced specific anti-NP antibody and cellular responses in recipient mice. These experiments suggested that transfer of antigen from transfected myocytes to APCs was the primary mechanism of priming the immune system after DNA immunization. It can be concluded that both mechanisms, transfection of myocytes and transfection of professional APCs, play a role in the generation of immunity through genetic immunization.

*Optimizing Strategies for DNA Vaccines*

A number of strategies have been proposed as possible means of optimizing the immune responses to DNA vaccines. These various strategies can positively alter the magnitude and orientation of the immune response by enhancing either cell mediated or specific antibody responses. Attempts to optimize DNA vaccines have included the use of conventional adjuvants, the addition of plasmids encoding specific cytokines or co-stimulatory molecules, specific targeting strategies, including DNA delivery systems and routes of administration, and lastly prime-boosting regimens (Leitner, et al. 1999; Babiuk, et al. 2003). It has been demonstrated that the use of a specific optimization strategy can polarize the response to a predominantly Th1 or Th2 immune response. Therefore, it is possible to stimulate the most desired immune response required to elicit protection from a specific pathogen (i.e., intracellular –
Th1, extracellular – Th2). Various DNA optimization strategies and the specific T helper response they polarize are shown in Figure 5.

Figure 5. Vaccination Strategies for a Predominant Th1 or Th2 Immune Response.

IL-4, IL-5, IL-10
(Geissler et al., 1997; Chow et al., 1998)

Aluminum Salts
(Wang et al., 2000)

Gene Gun, Intradermal, Intranasal Administration
(Fynan et al., 1993; Cohen et al., 1998; Weiss et al., 2002)

Encoded stimulatory molecules: CTLA-4, CD40L, CD4 T cell epitopes
(Deliyannis et al., 2000; Sin et al., 2001, Wilson et al., 2001)

IL-2, IL-12, IL-15, IL-18, IFN-γ
(Xiang et al., 1995; Iwasaki et al., 1997; Moore et al., 2002)

MPL, QS-21, Bacterial Toxins, CpG motifs
(Sasaki et al., 1997; Ban et al., 1997; Hamajima et al., 1998; Carson et al., 1997; Babiuk et al., 2003)

Intramuscular, Needle Administration
( Robinson, 1997; Weiss et al., 2002)

Encoded Stimulatory Molecules: B7, CD8 T cell epitopes
(Kim et al., 1998; Fujimara et al., 2001)
Polarization of specific or cell-mediated immune responses may be influenced by various immunization strategies. First, the addition of exogenous cytokines or encoded cytokines into a DNA expression vector can accelerate and augment the response in either Th1 or Th2 direction. The co-delivery of IL-12 or IFN-γ will enhance a Th1 response over a Th2 response, whereas co-delivery of IL-4 or IL-10 cytokines will enhance a Th2 immune response (Chow, et al. 1998). Second, the addition of a specific adjuvant will skew the desired immune response. Aluminum salts such as aluminum hydroxide and aluminum phosphate polarize Th2 responses, whereas lipid A derivatives such as monophosphoryl lipid A (MPL) and saponins such as QS-21 enhance Th1 responses. Immunostimulatory sequences such as non-methylated CpG (Cytosine-phosphate-guanine) motifs can be administered encoded in plasmid DNA or can be increased in numbers on the plasmid backbone. The addition of CpG motifs results in the switching of the response from Th2 to Th1 by activating monocytes, NK cells, and DCs (Carson and Raz 1997). As stated previously, the route of DNA administration (IM or gene gun) polarizes Th1 and Th2 responses as does the use of “prime-boost regimens” (Cohen, et al. 1998). Combining DNA immunization with subsequent boosters of other traditional forms of antigen (protein/polysaccharide) maximizes antibody responses resulting in increased protection. Third, APC capabilities of transfected cells can be increased by the co-delivery of genes encoding co-stimulatory molecules (Cohen, et al. 1998). The addition of plasmid encoded B7 co-stimulatory molecules has been shown to enhance antigen specific Th1 responses (Kim, et al. 1998). On the other hand, encoded co-stimulatory molecules such as CTLA-4 and CD40L stimulate Th2 responses by
enhancing the fusion of antibody responses (Deliyannis, et al. 2000; Sin, et al. 2001). The employment of any of these optimization strategies maximizes a particular immune response, thus making DNA advantageous to tailoring a unique response desired to protect against a specific pathogen.

**DNA Vaccine Safety Concerns**

A number of safety concerns have been raised about the use of DNA vaccines. These include: (1) integration of plasmid DNA into the host genome, (2) altered immune states such as autoimmunity and tolerance, and (3) the induction of anti-DNA antibodies. It has been suggested integration of DNA into the host genome may lead to insertional mutagenesis. This may result in the activation of protooncogenes, inactivation of tumor suppressor genes, and may cause aberrant cell growth leading up to carcinogenesis (Nichols, et al. 1995; Donnelly, et al. 1997; Hasan, et al. 1999).

To date there has been no clear evidence to indicate DNA integration into the host genome (Berglund, et al. 1998). Studies by Manam et al. (2000) examined the integration rate of plasmid DNA in mice and guinea pigs using different methods of administration (IM and gene gun) and various adjuvants (CpG motifs and aluminum salts). Under the various conditions tested, no integration of plasmid DNA into the host genome was detected in either animal models. It is speculated though, if integration of plasmid DNA into the host genome did occur, the rate would be 1,000 – 3,000 times less than that of spontaneous DNA mutations, and that using plasmids with little to no homology to the human genome would reduce that rate (Le, et al. 2000).
Altered immune states induced by DNA administration also have been a concern in DNA vaccine development. Autoimmunity is suggested to occur as a result of the immune destruction of cells expressing foreign encoded proteins (Donnelly, et al. 1997), as well as a result of the immunostimulatory activity of the CpG motifs naturally present in the plasmid backbone (Gurunathan, et al. 2000). Findings to date have indicated the level of immune destruction resulting in self auto-antibodies has been insufficient to induce auto-immunity. Mor et al. (1997) indicated auto-antibodies could be detected after DNA administration, but the levels of auto-antibodies were too low to promote autoimmune disease. Destruction of cells occurs with any viral or bacterial infection, as well as with tissue remodeling, therefore DNA vaccination should not pose a greater risk of inducing autoimmunity than a natural infection with a pathogen. Because the encoded antigen persists for a prolonged time after DNA vaccination, it is believed that immunological tolerance may result after immunization rather than protective immunity. This is especially important in neonates with immature immune systems. Since DNA is endogenously expressed and presented in context with self MHC class I molecules, a potential for tolerance could exist in an immature immune system where the peptide:MHC complex could be recognized as “self” (Silverstein and Segal 1975). Evidence for neonatal tolerance has been controversial and has been shown to be dependent on age as well as the particular species examined. Mor et al. (1996) and Ichino et al. (1999) studied tolerance induction in mice (< 8 d old) using a DNA plasmid encoding a circumsporozite protein of malaria. They found DNA immunization with this DNA plasmid induced tolerance formation in neonatal mice rather than protective immunity.
immunity. Conversely, studies by Liu et al. (1997) using African green monkeys as a model demonstrated that subimmunogenic doses of DNA encoding influenza A did not induce tolerance as initially suspected, but primed the monkeys for an anamnestic response for subsequent boosters. Other investigators (Klinman, et al. 1996; Prince, et al. 1997) have shown tolerance could be induced by DNA immunization in some species and not in others, but was critically dependent on the age of the subject. Continuing studies with DNA immunization in neonates using different models are being examined to determine the immune response induced in the very young.

The formation of anti-DNA antibodies in the host could possibly result from DNA administration. To date there has been no experimental evidence to suggest anti-DNA antibodies are induced (in mice and humans) even after repeated injections as detected by enzyme linked immunosorbant assays (ELISA) (Nabel, et al. 1993; Parker, et al. 1995). Purified double stranded DNA does not readily induce anti-DNA antibodies unless it is denatured and further coupled to other proteins (Gilkeson, et al. 1991). In addition, nonpathogenic anti-DNA antibodies that are bacterial specific and do not cross react with mammalian DNA are normally present in mice and humans. Therefore, it is unlikely that anti-DNA antibodies will be induced after DNA vaccination that could eventually lead to the formation of various altered immune states.

*Multiple Epitope DNA Constructs*

Recently DNA vaccine design has expanded to include multiple T and B cell epitopes in a “string-of-beads” fashion (Whitton, et al. 1993; An and Whitton 1997; Toes, et al. 1997; Ishioka, et al. 1999). The main advantage of this approach is the
opportunity to induce a multi-specific immune response directed at various immunologically relevant epitopes, in order to achieve adequate disease coverage against different strains (Ciernik, et al. 1996; Oseroff, et al. 1998; Thomson, et al. 1998). Studies (Hanke and McMichael 1999) have demonstrated the expression and presentation with as many as 15 epitopes in a single construct using epitopes from multiple pathogens. Multiple epitope DNA vaccination has included pathogens such as *Schistosoma mansoni* (Yang, et al. 2000), Influenza (Thomson, et al. 1998), *Listeria monocytogenes* (Yoshida, et al. 2001), and HIV (Hanke and McMichael 1999). The majority of multiple epitope vaccines have been targeted against viruses. However, the ability to modulate the immune response to specific epitopes also makes them ideal for use against encapsulated organisms where an immune response can be directed at polysaccharide epitopes.

Several factors must be considered when designing a multiple epitope DNA construct. First, conflicting results exist whether the exact orientation and arrangement of various epitopes in a construct are crucial for induction of an optimal response. Linking multiple epitopes in a single construct has been shown to be limited by the formation of negative flanking sequences from neighboring epitopes that suppress the presentation of another epitope. This includes determinants created at the junction between two epitopes by linking them together in tandem (Bergmann, et al. 1994; Hanke, et al. 1998b). However, studies by Velders et al. (2001) and others (Del Val, et al. 1991) have demonstrated the efficiency of processing and expression of epitopes can be increased by the use of defined flanking spacer sequences between neighboring epitopes. On the other hand, studies performed by
Chimini et al. (1989) and Hahn et al. (1991) have shown spacer sequences in a construct do not deleteriously nor positively influence the expression of multiple epitopes in vivo or in vitro. Second, the B cell epitope must be presented in correct conformation in order for it to adopt the shape of the nominal antigen. If the B cell epitope is presented out of its original context, antibodies elicited to the epitope will not recognize the targeted antigen. Third, due to the poor immunogenicity associated with short peptides, T cell help is needed to elicit an optimal immune response. Therefore, helper T cell epitopes need to be included in a multiple epitope DNA construct.

Taking into consideration the poor efficacy and immunogenicity of the current licensed meningococcal capsular polysaccharide vaccine in children <2 yr of age, and the limitations associated with the meningococcal conjugate vaccine, peptide mimics of the capsular polysaccharide may be a reasonable alternative vaccine candidate to induce a T-dependent immune response to \emph{N. meningitidis}. According to the literature several investigators have been successful in inducing functional anti-carbohydrate antibody responses through the use of peptides that mimic the capsular polysaccharide. A natural extension of the observation that peptides can mimic polysaccharides is the development of DNA constructs that induce anti-carbohydrate antibodies. DNA constructs can be designed to express peptide mimics as part of a fusion protein, or in combination with immunogenic B and T cell epitopes. This allows for the creation of a multi-epitope DNA construct that is able to induce a multispecific immune response directed at various immunologically relevant protein and carbohydrate epitopes.
The following studies will describe the evaluation of a peptide mimic of *N. meningitidis* serogroup C capsular polysaccharide (MCPS) selected through the biopanning of phage libraries compared with a peptide mimic of MCPS previously selected using anti-idiotypic technology. The experiments will further describe the evaluation of the immune response to vaccination with a multi-epitope DNA construct encoding the peptide mimic of MCPS.

The hypotheses of these studies are as follows:

1. Peptides that mimic the capsular polysaccharide of *N. meningitidis* serogroup C (MCPS) can be selected using various methods.

2. A peptide mimic of MCPS with a similar motif to a known peptide mimic of MCPS can elicit a similar protective anti-MCPS antibody response in mice.

3. A multi-epitope DNA construct encoding a peptide mimic of MCPS can induce a functional anti-MCPS antibody response that can protect mice against a lethal meningococcal challenge.

4. A multi-epitope DNA construct encoding a peptide mimic of MCPS can induce a T-dependent immune response with predominantly Th1 characteristics.
Two different methods result in the selection of peptides that induce a protective antibody response to *N. meningitidis* serogroup C

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**Running Headline**

An immunogenic peptide mimic of *N. meningitidis*
Abstract

*Neisseria meningitidis* is a leading cause of morbidity and mortality worldwide. The presently available capsular polysaccharide vaccine is poorly immunogenic in children under the age of two due to its T-independent (TI) nature. Efforts to overcome the TI response elicited by the polysaccharide vaccine have led to the development of polysaccharide-protein conjugate vaccines. Although a T-dependent (TD) response can be achieved in young children, the response to the polysaccharide still retains characteristics of a TI antibody response. An alternative method of potentially inducing a TD response to a carbohydrate antigen is through peptides that mimic the capsular polysaccharide. Our laboratory, through the production of an anti-idiotypic (anti-id) monoclonal antibody, designated 6F9, has previously identified a peptide mimic of the meningococcal serogroup C polysaccharide (MCPS). Using the same selecting monoclonal antibody (mAb), 1E4, we have screened a phage display library and identified thirteen unique peptides that bound specifically to mAb1E4. Two peptides, Pep1C and Pep2C, that demonstrated the highest binding to mAb1E4, were selected, complexed to proteosomes, and used to immunize Balb/c mice. Of the thirteen peptide motifs, only one peptide motif, that of Pep2C, was found to resemble the immunogenic peptide sequence of the anti-id selected with the same mAb, although many contained several similar amino acid residues. Immunization with Pep2C, but not Pep1C, induced a significant and functional anti-MCPS antibody response that conferred protection from a lethal challenge with meningococci. Our results indicate that immunization with a peptide of *N. meningitidis* serogroup C, screened with the same mAb that selected an anti-id
of MCPS, induces a functional and protective anti-MCPS immune response similar to that of the anti-id. This study demonstrates that two different selection methods, production of an anti-id and biopanning using a phage display library, can be used to select functional and protective peptides of MCPS with similar moieties.

Keywords

*Neisseria meningitidis*, Peptide Mimicry, Capsular Polysaccharides
1. Introduction

*Neisseria meningitidis* is a leading cause of bacterial meningitis and septicaemia worldwide (Moore 1992, Rosenstein et al. 1999, van Deuren et al. 2000). In developed countries the incidence of meningococcal disease varies from 1 to 3 cases per 100,000 individuals, whereas in developing countries the rate extends from 10 to 25 cases per 100,000 (Jackson et al. 1995, Raymond et al. 1997, van Deuren et al. 2000). Over 350,000 cases of meningococcal disease occur annually throughout the world, resulting in a case fatality rate of 10-13%. Forty-six percent of the cases occur in children less than two years of age (Lingappa et al. 2001), although recently an increased incidence has occurred in collegiate students (2000).

*N. meningitidis* is classified into many serogroups, serotypes, and subtypes based on differences in the capsular polysaccharide, lipopolysaccharides, and outer membrane proteins (Poolman et al. 1982, Frasch et al. 1985, Riedo et al. 1995). Thirteen different serogroups exist, of which serogroups A, B, C, Y and W-135 is responsible for more than 90% of all meningococcal disease cases each year (Jackson et al. 1995, Apicella 2000). More specifically, serogroup C is responsible for sporadic meningococcal disease in developed countries such as Canada and the United States, and alone causes 45% of bacterial meningitis infections in the US each year (Rosenstein et al. 1999).

The presently available quadrivalent meningococcal vaccine contains the capsular polysaccharides from the serogroups A, C, Y, and W-135 (King et al. 1996,
Ball et al. 2001), and is based on the observation that antibodies directed against the capsular polysaccharide protect individuals from infection by inducing complement-dependent bactericidal activity (Goldschneider et al. 1969, Gotschlich et al. 1969). The multivalent polysaccharide vaccine is currently recommended for controlling meningococcal disease outbreaks in individuals greater than two years of age, and for use in high-risk groups, such as those with complement deficiencies (1997, Ruben et al. 2001). Due to its poor immunogenicity in children less than two years of age, the polysaccharide vaccine is not routinely used in childhood vaccination. Past studies have demonstrated total antibody titers do not necessarily correlate with the level of functional antibodies (Cadoz 1998). The poor efficacy of the polysaccharide vaccine in young children is attributed to the TI nature of the capsular polysaccharide antigen. Studies have suggested the late development of an immune response to TI antigens may be attributed to an immature splenic marginal zone (Timens et al. 1989, Mond et al. 1995), low IgG2 production (Rijkers et al. 1998), and low complement activity (Davis et al. 1979).

Efforts to overcome the TI nature of the capsular polysaccharide have resulted in the development of polysaccharide-protein conjugate vaccines. By covalently linking a polysaccharide antigen to a protein antigen, such as tetanus or diphtheria toxoid (Peeters et al. 1991, Richmond et al. 2001), a TD immune response can be achieved including T cell help and immunologic memory. Presently, two bivalent meningococcal conjugate vaccines exist comprising serogroups A and C (Twumasi et al. 1995, Leach et al. 1997). Despite the ability of these conjugate vaccines to induce immunologic memory in children less than two, some studies have shown the
immune response elicited to the polysaccharide continues to show characteristics of a TI response (Heath et al. 2000, Breukels et al. 2001).

Another approach to induce a TD immune response to carbohydrate antigens is through the development of anti-idiotypic antibodies that mimic the capsular polysaccharide (Westerink et al. 1988, Greenspan and Bona 1993). By acting as a surrogate image of the carbohydrate antigen, the anti-idiotypic antibody can induce a specific functional antibody response targeted to the nominal antigen (Moe et al. 1999). Studies have demonstrated idiotypic vaccines can induce TD protective immune responses to *Pseudomonas aeruginosa* (Schreiber et al. 1991), *Neisseria gonorrheae* (Gulati et al. 1996), and *N. meningitidis* (Westerink et al. 1988).

Previously, our laboratory has produced an anti-idiotypic antibody (anti-id), designated 6F9, that mimics the capsular polysaccharide of *N. meningitidis* serogroup C (Westerink et al. 1990). The anti-id 6F9 elicits a functional TD anti-MCPS antibody response that confers protection from lethal challenge (Westerink and Giardina 1992). 6F9 was also shown to be a true mimic of MCPS as it was able to inhibit the binding of human hyperimmune sera to MCPS, in a solid phase assay. In addition, a peptide spanning the immunogenic CDR3 region of 6F9 was synthesized and induced protective anti-MCPS antibodies in a murine model (Westerink et al. 1995). These studies demonstrate the potential of peptides in mimicking carbohydrate antigens.

Recent studies have shown phage display libraries are capable of selecting peptide mimics that induce anti-carbohydrate immune responses. Large peptide libraries have been constructed consisting of many filamentous phage clones, each
displaying a unique peptide sequence on its virion surface (Scott and Smith 1990, Felici et al. 1991). These libraries encompass greater than $10^7$ different peptides, and have been successfully utilized for identification of epitopes that recognize antibodies (Cwirla et al. 1990, Devlin et al. 1990, Scott and Smith 1990), natural ligands of receptors (O'Neil et al. 1992), cultured cells, serum samples (Amersdorfer et al. 2002), and others (Cortese et al. 1996, Nakamura et al. 2002). The ability of this strategy to select for carbohydrate binding peptides has been demonstrated in studies involving Conavalin A (Oldenburg et al. 1992), Lewis Y antigen (Hoess et al. 1993), Cryptococcus neoformans (Valadon et al. 1996, Fleuridor et al. 2001), and streptococcal group A polysaccharide (Pinilla et al. 1998). Other studies by Phalipon et al (1997) and Pincus et al (1998) have further demonstrated the ability of peptide mimics to elicit anti-carbohydrate immune responses. The technology of screening a phage display library has also been used in our laboratory to select immunogenic peptide mimics for Streptococcus pneumoniae (Lesinski et al. 2001) and N. meningitidis serogroup A (Grothaus et al. 2000).

Both techniques, anti-id antibody production and biopanning a phage display library have been shown to produce functional peptide mimics of carbohydrate antigens, however phage display has several advantages. First, phage display is simpler and less time consuming that production of an anti-id. Secondly, large numbers of phage can be screened at one time to identify several mimotopes (Devlin et al. 1990, Cortese et al. 1995). It would be extremely laborious to identify several different anti-ids covering multiple epitopes of a complex antigen. Lastly, the
specific peptide response for mimicry can be readily defined in phage display without
the complicated technology necessary with anti ids.

In this study, we have screened a phage display library with a monoclonal
antibody, 1E4, previously used to produce an anti-idiotypic antibody that mimics
MCPS. The purpose of the study was to compare and evaluate the immunogenicity
of peptides selected by each method. Peptides selected from the library using
mAb1E4 were synthesized, complexed to proteosomes, and used for immunization.
One selected peptide was capable of inducing a protective anti-MCPS immune
response similar to the response elicited by the anti-id peptide mimic. This
immunogenic peptide also contained similar sequence motifs to that of the anti-id
produced with the same monoclonal antibody. This is the first study to demonstrate
that two different techniques that select for immunogenic peptides of carbohydrate
antigens can be used to produce similar peptides using the same selecting mAb.
2. Materials and Methods

2.1 Monoclonal antibody 1E4 and meningococcal serogroup C capsular polysaccharide (MCPS).

Meningococcal serogroup C capsular polysaccharide was obtained from NIBSC (Potters Bar, United Kingdom). The monoclonal antibody 1E4 specific for MCPS was previously produced in our laboratory as a tissue culture supernatant and used to select a monoclonal anti-idiotype antibody designated 6F9 (Westerink et al. 1995).

2.2 Origin of Peptide Library.

The phage library used in this study was a kind gift from Dr. G.P. Smith (Smith and Scott 1993). The phage library contains fifteen amino acid inserts at the N-terminal part of the pIII tailcoat protein of the phage fd (Parmley and Smith 1988). The phage fUSE5 was used as the vector (Oldenburg et al. 1992). The library was constructed by ligating a synthetic forty-five base pair BglII fragment into the fUSE5 vector and transfecting E.Coli K91/kan+ cells by electroporation. A fifteen-mer library was selected based on the idea that the length is similar to complementary determining regions (CDR) in antibodies. The CDR often confers mimicry capacity to many anti-idiotypic antibodies (Jerne 1974).

2.3 Affinity selection and amplification of the peptide library – biopanning.

Phage that bound the anti-MCPS monoclonal antibody 1E4 were isolated from the phage display library by successive cycles of selection and amplification. This procedure is known as biopanning and is based on that previously described by Valadon et al (Valadon and Scharff 1996). Briefly, one milligram of Dynabeads® M-
450 rat anti-mouse IgM coated beads (Dynal, Lake Success, NY) were labeled with 300 µg of mAb1E4 in 1ml 0.1% BSA/PBS at 4°C overnight. Unbound antibody was removed by washing four times with 0.1% BSA/PBS. The phage library was depleted of anti-mouse IgM binding clones by incubating 2x10^{11} phage transforming units (TUs) with 3 mg of washed Dynabeads® M-450 rat anti-mouse IgM coated beads in 1ml biopanning buffer (BPB) (10mM Tris-HCl, pH7.5, 150mM NaCl, 0.1% BSA, 0.1% Tween 20, and 0.02% NaN₃) for four hours at 4°C. The depleted library (unbound phage) was then added to 0.5 mg of mAb1E4 labeled beads and incubated at 4°C overnight on a rotating mixer. The beads were washed nine times with BPB. Phage were removed with elution buffer (0.1N HCl, pH2.2, 1mg/ml BSA, 0.1mg/ml phenol red) (Smith and Scott 1993). The eluted phage were titrated, amplified (Smith and Scott 1993), and subjected to two additional rounds of selection and amplification, as described above. Decreasing concentrations of mAb1E4 labeled beads were used in subsequent rounds to ensure the selection of high-binding phage (Valadon and Scharff 1996).

2.4 DNA sequencing of selected peptides.

Phage clones from the third round of biopanning were randomly selected and sequenced. Approximately 5 µg of single-stranded DNA was purified and sequenced with a Thermosequenase radiolabeled terminator sequencing kit (Amersham, Cleveland, OH). A 27-mer primer complimentary to a FUSE vector sequence derived from a region in the wild-type pIII common to all fd-tet derived vectors was used (5’-GTCATAGCAGACAGCCCTCATAG-3’).

2.5 Reverse phage ELISA.
Binding of the selected phage to mAb1E4 was determined by reverse ELISA as previously described by Valadon et al (Valadon and Scharff 1996). Ninety-six well microtiter plates (Nunc, PGC Scientific, Gaithersburg, MD) were coated overnight at 4°C with mAb1E4, at its previously determined saturation point (1:3200), in PBS. Wells were washed three times with PBS/0.05% Tween and blocked with PBS/1% BSA/0.05% Tween at 37°C for two hours. Duplicate serial dilutions of selected purified phage in PBS were added starting with 5x10^{12} TU/well and incubated at 37°C for two hours. Phage binding was detected with sheep anti-M13 phage antibody (5 prime, 3 prime, Boulder, CO) (1:4000) and incubated for one hour at 37°C. Anti-sheep IgG peroxidase conjugate (Southern Biotechnology Assoc, Birmingham, AL) (1:3000) was added to washed plates and incubated for one hour at 37°C. Plates were developed with substrate buffer containing o-phenylenediamine. Absorbance levels were read at 490nm with an EIA reader (Bio-Tek instruments, Burlington, VA).

2.6 Proteosome Synthesis.

Proteosomes were prepared from outer membrane complex vesicles from *N. meningitidis* serogroup B, strain M99, as previously described (Zollinger et al. 1979). Briefly, propagated bacteria, from overnight cultures, were collected by centrifugation at 8,000 rpm for fifteen minutes. Twenty-milliliters of TEN buffer (0.05M Tris-HCl, 0.15M NaCl, 0.01M EDTA, pH 7.4) was added for each liter of culture, and warmed to 56°C for thirty minutes. The bacteria were sheered by sonication and centrifuged at 13,000 rpm for fifteen minutes. The supernatant was subjected to two ultra-centrifugations at 30,000 rpm for 120 minutes. 10X Empigen BB buffer (0.5M TRIS, 0.5M NaCl, 0.1M EDTA, pH 7.4, and 10% Empigen BB)
was added to the pellet resuspended in TEN buffer. Proteosomes were precipitated by the addition of ammonium sulfate in a 1:1 vol/vol, and dialyzed against 0.1X Empigen BB buffer to remove excess detergent. Successful isolation of proteosomes was determined by SDS PAGE electrophoresis and protein concentration was measured using the Micro BCA™ protein assay reagent kit (Pierce, Rockford, IL).

2.7 Peptide synthesis and complexing to proteosomes.
Selected peptides were synthesized with a CYGG spacer at the amino terminus conjugated to a lauroyl group (Sigma Genosys, The Woodlands, TX). The addition of a lauroyl group allows for hydrophobic complexing of the peptide to the proteosomes. The cysteine present at the N-terminus appears to be essential for immunogenicity (Lowell et al. 1988a). The synthesized peptides were hydrophobically complexed to proteosomes on a 1:1 wt:wt basis in the presence of detergent. The peptide-proteosome complexes were dialyzed extensively for ten days against PBS, pH8.5, to remove excess detergent (Lowell et al. 1988b).

2.8 Peptide immunization studies.
Mice (n=6) were immunized intraperitoneally (i.p.) as described in Table 1. All mice were tail-vein bled on a weekly basis and serum tested for the presence of anti-MCPS antibodies. All care of animals was in accordance with institutional guidelines under the supervision of an Institutional Animal Care and Use Committee (IACUC).

2.9 MCPS ELISA.
Detection of an anti-MCPS antibody response was measured by ELISA. Briefly, ninety-six well microtiter plates were coated with 50 µl/well MCPS and methylated human serum albumin (NIBSC, United Kingdom) in PBS, at a final concentration of
10 μg/ml, overnight at 4°C. Wells were washed three times in PBS/0.1% Brij, and blocked at 37°C with 200 μl/well blocking buffer (10% heat inactivated calf serum/PBS/0.1% Brij) for two hours. Serial dilutions of post-immune sera were added to the plates in blocking buffer and incubated overnight at 4°C. Bound antibody was detected after an hour incubation with anti-mouse IgM and IgG peroxidase labeled conjugate antibodies (Southern Biotechnology Assoc, Birmingham, AL) (1:3000) in blocking buffer, and developed with substrate buffer containing 0.02% o-phenylenediamine. Absorbance levels were read with an EIA reader (Bio-tek instruments, Burlington, VA) at 490 nm. Anti-MCPS antibody titers were calculated as the dilution of serum corresponding to 25% of the maximum optical density obtained with the positive reference serum.

2.10 Bactericidal Assays.

Serum bactericidal assays were performed as previously described by Maslanka et al (Maslanka et al. 1997). Briefly, N. meningitidis serogroup C, strain C-11, was grown on brain heart infusion agar supplemented with 1% heat-inactivated horse serum (BHIA/S) at 37°C in 5% carbon dioxide. Working bacterial seed lots were prepared in Dulbecco’s phosphate buffered saline and 0.1% glucose to yield a final concentration of 2x10⁴ CFUs/ml. The assay was performed in ninety-six well tissue culture plates, using baby rabbit serum (Pelfreeze, Brown Deer, WI) as the source of complement. Dilutions of heat-inactivated post-immune sera (1:8 to 1:4096) and an equivalent cell:complement mixture were added to the micro wells. Controls included wells without post-immune sera, wells without complement, and wells containing positive control sera. Aliquots from the wells were taken at times zero and
sixty minutes, and plated out on BHIA/S plates using the agar tilt method, and incubated overnight at 37°C. The serum bactericidal titer was reported as the lowest reciprocal serum dilution taken at time 60 minutes yielding >50% killing as compared to time zero.

2.11 Live Challenge.

Mice immunized with 50µg peptide-proteosome complex, 50µg proteosomes, or 5µg MCPS were challenged with \textit{N. meningitidis} serogroup C, strain 35E, using the murine model for meningococcal infection as previously described (Calver et al. 1976, Salit and Tomalty 1984). Mice received 1000 mg/kg iron dextran i.p. seven days prior to challenge to enhance susceptibility to bacteria. The lethal dose killing fifty percent (LD50) was determined in a naïve mouse challenge with $10^3$-$10^5$ cfu of meningococci. Immunized mice treated with iron dextran were challenged i.p. with 10xLD50 under sterile conditions.

2.12 Pep2C binding to IgM mAbs.

Binding of Pep2C phage to several IgM mAbs with different specificities was determined by reverse ELISA as previously described in section 2.5. Ninety-six well microtiter plates were coated with mAbs specific for \textit{N. meningitidis} serogroup A polysaccharide and \textit{S. pneumoniae} serotypes 18C, 6A, 23F, 14, 4 and 19F in PBS.

2.13 Statistical Analysis.

Statistical analysis was performed using the Mann Whitney U non-parametric test (SPSS, Chicago, IL) for phage ELISA assays and pre and post immune sera antibody and bactericidal titers. A $P$ value <0.05 was considered statistically significant.
3. Results

3.1 Selection of MCPS specific peptides with mAb1E4.

Monoclonal antibody 1E4 was selected to screen the phage library because of its specificity for MCPS and its prior use to select an anti-idiotype antibody, designated 6F9. The anti-id, 6F9, was shown to elicit protection from a lethal dose of *N. meningitidis* serogroup C (Westerink and Giardina 1992). Subsequently, the CDR3 of the anti-id 6F9 was synthesized as a peptide and was shown to be immunogenic and confer mimicry of MCPS (Westerink et al. 1995). A fifteen-mer phage library was chosen based on the idea that the length is similar to the CDR in antibodies. Rat anti-mouse IgM coated immunogenic beads were labeled with mAb1E4 and used to screen the fifteen mer phage library for peptides that bound to the mAb. Phage that bound to the mAb1E4 coated beads were eluted and amplified in DH5 alpha F’ *E. coli* cells (Invitrogen, San Diego, CA). Subsequent rounds of biopanning were performed with decreasing concentrations of mAb1E4 (10nMol, 100pMol, and 1pMol) to select for high binding phage. Following the third round of biopanning fifty phage clones were selected, purified, and sequenced. The results of the sequence analysis identified thirteen different peptide sequences. Although a fifteen mer phage library was used, various peptide sequences had fewer than fifteen amino acid residues. The peptide sequences, including the anti-id 6F9, the frequency of phage clones, and common motifs are shown in Table 2. A common feature to the majority of sequences was the presence of hydrophobic aromatic amino acid residues such as tryptophan (W), tyrosine (Y) and proline (P).

3.2 Phage binding ELISAs.
To confirm the ability of the selected phage clones to bind to mAb1E4, phage clones representing each of the peptide motifs were purified and subjected to reverse ELISA. A phage clone, designated 19-1, known to bind a mAb specific for \textit{S. pneumoniae} was used as a negative control. Of the thirteen phage clones tested, the highest level of binding was demonstrated by Pep1C and Pep2C (Pep1C>Pep2C), shown in Figure 1. All other phage clones demonstrated significantly less binding (data not shown), similar to that of the negative control phage. This assay was repeated three times and gave similar results, thus indicating that phage specific for mAb1E4 were selected during the three rounds of biopanning. The translated peptide sequences of the phage demonstrating the highest degree of binding to mAb1E4, Pep1C and Pep2C, were compared to the sequence of the anti-id 6F9, produced using the same mAb, shown in Table 2. Pep2C shares a common motif comprising the three amino acids YYR with that of 6F9. Pep1C also contains a motif YVP that includes the hydrophobic amino acid tyrosine (Y) common to all peptides.

\textbf{3.3 Anti-MCPS antibody response to peptide immunization.}

To test the ability of Pep1C and Pep2C to elicit an anti-MCPS immune response, peptides were synthesized and complexed to proteosomes. Groups of eight week old, female Balb/c (n=6) mice were immunized on days zero, seven, and twenty-one with 50 or 100 µg peptide-proteosome complex. The positive control consisted of mice immunized with 5 µg MCPS on days 0 and 28, while negative control groups consisted of mice immunized with 50 or 100 µg proteosomes alone on days 0, 7, and 21. Sera were obtained on a weekly basis, and subjected to ELISA to determine the anti-MCPS antibody response. The results of these studies are shown in Figure 2 and
indicate that mice immunized with 50/100 µg Pep2C complexed to proteosomes produced an anti-MCPS IgM antibody response. The antibody response in mice immunized with 50/100 µg Pep2C-proteosome complex was significantly greater than mice immunized with 50/100 µg Pep1C-proteosome (p=.019), 50/100 µg proteosomes alone (negative control) (p=0.010), and MCPS (p=0.038). The antibody titers elicited by Pep2C immunized mice were more than six to twelve fold higher than the controls and Pep1C at day thirty-five. An anti-MCPS IgG antibody response was not detected in any of the groups. These data indicate that Pep2C, but not Pep1C, was capable of inducing an anti-MCPS antibody response.

3.4 Functional activity of post-immune sera – Bactericidal assays.

To determine the functional activity of the peptide induced anti-MCPS antibody response, complement-dependent bactericidal assays were performed on post-immune sera. Bactericidal activity of post-immune sera obtained from mice immunized with 50/100 µg Pep1C or Pep2C-proteosome complexes, positive and negative control mice was determined at day 35. The results of these assays are shown in Figure 3 and indicate that immunization with Pep2C-proteosome complex induced a functional antibody titer significantly higher than mice immunized with Pep1C-proteosome complex (p=0.029), MCPS (p=0.029), or proteosomes alone (p=0.010). Bactericidal antibody titers of mice immunized with 50 µg Pep2C were all >4096, while mice immunized with 100 µg Pep2C demonstrated titers ranging from 1024 to >4096. These bactericidal titers represent at least a four fold increase compared to all other groups. As expected, negative control mice showed very low levels of functional antibodies (<4 to 32), and mice immunized with MCPS had titers ranging from 32 to
128. All Pep1C immunized mice had functional antibody titers less than four. These assays indicate that immunization with Pep2C-proteosome complex results in the induction of antibodies that are bactericidal for \textit{N. meningitidis} serogroup C.

3.5 \textit{Live Challenge of Pep2C immunized mice results in protection.}

To test the ability of Pep2C to protect mice from infection, a live challenge study was performed. Additional groups of mice (n=6) were immunized at days zero, seven, and twenty-one with 50 $\mu$g Pep2C-proteosome complex. Controls consisted of mice immunized with 50 $\mu$g proteosomes alone using the same immunization schedule and mice immunized at days zero and twenty-eight with 5 $\mu$g MCPS. Previous studies performed in our laboratory have shown that mice immunized with MCPS must be boosted one week prior to challenge in order to survive challenge. To enhance the susceptibility to meningococcal infection, mice received 1000 mg/kg iron dextran one week prior to challenge. The mice were challenged i.p. with 10xLD50 of \textit{N. meningitidis} serogroup C strain 35E under sterile conditions. The results of this challenge study demonstrated that 5 out of 6 mice immunized with Pep2C-proteosome complex were protected against meningococcal infection, shown in Figure 4. Mice immunized with the positive control, MCPS, were protected and survived challenge, whereas mice immunized with proteosomes alone were not protected and all succumbed to meningococcal infection within thirty-six hours. These challenge studies indicate that immunization with a peptide that binds to a mAb specific for MCPS protected mice against a lethal challenge with serogroup C meningococcus.
3.6 Specificity of Pep2C.

To further support that binding of Pep2C was specific for MCPS and restricted to mAb1E4 binding, reverse ELISAs were performed using other murine IgM antibodies with different antigen specificities. The results of these assays are shown in Figure 5 and demonstrate that the Pep2C clone specifically bound to mAb1E4. Pep2C failed to significantly bind to the other IgM mAbs specific for various serotypes of *S. pneumoniae* and *N. meningitidis* serogroup A. The ELISAs were repeated and yielded similar results indicating that Pep2C is specific for mAb1E4. Pep2C was not cross-reactive for epitopes located outside of the antigen binding site i.e. those present on mAbs of the same isotype.

4. Discussion

In this study we have identified an immunogenic and protective peptide that was selected using a mAb specific for the capsular polysaccharide of *N. meningitidis* serogroup C. Using the technique of phage display, two peptides that were confirmed to bind to mAb1E4, were synthesized, complexed to proteosomes, and used for immunization in a murine model. The results of the immunization study demonstrated that the Pep2C-proteosome complex was capable of inducing a significant functional anti-MCPS antibody response. The protective nature of the immune response induced by Pep2C was subsequently confirmed by lethal challenge studies. Although many studies have demonstrated that peptides mimicking capsular polysaccharides can induce anti-carbohydrate antibodies (Phalipon et al. 1997, Pincus et al. 1998), the induction of a protective response has been rarely shown. This study
demonstrates the induction of a protective anti-carbohydrate response by a peptide selected for binding to a capsular polysaccharide specific mAb.

The mechanisms responsible for enabling a peptide to mimic a carbohydrate antigen remain to be elucidated. Several studies have postulated that structural similarity to the corresponding carbohydrate epitope is the basis for antigenic mimicry (Evans et al. 1994, Luo et al. 1998), whereas others have proposed antigenic mimicry is based on the antigen-antibody binding site of the anti-carbohydrate antibodies (Harris et al. 1997). Peptides that mimic capsular polysaccharides or bind anti-carbohydrate antibodies have been shown to have similar peptide structures. Sequences of carbohydrate mimics have demonstrated a preference for aromatic, acidic, hydrophobic, and hydrogen-bonding amino acid residues (Phalipon et al. 1997, Pincus et al. 1998, Glee et al. 1999). Hoess et al (Hoess et al. 1993) identified peptide mimics of Lewis Y antigen, and suggested the preference for certain amino acid residues may be attributed to their resemblance to sugar moieties of the carbohydrate epitopes along with a structural role similar to the branched nature of carbohydrates. Pincus et al (Pincus et al. 1998) selected a peptide mimic of group B streptococcal type III capsular polysaccharide (GBS) that contained aromatic amino acid residues, and suggested the presence of these residues to reflect sialic acid present in carbohydrates. Using x-ray crystallography to investigate the molecular interactions involved in antigenic mimicry of a carbohydrate, Luo et al (2000) and others (Oldenburg et al. 1992, Young et al. 1997) suggest the predominant forces involved include hydrogen bonding, stacking of aromatic side chains, and van der Waals forces. Young et al (1997) attribute the affinity of binding between an anti-
carbohydrate antibody and a peptide mimic to the presence of these H-bonds. Several investigators are concentrating their efforts on elucidating the nature of this complicated interaction.

Common motifs found in peptide mimics of carbohydrates have included repeats such as YPY in peptides that mimic Con A (Oldenburg et al. 1992, Scott et al. 1992), W/YXY in a peptide that mimics C. Neoformans (Valadon and Scharff 1996), and PWLY in a peptide mimic of Lewis Y antigen (Hoess et al. 1993). The peptides selected in this study also demonstrate the preference for aromatic residues such as tyrosine (Y) and tryptophan (W) and residues with cyclic side chains such as proline (P). Interestingly, we have previously demonstrated the motif YYR to be present in a peptide mimic, selected from the CDR3 of the anti-id 6F9, of N. meningitidis serogroup C. This YYR motif was shown to be crucial for the immunogenicity of the peptide (Westerink et al. 1995). Using the same selecting mAb, 1E4, to screen a phage display library, we were able to select an immunogenic peptide that contained a similar motif, YYR. Although both peptides share the similar YYR motif and induce protection, they elicit two different immune responses. Immunization with Pep2C resulted in an immune response characterized only by the presence of anti-MCPS IgM antibodies, indicative of a TI response. On the other hand, immunization studies with the 6F9 peptide resulted in a TD response, characterized by the secretion of IgG antibodies. The difference in the immune responses induced by the two peptides may be attributed to additional surrounding amino acid residues, other than YYR, that may be necessary to redirect the immune response from a TI into a TD response. Future studies will help to elucidate whether
the specific YYRP residues are crucial for the immunogenicity of Pep2C. This study provides continuing evidence that supports the suggestion that certain amino acid residues are required for a peptide to mimic a carbohydrate antigen.

Although many peptides that mimic carbohydrate antigens have been isolated, a correlation between mAb affinity, as measured by assay reactivity, antigenic mimicry, and immunogenic mimicry still remains to be addressed. Several investigators have demonstrated effective peptide mimics that induce high antibody titers to the original antigen also demonstrate high affinity binding to the antigen binding site of the isolating mAb, yet others have contradicted this relationship. Studies by Valadon et al (Valadon et al. 1998) using the glucoronoxylonmannan (GXM) portion of *C. neoformans* isolated peptides that bound with high affinity to the selecting mAb and even inhibited the interaction between antigen and antibody, as measured by ELISA, but failed to induce a significant anti-carbohydrate antibody response. Other investigators have demonstrated that high affinity binding to the isolating agent does not necessarily indicate that a peptide will be a good immunogen (Young et al. 1997, Adesida et al. 1999). Conversely, studies by Luo et al (2000) and others (Monafo et al. 1987, Raychaudhuri et al. 1990) investigating peptide and carbohydrate binding imply peptides with low affinity can still induce anti-carbohydrate immune responses.

These studies suggest that peptide affinity as measured by ELISA reactivity, may not be indicative of the antigenic or immunogenic capacity of the peptide. Our results support this postulate, as Pep1C demonstrated the highest binding to mAb1E4, yet failed to elicit an anti-MCPS antibody response. Pep2C, on the other hand,
showed lower reactivity to mAb1E4, yet demonstrated to be an immunogenic mimic by eliciting anti-MCPS antibodies resulting in protection from challenge. This may imply that Pep1C binds to mAb1E4 near or outside the carbohydrate antigen-antibody site, and fails to induce an anti-MCPS antibody response (Adesida et al. 1999). Our data supports the position that high reactivity to the isolating mAb is not sufficient for immunogenic or antigenic mimicry. Further studies, which would allow determination of Pep2C as a true mimic include examining the structural basis of the peptide-antibody and carbohydrate-antibody interactions.

We have shown that producing an anti-id and screening a phage display library with a specific anti-carbohydrate monoclonal antibody, can select for immunogenic peptides that induce anti-capsular polysaccharide antibodies to \(N. meningitidis\) serogroup C. These peptides share motifs common to several other peptides that mimic carbohydrates. Both peptides have been shown to induce the production of functional anti-MCPS antibodies that confer protection from a lethal challenge with meningococcus. This is the first study that compares these two different techniques, using a single mAb, and demonstrates that they can select for similar peptides.

**Acknowledgments**

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REFERENCES


Grothaus, M.C., Srivastava, N., Smithson, S.L., Kieber-Emmons, T., Williams, D.B.,
peptide mimic of the capsular polysaccharide of Neisseria meningitidis
serogroup A using a peptide display library. Vaccine 18, 1253-63.

immunization with a monoclonal anti-idiotope antibody that mimics the
Neisseria gonorrhoeae lipooligosaccharide epitope 2C7. J Infect Dis 174,
1238-48.

Harris, S.L., Craig, L., Mehroke, J.S., Rashed, M., Zwick, M.B., Kenar, K., Toone,
E.J., Greenspan, N., Auzanneau, F.I., Marino-Albernas, J.R., Pinto, B.M. and
Scott, J.K. (1997) Exploring the basis of peptide-carbohydrate crossreactivity:
evidence for discrimination by peptides between closely related anti-

Heath, P.T., Booy, R., Griffiths, H., Clutterbuck, E., Azzopardi, H.J., Slack, M.P.,
immunological risk factors associated with Haemophilus influenzae type b

which binds to the carbohydrate-specific monoclonal antibody B3. Gene 128,
43-9.

meningococcal outbreaks in the United States. An emerging threat. Jama 273,
383-9.


Table I

Eight week old, female Balb/c mice (n=6 per group) were immunized as described. The anti-MCPS specific antibody response was determined weekly by ELISA.

Table II

Fifty phage clones were selected and subjected to sequence analysis. The translated peptide sequence and occurrence in selected clones are shown. Similar moieties between selected peptides and the anti-id 6F9 are shown in bold.

Figure 1.

Binding of selected phage clones to mAb1E4 was tested using reverse ELISA. Serial dilutions of phage clones Pep1C, Pep2C, and an irrelevant control phage, 19-1, were added to plates coated with mAb 1E4 starting with 5x10^{12} TU/well. Absorbance levels were read at 490nm.

Figure 2.

Mice (n=6) were immunized as described in the text and the anti-MCPS antibody response was determined by ELISA. The geometric mean IgM antibody titer of each group is represented by a column, individual mouse titers are denoted by single circles. Some circles may be indicative of several mice in a group. * = Indicates statistical significance (p<0.05)

Figure 3.

Mice (n=6 per group) were immunized as described. Functional activity of post immune sera was determined by serum bactericidal assay. Geomean bactericidal titers for each group are represented by columns, individual mouse titers are denoted by
individual circles. Some circles may be representative of more than one mouse in a group.

* = Indicates statistical significance (p<0.05)

**Figure 4.**

Mice (n=6) were immunized as described and challenged on day 35 with 10xLD50 of *N. meningitidis* serogroup C strain 35E.

**Figure 5.**

Binding of Pep2C phage clone to various murine IgM mAbs varying in antigen specificity. Serial dilutions of the phage Pep2C were added to plates coated with the various mAbs starting with 5x10^{12} TU/well. Absorbance levels were read at 490 nm.

MAPS – Meningococcal serogroup A polysaccharide

PPS – Pneumococcal polysaccharide
Table I. Immunization dose and schedule.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (µg)</th>
<th>Immunizations (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50Pep1C</td>
<td>50µg Pep1C-Proteosomes</td>
<td>0, 7, and 21</td>
</tr>
<tr>
<td>50Pep2C</td>
<td>50µg Pep2C-Proteosomes</td>
<td>0, 7, and 21</td>
</tr>
<tr>
<td>50Proteos</td>
<td>50µg Proteosomes</td>
<td>0, 7, and 21</td>
</tr>
<tr>
<td>100Pep1C</td>
<td>100µg Pep1C-Proteosomes</td>
<td>0, 7, and 21</td>
</tr>
<tr>
<td>100Pep2C</td>
<td>100µg Pep2C-Proteosomes</td>
<td>0, 7, and 21</td>
</tr>
<tr>
<td>100Proteos</td>
<td>100µg Proteosomes</td>
<td>0, 7, and 21</td>
</tr>
<tr>
<td>MCPS</td>
<td>5µg MCPS</td>
<td>0 and 28</td>
</tr>
</tbody>
</table>
Table II. Sequence analysis of peptides selected with the MCPS specific mAb1E4.

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Peptide Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARIYYRYDGFAY</td>
<td>t Anti-id 6F9</td>
</tr>
<tr>
<td>AQAFDYV</td>
<td>PAWVV</td>
</tr>
<tr>
<td></td>
<td>Pep1C 2/50 clones (4%)</td>
</tr>
<tr>
<td>GFSYYR</td>
<td>PPWIL</td>
</tr>
<tr>
<td></td>
<td>Pep2C 2/50 (4%)</td>
</tr>
<tr>
<td>LSPYR</td>
<td>PSSWVLPGTSL</td>
</tr>
<tr>
<td></td>
<td>Pep3C 1/50 (2%)</td>
</tr>
<tr>
<td>AVAYPVSAAYV</td>
<td>PAWVS</td>
</tr>
<tr>
<td></td>
<td>Pep4C 2/50 (4%)</td>
</tr>
<tr>
<td>AVVAVPSYI</td>
<td>PTWIG</td>
</tr>
<tr>
<td></td>
<td>Pep5C 6/50 (12%)</td>
</tr>
<tr>
<td>YHAC</td>
<td>PPWVGIHRCEL</td>
</tr>
<tr>
<td></td>
<td>Pep6C 1/50 (2%)</td>
</tr>
<tr>
<td>VGLGDYGLD FAYVS</td>
<td>PSCRSGL</td>
</tr>
<tr>
<td></td>
<td>Pep7C 15/50 (30%)</td>
</tr>
<tr>
<td>GVMLCPQYF</td>
<td>SVYALPDSLVPWVR</td>
</tr>
<tr>
<td></td>
<td>Pep8C 1/50 (2%)</td>
</tr>
<tr>
<td></td>
<td>VHMIKPPWVRGVLSSG</td>
</tr>
<tr>
<td></td>
<td>Pep9C 3/50 (6%)</td>
</tr>
<tr>
<td></td>
<td>ESFPWVTVVPPPR</td>
</tr>
<tr>
<td></td>
<td>Pep10C 3/50 (6%)</td>
</tr>
<tr>
<td></td>
<td>APGRVPAALIPPWAW</td>
</tr>
<tr>
<td></td>
<td>Pep11C 2/50 (4%)</td>
</tr>
<tr>
<td></td>
<td>GLFVPPWGTSGDL</td>
</tr>
<tr>
<td></td>
<td>Pep12C 2/50 (4%)</td>
</tr>
<tr>
<td></td>
<td>GLFVPPWGTSGDL</td>
</tr>
<tr>
<td></td>
<td>Pep13C 10/50 (20%)</td>
</tr>
</tbody>
</table>
Figure 1.
D. Prinz et al, Fig. 2

Figure 2.
Figure 3.
Figure 4.
Figure 5.

D. Prinz et al, Fig. 5
Induction of a Protective Capsular Polysaccharide Antibody Response to a Multi-
Epitope DNA Vaccine Encoding a Peptide Mimic of MCPS

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Keywords
Neisseria meningitidis, DNA vaccines, Capsular Polysaccharides, Bactericidal Activity, Protective Immunity

Running Headline
A multiple epitope DNA vaccine for N. meningitidis
SUMMARY

Systemic infection by encapsulated organisms, such as *Neisseria meningitidis*, is a major cause of morbidity and mortality worldwide, especially in individuals less than two years of age. Antibodies directed at the capsular polysaccharide are shown to be protective against disease by inducing complement-dependent bactericidal activity. The current polysaccharide vaccine has been shown to be poorly immunogenic in high-risk groups and is likely related to its T-independent properties. An alternative approach to eliciting a T-dependent serum IgG antibody response to encapsulated pathogens is DNA vaccination. We assessed the immunogenicity of a multi-epitope DNA vaccine encoding a T cell helper epitope and a peptide mimic of *Neisseria meningitidis* serogroup C. The DNA construct induced a significant anti-polysaccharide antibody response that was bactericidal. Mice immunized with the DNA construct were subsequently protected against a challenge with a lethal dose of *N. meningitidis* serogroup C.
INTRODUCTION

*Neisseria meningitidis*, a Gram-negative encapsulated bacterium, is a major cause of mortality and morbidity throughout the world (van Deuren et al. 2000). *N. meningitidis* is a leading cause of bacterial meningitis and septicemia in young children, especially those under the age of two (Moore 1992, Rosenstein et al. 1999). Despite the availability of antibiotics and a polysaccharide vaccine, over 350,000 cases of meningococcal disease occur annually throughout the world (Jackson et al. 1995, Raymond et al. 1997). Under optimal conditions of identification and therapy, the mortality rate is at least 10% and morbidity is as high as 30% (2000, Lingappa et al. 2001). Meningococci are classified into serogroups according to the structure of their capsular polysaccharides (Poolman et al. 1982, Frasch et al. 1985, Riedo et al. 1995). To date, 13 serogroups have been identified, however four serogroups, namely A, B, C and Y account for about 90% of the cases (Jackson et al. 1995, 2000).

In the U.S., serogroup C alone is responsible for 45% of the cases of meningococcal meningitis each year (2000). The group C meningococcal polysaccharide vaccine is based on the observation that antibodies directed at the polysaccharide capsule protect against disease by inducing complement-dependent bactericidal activity (Goldschneider et al. 1969, Gotschlich et al. 1969, King et al. 1996, Ball et al. 2001). Although it is currently recommended for controlling meningococcal outbreaks and epidemics, the polysaccharide vaccine has several limitations. First, group C polysaccharide fails to elicit protective levels of antibodies in children less than 2 years old, the age group with the highest incidence, mortality, and morbidity. Second, re-immunization in this age group causes
suppression rather than enhancement of the immune response (Fattom et al. 1999, Borrow et al. 2000a, Nurkka et al. 2000). The poor efficacy and limited duration of the immune response in young children is attributed to the T-independent nature of the polysaccharide vaccine, as the response to these antigens develops late in ontogeny (Timens et al. 1989, Mond et al. 1995, Rijkers et al. 1998, English et al. 2000). A polysaccharide-protein conjugate converts group C polysaccharide from a T-cell independent antigen into an immunogenic and effective vaccine at all ages (Robbins and Schneerson 1990, Twumasi et al. 1995, Leach et al. 1997, English et al. 2000, MacLennan et al. 2001, Richmond et al. 2001). Limitations of conjugates are the requirement of at least 3, and possibly 4 immunizations in infancy to achieve optimal efficacy. In addition, studies have shown that the antibody response to the polysaccharide component of the conjugate continues to possess characteristics of a T-independent immune response (Seppala et al. 1988, Heath et al. 2000, Breukels et al. 2001).

An alternative approach to the development of a T-dependent immune response to a carbohydrate is the use of anti-idiotypic antibodies that mimic the polysaccharide (Westerink et al. 1988, Greenspan and Bona 1993, Harris et al. 1997, Moe et al. 1999). Anti-id antibodies can act as surrogate images of the carbohydrate antigen and can induce a functional, carbohydrate antibody response (Scott and Smith 1990, Gulati et al. 1996). Previously, we have identified an anti-id antibody that mimics the polysaccharide of *N. meningitidis* serogroup C (Westerink et al. 1990). This anti-id elicited a T-dependent anti-polysaccharide antibody response that was protective in animal studies (Westerink et al. 1990, Westerink and Giardina 1992).
Further immunization studies demonstrated that a peptide, spanning the immunogenic CDR3 region of the anti-id antibody, complexed to proteosmes induced protection against a lethal challenge with meningococci (Westerink et al. 1995). These findings illustrate that a peptide mimic of a capsular polysaccharide can induce a T-dependent immune response.

A natural extension of the observation that peptides can mimic polysaccharides is the development of DNA constructs that induce polysaccharide antibodies. DNA immunization is based on the concept that endogenous expression of an encoded peptide by host cell machinery will stimulate an immune response similar to that induced by a pathogen (Donnelly et al. 1997, Robinson 1997, Babiuk 1999). Several studies have demonstrated the potential advantages of DNA immunization (Donnelly and Liu 1998, Leitner et al. 1999). First, DNA vaccines are inexpensive to produce and easily constructed. Secondly, DNA vaccines are thermally stable at high and low temperatures, avoiding the need for cold chain transport. This is essential for delivering the vaccine to areas of endemic disease. Third, DNA vaccines are malleable and can be altered to modulate the magnitude and orientation of the immune response (Cox and Coulter 1997, Cohen et al. 1998, Hasan et al. 1999). This allows the insertion of multiple DNA encoded epitope sequences representing protein as well as carbohydrate antigens into the DNA vector (Whitton 1994, Hanke et al. 1998b, Thomson et al. 1998, Ishioka et al. 1999, Velders et al. 2001). An advantage to this approach is the induction of a multi-specific immune response directed at various immunologically relevant epitopes (Whitton et al. 1993, An and Whitton 1997). The ability to modulate the immune response to specific protein and
carbohydrate epitopes makes DNA vaccines ideal for use against encapsulated organisms, where antibodies can be directed at both the polysaccharide and relevant protein epitopes. Including epitopes that encode peptide mimics of various serogroup polysaccharides, and those encoding conserved cell wall components (i.e. proteins), allows the construction of a multi-epitope DNA vaccine that has the potential of protecting against various meningococcal strains.

In this present study, we constructed a multi-epitope DNA vaccine, encoding a peptide mimic of meningococcal serogroup C capsular polysaccharide (MCPS), a universal T-cell helper epitope, and a secretory leader sequence. Immunization with this DNA construct resulted in a functional anti-MCPS antibody response. Live challenge studies demonstrated the immune response elicited by the DNA construct was protective against a lethal dose of meningococci.
MATERIALS AND METHODS

Capsular Polysaccharides and Bacteria Strains

Meningococcal serogroup C capsular polysaccharide (MCPS) was purchased from the National Institute for Biological Standards and Control (United Kingdom). Meningococcal strains C-11 and C-35E were kind gifts from G.M. Carlone (CDC and prevention, Atlanta, GA), and were used for bactericidal assays and lethal challenge. DNA Construct

The DNA expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA) was used for the construction of our multi-epitope design. Sense and anti-sense oligonucleotides encoding 1) secretory leader sequence derived from the adenovirus E3, MRYMILGLLALAAVCSAA (Persson et al. 1980), 2) a T-cell helper epitope from HIV gp120, KQIINMQAVGKAMYA (Ahlers et al. 1997), 3) T cell helper epitope from HIV gp 120 in tandem with the B-cell epitope (MCPS peptide mimic), KQIINMQAVGKAMYAARIYYRYDGFAY, were synthesized (Integrated DNA Techniques, Coralville, IA). Purified oligonucleotides were resuspended in distilled water to a final concentration of 1nmole/µl. For the P3A DNA construct, the leader sequence oligonucleotide was cloned into the pcDNA3.1 vector at the Hind III and Not I restriction sites. Subsequently, the T- and B-cell epitope encoded oligonucleotide was cloned into the vector between the Not I and Xba I restriction sites. For the P3C DNA construct, the oligonucleotides encoding the secretory leader sequence and T-cell epitope were cloned into the vector as described above. Briefly, the pcDNA3.1 expression vector was double digested at 37°C with the appropriate restriction enzymes. Annealed sense and anti-sense strands of the oligonucleotides
were ligated into the digested vector for sixteen hours at 16°C. Transformation of the ligate was performed in Top 10F’ E. Coli competent cells (Invitrogen, Carlsbad, CA) and plated out on agar plates containing 50 µg/ml ampicillin. Transformed bacterial clones were screened for an insert by PCR using appropriate primers. Positive clones were further screened for correct orientation of the insert by DNA sequencing using a Thermosequenase radiolabeled terminator sequencing kit (Amersham, Cleveland, Ohio). Plasmid DNA for immunizations was prepared using the endofree Qiagen-tip 500 (Maxi) kit (Qiagen Inc., Santa Clarita, CA) according to the manufacturer’s protocol. Endotoxin free PBS was used to resuspend DNA.

**DNA immunization studies**

Eight week old, female BALB/c mice (n=6) were immunized intramuscularly (i.m.) in the anterior tibialis with purified DNA or intraperitoneally (i.p.) with MCPS. Mice were sedated before immunization using a mixture of Ketamine HCl and Xylazine at 80/16 mg/kg. Mice were immunized at days zero, twenty-one, and sixty-three with 100 µg DNA suspended in 100 µl PBS and 40 µg aluminum phosphate gel adjuvant (Superfos Biosector a/s, Vedbaek, Denmark). The test group consisted of mice immunized with the P3A construct containing the leader sequence, T- and B-cell epitopes. Controls were mice immunized with the P3C construct, containing the leader sequence, T-cell epitope, but lacking a B-cell epitope and mice immunized i.p. with 5 µg MCPS at days zero. All mice were tail-vein bled at day 0 (pre-immunization) and at days 28, 42, 56, 70, and 84 post-immunization and sera was screened for anti-MCPS antibodies.
**MCPS ELISA**

MCPS antibodies were measured by ELISA. Briefly, ninety-six well microtiter plates were coated overnight at 37°C with 50 µl/well MCPS and methylated human serum albumin (NIBSC, United Kingdom) at a final concentration of 10 µg/ml. Wells were washed three times in PBS/0.1%Brij, and blocked with 200 µl of blocking buffer (10% heat inactivated calf serum/PBS/0.1%Brij). Serial dilutions of post-immune sera, in blocking buffer, were added to the microtiter wells, and incubated overnight at 4°C with positive and negative control sera present on each plate. Bound antibody was detected after an hour incubation with peroxidase-conjugated anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 antibodies (Southern Biotech, Birmingham, Alabama) in a 1:3000 dilution in blocking buffer, and developed with substrate buffer containing 0.02% o-phenylenediamine. Absorbance levels were read at 490nm with an EIA reader (Bio-Tek instruments). Anti-MCPS antibody titers were calculated as the dilution of serum corresponding to 25% of the maximum optical density obtained with the positive reference serum.

**Serogroup C Bactericidal Assays**

The serum bactericidal assay was performed as previously described (Maslanka et al. 1997). *N. meningitidis* serogroup C strain C-11 was grown overnight at 37°C in 5% carbon dioxide on brain heart infusion agar supplemented with 1% heat inactivated horse serum (BHIA/S). A working bacterial seed lot was prepared by resuspending overnight colonies in Dulbecco’s phosphate buffered saline and 0.1% glucose, to yield a final concentration of $2 \times 10^4$ CFUs/ml. Dilutions of heat inactivated post-immune sera, ranging from 1:8 to 1:4096, were added to a ninety-six
well tissue culture plate. An equivalent cell: complement mixture (baby rabbit serum – Pelfreeze, Brown, Deer, WI) was added to all wells of the tissue culture plate, excluding the control wells, and incubated at 37°C. Controls included wells without post-immune sera, wells without a complement source, and wells with positive control sera. At times zero and sixty, aliquots from the wells were plated out on BHIA/S plates using the agar tilt method and incubated overnight at 37°C with carbon dioxide. The bactericidal titer was reported as the lowest serum dilution yielding >50% killing compared to time zero.

**Challenge Experiments**

Additional groups of mice (n=6) were immunized with DNA or MCPS as shown in Table 1. Mice were challenged at day 56 with *N. meningitidis* serogroup C, strain 35E, using a murine model for meningococcal infection (Calver et al. 1976, Salit and Tomalty 1984). Seven days prior to challenge, mice received 1000 mg/kg iron dextran i.p. to enhance susceptibility to the bacteria. The lethal dose killing fifty percent (LD 50) was determined in iron loaded naïve mice challenged with 10^3-10^5 cfu of meningococci. Immunized iron treated mice were challenged i.p. with 10×LD50 under sterile conditions with strain 35E. Ninety-six hours post challenge, survivors were euthanized and sera were collected for analysis.

**Statistical Analysis**

Analysis of pre- and post-immune sera antibody and bactericidal titers was performed by non parametric Mann-Whitney U test (SPSS, Chicago, IL). Statistical analysis on post challenge sera was performed by Fisher’s exact test (SPSS). A p<0.05 was considered statistically significant.
RESULTS

Construction of DNA vaccines

For the purpose of our study, a secretory leader sequence from Adenovirus E3 was included into the DNA construct to increase exogenous expression of our B- and T-cell encoded peptides. The T-cell helper epitope from HIV gp120 is considered a “universal” T-cell epitope due to the lack of HLA restriction and its ability to bind to both MHC class I and II molecules (Ahlers et al. 1997).

Oligonucleotides encoding the leader sequence, T-cell helper epitope, and the peptide mimic of MCPS, B-cell epitope, were cloned into the appropriate restriction sites of the pcDNA3.1 expression vector. Bacterial colonies containing inserted oligonucleotides were identified using PCR. To ensure the oligonucleotides were inserted in the correct orientation and reading frame, clones selected positive by PCR were sequenced. A plasmid vector that demonstrated correct orientation and insertion was selected for further studies. Plasmid DNA for immunizations was prepared and resuspended in endotoxin-free PBS.

MCPS antibody response to DNA immunization

Groups of BALB/c mice (n=6) were immunized on days zero, twenty-one, and sixty-three with 100 µg of the indicated DNA construct with aluminum phosphate gel adjuvant. The positive vaccine control consisted of mice immunized at day zero with 5 µg MCPS. Sera were obtained and an anti-MCPS antibody response determined by ELISA throughout the course of the study. The results of these studies are shown in figure 1 and indicate that mice immunized with P3A DNA produced an anti-MCPS IgM antibody response that was significantly higher (p<0.05) than mice
immunized with P3C DNA (negative control). The increase in antibody levels after subsequent boosts in the negative group are attributed to the administration of adjuvant, which enhances non-specific immune responses. The antibody response in the P3A DNA immunized mice was higher than the MCPS immunized group (positive control), but this difference did not reach statistical significance. An anti-MCPS IgG antibody response was not detected in the P3A or P3C DNA groups despite the presence of a T-cell epitope. A minimal anti-MCPS IgG3 antibody response (titers from 5-30) was detected in two MCPS immunized mice at day 56 but the results were not statistically significant (data not shown). The immunization study was repeated three times, yielding similar antibody titer results.

**Bactericidal antibodies**

To determine the functional activity of post-immune sera, bactericidal assays were performed. Immunization with the P3A DNA construct induced significantly greater bactericidal titers at day 56 than mice immunized with P3C DNA (p=0.002) (Figure 2). Bactericidal titers for mice immunized with P3A DNA were calculated and ranged from 8 to 256, whereas all mice immunized with P3C DNA had titers less than four. Mice immunized with MCPS as positive controls also had significantly higher bactericidal titers (32-128) than the negative control (p=0.005), but not significantly different from levels elicited by mice immunized with P3A DNA. These data indicate that immunization with P3A DNA induces antibodies that are functional and bactericidal against *N. meningitidis* serogroup C.

**Protective efficacy of DNA immunized mice**
A challenge experiment was performed to further assess the functional activity of the immune response induced in mice by immunization with DNA. Groups of mice were immunized as described. Mice immunized with MCPS were boosted with 5µg MCPS seven days prior to challenge, as mice immunized with polysaccharide at day 0 only, succumbed to infection. Mice received 1000 mg/kg iron dextran one week prior to challenge to enhance susceptibility to meningococcal infections, and were subsequently challenged with 10×LD50 dose of *N. meningitidis* serogroup C strain 35E. The results of these studies demonstrate that mice immunized with P3A DNA and MCPS were protected against meningococcal infection, with 100% survival. Five of the six mice immunized with P3C DNA (negative control) died within twenty-four hours post-challenge. This challenge was repeated and yielded similar results.

**Antibody Isotype Profiles Post-Challenge**

To evaluate the anti-MCPS antibody levels post-challenge among survivors in both P3A and P3C DNA groups, sera were collected ninety-six hours after challenge. ELISAs were performed to measure the antibody isotypes present in the collected sera. The results of these assays demonstrate no significant difference in anti-MCPS IgM antibody levels between the survivors immunized with P3A DNA, MCPS and the one survivor immunized with P3C DNA (data not shown). Prior to challenge, IgG levels were not detectable in P3A DNA immunized mice, whereas post-challenge anti-MCPS IgG antibodies were detected in all sera. ELISAs evaluating the different IgG antibody isotypes in sera, indicate high levels of IgG1 and IgG3 with moderate levels of IgG2a and IgG2b antibodies in mice immunized with P3A DNA (Figure 3a).
The survivor immunized with P3C DNA had no detectable IgG1, IgG2a and IgG2b, with a low level of IgG3 antibody (Figure 3b). Two mice immunized with MCPS demonstrated low levels of IgG3 antibodies pre-challenge, whereas the presence of IgG1, IgG2b, and IgG3 antibodies were detected post-challenge in the majority of the sera (Figure 3c). No anti-MCPS IgG2a antibodies were detected in survivors immunized with MCPS. Overall, the anti-MCPS IgG1, IgG2a, IgG2b, and IgG3 antibody titers were significantly greater in mice immunized with P3A DNA compared to the survivor immunized with P3C DNA (p=0.003, 0.007, 0.007, and 0.014 respectively). In addition post-challenge anti-MCPS IgG antibody titers, of all isotypes tested, were also significantly greater in mice immunized with P3A DNA compared to those immunized with MCPS (p=0.026, 0.030, 0.0001, 0.003 respectively).
DISCUSSION

Capsular polysaccharides induce a T-independent immune response characterized primarily by the presence of IgM anti-polysaccharide antibodies, lack of isotype switching and a memory response, and are poorly immunogenic in the very young (Howard 1987, Stein 1992). Alternative approaches to eliciting a T-dependent response to carbohydrate antigens consist of conjugate vaccines, anti-idiotope vaccines, and potentially DNA vectors that encode peptide mimics of the polysaccharide capsule. A peptide based DNA immunization targeted to carbohydrate antigens has distinct advantages. First, peptide encoded DNA vaccines have the ability to induce carbohydrate cross-reactive humoral and cellular immune responses (Monzavi-Karbassi et al. 2001a). Secondly, DNA vaccines can redirect the immune response to a Th1 response characterized by the presence of IgG2a in mice (Kieber-Emmons et al. 2000). IgG2a is a complement fixing and opsonizing antibody crucial for preventing infection by a bacterial pathogen. It has previously been noted that the redirection of a Th2 to a Th1 immune response induced by a peptide-based DNA vaccine may help to overcome the immune tolerance to polysaccharide antigens seen in neonates (Monzavi-Karbassi et al. 2001a). Finally, several peptide mimics from different capsular polysaccharides may be incorporated into a single DNA vector in order to elicit anti-polysaccharide antibodies targeted to other meningococcal serogroups.

In a multi-epitope DNA construct several factors must be taken into consideration with design. First, B-cell epitopes must assume and be presented in correct conformation in order to adopt the shape of the nominal antigen. Otherwise,
peptide epitopes may induce antibodies that do not recognize the bacterial antigen. Secondly, short peptide sequences are unstable and poorly immunogenic in vivo. T-cell help is required to induce an optimal immune response. The polymorphism of HLA molecules, to which peptides bind, affects the magnitude and direction of the T-cell help provided. Therefore, an “universal” T helper epitope should be included in the multi-epitope construct, eliminating HLA restriction. Lastly, linking multiple epitopes in a single construct may form deleterious flanking sequences from neighboring epitopes potentially suppressing the presentation of the target epitopes (Eisenlohr et al. 1992, Toes et al. 1997, Hanke et al. 1998a, Yoshida et al. 2001). This includes determinants created at the junction between two neighboring epitopes, when linking peptides in tandem (Bergmann et al. 1994). Several studies present conflicting results concerning the role of neighboring sequences on the efficiency of expression (Del Val et al. 1991, Thomson et al. 1996).

We have developed a multiple epitope DNA vaccine encoding a “universal” T-cell helper epitope and a peptide that mimics the polysaccharide capsule of *N. meningitidis* serogroup C. This construct was designed to increase exogenous expression of our peptide mimic and to simultaneously induce T-cell help, thus stimulating both a humoral and cellular immune response. The leader sequence assists in the targeting and transport of the B-cell epitope into the endoplasmic reticulum (ER) thereby enhancing translation and endogenous expression of the peptide (Ciernik et al. 1996). Likewise, a T-cell helper epitope provides sufficient T-cell help to antibody producing B-cells. By inserting a “universal” T-cell epitope,
HLA restriction is avoided and MHC class I and II molecules are targeted with the same affinity (Panini-Bordignon et al. 1989, Joshi et al. 2001).

In this study we have shown that immunization with a DNA construct encoding a carbohydrate peptide mimic and a “universal” T-cell helper epitope induces protective levels of anti-MCPS antibodies. The feasibility of inducing polysaccharide antibody responses through DNA immunization has been reported in several studies (Lesinski et al. 2001, Monzavi-Karbassi et al. 2001b), but the functional activity of the antibody response was not characterized. Primary and booster immunizations with the P3A DNA construct elicited anti-MCPS antibodies with bactericidal activity. Mice immunized with P3C DNA produced significantly lower antibody titers that lacked detectable functional activity. Studies by Maslanka et al (Maslanka et al. 1998) and others (Borrow et al. 2000b) have demonstrated that standard serogroup C ELISAs measures both low avidity non-functional antibodies as well as high avidity antibodies that correlate with protection. In addition, a relationship has been shown by Goldschneider et al (Goldschneider et al. 1969) between the presence of bactericidal antibodies and levels of protection against infection. It is likely that the P3C DNA vector induced low avidity, non-specific and consequently non-bactericidal antibodies that cross-react with MCPS. Therefore, even though moderate levels of anti-MCPS antibodies were elicited by P3C DNA mice, the bactericidal data that measured high avidity, functional anti-MCPS antibodies, was a more accurate measurement of future protection.

Subsequently following a challenge with 10xLD50 dose of live meningococci, mice immunized with the P3A DNA construct and MCPS survived. In contrast one
out of six P3C immunized mice (negative control) survived lethal challenge. A possible explanation for survival of this mouse is that meningococci tend to aggregate in solution. Despite our attempts to standardize the challenge procedure and dose, it is likely that the surviving mouse in the negative control group did not receive the 10xLD50 dose of meningococci. This suspicion is supported by the finding that six hours post-challenge the mouse was not bacteremic, whereas bacteria was detected in blood of P3A and MCPS immunized mice.

Pre-challenge antibody studies showed a predominant anti-MCPS IgM antibody response, indicating the stimulation of a T-independent response. However, at ninety-six hours post-challenge, the anti-MCPS IgG1, IgG2a, IgG2b, and IgG3 antibody titers were significantly greater in P3A DNA immunized mice compared to the survivors in both control groups. The absence of anti-MCPS IgG antibodies in P3A DNA mice and low levels of IgG3 antibodies in MCPS mice pre-challenge suggests that IgM antibodies were protective against challenge, and that anti-MCPS IgG antibodies were not required for survival. The unexpected presence of various IgG isotypes in post challenge sera from mice immunized with DNA indicates the induction of a mixed Th1 and Th2 immune response. The presence of anti-MCPS IgG2a antibodies was only seen in mice immunized with the multi-epitope DNA construct. The data also indicate that immunization with DNA resulted in a greater memory response in P3A DNA mice than mice immunized with MCPS due to the presence of greater anti-MCPS IgG levels post-challenge. These results support previous observations by other investigators that DNA immunization can redirect the
immune response from a Th2 response, primarily seen with polysaccharide vaccines, to a more desirable Th1 immune response.

In a T-independent immune response carbohydrate antigens stimulate B cells by crosslinking surface IgM, leading to the production of antigen-directed antibodies. These antibodies are of relatively low affinity to antigen with limited evidence for somatic hypermutation. T helper cells support the differentiation and proliferation of B cells into plasma or memory B cells in a T-dependent immune response. This is characterized by somatic hypermutation, and isotype switching from IgM to IgG or IgA antibodies. The immune response induced by our DNA construct was uniquely different from a “true” T-independent or T-dependent response. Prior to challenge, characteristics of a T-independent response were detected, whereas post-challenge a T-dependent like response was observed with the presence of high levels of IgG and evidence of a memory response. Previous studies using carbohydrate antigens have described the evidence of T cell responses in a predominantly characterized T-independent response to the polysaccharide (Boswell and Stein 1996, Sverremark and Fernandez 1998, Lucas and Reason 1999). Studies from other investigators indicate that immunization with DNA encoding carbohydrate mimotopes could result in a T-dependent response with isotype switching (Kieber-Emmons et al. 2000). In addition, cellular response studies of carbohydrate mimicking peptides indicate that mimotopes can activate T cell subsets of Th1 and Th2 phenotypes (Cunto-Amesty et al. 2001a, Cunto-Amesty et al. 2001b, Monzavi-Karbassi et al. 2001a, Monzavi-Karbassi et al. 2001b). Our results imply that B cell memory and B cell activation can occur without standard effector responses normally seen in a T-dependent immune response. This
was also recently demonstrated by Laylor et al (Laylor et al. 1999), where DNA vaccination resulted in a significant B cell memory response in the absence of an overt effector response. Similarly, Klinman (Klinman 1997) reported that, in limiting dilution fragment cultures, memory B-cell clones arise without detectable primary antibody production.

Previous immunization studies in our laboratory using the MCPS peptide mimic complexed to proteosomes, resulted in a T-dependent immune response. However, the response to the same peptide, when encoded in a DNA vector, was markedly different. The results from our present study indicate that T cell help is not adequately being provided prior to challenge. This may be attributed to the design and efficacy of the multi-epitope DNA construct. First, the T cell epitope included in the vector may not be effectively targeting T cell help. A T helper epitope from *N. meningitidis*, may be more suitable for stimulating T helper cells and improving the immune response. Secondly, specific flanking sequences may be required to allow optimal expression of the T cell epitope. These considerations will be addressed in the near future in order to optimize our DNA vector design.

We have shown that DNA immunization with a peptide mimic of MCPS results in a strong B-cell memory response in the absence of a strong effector response. We are currently expanding these studies and performing in depth analysis of the nature of the immune response to DNA vaccines expressing carbohydrate peptide mimics. Our future results may influence the decisions regarding appropriate methods used for evaluating the efficacy of DNA vaccines.
ACKNOWLEDGMENTS

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REFERENCES


15 Nurkka, A, MacLennan, J, Jantti, V, Obaro, S, Greenwood, B, Kayhty, H. Salivary antibody response to vaccination with meningococcal A/C

16 Fattom, A, Cho, YH, Chu, C, Fuller, S, Fries, L, Naso, R. Epitopic overload at the site of injection may result in suppression of the immune response to combined capsular polysaccharide conjugate vaccines. Vaccine 1999;17:126-133.


36 Westerink, MAJ, Giardina, PC, Campagnari, AA, Apicella, MA. The thymus dependent nature of the murine antibody response to a monoclonal anti-
idiotypic antibody to the *Neisseria meningitidis* serogroup C capsular polysaccharide. Microb Pathog 1990;8:411-419.


40 Babiuk, L. Broadening the approaches to developing more effective vaccines. Vaccine 1999;17:1587-1595.


55 Ahlers, JD, Takeshita, T, Pendelton, D, Berzofsky, J.A. Enhance immunogenicity of HIV-1 vaccine construct by modification of the native peptide sequence. Proc Natl Acad Sci 1997;94:10856-10861.


72 Joshi, SK, Suresh, PR, Chauhan, VS. Flexibility in MHC and TCR recognition: degenerate specificity at the T cell level in the recognition of promiscuous Th epitopes exhibiting no primary sequence homology. J Immunol 2001;166:6693-6703.


**Figure 1.** Mice (n=6) were immunized with 100 µg DNA and 40 µg AP on days 0, 21, and 63. Positive controls were immunized with 5 µg MCPS on days 0. The anti-MCPS antibody response was determined by ELISA. The geometric mean IgM antibody titer of each group is represented by a column.

* = Indicates statistical significance of groups compared to negative control (p<0.05)

**Figure 2.** Functional activity in post immune sera was determined by serum bactericidal assays at day 56. Geometric mean bactericidal titers for each group are represented by columns; individual mouse titers are denoted by circles. Some circles may be representative of more than one mouse in a group.

* = Indicates statistical significance of groups compared to negative control (p<0.05)

**Figure 3 a, b and c.** Mice immunized with DNA and MCPS were challenged with a lethal dose of *N. meningitidis* serogroup C strain 35E. Ninety-six hours post-challenge sera were collected from survivors and anti-MCPS IgG1, IgG2a, IgG2b, and IgG3 determined by ELISA. Geometric mean pre- and post-challenge antibody titers for each isotype are represented by columns; individual mouse titers are denoted by circles. Some circles may be representative of more than one mouse.

* = Indicates statistical significance of test group compared to controls (p<0.05)
Figure 1.
Figure 2.
Figure 3c.

Reciprocal titer

MCPS IgG Isotypes
Brief Report

A DNA Construct Encoding a Peptide Mimic of MCPS Favors a Th1 Response

in the absence of an IgG effector response

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Running Headline

DNA vaccination favors a Th1 immune response

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Abstract

Previous studies have shown that immunizations with a DNA construct encoding a T cell epitope and a peptide mimic of meningococcal serogroup C capsular polysaccharide (MCPS) resulted in a protective immune response in the absence of a specific anti-MCPS IgG response. In this study, we have analyzed specific cytokine expression using real-time RT-PCR and demonstrated that a Th1 immune response was induced by DNA vaccination. These studies suggest that despite the lack of a strong effector response post-immunization, the DNA construct induced a T-dependent immune response, characterized by the presence of IL-2 and IFN-γ.

Keywords

*Neisseria meningitidis*, DNA vaccines, Capsular Polysaccharides, Cytokines, Th1/Th2 immune responses, Immunologic memory
**Introduction**

*Neisseria meningitidis* serogroup C is a major cause of mortality and morbidity worldwide. In the United States alone, this pathogen is responsible for 45% of all meningococcal meningitis cases occurring each year (2000). Presently available vaccines for meningococcal serogroup C disease include the capsular polysaccharide vaccine. The polysaccharide vaccine is poorly immunogenic in children less than two years of age due to its T-independent nature (Howard 1987, King et al. 1996). In order to overcome the poor immunogenicity associated with the group C polysaccharide, alternative strategies such as polysaccharide-protein conjugate vaccines have been developed. These vaccines have been demonstrated to induce a T-dependent immune response, characterized by immunologic memory (English et al. 2000, Richmond et al. 2001).

An alternative approach to eliciting a T-dependent immune response to encapsulated antigens such as *N. meningitidis* serogroup C is through the administration of DNA constructs. Several studies have shown that DNA vaccines can induce T-dependent immune responses directed at a variety of infectious agents and induce immunologic memory (Kieber-Emmons et al. 2000, He et al. 2001). DNA vector construction is highly malleable and easily altered to modify the magnitude and orientation of the desired immune response (Cohen et al. 1998). This advantage allows for the insertion of multiple epitope sequences representing proteins, as well as peptide mimics of carbohydrate antigens (Velders et al. 2001). The ability to direct the immune response to specific protein and carbohydrate
epitopes makes a DNA vaccine an ideal method for inducing a T-dependent immune response to encapsulated organisms.

We have previously demonstrated that immunization with a multi-epitope DNA construct encoding a universal T cell epitope and a peptide mimic of meningococcal C polysaccharide (MCPS) induced a functional anti-MCPS IgM antibody response in mice, as detected by ELISA and serum bactericidal assays (Prinz et al. 2003). Immunized mice survived lethal challenge with \textit{N. meningitidis} serogroup C. Furthermore, forty-eight hours post-challenge, sera contained high anti-MCPS IgG1 and IgG2 antibody titers indicative of a mixed Th1 and Th2 response. These data suggested that immunologic memory was present in the absence of the standard effector responses normally seen in a T-dependent immune response. In the present study, cytokine profiles characteristic of both Th1 and Th2 responses were investigated in mice immunized with either a multi-epitope DNA construct, MCPS, or the meningococcal group C conjugate vaccine in order to examine the nature of the induced anti-MCPS immune response.

**Materials and Methods**

Eight week old, female Balb/c mice (n=6) were immunized as previously described (Prinz et al. 2003). Groups consisted of mice immunized with: 1) DNA construct encoding a T cell epitope and a peptide mimic of MCPS (designated P3A), 2) DNA construct encoding the T cell epitope without the B cell epitope (negative control, designated P3C), 3) MCPS (National Institute for Biological Standards and Control, UK), and 4) meningococcal C conjugate vaccine (Chiron, Emeryville, CA), as a T-dependent positive control. Mice were immunized as outlined in Table 1.
Tail-vein bleeds were performed at week 2 and 4. ELISAs were used to test sera for the presence of anti-MCPS antibodies.

Mice were sacrificed at week five and spleens removed. Splenocytes were isolated, resuspended in tissue culture media supplemented with heat inactivated fetal bovine serum, and coated on a 12-well microtitre plate at a final concentration of $1.0 \times 10^6$ cells/well. Cells were stimulated with 20µg MCPS or a crude mixture of heat-inactivated *N. meningitidis* serogroup C (HICM), strain 35E, and harvested forty-eight hours later. RNA was isolated using Trizol reagent (Gibco Life Technologies, Gaithersburg, MD), and reverse-transcribed using Supercrypt™ first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA)).

Levels of cytokine expression in mRNA were analyzed using quantitative real-time RT-PCR. Selected primers were designed specific for IL-2 (FW-5’-CCTGAGCAGGATGGAGAATTACA-3’ and RV-5’TCCAGAACATGCCGCAGAG-3’), for IL-4 (FW-5’-ACAGGAAGGGACGCGCCAT-3’ and RV-5’-GAAGCCCTACAGACGAGCTCA-3’), for IFN-γ (FW-5’TCAAGTCATCATGATGGAAGAA-3’ and RV-5’TGGCTCTCGAGGATTTTCATG-3’), and Beta-actin (FW-5’AGAGGGAAATCGTGCAGAC-3’ and RV-5’CAATAGTGATGACCTGCGCCGT-3’).

PCR amplifications were performed in a total volume of 10µl containing 1.0µl cDNA (concentrations 0.25ng, 1ng, 4ng, or 8ng total cDNA), 1µl 2mM dNTPs, 0.2µl 10µM forward primer, 10µM reverse primer, 0.5µl SYBR green I (diluted 1:1000) (Molecular Probes, Eugene, OR), 1µl 10X PCR Buffer, 0.1µl Platinum Taq DNA...
polymerase (Invitrogen). Each PCR amplification was performed in duplicate using the Roche Lightcycler (Roche Diagnostics, Indianapolis, IN). Denaturation of the template occurred at 95°C for 10 min, followed by a total of 40 cycles of amplification and quantification at 95°C for 15s, 60°C for 10s, and 72°C for 6s, and finally cooling to 40°C. The relative quantification data of murine cytokine expression in comparison to a reference gene was generated on the basis of a mathematical model for relative quantification in real-time RT-PCR as described by Pfaffl et al (Pfaffl 2001). Beta-actin was used as the reference gene transcript, and levels of IL-2, IL-4 and IFN-γ were determined as the fold difference of expression in cells stimulated with MCPS or HICM versus baseline expression (calculated from unstimulated cells).

Statistical analysis on intra-assay (duplicate repeats within one amplification run) and inter-assay (different experimental runs) was performed using the non-parametric Mann-Whitney U test (SPSS).

Results

Immunization of mice with P3A, MCPS, and the conjugate vaccine elicited anti-MCPS IgM antibodies as detected by ELISA, comparable to IgM levels detected in all previous studies (Prinz et al. 2003). Mice immunized with P3C demonstrated significantly lower anti-MCPS IgM antibody titers than those immunized with P3A (p=0.05), data not shown. Anti-MCPS IgG antibodies were only detected in mice immunized with the conjugate vaccine.

The levels of cytokine mRNA expression in immunized mouse spleen cells stimulated with MCPS or heat inactivated serogroup C meningococci (HICM) are
shown in figure 1. Stronger levels of IL-2 expression were observed in mice immunized with P3A and the conjugate vaccine following both MCPS and HICM stimulation (fold differences from baseline for MCPS 25.15 and 5.735, HICM 30.3 and 11.7 respectively). Mice immunized with MCPS demonstrated lower expression of IL-2 compared to P3A and conjugate immunized mice (MCPS 1.0, HICM 1.5), whereas P3C immunized mice had no detectable expression of IL-2 to HICM stimuli with weak levels of IL-2 to MCPS stimuli (2.12).

IL-4 mRNA expression to MCPS and HICM stimuli was primarily observed in conjugate and MCPS immunized mice (fold differences from baseline for MCPS 30.36 and 31.3, HICM 62.3 and 3.552 respectively). Mice immunized with P3A demonstrated lower expression of IL-4 than conjugate and MCPS immunized mice to MCPS stimuli (MCPS 5.848). Stronger levels of IL-4 were seen to HICM stimulus in P3A mice in comparison to MCPS immunized mice (HICM 4.703). No IL-4 mRNA expression was detected in mice immunized with P3C to either MCPS or HICM stimuli.

Lastly, IFN-γ mRNA expression was only detected in mice immunized with P3A and the conjugate vaccine (MCPS 5.68 and 0.499, HICM 9.87 and 1.483). Expression of IFN-γ was not detected for either stimuli in MCPS or P3C immunized mice.

Discussion

Previously, we designed a multi-epitope DNA construct that induced a functional anti-MCPS antibody response in mice (Prinz et al. 2003). The immune
response, although protective in nature, was characterized by anti-MCPS IgM antibodies alone, with no detectable anti-MCPS IgG. These results were indicative of a T-independent immune response. However, challenge with live group C meningococci induced high levels of IgG in DNA immunized mice within forty-eight hours post-challenge, suggesting the formation of memory B cells despite the absence of an IgG effector response prior to challenge.

In the current study we wished to clarify the nature of the DNA induced anti-MCPS immune response by examining the Th1/Th2 cytokine profile produced by spleen cells from previously immunized mice, stimulated with heat-inactivated N. meningitidis serogroup C or MCPS. The presence of cytokines IL-2 and IFN-γ were studied as representatives of a Th1 response, and IL-4 was used as a Th2 indicator. Based on analysis of the cytokine profiles, we have shown that immunization with a DNA construct encoding a carbohydrate peptide mimic induces predominantly a Th1 immune response. This was supported by the detection of significant levels of IL-2 and IFN-γ cytokine expression. In contrast, mice immunized with the conjugate vaccine demonstrated a mixed Th1/Th2 response to MCPS and heat-inactivated whole organism, with IL-2 and IFN-γ expression approximately equivalent to IL-4 levels. Mice immunized with MCPS primarily generated a Th2 immune response with higher levels of IL-4 in comparison to IL-2, and lack of IFN-γ expression. Lastly, mice immunized with the negative control, P3C, showed slight upregulation of IL-2 expression to MCPS stimuli, but IL-4 or IFN-γ expression was not detected in response to either stimulator. These data suggest the immune response to the P3A DNA construct shares characteristics of a T-dependent immune response and induces
a Th1 cytokine profile in contrast to the conjugate vaccine that induces a mixed Th1/Th2 response.

Unlike protein antigens, the exact mechanism of immune stimulation and generation of memory by DNA vaccination remains unclear. Several studies by Laylor et al. and others (Laylor et al. 1999, Kieber-Emmons et al. 2000, Cazeaux et al. 2002) have likewise demonstrated that intramuscular DNA vaccination promoted memory formation without an overt effector immune response, characteristically seen following immunization with a protein antigen. The magnitude and direction of the immune response induced by DNA vaccination can be affected by many factors such as method and site of inoculation, use of adjuvant, transfection efficiency and antigen presentation. Intramuscular immunization with DNA has been demonstrated to result in prolonged expression of antigen at the site of inoculation. It is suggested that formation of memory by this route may be due to a high degree of “pure priming” with DNA (Corr et al. 1999). The presence of a persistent antigen source could serve to stimulate memory cells. Low levels of secreted antigen do not necessarily result in an overt effector immune response, but would select mainly for B cells producing high rather than low avidity antibodies. These specific B cells may account for the long-lived immune responses and immunologic memory that has been demonstrated after DNA immunization.

Previously, we have demonstrated that a DNA construct encoding a carbohydrate peptide mimic induces an anti-polysaccharide antibody response that initially appears T-independent in nature. However, upon further examination of T cell activation, it is apparent the immune response is T-dependent with a strong Th1
component. This implies that B cell stimulation, specifically by peptide antigens, may occur without standard effector responses normally seen in a T-dependent response induced by protein antigens. These results may influence the way we analyze the efficacy of an immune response induced by DNA immunization.
References


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### Table 1. D Prinz et al

<table>
<thead>
<tr>
<th>Groups (n=6)</th>
<th>Dose</th>
<th>Immunization Schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>P3A</td>
<td>100µg DNA and 40µg AP</td>
<td>W0, 3</td>
</tr>
<tr>
<td>P3C</td>
<td>100µg DNA and 40µg AP</td>
<td>W0, 3</td>
</tr>
<tr>
<td>Conj</td>
<td>2µg Ag and 0.2mg AH</td>
<td>W0, 3</td>
</tr>
<tr>
<td>MCPS</td>
<td>5µg MCPS</td>
<td>W0</td>
</tr>
</tbody>
</table>

Immunization schedule.

Note: AP = Aluminum phosphate gel adjuvant (Superfos Biosector a/s), MCPS = Meningococcal serogroup C polysaccharide, Ag = antigen, AH = Aluminum hydroxide gel adjuvant (Superfos Biosector a/s)
Figure 1 D. Prinz et al

A.

B.
Cytokine analysis using quantitative real-time RT-PCR to determine Th1/Th2 phenotype. The relative mRNA expression of IL-2 (A), IL-4 (B), and IFN-\(\gamma\) (C) in mouse spleen cells stimulated with MCPS or heat-inactivated *N. meningitidis* serogroup C (HICM) was calculated as the fold difference of expression versus baseline (unstimulated cells). The expression of the various cytokines was normalized to the expression of Beta-actin. Data shown is relative mRNA expression in 1 ng/ul cDNA input.
DISCUSSION/SUMMARY

T-dependent immune responses to carbohydrate antigens have been successfully induced by peptides that mimic capsular polysaccharides. Peptides that mimic carbohydrate antigens can be selected using either anti-idiotypic antibodies or phage display libraries. The mechanisms that enable a peptide to elicit anti-carbohydrate immune responses remain to be elucidated, but is suggested that this is based on the peptides inherent chemical structure. Important for vaccine development to carbohydrate antigens, peptides are recognized and presented by MHC molecules to B and T cells, thus eliciting a desired T-dependent immune response. Recently, several investigators have identified peptide mimics capable of eliciting protective anti-carbohydrate antibody responses, demonstrating that this technology may be used for vaccine development against encapsulated organisms.

We hypothesized that peptides that mimic the capsular polysaccharide of *N. meningitidis* serogroup C (MCPS) can be selected using various methods. The selection of an immunogenic peptide of MCPS has been described in Manuscript 1 (Prinz, et al. 2004). Previously our laboratory has identified a functional and protective peptide mimic of MCPS through the production of an anti-idiotypic antibody, designated 6F9 (Westerink, et al. 1990, 1995; Westerink and Giardina 1992). The anti-MCPS monoclonal antibody 1E4 (mAb 1E4) used to produce the anti-id 6F9 was subsequently used in Manuscript 1 to screen a phage display library to select for peptides that bound to the mAb 1E4 and could potentially mimic MCPS. The biopanning process used resulted in the selection of ten different peptide motifs that bound to mAb 1E4. Based on reverse ELISA binding studies, we selected two
peptides, designated as Pep1C and Pep2C, for further studies. Binding of the peptides to the selecting mAb or ligand does not ensure that these peptides are either immunogenic or protective. Therefore, immunizations with the peptides complexed to outer membrane proteins, or proteosomes, were performed to test the ability of Pep1C and Pep2C to induce anti-MCPS immune responses. The results of the immunization study demonstrated that the Pep2C-proteosome complex, but not the Pep1C-proteosome complex, was capable of inducing an anti-MCPS IgM antibody response significantly greater than positive (MCPS immunized) and negative (proteosomes alone) control antigens. An anti-MCPS IgG antibody response was not detected, suggesting the immune response to the Pep2C-proteosome complex was T-independent in nature.

To determine the functional activity of the peptide induced anti-MCPS IgM antibody response, complement-dependent bactericidal assays were performed on post-immune sera. The results indicated the Pep2C-proteosome complex was capable of inducing functional antibody titers significantly greater than titers induced in all other immunized groups, including positive controls. To further assess the functionality of the anti-MCPS antibodies a lethal challenge was conducted (10xLD50 serogroup C dose). The results confirmed that the anti-MCPS immune response induced by Pep2C-proteosomes protected against lethal infection with meningococci. In these challenge studies, five out of six mice immunized with the Pep2C-proteosome complex survived, compared to zero out of six mice immunized with proteosomes alone. These data support the hypothesis that immunization with a
peptide selected for binding to a specific anti-MCPS mAb can induce a protective anti-polysaccharide antibody response in a murine model.

In Manuscript 1 we demonstrate that Pep2C is an immunogenic mimic of MCPS. This is likely related to the peptides ability to specifically bind to the anti-MCPS mAb 1E4 and to elicit a specific anti-MCPS antibody response in an animal model. Previously the anti-id 6F9, selected with the same anti-MCPS mAb 1E4 used in Manuscript 1, was shown to be a true mimic of MCPS (Westerink and Giardina 1992). Westerink et al. (1992) demonstrated the ability of 6F9 to inhibit the binding of human hyperimmune sera to MCPS in a solid phase assay. In addition, a peptide spanning the immunogenic CDR3 region of 6F9 was synthesized and induced a T-dependent anti-MCPS antibody response that subsequently conferred protection from lethal challenge (Westerink, et al. 1995). The motif YYR was shown to be present in the 6F9 peptide mimic and was demonstrated to be crucial for the immunogenicity of the peptide. Interestingly Pep2C contained the same motif YYR. This coincides with findings of other investigators (Oldenburg, et al. 1992, Hoess, et al. 1993, Valadon, et al. 1996) that have demonstrated the preference for aromatic residues such as tyrosine (Y) and tryptophan (W) and residues with cyclic side chains such as proline (P) in peptides that mimic carbohydrates. Although the studies in Manuscript 1 support the hypothesis that a peptide mimic of MCPS with a similar motif to a known peptide mimic of MCPS can induce a similar protective anti-MCPS antibody response, it has been demonstrated that the immune responses are different in nature. The immunogenic mimic Pep2C induced primarily anti-MCPS IgM antibodies indicative of a T-independent immune response, whereas the 6F9 peptide induced a T-
dependent immune response, characteristic by the presence of anti-MCPS IgG antibodies. The difference in the immune response induced by the two peptides may be attributed to amino acid residues surrounding the YYR motif. These amino acids may be required to direct the immune response from a T-independent to a T-dependent response, and may play a role in antigenic mimicry. Despite these differences, our study supports the hypothesis that peptide mimics of capsular polysaccharides can be selected using various methods and provides continuing evidence that certain amino acids are required for successful carbohydrate peptide mimicry. Several other investigators (Phalipon, et al. 1997, Pincus, et al. 1998) studying the mechanisms behind carbohydrate mimicry have described similar findings.

The primary advantage of using peptide mimics of carbohydrate antigens is their potential to stimulate T-dependent immunity. However, several concerns associated with peptide antigens as vaccine candidates remain to be resolved. Small peptides are unstable and poorly immunogenic. They are often degraded rapidly affecting their antigenicity in vivo. In Manuscript 1, Pep1C and Pep2C were complexed to proteosomes to increase their stability and immunogenicity. As a result, polyclonal B cell activation occurred that increased non-specific background immune responses demonstrated in the negative control group. Peptide mimics of carbohydrate antigens have been shown to be extremely hydrophobic in nature. This complicates the peptides conjugation to proteosomes, and can affect the overall conformation of immunogenic residues in vivo. Therefore, to resolve some of the
issues associated with peptide antigens, construction of a DNA vector encoding the peptide mimic of MCPS was considered.

Peptide-based DNA immunization targeted to carbohydrate antigens has several advantages. First, peptide-encoded DNA constructs have the ability to induce carbohydrate cross-reactive humoral and cellular immune responses, thus inducing a T-dependent response to a carbohydrate antigen (Monzavi-Karbassi, et al. 2001b). Second, several studies (Cox and Coulter 1997, Cohen, et al. 1998) have demonstrated that DNA constructs can easily be altered to modulate the magnitude and orientation of the immune response. It has been shown the immune response to a DNA construct can be redirected to a T helper 1 (Th1) response. This type of response may help to overcome the immune tolerance to polysaccharide antigens present in children less than 2 yr of age(Monzavi-Karbassi, et al. 2001a). Third, DNA constructs can be designed to express multiple immunologically relevant B and T cell epitopes (Whitton, et al. 1993, An and Whitton 1997). The main benefit of this is the ability to include epitopes that encode peptide mimics of various serogroup polysaccharides and those encoding cell wall components (OMPs). This allows the construction of a multiepitope DNA construct that has the potential of protecting against various meningococcal serogroups.

Most of the literature has demonstrated the success of DNA vaccines targeted at intracellular bacteria and viruses. Few investigators have shown the use of DNA constructs encoding peptide mimics of capsular polysaccharides. However, studies by Lesinski et al. (2001) have demonstrated the ability of a DNA construct encoding a peptide mimic of S. pneumoniae capsular polysaccharide to induce anti-
polysaccharide antibodies in a mouse model. Likewise studies by Kieber-Emmons et al. (2000) and Velders et al. (2001) have reported the ability of DNA vaccines encoding peptide mimics to induce T-dependent immune responses as characterized by isotype switching.

Based on these studies, we hypothesized that a multi-epitope DNA construct encoding a peptide mimic of MCPS could induce a functional anti-MCPS antibody response that could protect mice against lethal meningococcal challenge. The construction of a multi-epitope DNA construct encoding a peptide mimic of MCPS has been described in Manuscript 2 (Prinz, et al. 2003). The DNA construct, designated P3A DNA, was designed to include a secretory leader sequence derived from Adenovirus E3, a universal T cell helper epitope from Human Immunodeficiency Virus (HIV) glycoprotein 120 (gp120) in tandem with a B cell epitope, the MCPS peptide mimic. The peptide mimic selected to be incorporated into the DNA construct was the 6F9 peptide. This peptide was selected based on its ability to elicit a protective T-dependent immune response. The DNA construct was designed with the purpose of increasing exogenous expression of the peptide mimic and to simultaneously induce T cell help.

Immunization studies with the DNA construct demonstrated that P3A DNA induced a significantly greater anti-MCPS IgM antibody response than that of the negative control group (mice immunized with vector alone, designated P3C DNA). The antibody response induced in P3A DNA immunized mice was higher than MCPS immunized mice (positive control), but this did not reach statistical significance. An anti-MCPS IgG antibody response was not detected in the P3A DNA immunized
mice despite the presence of a T helper epitope in our construct. These data suggested that the P3A DNA construct induced a T-independent antibody response.

To determine the functional activity of post-immune sera, complement-dependent bactericidal assays were performed. The results indicated that P3A DNA was capable of inducing anti-MCPS IgM antibodies that were functional in nature. The sera of P3A DNA immunized mice showed significantly greater bactericidal antibody titers than negative control mice. To further assess that the functional antibody response could protect mice against meningococcal infection, a lethal challenge was conducted. The results of these studies showed the anti-MCPS immune response induced by P3A DNA protected against challenge with a 10xLD50 dose of live meningococci. All mice immunized with P3A DNA or MCPS survived, whereas five of the six mice in the negative control succumbed to infection within 24 h post challenge. A possible explanation for survival of this mouse is that meningococci tend to aggregate in solution. Despite attempts to standardize the challenge procedure and dose, it is likely that the surviving negative control mouse did not receive the full lethal dose of meningococci. This is supported by the fact that at 6 hours post challenge the mouse was not bacteremic, whereas all mice immunized with P3A DNA or MCPS were bacterimec at this time point. The immunization and challenge studies supported the hypothesis that a multi-epitope DNA construct encoding a peptide mimic of MCPS could induce a protective anti-MCPS antibody response.

Despite the ability of the P3A DNA construct to protect from lethal challenge, the immune response induced prior to challenge was characteristic of a T-independent
response. In order to determine the presence of a memory response and to eliminate the possibility that high levels of circulating antibodies protected DNA immunized mice, post-challenge sera were tested for the presence of anti-MCPS IgG antibodies. At 96 hours post challenge, anti-MCPS IgG1, IgG2a, IgG2b, and IgG3 antibody titers were significantly greater in the P3A DNA immunized mice than that of the survivor in the negative control group. Anti-MCPS IgG1, IgG2b, and IgG3 antibodies were detected in MCPS immunized survivors, but at significantly lower levels than P3A DNA mice. The presence of the various IgG isotypes in post challenge sera from mice immunized with P3A DNA indicates the induction of a mixed Th1 and Th2 immune response. Anti-MCPS IgG2a antibodies were only detected in P3A DNA immunized mice. IgG2a is an important complement fixing and opsonizing antibody crucial for preventing infection by a bacterial pathogen. The results also indicated that P3A DNA immunized mice elicited a greater memory response than all other groups, as demonstrated by higher anti-MCPS IgG levels post challenge. These studies provide evidence that a B cell memory response was induced by P3A DNA despite the absence of a strong effector response, and support the hypothesis that a multi-epitope DNA construct encoding a peptide mimic of MCPS can induce a T-dependent immune response.

T-independent immune responses to carbohydrate antigens are characterized by isotype restriction, lack of affinity maturation, and lack of a memory response (Lesinski and Westerink 2001). In Manuscript 2 the response to the P3A DNA construct appeared to be T-independent in nature prior to challenge. However, post-challenge survivors immunized with the P3A DNA construct demonstrated an
antibody response indicative of a T-dependent immune response with evidence of memory. The immune response induced was uniquely different from a “true” T-independent or T-dependent response. Investigators (Boswell and Stein 1996, Sverremark and Fernandez 1998, Lucas and Reason 1999) using carbohydrate antigens have described T cell responses in a predominantly characteristic T-independent response to a polysaccharide antigen. Recently, studies (Laylor, et al. 1999) have demonstrated DNA vaccination could result in significant B cell memory responses in absence of overt effector responses. Furthermore, it was shown in limiting-dilution culture fragments, memory B cell clones could arise without detectable primary antibody production (Klinman 1997). Our results support these findings that B cell memory and B cell activation can occur without standard effector responses normally seen in a T-dependent immune response. To further examine the anti-MCPS immune response induced by P3A DNA prior to challenge, cytokine profiles characteristic of both Th1 and Th2 responses were investigated.

It was hypothesized that a multi-epitope DNA construct encoding a peptide mimic of MCPS can induce a T-dependent immune response with predominantly Th1 characteristics, as suggested by the presence of IgG2a post-challenge. The analysis of Th1 and Th2 cytokine profiles in mice immunized with a multi-epitope DNA construct has been described in Manuscript 3 (Prinz et al., submitted). Splenocytes were isolated from mice immunized with the P3A DNA construct, P3C DNA, MCPS, or the polysaccharide-protein conjugate vaccine, and stimulated with MCPS or heat inactivated *N. meningitidis* serogroup C (HICM). Levels of specific cytokine expression in mRNA were analyzed using relative quantitative real-time RT-PCR.
The presence of cytokines IL-2 and IFN-λ were studied as representatives of a Th1 response and IL-4 was used as a Th2 indicator.

The results from these studies demonstrated that immunization with the P3A DNA construct encoding a peptide mimic of MCPS induced predominantly a Th1 immune response to both MCPS and HICM stimulators, characterized by high levels of IL-2 and IFN-λ. In contrast mice immunized with the conjugate vaccine showed a mixed Th1/Th2 response with equivalent levels of IFN-λ, IL-2, and IL-4, whereas mice immunized with MCPS demonstrated a Th2 response with high levels of IL-4 mRNA expression. Mice immunized with the negative control P3C DNA showed slight levels of IL-2, but IL-4 and IFN-λ mRNA expression were not detected, suggesting a T-independent response to the vector. These data support the hypothesis that a multi-epitope DNA construct can induce a T-dependent immune response with predominantly Th1 characteristics (IL-2 and IFN-λ).

The precise mechanism by which DNA immunization stimulates an immune response and generates memory remains under investigation. To date the majority of studies using DNA as a vaccine candidate have focused on intracellular viral, bacterial, and parasitic pathogens. Limited knowledge regarding the mechanism of DNA constructs encoding peptide mimics of extracellular bacteria is available. Studies by Kieber-Emmons et al. (2000) have shown that an anti-carbohydrate T-dependent (Th1) immune response could be induced by a DNA vaccine encoding a peptide mimic of the carbohydrate tumor antigen Lewis Y. This was evident by IgG2a antibody production post-immunization. The results of our studies involving a multi-epitope DNA construct encoding a peptide mimic of MCPS demonstrate the
ability of DNA to induce a functional T-dependent immune response that supports B cell memory formation. Although the response is not a “true” T-dependent immune response in the sense that a post-immunization antibody effector response is absent, we have demonstrated that memory is being stimulated with a strong Th1 component. Other investigators (Laylor, et al. 1999, Cazeaux, et al. 2002) have likewise demonstrated that intramuscular DNA immunization promoted memory formation without apparent effector immune responses.

The magnitude and direction of the immune response induced by DNA immunization can be affected by many factors. Studies with DNA constructs encoding protein antigens have shown the method and site of inoculation, including the use of various adjuvants, transfection efficiency, and antigen presentation affect the desired immune response of DNA immunization. These same strategies may not necessarily apply to DNA constructs encoding peptide mimics of carbohydrate antigens. Previous immunization studies using the MCPS peptide mimic resulted in an overt T-dependent immune response. However, the response to the same peptide, when encoded in a DNA vector, was markedly different. This may be attributed to the design and efficacy of the multi-epitope DNA construct. The construction of the vaccine may not be efficient at providing adequate T cell help to result in an overt effector IgG response post-immunization. Alternative construct design, including the use of different adjuvants, needs to be investigated to improve the post-immunization response.

Despite the lack of an overt effector response, studies with the multi-epitope DNA construct have demonstrated the ability to generate a memory response. In a T-
dependent response to a protein antigen, T helper cells support the differentiation and proliferation of B cells into plasma or memory cells. However, it has been suggested that intramuscular immunization with DNA may prime for the formation of memory cells at the site of inoculation (Corr, et al. 1999). Intramuscular immunization of DNA results in prolonged expression of low levels of antigen. This may result in priming of memory cells without strong effector responses associated with high levels of antigen. The presence of a continuous “antigen depot” at the site of inoculation could select for B cells producing high and not low avidity antibodies. Conversely, a T-independent response selects for low-avidity IgM antibodies. The specific DNA primed B cells may account for the long-lived immune responses and immunologic memory that has been demonstrated after DNA immunization.

In conclusion, studies with a peptide mimic of MCPS and a multi-epitope DNA construct encoding a peptide mimic of MCPS have demonstrated that anti-MCPS immune responses can be induced following immunization with these potential vaccine candidates. The responses induced are functional and are protective against lethal infection in a murine model. Furthermore, a multi-epitope DNA construct encoding a peptide mimic of MCPS can induce a T-dependent immune response, more specifically a Th1 response, with evidence of B cell memory formation. This implies that B cell stimulation, specifically by DNA constructs encoding peptide mimics of carbohydrate antigens, may occur without standard effector responses normally seen in immunization studies with protein antigens. These results demonstrate the complex nature of the immune response induced by
DNA immunization encoding a peptide mimic, and may influence future methods of vector design and evaluation of DNA vaccine efficacy.

The studies presented describe the selection and evaluation of an immunogenic peptide mimic of the capsular polysaccharide from *N. meningitidis* serogroup C (MCPS). As a natural extension of peptide mimicry, a multi-epitope DNA construct encoding a peptide mimic of MCPS was subsequently investigated.

The anti-MCPS mAb 1E4 used to previously identify the anti-id immunoglobulin 6F9, was used to screen a phage display library. Sequence analysis demonstrated that mAb 1E4 could select for a peptide (Pep2C) with a similar motif as 6F9. Immunization studies in a murine model with Pep2C complexed to proteosomes showed that Pep2C could induce a functional anti-MCPS antibody response that protected mice against lethal meningococcal infection. However, the response to the Pep2C-proteosome complex was T-independent in nature. Similar immunization results were previously found with a peptide spanning the region of the anti-id 6F9, except the functional nature of the response was T-dependent. This was the first study to show that two different techniques resulted in the selection of MCPS peptide mimics, similar in sequence, but different in nature.

To overcome some of the complexities associated with using peptides as immunogens, a multi-epitope DNA construct encoding the 6F9 peptide mimic of MCPS was designed. Immunization studies with the DNA construct resulted in the induction of functional anti-MCPS antibodies that protected against lethal meningococcal infection. Prior to challenge, the immune response appeared to be T-independent in nature, however, post-challenge results demonstrated a strong T-
dependent immune response with evidence of B cell memory. Further analysis of the nature of the induced anti-MCPS immune response showed that a specific Th1 immune response was being stimulated by the multi-epitope DNA construct.

The results of these studies indicate that a peptide-mimic encoding DNA construct can induce a functional T-dependent immune response, including B cell memory. This response is unique in that it occurs without an overt IgG effector response normally seen with immunization with protein antigens. Furthermore, this study demonstrates the complexity of the mechanisms behind the immune response associated with DNA immunization encoding peptide mimics of carbohydrate antigens. This may influence the decisions regarding appropriate methods used for evaluating the efficacy of DNA immunization.
BIBLIOGRAPHY


Banerjee-Bhatnagar, N.; and Frasch, C.E. 1990. Expression of Neisseria meningitidis iron-regulated outer membrane proteins, including a 70-kilodalton transferrin
receptor, and their potential for use as vaccines. Infect. Immun., 58:2875-2881.


class I molecules depends on its neighboring residues in the protein. Cell, 66:1145-1153.


Evans, S. V.; Rose, D.R.; To, R.; Young, N.M.; and Bundle, D.R. 1994. Exploring the mimicry of polysaccharide antigens by anti-idiotypic antibodies. The
crystallization, molecular replacement, and refinement to 2.8 Å resolution of an idiotope-anti-idiotope Fab complex and of the unliganded anti-idiotope Fab. J. Mol. Biol., 241:691-705.


Ferry, N. S.; and Steele, A.H. 1935. Active Immunisation with Meningococcus toxin. JAMA, 104:983-984.


Gold, R.; Lepow, M.L.; Goldschneider, I.; Draper, T.F.; and Gotshlich, E.C. 1979. Kinetics of antibody production to group A and group C meningococcal polysaccharide vaccines administered during the first six years of life:


Granoff, D. M.; Anderson, E.L.; Osterholm, M.T.; Holmes, S.J.; McHugh, J.E.; Belshe, R.B.; Medley, F.; and Murphy, T.V. 1992. Differences in the


Intranasal administration of a meningococcal outer membrane vesicle vaccine
induces persistent local mucosal antibodies and serum antibodies with strong

Hanke, T.; Blanchard, T.J.; Schneider, J.; Ogg, G.S.; Tan, R.; Becker, M.; Gilbert,
of intravenous and intramuscular administrations of modified vaccinia virus
Ankara-based multi-CTL epitope vaccine for human immunodeficiency virus

Hanke, T.; and McMichael, A. 1999. Pre-clinical development of a multi-CTL
epitope-based DNA prime MVA boost vaccine for AIDS. Immunol. Lett.,
66:177-181.

Hanke, T.; Schneider, J.; Gilbert, S.C.; Hill, A.V.; and McMichael, A. 1998b. DNA
multi-CTL epitope vaccines for HIV and Plasmodium falciparum:

Harris, S. L.; Craig, L.; Mehroke, J.S.; Rashed, M.; Zwick, M.B.; Kenar, K.; Toone,
E.J.; Greenspan, N.; Auzanneau, F.I.; Marino-Albernas, J.R.; Pinto, B.M.; and
Scott, J.K. 1997. Exploring the basis of peptide-carbohydrate crossreactivity:
evidence for discrimination by peptides between closely related anti-


Hsu, S. C.; Schadeck, E.B.; Delmas, A.; Shaw, M.; and Steward, M.W. 1996. Linkage of a fusion peptide to a CTL epitope from the nucleoprotein of measles virus enables incorporation into ISCOMs and induction of CTL responses following intranasal immunization. Vaccine, 14:1159-1166.


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Joshi, S. K.; Suresh, P.R; and Chauhan, V.S. 2001. Flexibility in MHC and TCR recognition: degenerate specificity at the T cell level in the recognition of
promiscuous Th epitopes exhibiting no primary sequence homology. J. Immunol., 166:6693-6703.


Leach, A.; Twumasi, P.A.; Kumah, S.; Banya, W.S.; Jaffar, S.; Forrest, B.D.; Granoff, D.M.; LiButti, D.E.; Carlone, G.M.; Pais, L.B.; Broome, C.V.; and


association with universities providing relatively large amounts of catered hall accommodation. Epidemiol. Infect., 122:351-357.


Olcen, P.; Kjellander, J.; Danielsson, D.; and Lindquist, B.L. 1981. Epidemiology of Neisseria meningitidis; prevalence and symptoms from the upper respiratory


Salmi, I.; Pettay, O.; Simula, I.; Kallio, A.K.; and Waltimo, O. 1976. An epidemic due to sulphonamide-resistant group A meningococci in the Helsinki area.


Scherp, H. W.; and Rake, G. 1945. Studies on meningococcal infection. XIII. Correlation between anti-polysaccharide and the antibody which protects mice against infection with type 1 meningococi. J. Exp. Med., 8:85-92.


Seleste, E. 1941. Uber das Meningitis Epidemica - Syndrom und damit verbundene Nachkrankheiten in Finland. Duodecim., 41:131-134.

Seppala, I.; Sarvas, H.; Makela, O.; Mattila, P.; Eskola, J.; and Kayhty, H. 1988. Human antibody responses to two conjugate vaccines of Haemophilus...


serogroup B outer-membrane protein meningococcal vaccines: a randomized controlled trial in Chile. JAMA, 281:1520-1527.


ABSTRACT

Carbohydrate antigens, such as the capsular polysaccharide of *Neisseria meningitidis*, are considered T-independent in nature. T-independent antigens do not require T cell help to elicit an immune response and do not generate immunologic memory formation. Carbohydrate antigens have limited responses as immunogens and fail to elicit protective levels of antibodies in children less than 2 yr of age. In order to increase the immunogenicity of a capsular polysaccharide, it must be converted into a T-dependent antigen. T-dependent antigens have the ability to associate with major histocompatibility complex (MHC) molecules and be presented to T cells. This generates a memory response and overcomes the immune tolerance associated with carbohydrate antigens in the young. One method of converting a carbohydrate into a T-dependent antigen is through the use of molecular mimicry. Molecular mimicry is defined as the ability of structurally unrelated molecules to exert the same biological effect. The concept of mimicry is based on the idiotypic network that suggests mimicry is the function of reproducing the binding interaction between an antibody and antigen. Recent studies have extended this concept by demonstrating the potential of phage display libraries in selecting peptides capable of mimicking the capsular polysaccharide, and thus eliciting anti-polysaccharide antibodies when used as immunogens. A natural extension of the observation that peptides can mimic polysaccharides is the development of DNA constructs that encode peptide mimics of capsular polysaccharides. DNA constructs have been shown to induce long lasting humoral and cellular responses, and can easily be altered to manipulate the magnitude and orientation of the desired immune response.
Therefore, multiple DNA encoded epitope sequences representing specific carbohydrate and protein epitopes can be included in the construct design. Therefore, DNA immunization may be useful against encapsulated organisms by directing the response to specific polysaccharide as well as protein epitopes. The following studies will describe the selection and evaluation of an immunogenic peptide mimic of \textit{N. meningitidis} serogroup C capsular polysaccharide (MCPS). The studies will further describe the design of a multi-epitope DNA construct encoding a peptide mimic of MCPS, and will evaluate and characterize the anti-MCPS immune response in a murine model.