STUDIES OF PEPTIDE MIMICRY OF THE GROUP B STREPTOCOCCUS TYPE III CAPSULAR POLYSACCHARIDE ANTIGEN

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of

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August 2007
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Rattanaruji Pomwised

August, 2007
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ABSTRACT

Capsular polysaccharide (CPS) of Streptococcus group B (GBS) is a poor immunogen and functions as T cell independent antigen, eliciting low IgG antibody with deficient immunologic memory. We previously identified a peptide, S9, which mimics CPS of type III GBS. Here we have taken steps to develop the mimetic peptide as a vaccine against GBS group III. We enhanced the immunogenicity of the peptide by presenting it on the coat protein of Cowpea Chlorotic Mottle Virus (CCMV). And we searched for better mimetic peptides by constructing a secondary phage display library. To accomplish the first goal, DNA encoding S9 was cloned into five constructions CCMV coat protein loops using recombinant DNA techniques. The results indicated that inserting the S9 peptide sequence into CCMV coat protein loops disrupted virus and virus-like particle assembly. Therefore the S9 peptide was conjugated to CCMV coat protein using chemical linkers. The CCMV-S9 conjugation products remained intact as monomer virions. Mice were immunized with the CCMV-S9, SubE-S9 (a mutant that does not assemble virions), CPMV-S9 (S9 conjugated to Cowpea Mosaic Virus) and S9 conjugated to the carrier protein KLH, with and without Freund’s adjuvant. The CCMV-S9, CPMV-S9 and KLH-S9 induce anti-S9 antibody even without the adjuvant whereas SubE-S9 induced an anti-S9 response only with adjuvant. The CCMV-S9 and CPMV-S9 predominantly induced a Th1 response with antigen-specific IgG2a and IFN-γ production, whereas the KLH-S9 predominantly induced a Th2 response with antigen-specific IgG1 and IL4/IL10 production. To accomplish the second goal, a DNA sublibrary was designed to have approximately one mutation in each displayed peptide sequence. Peptides with higher affinity to the S9 mAb were identified by affinity selection. ELISA analysis from randomly selected phage clones indicated that amino acid residues 3-5 and 7-10 of S9 peptide are important in specific binding activity to S9 monoclonal antibody. These studies identified peptides with greater affinity for the selecting antibody, i.e. enhanced antigenicity.
INTRODUCTION

**Streptococcus**

The streptococci are gram-positive spherical bacteria that characteristically form pairs or chains during growth. They are a heterogeneous group of organisms that include commensal bacteria as well as virulent pathogens. These organisms are of agricultural and medical importance. Major human pathogens include group A streptococci (*S. pyogenes*), group B streptococci (*S. agalactiae*), and the pneumococcus (*S. pneumoniae*), but other species are capable of causing disease. Table 1.1 and 1.2 list the major characteristics of the principal streptococcal species (2).

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<th>Common and Important Diseases</th>
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<td><em>Streptococcus pyogenes</em></td>
<td>A</td>
<td>Beta</td>
<td>Throat, skin</td>
<td>PYR test positive, inhibited by bacitracin</td>
<td>Pharyngitis, impetigo, rheumatic fever, glomerulonephritis Neonatal sepsis and meningitis</td>
</tr>
<tr>
<td><em>Streptococcus agalactiae</em></td>
<td>B</td>
<td>Alpha and Beta</td>
<td>Female genital tract, colon</td>
<td>Hippurate hydrolysis, CAMP-positive (Christie, Atkins, Munch-Peterson test)</td>
<td>Pneumonia, meningitis, endocarditis</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>None</td>
<td>Alpha</td>
<td>Throat</td>
<td>Susceptible to optochin. Colonies soluble in bile, Quellung reaction-positive</td>
<td>Dental caries (S mutans), endocarditis</td>
</tr>
<tr>
<td>Viridans streptococci</td>
<td>Usually not typed or untypable</td>
<td>Alpha, none</td>
<td>Mouth, throat, colon, female genital tract</td>
<td>Optochin-resistant. Colonies not soluble in bile. Carbohydrate fermentation patterns</td>
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Table 1. 2 Characteristics of Medically Important Streptococci Infrequently Associated with Disease

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<th>Associated Diseases</th>
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<tr>
<td><em>Enterococcus faecalis</em> (and other enterooccus) (Now is grouped into genus <em>Enterococcus</em> (3))</td>
<td>D</td>
<td>None, alpha</td>
<td>Colon</td>
<td>Growth in presence of bile, hydrolyze esculin, growth in 6.5% NaCl, PYR-positive (hydrolysis of L-pyrrolidonyl-2-naphthlamide)</td>
<td>Abdominal abscess, urinary tract infection, endocarditis</td>
</tr>
<tr>
<td><em>Streptococcus bovis</em> (nonenterococcus)</td>
<td>D</td>
<td>None</td>
<td>Colon</td>
<td>Growth in presence of bile, hydrolyze esculin, no growth in 6.5% NaCl, degrades starch</td>
<td>Endocarditis, common blood isolate in colon cancer</td>
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<td><em>Streptococcus anginosus</em></td>
<td>F (ACG) and untypable</td>
<td>Beta</td>
<td>Throat, colon, female genital tract</td>
<td>Small colony variants of beta-hemolytic species. Group F are bacitracin-resistant and PYR-negative. Obligate anaerobes</td>
<td>Pyogenic infections, including brain abscesses</td>
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<tr>
<td><em>Peptostreptococcus</em> (many species)</td>
<td>None</td>
<td>None, alpha</td>
<td>Mouth, colon, female genital tract</td>
<td></td>
<td>Abscesses (with multiple other bacterial species)</td>
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Growth and Classification

Streptococci characteristically form chains of two or more organisms. The lengths of the chains vary and depend upon environmental factors. They lose their gram-positive appearance and appear to be gram-negative as the culture ages. They are facultative anaerobes and grow best at 37°C. Most streptococci produce a capsule resulting in mucoid colonies. Variants of the same strains may show different colony forms. For example, in group A *streptococci*, strains which produce much M protein appear as matte colonies, and strains which produce little M protein form glossy colonies. Phase variation in colony morphology has been described for streptococcal species (4-8).
No one system suffices to classify all streptococci. Therefore, their classification is based on different methods: hemolysis pattern, biochemical, physiological and morphological characteristics, and immunological grouping according to Lancefield’s method. This has led to a somewhat confusing nomenclature that includes species names, immunological grouping (e.g. group A streptococci), and/or hemolysis pattern. The hemolysis pattern is the effect that the bacteria have on sheep red blood cells when grown on blood agar. Many streptococci hemolyze red blood cells in vitro in varying degrees. Complete disruption of erythrocytes is called β-hemolysis, resulting in a clear zone around a colony. Incomplete lysis of erythrocytes with a green pigment zone around a colony is called α-hemolysis. The formation of a greenish zone around colonies on blood agar is due to discoloration and loss of potassium from the red cells and peroxide production by bacteria (9). Hemolysis is the result of the secretion of soluble hemolysins; examples of streptococcal hemolysins are streptolysin O and S of group A streptococci. Streptolysin O is an antigenic protein (MW 60,000) that is hemolytically active in the reduced state but inactive in the presence of oxygen (10). An antibody response to streptolysin O suggests either recent infection with streptococci or persistently high antibody levels due to an exaggerated immune response in a hypersensitive person (11). Streptolysin S is not antigenic. It may be inhibited by a nonspecific inhibitor present in human sera (9, 12).

Speciation of streptococci is based on biochemical reactions. Biochemical tests include sugar fermentation reactions, tests for the presence of enzymes, and tests for susceptibility or resistance to certain chemical agents. PYR is the test for hydrolysis of L-
pyrrolidonyl-2-naphthylamide. Enzymatic hydrolysis of L-pyroglutamic acid β-naphthylamide releases free β-naphthylamine, which is detected and shown by a color change after adding hydrochloric acid (13). This test distinguishes group A *Streptococcus* from other β-hemolytic streptococci. The physiological tests include CAMP test (Christie, Atkins, Munch-Peterson test). It was designed to aid in the identification of *S. agalactiae*. This test relies on the fact that most strains produce a diffusible, extracellular compound that will, in conjunction with a specific β-hemolysin of *Staphylococcus aureus*, cause complete lysis of sheep red blood cells in an agar medium (14, 15). The morphological tests include the Quellung reaction tests for the presence of capsule. The test principle is based upon the fact that capsule can prevent India ink from penetrating into bacteria. Encapsulated cells appear to have halo around them. The reaction between bacteria and antiserum raised against the bacterial capsule causes capsular swelling and make it easily visualized in India ink suspension (16). Streptococcal species may also be distinguished on the basis of starch hemolysis. Bacteria are cultivated on a plate containing starch. Gram’s iodine is then flooded onto the plate. Clear zones around colonies that hydrolyze starch will be observed on a blue background. Group D degrades starch while group A, B and F don’t (17). The carbohydrate fermentation test is used to differentiate bacteria based on their ability to oxidize or ferment specific sugars. For example *S. anginosus* (viridans streptococcus) is capable of a 2,3-butanediol fermentation as a continuation of mixed-acid fermentation (18). Group E streptococci can produce acid from lactose but are unable to ferment trehalose and sorbitol (19).
The Lancefield classification is based upon immunologic differences among the group carbohydrate structures of streptococci. Classically this is performed using precipitin reactions between hot acid or enzyme extracts of the bacteria and specific antisera (20, 21). Using this method, Lancefield characterized streptococci into groups A through T. The serologic specificity of the group-specific carbohydrate is determined by an amino sugar. For group A streptococci, this is rhamnose-N-acetylglucosamine; for group B, rhamnose-glucosamine polysaccharide, for group C, rhamnose-N-acetylgalactosamine; for group D, glycerol teichoic acid containing D-alanine and glucose; for group F, glucopyranosyl-N-acetylglactosamine.

**Group A Streptococcus**

Most streptococci that contain the group A antigen are *S. pyogenes*. These organisms produce a number of factors that may serve to enhance their virulence and cause disease. They can be further classified into serotypes based on antigenicity of M protein (22, 23).

M protein is a major virulence factor of group A streptococci. It appears as hair-like projections of the streptococcal cell wall (24). Group A streptococci which lack M protein are not virulent (25). Antibody to M protein is type specific. M protein binds to fibrinogen, fibrin and their degradation products to coat the bacterial surface in order to block complement deposition on their surface (26, 27). The secreted M protein can bind to fibrinogen. The aggregates bind to integrin receptors on the surface of polymorphonuclear leukocytes (PMN) leading to the activation of the host defense mechanism of these cells, including the secretion of toxic oxygen metabolites, a variety
of proteolytic and glycolytic enzymes, and proinflammation cytokines, such as tumor necrosis factor and IL-6 (27, 28). This in turn causes damage to host tissues leading to bacteria invasion into host cells. Systemic activation of this proinflammatory pathway may lead to toxic shock syndrome.

The capsule, which is composed of hyaluronic acid and containing repeating units of glucuronic acid and N-acetylglucosamine is considered to be an antiphagocytic structure (29). The capsule is also an important adherence factor because it binds to CD44 on epithelial cells and interferes with the attachment of phagocytes (29-32).

Group A streptococci also produce plasminogen-binding proteins: glyceraldehydes-3-phosphate dehydrogenase, enolase, and streptokinase (33-38). These proteins bind to extracellular matrix metalloproteases or collagenases to facilitate tissue invasion. This strategy enhances bacterial invasion through normal tissue barriers.

Streptococcus group A produces C5a peptidase, which cleaves six amino acids from the carboxy terminus of C5a, which is the chemotactic factor generated by the complement cascade (39). This event inhibits the recruitment of phagocytic cells to the site of infection.

Group A streptococci also posses streptococcal proteinase (streptococcal exotoxin B) which is an extracellular cysteine proteinase. It can convert IL-1β precursor to active IL-1β, an inflammatory cytokine (40-42). The inflammatory cytokines in turn injure host cells leading to increasing bacterial invasion (43). Group A streptococci produce a variety of toxins such as streptolysins S and O and pyrogenic exotoxins A, B and C and also mitogens such as streptococcal superantigen (SSA), streptococcal mitogenic exotoxin Z
(SMEZ). Streptolysins S and O are hemolysins, which lyse red blood cells and possibly other cell types. The three streptococcal pyrogenic exotoxins (SPEs) A, B and C are T cell superantigens, which enhance delayed-type hypersensitivity and suppress the antibody response (9). The superantigens are capable of binding to the beta chain (Vβ-chain) of a characteristic set of T-cell receptors and also to the MHC class II molecule expressed on resting B cells, monocytes and dendritic cells. Binding to the T-cell receptor and MHC class II molecule causes T cells to proliferate and release inflammatory cytokines involved in toxic shock syndrome(44-46).

Group A streptococci live on human skin and mucous membranes and can spread from person to person. The first step in the establishment of a streptococcal infection is the adherence of the bacteria to epithelial cells. Lipoteichoic acid (LTA) is an important adhesin of group A streptococci (47). LTA can interact specifically with fibronectin, a host protein that coats the epithelial cells of the oropharynx (48). The LTA molecules act as an adhesin by bridging molecules on the streptococcal surface, such as the M protein, with cell surface proteins (49). LTA interacts with the streptococcal proteins through its negatively charged polyglycerolphosphate backbone, which is attracted to positively charged residues of the surface proteins. The lipid moiety of LTA projects outward and interacts with hydrophobic fatty acid-binding sites on fibronectin and epithelial cells (49, 50). The spread of organisms depends on how the infection is acquired. Infection of the skin or mucous membranes may be localized and associated with necrosis and abscess formation. However the bacteria are also capable of rapid spread through tissues, earning it the lurid nickname “flesh eating bacteria”. The spread of bacteria occurs due to the
secretion of digestive enzymes including proteases, hyaluronidase, DNAase and streptokinaes (51). The streptokinase binds to host plasminogen degrading it to plasmin and leading to fibrin degradation (34, 35). Streptococcus Group A evokes an intense inflammatory response in tissues, resulting from the production of proinflammatory cytokines from PMNs and monocytes.

Group A Streptococci cause disease in several ways. They may cause damage by direct infection: “Strep” throat, pyoderma (skin infections), necrotizing fasciitis and septicemia. The elaboration of streptococcal toxins causes scarlet fever and toxic shock syndrome (23, 44, 52). There may also be nonsuppurative sequelae of streptococcal infections such as acute poststreptococcal glomerulonephritis and acute rheumatic fever. The toxin-mediated and post-infectious syndromes result from inappropriate stimulation of the immune system. Streptococci possess a number of antigens that cross-react with human tissues; heart muscle and valvular connective tissue (23, 45, 53, 54). Some of these epitopes are an integral part of M protein molecule. Antibodies to certain purified M proteins react with myosin, phosphorylase and several other unidentified proteins in heart tissue (55-57). Streptococcal antigens may also evoke cross-reactive T cells. These antigens stimulate the production of cytotoxic T cells active against cultured cardiac myocytes. These cardiac reactive antibodies and T cells may be involved in the pathogenesis of rheumatic fever. The toxin SPEs are potent mitogens; they stimulate T-cell proliferation. The SPEs are superantigens interacting with conserved regions of the V domain of the T-cell receptor rather than with hypervariable regions (46, 58, 59).
Therefore, polyclonal activation results in an inappropriate immune response that may be the major cause of the toxic shock syndrome.

**Pneumococcus (Streptococcus pneumoniae)**

Pneumococcus is placed in the viridans group. Lancefield grouping is not useful for discriminating between the viridans streptococci therefore subdivision of the viridans is based on biochemical and physiological characteristics. The optochin susceptibility test is used to differentiate the pneumococci from other viridans.

Pneumococci are the major cause of bacterial pneumonia. They are surrounded by polysaccharide capsules. The polysaccharide capsule is a potent antiphagocytic factor. Antibodies against capsular polysaccharide have differentiated pneumococci into 90 serotypes (60). Antibodies against capsular polysaccharide are not protective (61). Pneumococci also contain C-substance, which contains teichoic acid, a part of the cell wall. C-substance reacts with C-reactive protein, a nonantibody β globulin from serum, which acts as an acute phase reactant. The complex of C-substance and C-reactive protein induces the complement cascade, leading to the release of inflammatory mediators and opsonizing the organisms to enhance phagocytosis (62-64). Pneumococci also contain pneumococcal surface protein A (PspA), which is located on the cell wall. PspA is a surface protein with variable molecular size ranging from 67 to 99 kDa. It appears to protect against the host complement system (65). PspA is highly polar, which results in capsular charge stabilization through the electropositive end of the molecule and prevention of complement activation through the predominant electronegative part of PspA (65). Pneumococcus possesses hyaluronate lyase, which is part of a group of
enzymes called hyaluronidases (66, 67). The hyaluronidase enzyme facilitates invasion by breaking down the extracellular matrix. The bacteria also contain pneumolysin, which is a cytoplasmic enzyme that is released due to the action of surface pneumococcal autolysin (68). The enzyme is cytotoxic to ciliated bronchial epithelial cells. It disrupts tight junctions and the integrity of the bronchial epithelial monolayer. Due to the enzyme’s function, the ability of ciliated bronchial cells to clear mucus from the lower respiratory tract is reduced. The cytotoxic activity of pneumolysin can directly inhibit phagocyte and immune cell function, which leads to suppression of the host inflammatory and immune responses (69).

Humans are reservoirs of pneumococci. Transmission occurs from direct contact and also airbone. Colonization by bacteria occurs in the nasopharynx (70). The outcome of colonization is determined by the bacterial virulence and the efficiency of host defense mechanisms. Pneumococcal adherence to host cells has been proposed to be a two-step process. The first step is targeting an anatomic niche of the host, such as the nasopharynx, and binding to the host cell-surface glycoconjugates on respiratory epithelial and endothelial cells (68). The second step involves cytokine production by the host cell. This cytokine activation results in expression of novel glycans on the surface of activated cells and increased pneumococcal adherence (71-73).

Pneumococci cause life-threatening diseases such as pneumonia, bacteremia and meningitis. Disease rates are high in young children, the elderly and patients with predisposing conditions such as asplenia, chronic medical conditions or
immunosuppressive illnesses, particularly AIDS. Vancomycin and penicillin are the
drugs of choice for treatment.

**Other Streptococci**

Group C and G streptococci occur sometimes in the nasopharynx and may cause
sinusitis, bacteremia or endocarditis (74-76). Group D streptococci are the divided into
two groups, enterococci and nonenterococci. Current classification groups enterococci
into a new genus *Enterococcus* based on 16 rRNA gene sequence analyses (3). The
enterococci are normal flora of the intestinal tract and genitourinary tract. They can grow
in high salt concentrations and in detergent including 60% bile (77). Most infections are
urinary tract infections. Antibiotic synergism (combination use of different antibiotics) is
recommended for Enterococcal infection (78). The nonenterococcal species do not grow
in high salt concentrations and are sensitive to penicillin (79). Group F streptococci such
as *S. anginosus* and *S. milleri* are part of the normal flora. Viridans streptococci include
*S. mutans*, *S. salivarius* and *S. mitis* (80). Typically they are α-hemolytic but they may be
nonhemolytic. The viridans streptococci are the most prevalent members of the normal
flora of the upper respiratory tract and are important for the healthy state of the mucous
membranes there. They may reach the bloodstream as a result of trauma and are a
principal cause of endocarditis on abnormal heart valves. Some viridans streptococci such
as *S. mutans* synthesize large polysaccharides such as dextrans or levans from sucrose
and contribute importantly to the genesis of dental caries.
Group B Streptococcus (GBS)

Group B streptococci cause serious disease in newborn infants and in immunocompromised adults. Despite the fact that the bacteria are exquisitely sensitive to antibiotics, the aggressive nature of infection leads to high morbidity and mortality. The development of a vaccine to prevent infection in targeted groups is considered a public health priority (81).

Identification and Morphology

Lab Tests Group B streptococci (Streptococcus agalactiae) are spherical gram-positive organisms arranged in chains. GBS can be either α or β hemolytic, if β hemolytic, then weakly so. GBS can be differentiated from other β-hemolytic streptococci on the basis of the hippurate test, CAMP test and serological tests. GBS are able to produce the enzyme hippuricase, which hydrolyses sodium hippurate to glycine and sodium benzoate. Ninhydrin reagent forms a purple color when exposed to glycine and serves as the indicator for the assay. GBS produce a diffusible extracellular compound called CAMP factor, that will, in conjunction with a specific β hemolysin of Staphylococcus aureus act synergistically to cause complete lysis of red blood cells (14, 15). GBS contain a group specific carbohydrate antigen, that serves to distinguish it from other groups of streptococci, and a polysaccharide capsule, which is the basis of serotype specificity. A variety of different immunological assays have been used to define both the group and type of GBS, latex agglutination being the most commonly used (82). Polymerase chain reaction (PCR) can also be used for GBS identification. Primers for the
The cfb gene, encoding CAMP factor, were used in the PCR reaction. Real-time PCR assay for the rapid detection of GBS was developed using the same set of primers to shorten time and increase sensitivity (83, 84).

**Capsule and Group Antigen** The cell wall of GBS is composed of protein antigens such as R, T and Rib antigen, peptidoglycan and carbohydrate (group antigen). The capsule contains the serotyping antigen that subdivides GBS into nine subgroups and may be a major virulence factor (85).

The group B carbohydrate is a common antigen, which is found in all strains and serotypes of GBS. The group B carbohydrate is chemically composed of rhamnose, galactose, N-acetylglucosamine, and glucitol. These sugars are linked by phosphodiester bond to form four different oligosaccharide units resulting in a complex and branched subunit structure (86). The terminal position of rhamnose is considered to be part of the immunodominant epitope (87). Antibody to the group B carbohydrate is unable to protect mice from lethal challenge with viable GBS (88). All strains of GBS isolated from humans are encapsulated and can be classified on the basis of serology and capsular polysaccharide (CPS) structure. Nine GBS serotypes have been so far identified: Ia, Ib, II, III, IV, V, VI, VII and VIII (85). The repeating unit of CPS contains combinations of these saccharides: glucose, galactose, N-acetylglucosamine, rhamnose, and sialic acid. There is a different arrangement of the saccharides in the repeating unit of each CPS. The CPSs of GBS type Ia and Ib are structurally similar, differing only in the linkage of the side chain galactose to N-acetylglucosamine. Type Ia CPS has a \( \beta-(1\rightarrow4) \) linkage and Ib CPS has a \( \beta-(1\rightarrow3) \) linkage (89). Type II CPS is composed of glucose, galactose, N-
acetylglucosamine, and sialic acid with ratio 2:3:1:1 (90). Type III CPS has a trisaccharide backbone of glucose, N-acetylglucosamine and galactose and a disaccharide side chain of sialic acid and galactose linked $\beta-(1\rightarrow4)$ to backbone N-acetylg glucosamine (91). Type IV CPS is composed of glucose, galactose, N-acetylglucosamine, and sialic acid with ratio: 2:2:1:1 (92). Type V CPS is composed of trisaccharide backbone of glucose, galactose and glucose and two side chains; one side chain is composed of glucose linked $\beta-(1\rightarrow3)$ to backbone galactose and the other side chain linked $\beta-(1\rightarrow6)$ to a backbone glucose in a trisaccharide of N-acetylglucosamine, galactose and sialic acid (93). Type VI CPS contains galactose, glucose and sialic acid in a 2:2:1 molar ratio (94, 95). Type VII CPS contains a trisaccharide backbone and trisaccharide sidechain (96). Type VIII CPS lacks N-acetylglucosamine. The subunit contains glucose, galactose, rhamnose and sialic acid in 1:1:1:1 molar ratio (97).

**Phase Variation** Group B streptococci demonstrate high-frequency phase variation. Colony opacity variants were detected for GBS type III (8). Occasional transparent colonies with one or more opaque regions were detected on lucid solid medium. Opaque-transparent switches were observed in both directions. Stable opaque and transparent variants have been isolated and compared to each other. Transparent variants are more virulent compared to opaque variants (98). Opaque variants grow poorly in Todd-Hewitt medium, the standard medium used to grow GBS. Opaque variants also have differentiation among their groups. One opaque variant produces unique or overexpressed proteins of 46 and 75 kDa. One variant has capsule alteration with decreased type III antigen, increasing buoyant density. Another variant has a thinner
extracellular matrix and lacks the group B antigenic structure. Differences between transparent and opaque variants, and the genetic mechanism responsible for phenotypic switching, remain to be elucidated. Phase variation may play a role in the pathogenesis of GBS infection.

**GBS Infections**

**Disease Caused by GBS** GBS is a normal member of the human vaginal and lower intestinal flora. About 10-30% of pregnant women are colonized with GBS (81, 99, 100). GBS are important causes of serious bacterial disease in neonates, pregnant women, and in immunocompromised adults. In newborns, GBS diseases are characterized as either early onset or late onset. Early onset GBS disease occurs in infants less than 7 days of age, typically 1 to 2 days old. In early-onset disease, the neonate is infected by exposure to GBS before or during birth. The early-onset disease is probably caused by spread of bacteria from the maternal genital tract through ruptured membranes into the amniotic fluid. Bacteria amplify and colonize the respiratory tract of the newborn. In some cases the spread of bacteria occurs even without premature rupture of the membrane. Late onset, which is less common than the early-onset disease, occurs in infants older than 7 days of age. GBS disease in newborn babies includes sepsis, meningitis, pneumonia, cellulitis, osteomyelitis and septic arthritis. In late-onset disease, the two most common clinical manifestations are meningitis and bacteremia. The most common syndrome in adults are skin infections (101, 102). Bacteremia can also occur. GBS can also lead to pneumonia, which is a severe and has a high mortality rate (103). Endocarditis and epiglottitis also have been reported in older children (104). A
substantial proportion of newborns who survive GBS infection suffer from sequelae. Neurological sequelae occur in up to 50% of the survivors and include mental retardation, cortical blindness, deafness, uncontrolled seizures, hydrocephalus, hearing loss and speech and language delay. In pregnant women GBS cause urinary tract infection, amnionitis, endometritis and wound infection (105). In nonpregnant adults infection is most commonly seen in patients with diabetes mellitus, cancer, and AIDS. In these people GBS can cause skin or soft tissue infection, bacteremia, genitourinary tract infection, and pneumonia. The fatality rate for GBS disease can be as high as 4% in newborns (106). Fatality rates are higher in adults older than 65 than in younger adults.

**Epidemiology and Transmission** GBS is a commensal of the genitourinary tract in 15-40% of adult women (107). It is the leading cause of serious bacterial infection in newborn babies. Infected newborns are exposed to the microorganism carried by their mothers either just prior to parturition or during the birth process. The risk of neonatal colonization correlates with the intensity of maternal colonization. GBS colonization rates in pregnant women have remained constant over the past decade (25-30%) (108, 109). When mothers were GBS culture positive at delivery, the vertical transmission rate (of GBS colonization of the newborn) is between 29-72% with a mean rate of 51% (100). If the maternal GBS culture is negative during labor, only 3% of infants become asymptotically colonized (110). Neonates are exposed to GBS either via GBS ascension through ruptured amniotic membranes, aspiration of contaminated amniotic fluid, or through exposure to colonized tissues while passing through the birth canal. Bacteria crossing intact amniotic membranes have also been reported to cause disease.
Organism acquisition during passage through birth canal is thought to cause late onset disease. Horizontal transmission from the environment occurs but is rare (110). GBS type Ia, III, and V are most prevalent in early-onset disease. Serotype III GBS are important because they account for 36% of early onset and 71% of late onset GBS infection (111).

The genetic epidemiology of disease-causing GBS has been studied by using Hind III restriction endonuclease digest patterns (RDPs) of chromosomal DNA. Bacteria have been classified into RDP type 1, 2, 3 for GBS type III and 1, 2, 3 and 4 for GBS type I (112). GBS strains causing early onset septicemia belong to RDP types Ia-3 and III-3. GBS strains of RDP type Ia-1 and III-1 have low capsular sialic acid levels and are susceptible to tetracycline. Half of the strains in the study causing meningitis belong to type III-2 or III-3. According to RDP typing, GBS type III shares the same clonal origins with some strains of type II and V (112).

Pathophysiology of Infection

GBS infection in newborns can lead to death and disability. In pregnant women, GBS causes clinical illness ranging from mild urinary tract infection to life-threatening sepsis and meningitis. Most invasive maternal infections are bloodstream infections, osteomyelitis, endocarditis and meningitis. Noninvasive syndromes in pregnancy include amnionitis, endometritis, wound infections fascitis and cellulitis. In nonpregnant adults invasive GBS disease starts with bacteremia. The skin is the next most common site of infection.

Newborns are easily infected by GBS because they are quantitatively and qualitatively deficient in host defenses. The interaction between bacteria and host leading
to the disease can be divided into steps, including: adherence to epithelial surfaces, invasion of epithelial and endothelial cells, direct injury to host tissues, avoidance of host immunological defenses, and induction of the sepsis syndrome.

**Adherence** Adherence to epithelial surfaces is considered the first step in pathogenesis. Adherence of GBS to epithelial cells plays an important role in colonization of the rectum and vagina, as well as in initiating invasive infections of newborns. GBS can adhere to multiple cell surface components through both protein and non-protein adhesins (ligands) on the GBS surface. Interactions through opsonin-mediated processes are also possible. An example of a nonprotein ligand in GBS adherence is lipoteichoic acid (LTA). LTA is an amphiphilic glycolipid polymer extending from bacterial cell wall. Enzymatic treatment of fetal epithelial cells with trypsin or periodate destroys LTA binding to cells, indicating the presence of a glycoprotein receptors on the fetal cells (113). A later study shows that the LTA may have cytotoxic functions as well (114). Pretreatment of GBS with protease can decrease their ability to attach to epithelial cells, implying that bacterial surface proteins are also important in bacterial-host adherence.

Adhesins can also bind the extracellular matrix (ECM), which is a macromolecular structure underlying epithelial and endothelial cells. ECM is composed of a variety glycoproteins: collagen, laminin, fibronectin and fibrinogen. Several interactions between GBS surface proteins and receptors in ECM have been reported to play roles in the adherence process. Laminin binding protein (Lmb) has been identified in GBS. Lmb is a putative lipoprotein, which has significant homology to group A
streptococcal Lra I protein family, which mediates bacterial-host cell attachment. Lmb protein is expressed by most GBS strains. Mutation in the lmb gene has been reported to reduce adherence to immobilized human laminin (115).

Two fibrinogen binding proteins, FbsA and FbsB, have been identified. The FbsA has a signal peptide, a repeat region and a C-terminal wall-anchoring region with an LPXTG motif. The repeat region is responsible for the fibrinogen binding. Deleting the fbsA gene causes a reduction in the binding activity to soluble fibrinogen, as well as adherence to and invasion of epithelial cells. Adherence, but not invasion, is partly restored by reintroducing the fbsA gene (116, 117).

GBS is reported to bind to cytokeratin 8, which is a constituent of plasma membranes (118). The bacterial protein ligands responsible for binding to cytokeratin 8 are not yet identified. Hydrophobicity of surface proteins also plays a role in mediating adherence. Protein surface antigens are highly hydrophobic whereas polysaccharide antigens containing sialic acid are highly hydrophilic. Hydrophobic strains adhere to epithelial cell better than hydrophilic strains. Treatment with neuraminidase enhances the surface hydrophobicity of hydrophilic GBS strain whereas the hydrophobic strain is unaffected by this treatment (119).

GBS expresses a cell-surface-associated protein A (CspA) encoded by cspA gene. Csp A contains a putative signal peptide and a cell-wall anchor motif (120). Sequence analysis indicates that it shares functional and structural domains of the cell envelope-associated protease (CEP) family. The 153 kDa CspA protein is a subtilisin-like extracellular serine protease homologous to streptococcal C5a peptidase and caseinases
of lactic acid bacteria, but it does not cleave C5a or casein. CspA cleaves fibrinogen. It degrades the lower α band of fibrinogen (120). The function of fibrinogen cleavage by CspA is not clear. It has been postulated that GBS may be exploiting the adhesive properties of a fibrin-like substance. Proteolysis of the N termini of the α and β chains of fibrinogen lead to polymerization of cleaved fibrinogen, forming fibrin, which is highly adhesive (120). Therefore the cleavage of fibrinogen by CspA may lead to fibrin-like substance formation. This may promote the aggregation of GBS or the coating of GBS with fibrin. It has been shown that cspA mutants are significantly more sensitive than wild-type strains to opsonophagocytic killing by human neutrophils in vitro (120).

**Invasion** The second step in pathogenesis is invasion of epithelial and endothelial cells. Organisms access the blood stream by direct invasion of the epithelial cell barrier. GBS can penetrate and survive within various tissues, including placental membranes, the alveoli and the brain microvascular endothelial cells (BMEC), the single-cell layer which constitutes the blood brain barrier (121). GBS invade more efficiently into pulmonary microvascular endothelial cells than endothelial cells of the pulmonary artery suggesting a tropism for particular endothelial surfaces (122, 123) and also implying that lung endothelium may be the original site of bacterial invasion in neonates. GBS has been shown to invade chorion and amnion epithelial cells in vitro (124). The chorion-amnion is a multilayered structure comprised of a monolayer of chorion epithelial cells that line the maternal side of the placenta. Amniotic fluid infections most often occur through a ruptured chorioamniotic membrane. It is also possible that bacteria go through intact placental membranes and then replicate in amniotic fluid. Exposure to high
bacterial concentrations probably leads to colonization of the lung and airways, and subsequent pneumonia, sepsis and meningitis. Even though GBS is considered an extracellular bacterium, it can survive in the host cell in the cytoplasm or in intracellular vacuoles (125). However, evidence of intracellular replication has yet to be reported. Active bacterial DNA, RNA, and protein synthesis appears to be necessary for invasion (126). The bacterial invasion mechanism also requires host microtubular and cytoskeletal elements. Invasion is inhibited in a dose-dependent manner by decreasing extracellular Ca$^{2+}$ level and by substances known to interfere with eukaryotic calcium regulatory systems (125). The result implies that GBS may invade cells by triggering calcium-dependent phagocytosis-like internalization mechanisms.

GBS surface proteins play significant roles in the invasion process. Surface proteins interact with host protein receptors in the ECM and on cells. Recently, it has been reported that the alpha ($\alpha$) C protein (ACP) on the surface of GBS mediates GBS entry into cervical epithelial cells and GBS translocation across layers of these cells (127). The ACP is characterized as one of the two proteins that constitute the C antigen. C antigen has been found in many strains except clinical type III isolates of GBS. Based on trypsin sensitivity, C antigen is composed of two proteins, a trypsin-resistant alpha-protein and a trypsin-sensitive betaprotein (the $\alpha$-C protein, ACP, and $\beta$-C protein). The ACP is unrelated to the $\beta$-C protein. ACP is grouped into the alpha like protein family (Alp). The members of the Alp family are ACP, Rib, R28 and Alp2 protein. Rib is identified from GBS type III (128), R28 is found on the surface of type V (129) and alp2 is identified in type V (130). ACP is composed of an N-terminal signal sequence
followed by a nonrepeated N-terminal region, a repeat region, a wall-anchored region with an LPXTG motif, a short hydrophobic region that may span the cellular membrane, and a charged C-terminal tail. It has been predicted that the repeats in Alp have structures relate to immunoglobulin (Ig) implying the possible function of Alp in recognition (ligand-host receptor recognition). ACP interacts with host cell glycosaminoglycan (GAG). The data indicate that ACP interacts with GAG and enters the cell cytosol by a mechanism that involves Rho GTPase-dependent actin rearrangement (127). The predicted 53 kDa Spb1 protein (surface protein of group B streptococcus-1) may be involved in the invasion process as well. Mutation in gene spb1 leads to less invasion in the HeLa cervical epithelial cell, and restoration of spb1 in trans corrected the defect (131). The other Fbs protein, FbsB (FbsA was discussed above in adherence), also promotes invasion into epithelial cells. Deletion of the fbsB gene severely impairs the invasion of mutant GBS into lung epithelial cells (132). C5a peptidase is a surface-associated serine protease that specifically cleaves the complement component C5a, a chemotaxin for polymorphonuclear leukocytes (PMN). C5a peptidase may play roles in both the invasion process and avoidance of the host immune system. All group A streptococcal (GAS) serotypes, GBS, as well as groups C and G streptococci of human origin produce the streptococcal C5a peptidase (SCP) (133). The protein is highly conserved across species. The SCP from GAS (ScpA) is 95 to 98 % (residue) identical to SCP from GBS (ScpB) (134). The ScpB promotes invasion by interacting with fibronectin. Mutations cause a reduction in epithelial cell invasion that can be fully restored by trans complementation with the cloned wild-type gene (135).
GBS capsular polysaccharide (CPS) may have a negative effect on the invasion process. The main function of CPS is generally considered to be prevention of phagocytosis by host immune cell. Wild-type encapsulated clinical isolates, an asialo and unencapsulated isogenic transposon mutants invade epithelial cells to different degrees (136). This observation indicates that capsular polysaccharide is not essential for invasion of respiratory epithelial cells. The isogenic strains of GBS, which lack capsular sialic acid or type III capsular polysaccharide, invade and injure human umbilical vein endothelial cells three-to five fold more than the wild-type strains (126). The capsule-deficient strains also invade lung endothelial cells more than wild-type strains (137). The results indicate that capsular polysaccharide attenuates invasion. The attachment and invasion processes are regulated by oxygen and bacterial growth phase (138), as is the production of CPS. It is possible that in the beginning of the process, bacteria produce less CPS to facilitate attachment and invasion, and then later switch to high production of CPS to mask the surface proteins preventing them from being recognized by antibody and to inhibit opsonophagocytosis.

**Host Injury** After invasion, bacteria proliferate. The proliferation is associated with injury to host tissues. However, proliferation is not essential for injury. Direct cell injury facilitates penetration and spread of the organisms. GBS products, including cytotoxins and enzymes, can directly mediate injury. GBS β-hemolysin has been shown to be directly cytotoxic to lung epithelial cells (139). It acts as pore-forming cytolysin (137). Nonhemolytic variants didn’t induce cell injury in this model, while hyperhemolytic variants derived by chemical or transposon mutagenesis are extremely
toxic; resulting in disruption of cytoplasmic and nuclear membranes, loss of microvillus architecture, and marked swelling of cytoplasm and organelles (139). GBS also produce several proteinases, which can degrade tissue. Cell-associated collagenase activity has been postulated. It has been shown that antibodies against collagenase from *Clostridium histolyticum* cross-react with cell-associated proteins produced by GBS, and inhibit GBS hydrolysis of a synthetic peptide collagen analog (140). GBS also contain hyaluronan lyase. This enzyme degrades extracellular hyaluronan, which is an important component of ECM and may promote persistent colonization of the vagina (141, 142). Neutrophils and macrophages have hyaluronan receptors, CD44. Thus this enzyme may inhibit their ability to interact with infected host cells. A GBS hyaluronate lyase has been identified, which shares 50.7% amino acid identity with hyaluronidases from *S. pneumoniae* but not from GAS and *Clostridium perfringens* (141).

**Avoidance of Immune System** To firmly establish infection, GBS must avoid immune clearance by both innate and acquired immune mechanisms. After penetration into lung tissue or the blood stream, an immunological response is recruited to clear the organism. The central elements of the response are neutrophils, although macrophages are also involved in bacterial clearance. Effective phagocytosis of GBS by neutrophils requires opsonization, requiring serotype-specific anti-GBS immunoglobulin, and both classical and alternative complement pathways. In order to avoid opsonophagocytosis GBS possess several factors that interfere with the phagocytosis mechanism. The main factor is the type-specific polysaccharide capsule. Sialic acid rich CPS inhibits complement alternative pathway activation, as seen in adult sera deficient in type-specific
anticapsular antibodies (143). Resistance of serotype III GBS to opsonophagocytosis is proportional to the sialic acid content of the capsular polysaccharide. Removal of sialic acid by treatment with neuraminidase or by transposon-insertional mutagenesis increases deposition of opsonic C3 (144). A GBS type III mutant strain, which is capsule-deficient, binds to C3b, the active form of C3, in greater amounts than the non-mutant parent strain. The parent strain binds predominantly to C3bi, the inactive form leading to reduction of C3b deposition on bacterial cell surface. The sialic acid rich capsule prevents C3b deposition, presumably through acquisition of Factor H (FH) from host plasma (144). Capsular sialic acid limits C5a production (145). As noted earlier, C5a is an important chemoattractant that recruits neutrophils and macrophages. The mechanism by which the capsule inhibits C5a production is yet to be defined. The ability of GBS to inhibit C5a production was observed in type III GBS, which have high capsular sialic acid. The C5a-ase might not be necessary for type III strains to cause invasive disease because the high sialic acid content inhibits C5a production.

Several GBS surface proteins are also involved in escaping host immunity. C5a peptidase functions both in invasion, as described above, and in immune avoidance. ScpA and ScpB cleave the human C5a molecule between residues His-67 and Lys-68, causing the release of a short C-terminal C5a fragment (135). An ScpB-negative mutant shows increased sensitivity to phagocytosis by immune cells in the presence of complement. GBS surface protein Alp (alpha-like protein, described earlier under bacterial invasion) also functions in avoiding the host immune system. The repeat regions of Alp (described earlier) affect immune escape by reducing the immune response to the
whole protein and in particular to the N-terminal region. The \( \beta \) component of the C protein can bind to IgA (146). This binding can interfere sterically with deposition of opsonically active complement protein C3 on the GBS surface. The \( \beta \) component of the C protein also binds to the soluble complement inhibitor factor H (fH) (147). The fH-binding sites on the \( \beta \) component consisted of discontinuous and partially homologous sequences. Therefore, GBS bind multiple sites on fH, making the association more stable. Binding of the GBS \( \beta \) component does not block active sites on fH. Thus active sites on fH are free to inhibit C3b deposition and opsonophagocytosis (147).

The lipoteichoic acid (LTA) has been shown to play a role in avoidance of the immune system. LTA mutant strains display an increased susceptibility to human defensins. Moreover, the mutant strains are less virulent than the wild-type (148). Killing mechanisms of phagocytic cells involve production of reactive oxygen metabolites (oxidative burst). The production of reactive oxygen intermediates including superoxide anion, hydrogen peroxide and hydroxyl radicals are toxic to GBS and to host cells, damaging DNA, RNA, protein and lipid. GBS has mechanisms to evade the oxidative burst by using enzyme Mn-cofactored superoxide dismutase (SodA) (149). The enzyme converts the superoxide anion to molecular oxygen and hydrogen peroxide, which can be metabolized by bacterial catalases and/or peroxidases. SodA mutants display an increase in susceptibility to killing by macrophages.

GBS has been shown to induce apoptosis in macrophages (150-152). GBS does not need to be in the macrophage cytoplasm to promote apoptosis. The apoptotic pathway initiated by GBS is dependent on host protein synthesis (but independent of caspase-1
and –3). Apoptosis of macrophages can decrease antigen presentation and inhibit the generation of immune responses.

**Induction of Sepsis Syndrome** The last stage in disease pathogenesis is induction of the sepsis syndrome. The sepsis syndrome arises from the body’s extreme attempts to eliminate the GBS. While attempting to clear the pathogen, overreaction of the immune system may cause damage to the host.

If the epithelial barrier and immunological clearance fail, GBS establish bacteremia in newborns. These infants may develop septic shock as consequence. Severe early-onset GBS disease is clinically different from septic shock syndrome resulting from infection with gram-negative bacteria. GBS sepsis syndrome includes systemic hypotension, persistent pulmonary hypertension, tissue hypoxemia and acidosis, temperature instability, neutropenia and multiple-organ system failure. GBS induce many cytokines. Predominant among these are Th1 and proinflammatory cytokines, and less Th2 cytokines (153, 154). GBS infection has been reported to stimulate TNF-α, Interleukin-1 (IL-1), IL-2, gamma-interferon, IL-6, IL-8, and IL-12 production. TNF is a key mediator, but other cytokines also play a role in the unique GBS sepsis syndrome (155).

GBS also induces TNF-α production from macrophages and monocytes. TNF-α release is relevant to the pathophysiology of neonatal sepsis. TNF-α is believed to play an important role in morbidity and mortality. It is a proinflammatory cytokine that produces fever, metabolic acidosis, capillary leak and cardiovascular collapse leading to systemic hypotension. TNF-α production is inhibited by monoclonal antibodies against
CR3 and CR4, which are receptors for C3 and C4 complement. Therefore the ability of plasma and serum to amplify TNF-α production in response to GBS is dependent on the activity of the complement pathway (156). It has been shown that group B polysaccharide and GBS peptidoglycan, which are cell wall components, induce greater production of TNF-α than CPS or LTA (156, 157). GBS induce TNF-α independently from CD14 (158). Studies have shown that heat-killed GBS type III induces host cell signal transduction that leads to activation of transcription factors NF-kB and AP-1 that can lead to TNF production. The MAPK (p38 mitogen-activated protein kinase) pathway is also involved in TNF-α production by GBS (155). Mutant strains lacking CPS can induce TNF-α production to the same degree as wide type strains (155).

GBS also induce production of other cytokines. The production of many of these cytokines follows TNF-α release (153). IL-1 may induce septic shock. Treatment with an IL-1 receptor antagonist can reduce the symptoms. GBS also induces nitric oxide production in respiratory epithelial cell leading to lung injury (159). The nitric oxide may also play an important role in meningitis because GBS induce a respiratory burst in the CNS. The resulting production of hydrogen peroxide and superoxide leads to CNS damage. GBS also induce human monocytes to express cyclooxygenase 2 (COX 2), which is involved in many inflammatory states (160).

IL-12 also may play a role in the induction of neonatal sepsis and clearance of organisms. Pretreatment with anti IL-12 increases lethality in a mouse model (161). Administration of recombinant IL-12 improves survival time and decreases GBS in blood.
GBS may induce membrane injury indirectly by alteration of host cell processes. The presence of GBS in the lower uterine cavity promotes oxygen radical-induced damage to adjacent fetal membranes.

Use of Antibiotics to Prevent and Treat GBS Infection

The Centers for Disease Control and Prevention (CDC) recommends screening pregnant women for Group B strep two to four weeks before labor by using the standard culture method. Women who have a positive GBS result will be given four hours of antibiotic treatment during labor (162).

GBS vertical transmission can be inhibited by ampicillin chemoprophylaxis administered at least 4 hours before delivery to achieve bactericidal effect. Giving penicillin G 4 hours before delivery (intrapartum antibiotics) is also effective (163). Penicillin administration to infants shortly after birth (postpartum prophylaxis) reduces early-onset GBS. Antibiotics used preferentially are ampicillin, clindamycin, gentamycin and penicillin. The combination of penicillin and gentamycin reduces the rate of GBS infection (164). The CDC recommends intravenous antibiotics during labor for women who have maternal risk factors (162). These risk factors include premature delivery, maternal fever, and prolonged rupture of the amniotic membranes. The use of vaginal disinfectants such as Chlorhexidine was investigated in Sweden. The result shows that vaginal disinfectants are less effective than antibiotic prophylaxis. However, the use of vaginal disinfectants leads to less resistance when compared to the use of antibiotics.

Penicillin is the drug of choice for known GBS infections. However, because symptoms and clinical suspicion often precede the definitive bacteriologic diagnosis,
empirical therapy for neonatal meningitis is established, often with a combination of ampicillin, a third generation cephalosporin that penetrates the CNS, and/or an aminoglycoside. General antibiotics are administered for the first 48-72 hours until species are identified, susceptibility testing is completed, and a clinical response is observed. Penicillin is then used for a total of 10 to 14 days (164). In neonates, the recovery rate is about 75-80% without sequelae and 5-10% with sequelae (165). Neonatal fatality rate after treatment is 5-7% (164). In adult infection with GBS and in late-onset neonatal disease, penicillin is also the drug of choice. The recovery rate (50-60%) is lower compared to the early onset disease (166).

Even though penicillin is the drug of choice for prophylaxis and treatment of GBS disease and resistance to penicillin has not been reported, other antibiotics such as macrolides have been recommended as secondary agents for the patients with penicillin allergy. It has been reported that clinical GBS isolates exhibit degrees of resistance to antibiotics such as erythromycin (18%), clindamycin (8%) and tetracycline (>80%) (167). Resistance mechanisms include ribosomal modification by methylase encoded by an *erm* gene and drug efflux by membrane-bound protein encoded by a *mef* gene.

Hypothermia has been proposed as an adjunctive treatment for severe bacterial meningitis in a rabbit model of severe GBS meningitis (164). The application of hypothermia initiated shortly after antibiotic therapy improves short-term physiologic measures associated with brain injury.
Protective Immunity to GBS

The absence of maternal antibody to GBS capsular antigens has been identified as an important factor in the pathogenesis of GBS infections in newborns (168). Presumably, transplacental transfer of maternal anti-GBS antibody results in sufficient immunity in the neonate to protect against the development of infection. The absence of maternal antibody in the face of vaginal colonization with GBS places the infant at highest risk. Why some women who carry GBS fail to mount an antibody response is still a matter of conjecture. Bacterial factors may play a role. Although host factors play an important role, it has been shown that women whose infants have had GBS infection are able to produce anti-GBS antibodies in response to immunization with GBS carbohydrate (169).

Lancefield originally demonstrated the protective efficacy of anti-GBS antibodies using rabbit antisera (170). There are eight serotypes of GBS. Lancefield established that protective efficacy is type specific (85). Antisera raised against type II or III GBS do not protect against infection with type I bacteria (171). These experiments also show that antibodies to group B determinants (i.e. expressed on all GBS) are not protective. Using monoclonal antibodies Pincus, et al. have confirmed these findings, and demonstrated that the protective epitope on the type III capsular polysaccharide (CPSIII) contains sialic acid (172-174). Monoclonal antibodies directed against non-sialic acid determinants on CPSIII are not protective (175). Although there have been reports that group specific antibodies can protect (176), most studies confirm Lancefield’s original observation. Studies of Pincus, and those of others, indicate that one reason for the failure of group specific antibodies to protect is that
there is limited expression of these epitopes on the surface of intact GBS (172). Protein antigens of GBS can also elicit protective antibodies (177, 178).

The role of CPS$_{III}$ in bacterial virulence has been studied, in part because the protective antibody response to the capsular antigen is so well characterized. Variants lacking the type III capsule have markedly reduced virulence (136, 144, 179). Transposon mutagenesis has demonstrated that sialylation of CPS$_{III}$ is critical for pathogenicity (179). The type III capsule is anti-complementary, inhibiting the activation of the alternate pathway of complement activation. Phase variants and transposon mutants lacking the capsule spontaneously activate complement. Sialic acid plays an important role in the ability of CPS$_{III}$ to inhibit complement deposition (145, 180). It has been postulated that the reason antibodies against sialic acid containing epitopes of CPS$_{III}$ are protective is that they may block this complement-inhibiting activity of CPS$_{III}$, thus facilitating C3 deposition, perhaps by both classical and alternate pathways, as well as interacting with Fc receptors on neutrophils (181).

Protective antibodies to CPS$_{III}$ appear to be directed at a single, repeating epitope containing sialic acid (181). This structure also appears to function as a virulence factor, in part because it is able to prevent activation and deposition of complement by the bacterial surface. Antibodies that bind to this target block the anti-complementary activity, as well as serve to opsonize GBS. The capsular polysaccharides of other GBS serotypes express similar critical epitopes against which protective antibody is directed.

GBS surface proteins play important roles during different stages of an infection. Several GBS surface proteins have been purified and characterized. Some of them have been shown to induce protective antibody against GBS. The first surface protein antigen
identified in GBS was the C protein, which we have described earlier. Lancefield et al. have shown that antibodies to the C antigen are protective against GBS in a mouse model (182, 183). All of the alpha-like proteins (Alps) confer protective immunity against GBS in animal models (184, 185). With the exception of ACP, biological functions of these proteins still are yet to be defined. It has been shown that immunization with ScpB induces protective antibody in mice (186). The 45 kDa Sip protein has a wall-anchoring motif and induces protective immunity against lethal infection with GBS (187). It is interesting that Sip from all nine GBS serotypes is highly conserved; implying Sip could be a good candidate for vaccine development. The function of Sip protein is not known. Another surface protein, which has been shown to elicit a protective antibody, is R antigen. Like C antigen, the R antigen is immunogenic on intact GBS. The biological function of R antigen is still not known. Antibodies raised against a R antigen protect mice against infection with R-antigen positive strain of GBS, but not from R negative strains (188, 189).

Development of a GBS Vaccine

Protective immunity of GBS can be achieved by maternal antibodies to the CPS of GBS, which is transferred through placenta. Like other bacterial CPS, GBS CPS is a poor immunogen and acts as a T-independent antigen resulting in IgM production instead of IgG and lack of immunological memory. An initial vaccine consisting of CPS\textsubscript{III} underwent both animal and then human testing (190). Although the vaccine elicited antibodies in only 60% of pregnant women immunized, the passive transfer of anti-GBS antibodies to the neonate was shown, thus demonstrating the feasibility of maternal
immunization (191). Due to the low immunogenicity of the purified CPS, alternative approaches are being examined.

One strategy to improve immunogenicity of the CPS is to couple it to carrier proteins, to obtain a CPS-protein conjugate vaccine. Different coupling strategies and protein carriers have been proposed. Tetanus toxoid (TT), cholera toxin B subunit, and GBS proteins have been used as protein carriers. Tetanus toxoid has been most thoroughly evaluated. Coupling procedures include binding cyanogen bromide-activated CPS to TT via the spacer adipic acid dihydrazide (192, 193). Another strategy is to conjugate CPS to TT without linkers (194, 195). GBS type III CPS is treated with periodate oxidation method to form free aldehyde groups on the sialic acid side chain. Then the treated polysaccharide is coupled with TT via the aldehyde groups.

TT-CPS conjugates have been tested in animals and in humans. The ability of CPSIII-TT, administered to the mother, to protect GBS-infected neonates was demonstrated in animals (193, 196). Subsequent clinical trials of this vaccine have demonstrated an opsonic antibody response in approximately 90% of recipients (195). At the same time, experimental studies conjugating the CPS of other GBS serotypes to tetanus toxoid have been performed. The protective efficacy of a tetravalent (types Ia, Ib, II, and III, which account for >90% of clinical infections) polysaccharide-tetanus toxoid conjugate vaccine has been demonstrated in animals (197, 198). The conjugate vaccine produces predominantly IgG1, in a mouse model, implying a Th2 bias of the vaccine. Prior exposure to GBS and TT can affect the antibody response (195).
Since colonization of genital and lower intestinal tracts is critical in GBS transmission, effective mucosal immunity may play an important role in vaccine-mediated protection by limiting GBS colonization. IgA is the main protective antibody in mucosal immunity. CPS_{III} was coupled to cholera toxin B subunit (CTB) by reductive amination (199). The conjugated vaccine was administered to mice. Immunization elicits both mucosal and systemic immune responses with high IgA, and IgG antibody against CPS and GBS in sera and at mucosal sites. The immunization route affects the immune response obtained: intranasal immunization elicits the highest level of IgA and IgG in the lungs, peroral immunization leads to highest antibody levels in the intestine, vaginal vaccination generates highest antibody levels in vagina, and rectal administration produces the highest response in the rectum (200, 201).

A second approach to generate a protective anti-GBS antibody response is by using GBS antigenic surface proteins, which are expressed on most or all GBS serotypes, as vaccine candidates. GBS surface antigenic proteins were identified and tested for ability to elicit protective antibody against GBS, as discussed above. Surface antigenic proteins, ACP protein, β-C protein, Sip and Rib have been tested in animals as potential candidate antigens for a vaccine (128, 187, 202, 203). All of them confer protective antibody against GBS, and the sera from vaccinated animals protecta mice from GBS lethal challenge. In an effort to elicit the greatest degree of protection by using multiple GBS antigens, vaccines have been designed in which the CPS is conjugated to a GBS protein. The alpha-C protein is conjugated to CPS_{III} using the reductive amination method (202). Low titers of antibody to type III GBS are elicited. Adjuvant is required
for immunization. Compared to GBS-TT vaccine, these proteins provide less impressive protection. Moreover, some proteins are not expressed in all clinical isolates. For example, beta-C protein is expressed in type Ia and Ib, but not in III. Even though Rib and Sib are more abundantly expressed in clinically significant strains, not all of them contain these proteins. Generally GBS, which do not contain Rib, do express alpha-C protein. The ScpB protein, found in all clinical GBS isolates is also believed to be good candidate for vaccine development. Immunization with purified ScpB increases the bacterial clearance in lung (204).

**Mimotopes**

**Antigen**

An antigen is a substance that can be bound specifically by an antibody, T-cell receptor, or other specific immune recognition molecule. In this thesis, we will confine our discussion of antigenicity to recognition by antibody. Some antigens can induce antibody production; some do not. An antigen, which is able to induce an immune response, is called an immunogen. Thus not all antigens are immunogenic, but all immunogens are antigenic. For example, a hapten is a small molecular weight molecule that when bound to a carrier protein can elicit antibodies that bind to the hapten. Thus the hapten alone is antigenic, that is it can be bound by antibody, but not immunogenic. When coupled to a carrier it is both. Immunogenicity results from four properties of the immunogen: foreignness, molecular size, chemical composition, and susceptibility to antigen processing and presentation. An immunogen has to be recognized as nonself by
the immune system. The best immunogens generally have molecular weights greater than 25 kDa, molecules less than 5 kDa have poor immunogenicity (205). Antigens with high complexity in chemical composition generally have high immunogenicity. Proteins are good immunogens, simple carbohydrates with repeated chains are poorly immunogenic, as are lipids and DNA. Molecules that are not degraded or processed by antigen presenting cells are poorly or not at all immunogenic, for example plastics.

**Epitope**

Antibody binds to specific sites on the antigen, which are referred to as antigenic determinants or epitopes. Epitopes can be bound directly by antibodies, which are also the antigen receptors on B lymphocytes. B cells and T cells often recognize different epitopes on the same molecule. The T-cell receptor does not recognize native antigen as do B cells, but recognizes antigen that has been processed into antigenic peptides and presented in association with MHC molecules. Therefore destruction of the conformation of a protein does not affect its T cell epitopes. Antigens recognized by T cells must possess two different interaction sites: the epitope, which interact with the T cell receptors, and the agretope, which interacts with an MHC molecule.

Properties of epitopes for antibody/B cells include accessibility of the epitope, hydrophilicity, and structural mobility. Antigenic sites should be accessible on the surface of the molecule to be recognized by antibodies binding to the native protein. In general the native conformation of proteins in aqueous environments is to fold with the hydrophobic residues buried in the interior and hydrophilic residues exposed on the
Segmental mobility of the polypeptide backbone also contributes to antigenicity.

**Antigen-Antibody Interaction**

Detailed structural analyses of the interactions between epitopes and antibodies have been reported. Here we will discuss the interaction of Fab antibody fragments to protein antigens: hen egg white lysozyme (HEL) (207-210), influenza virus neuraminidase (211, 212) and angiotensin II (213). Interactions of several of these antibodies with anti-idiotypic antibodies have also been studied and compared to the interaction with antigen (214, 215). B cell epitopes can contain sequential or nonsequential amino acids. In the latter case, the native protein is folded to bring the amino acids into close juxtaposition.

The interaction between antigen and antibody is influenced by the structure of the antigen, as well as the sequence of the portions of the antibody that make contact with the antigen, the so-called complementarity determining regions (CDRs). Angiotensin II is a small antigen, folded into a compact structure that interacts with the antibody within a deep and narrow cleft (216). Epitopes of globular proteins, such as HEL interact with antibody on the surface of the protein and in a very shallow binding cleft (209). The binding interaction between antibody and antigen requires good complementarity between proteins and reasonable juxtapositions of polar residues to permit hydrogen bond formation. Studies of antigen/antibody binding in HEL reveal water molecules in cavities between the two that provide a bridge for hydrogen bonds (207, 209). Two monoclonal antibodies (mAb) specific for the influenza neuraminidase, NC41 and NC10, have been
studied (211, 212). The neuraminidase epitope for NC10 overlaps that for NC41. These two mAbs bind differently to antigen. Five of the six CDRs of NC41 make contact with antigen while four CDRs of NC10 make contact. Thus antibody/antigen interactions reflect both structures (212).

Anti-Idiotype Antibody (Anti-Id)

The amino acid variability of the antibody CDRs allows them to function as antigenic determinants that can in turn lead to the production of new antibodies, termed anti-idiotypic antibodies (anti-Id). The interactions between the anti-lysozyme antibody D1.3 (Ab1) and its anti-Id (Ab2) and its antigen HEL were studied (215). The anti-Id antibody binds to 13 amino acids on D1.3 mainly from the CDRs; whereas seven amino acids make contact with the nominal antigen, HEL. The main chain conformation of the D1.3 CDRs in the anti-Id Ab complex shows significant differences from the complex with HEL, especially the side chains of the CDRs. Thus the conformation of the antibody binding site is influenced by the antigen it binds. As discussed below, anti-idiotypic antibodies have been used as mimics of antigen, since both bind the same antibody. In this case it has been said that the anti-idiotype carries the internal image of the antigen, although as the HEL data show, this internal image is not perfect (215, 217). An example of internal image mimicry is seen in the angiotensin system. The anti-anti-Id (Ab3) binds strongly to the antigen angiotensin (218, 219). Crystallographic studies show that when complexed with Ab3, the angiotensin adopts a conformation that resembles the CDR3 of a light chain (220). This suggests a possible mechanism for the interactions; in which a
CDR of Ab2, presumably CDR3 of the light chain, would carry a similar conformation to that of the angiotensin

**Polysaccharide Antigen**

The repeating polymeric structures of polysaccharide and DNA are poor immunogens. They function as T-independent antigens eliciting primarily low affinity IgM antibodies and failing to induce immunologic memory. Cross reactions among these structures demonstrate how similar epitopes, having different chemical structures on very different molecules can mimic each other. Capsular polysaccharide from group B meningococcus and *Escherichia coli* K1 are poorly immunogenic (221-224). The α(2→8)-linked N-acetyl neuraminic acid (Neu Nac) polymer, which is found in the *E. coli* K1, *Klebsiella*, and group B meningococcus CPS component can be bound specifically by monoclonal IgM antibody (NOV) (223, 224). The antibody is protective against *E. coli* in newborn rats. The mAb also cross-reacts with polynucleotides and denatured DNA. The cross-reactions might result from a similar distribution of negative charges between the carboxyl group of α(2→8)Neu Nac and the phosphates of the denatured DNA or polynucleotides (224). This finding suggests that the distribution of negative charges on certain antigens is the basis of cross reactivity in this system. More generally, it suggests that antibody cross reactivity is based on structural similarities between antigens. The amino acid sequence of IgM[^NOV] V<sub>H</sub> is 106 residues and belongs to subgroup V<sub>B</sub> III, and V<sub>L</sub> is 109 residues and belongs to subgroup II (222).

Antigenic cross-reactions could lead to disease. A human anti-DNA monoclonal autoantibody, 16/6, is found to share an idiotypic determinant with antibody from patients
with active systemic lupus erythematosus (SLE), and with other anti-DNA mAb from unrelated patients (225). Amino acid analysis reveals that first 40 light chain amino terminal residues are identical among the antibodies sharing this idiomotype. Six mAb from patients with Waldenstrom’s macroglobulinemia that react with *Klebsiella pneumoniae* polysaccharides were tested for their ability to bind to nucleic acid antigens (225). The study shows that one of these IgMs bound to polynucleotides and to single stranded DNA. This antibody is also the only one to share this idiomotype. The binding between this IgM and the polynucleotides is inhibited by *Klebsiella* polysaccharide 30 (K30). The 16/6 mAb is found to react weakly with the K30. The results indicate that anti-polysaccharide antibodies share an idiomorphic determinant with anti-nucleic acid antibodies and immunoglobulin from SLE and Waldenstrom’s macroglobulinemia, a cancer of IgM-secreting plasma cells.

Dextran is a polysaccharide consisting of repeating d-glucose residues. It has also been shown to be a T-independent antigen. In classic experiments that defined the size of the antigen-combining site of immunoglobulin, the combining sites of antibodies to \( \alpha(1\rightarrow6) \) dextran are shown to have an upper size limit of six to seven oligosaccharide glucose residues (226). The lower limit, based on the ability of the antibody to cross-react in precipitin tests with isomaltose oligosaccharide coupled to BSA, is between one and two glucoses (226, 227). Synthetic glycolipids prepared by coupling isomaltose oligosaccharide to steary-lamine are T-independent antigens in mice as well (228).
**Epitope Mapping**

The nature of peptide and protein antigenic determinants has also been studied and has led to useful approaches to making specific antisera, to designing vaccines, and for mapping the antibody response to protein antigens. A key concept underlying much of this work is that a short peptide can serve as a surrogate for the epitope on the intact protein. Using epitope mapping techniques, both continuous and discontinuous peptide epitopes have been defined (229, 230). In continuous epitopes, the amino acids encoding the antigenic determinant are adjacent to each other in the primary protein structure. Discontinuous epitopes have amino acids that are brought together by the folding of the native protein. Denaturation may destroy discontinuous epitopes, and monoclonal antibodies recognizing such epitopes will not generally function in SDS-PAGE immunoblotting techniques (Western blots) that require protein denaturation.

In the epitope mapping procedure for continuous epitopes, overlapping peptides covering the length of a protein are designed and tested for antibody binding. For example, a total of 208 overlapping hexapeptides covering the total 231 amino acid sequence of the immunologically important coat protein VP1 of foot and mouth disease virus (FMDV), type O, were synthesized on a solid phase (230). The attached peptides were tested for antigenicity by an ELISA using a variety of antisera. The result indicates that an immunodominant epitope is located between amino acids 146-152 of that protein. Then 120 hexapeptides representing complete single point amino acid replacement sets were synthesized and tested for retention of antigenicity. The result indicates that leucine
residues at position 148 and 151 were essential for binding of antibodies in antisera raised against FMDV (230).

An example of a discontinuous epitope was identified with a mAb raised against FMDV type A$_{10}$ (229). The peptides mimicking the epitope were identified. No sequence homology was found in capsid protein VP1 of FMDV type A$_{10}$. The result suggests that protein folding, in which amino acid residues from three regions distant from one another in the primary sequence, are brought into close proximity at this epitope.

Mimotope

Identifying peptides that represent epitopes suggested the mimotope concept, in which molecules that mimic the structure of antigenic determinants are constructed and tested for antigenicity and for the ability to elicit cross-reactive antibodies to the original antigen. Just as many pharmacologically active drugs are considered to be mimics of the natural ligands for protein receptors, mimotopes are mimics of antigenic determinants for protein antibodies. Because the universe of peptide mimotopes could not readily be constructed from the primary amino acid sequence of a protein antigen, the attempt to identify mimotopes requires large libraries of peptides, particularly of discontinuous epitopes. This has been accomplished through combinatorial chemistry and through genetic techniques such as phage display.

An alternate approach to designing mimotopes for antibodies is to use the resemblance of anti-idiotype (anti-Id) to antigen. The idiotype (Id) of an antibody is that part which is antigenically unique as determined by a second set of antibodies made against the first one. Thus idiotypes are in the variable region in, at, or near the
combining site. Thus the combining site defines both antigen specificity and the idiotype of an antibody. In 1964, Jerne proposed that the immune system is regulated by networks of anti-idiotypic antibodies acting as a self-contained homeostatic network (231). According to this hypothesis, the Id-anti-Id interactions regulate the immune response of a host to a given antigen. The Id regulatory mechanism influences the prevalence of different combining sites and antibody specificity. Based upon the structural studies we have previously described, the anti-idiotypic may resemble the antigen. According to the network concept, immunization with a given antigen will generate the production of Ab1 against the antigen. When sufficient Ab1 is produced, it then induces an Ab2 (anti-Id) against its idiotype. Some of these Ab2 can mimic the structures of external antigens. Thus by manipulating the idiotypic network it may be possible to elicit antibodies that cross react with the original immunogen. In this case the anti-Id is said to contain the internal image of the antigen. Immunization with anti-Id Ab2 generates anti-anti-Id Ab3, which recognizes the original antigen. However, there may be differences in the structure of Ab3 and Ab1. Based upon the ability to mimic the internal image of antigen, anti-idiotypic immunization has been studied extensively for vaccines against cancer (232) and infectious agents. Anti-Ids elicit antibodies to microbial antigens, including proteins, LPS, and capsular polysaccharides (233-235). More about the use of anti-idiotypes to mimic microbial polysaccharides is detailed below.

Combinatorial protein-display libraries permit the examination of an extremely large number of possible sequences (libraries), having as many as $10^9$ individual members. This may be done biologically using genetic approaches or chemically through
directed synthesis. Biological approaches include displaying peptide sequences on phages, microbes, and even eukaryotic cells. Combinatorial design provides a complementary approach for understanding sequence and structure compatibility and discovering novel sequences that have a specific structure. In addition to creating a library with a high degree of diversity, the combinatorial approach provides materials which can be assayed for structure (e.g. sequence if protein) or function. Synthetic combinatorial libraries are not restricted to proteins that can be made by living organisms, but rather may be made with D-amino acids, carbohydrates, novel molecules (e.g. dendrimers), and so forth. Thus synthetic libraries cover a larger portion of chemical space (the range of chemical compounds that can have biological effects) than do biological expression systems. Moreover they may be used to select for materials that function in non-natural environments that would kill the biological vector. However, in our studies we have used phage display technology because of the ready availability of peptide-display libraries (e.g. Burritt (236), Smith (237)).

**Phage Display Technology**

Based on the mimotope concept, phage-displayed technology was introduced by Smith (238). The construction of random peptide or gene fragment display libraries allows researchers to map contact points in protein-protein interactions. The technology depends upon an inserted random mutation at an appropriate location and the expression of the mutated sequence on the viral coat protein of viable phages.

The filamentous bacteriophage M13 and its derivatives have often been used as library vehicles. The phage consists of a stretched-out loop of single-stranded DNA
sheathed in a tube composed of several thousand copies of the major coat protein pVIII. At the tips of the virion, pIII and pVI minor coat proteins are involved in host-cell binding and in termination of the assembly process. Genes II and X encode replication proteins. Gene V encodes a single-stranded DNA binding protein. Gene IX encodes amino coat protein responsible for assembling pIII in the viral coat. Genes I and IV encode morphogenetic proteins. The three structural proteins, pIII, pVI and pVIII have been used for insertion of foreign sequences in the peptide libraries (237, 239). PIII is most commonly used, followed by pVIII, and very rarely pVI. Proteins pVIII are expressed at very high levels (2700 copies/phage), whereas protein pIII is expressed in only three to five copies/phage. Because pVIII represents the majority of the virion, marked constraints are placed on the insertions that may be placed in this protein. Either the length of the insert or the number of copies of the pVIII expressing the mutant protein must be limited. Insertion of greater than nine amino acid residues abolishes the viral assembly process (239). The incorporation of peptides in the pVIII protein without significant reduction in viral assembly requires either co-infection of the host *E. coli* with a helper phage, which encodes a wild type pVIII protein or the expression of wild-type pVIII protein on a plasmid in the infected cell (240).

The pIII protein is less critical in viral assembly and still binds to the phage receptor even with large inserts. The pIII can bear fusion proteins containing several hundreds residues at or near the amino terminus without significant loss of function. The expression of foreign peptides on pIII proteins has a minimal effect on the infectivity or assembly of virions (237, 239).
The assembly process of phages requires interaction between their structural proteins and two extra non-structural proteins (pI and pIV) and also host \((E. coli)\) proteins (241). Displayed proteins fused to the amino-terminus of pIII and pVIII fold in the periplasm before incorporating into the phage coat. The displayed protein, which can contain cysteine residues, may form disulfide bonds preventing incorporation of coat protein into the viral particle. Study of “random” insertions into pVIII indicates a bias for certain peptides, presumably those with a suitable structural conformation (242, 243).

M13 phages infect \(E. coli\) through fertility pili (F factor). They attach to pili prior to infection. The model for initiation of infection has been studied (244). The extreme amino-terminal end of pIII (g3p-D1) is pulled off the next upstream domain (g3p-D2) by interaction with the bacterial cell surface protein TolA. The interaction allows for viral disassembly and pilus retraction. Amino-terminal inserts on pIII do not greatly affect this process.

Construction of a phage-display library requires manipulation of the double-stranded replicative form (RF) of the vector. The RF can be harvested in the same manner as plasmids. The DNA library is inserted into the vector and then complete RFs with the DNA insertions are electroporated into competent \(E. coli\) cells. Peptide sequences to be displayed are encoded by the insertions into the genes encoding the phage coat proteins. These may be random sequences, DNA obtained from sources that one is attempting to mimic (e.g. epitope libraries constructed from genomes of pathogenic microbes), or mixtures of constrained and random sequences. Diversity in the library can be estimated from the number of transfection events following the introduction of the recombinant
vector DNA into host cells. The estimation is based on the assumption that few displayed-peptides are represented more than once.

Antibody has been used to select display libraries in attempts to identify antigenic determinants recognized by these antibodies. Initially this was restricted to identifying peptides bound by anti-protein antibodies. These approaches are able to define not just linear epitopes, but also conformational epitopes. The results demonstrate immunodominant regions of proteins, and also identify potential vaccine antigens. Interestingly, phage display has also been used to identify peptides that mimic carbohydrate and other non-protein structures.

Peptide Mimicry of Carbohydrate Structure

Although it may seem odd to seek peptides that mimic carbohydrates, naturally occurring carbohydrate-mimetic peptides can be found. For example, the tendamistat and other proteins that function as inhibitors of α–amylase have WRY tripeptide sequences that bind in the enzyme’s active site (245, 246), whose “natural” substrate is the carbohydrate amylose. Phage display technology has been used to identify peptide ligands for the carbohydrate-binding protein concanavalin A (Con A) (247). Peptides with a consensus sequence, YPY, bind Con A with affinity comparable to the carbohydrate ligand. The mimetic peptide has been shown to competitively inhibit carbohydrate-specific binding.

Initial studies that demonstrated peptide mimicry of microbial polysaccharides were based upon the anti-idiotypic approach. Westerink et al. developed a monoclonal anti-Id that contains the internal image on its combining site, which mimics
meningococcal group C capsular polysaccharide (248, 249). The anti-Id elicits a T-dependent antibody response in mice directed toward CPS of meningococcal group C. The sequences of the variable regions of heavy and light chains of the anti-Id reveal that the CDR 3 is unique, in that the sequence tract YRY is exposed on the surface. Immunizing mice with the mimetic peptide results in significant anti-meningococcal group C antibody response. Immunized mice are protected against infection with a lethal dose of bacteria. On the basis of these studies, other groups, including our own, have used a variety of methods to elicit antibodies to microbial carbohydrate and LPS antigens. These are summarized in table 1.3.

Epitope mapping techniques and sequence analysis from anti-Id and phage-displayed libraries demonstrate that antibodies bind to epitopes of three to five amino acids within a sequence. The fine specificity of some carbohydrate-binding proteins maps to peptides containing central W/YXY residues. Peptides with the aromatic motif W/YXY are defined to mimic several carbohydrate subunits. These include YPY as a mimic of mannose (ConA (247)), WRY found to mimic α(1→4) glucose, WLY (amylase inhibitor (245) which mimics Lewis Y (250), and YRY as a mimic of the major C polysaccharide α(2-9) sialic acid of Neisseria meningitidis(251). A protective antibody against Cryptococcus neoformans glucuronoxylomanna was selected that displays a binding motif of TPXW(M/L)(M/L) (252). The peptide doesn’t interact with non-protective anti carbohydrate antibodies to Cryptococcus which indicates both the specificity of selected peptides and the potential for peptide structures to differentiate between protective and nonprotective antibodies (253).
<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Mimetic</th>
<th>Comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-D-Mannopyranoside</td>
<td>Peptide from phage-displayed peptide library</td>
<td>Similar consensus sequence obtained independently by two groups</td>
<td>(242)</td>
</tr>
<tr>
<td>Meningococcal group C polysaccharide</td>
<td>Anti-idiotype and phage-displayed peptide library</td>
<td>Elicits protective antibody when used anti-Id and peptide derived from anti-Id mAb as an immunogen</td>
<td>(248, 249)</td>
</tr>
<tr>
<td>Siayl Lewis X antigen</td>
<td>Peptide from phage-displayed peptide library</td>
<td>Peptide is a specific inhibitor of neutrophil adhesion</td>
<td>(254)</td>
</tr>
<tr>
<td>Group A streptococcal polysaccharide</td>
<td>Anti-idiotype</td>
<td>Elicits polysaccharide cross-reactive antibody when used as an immunogen</td>
<td>(234, 255)</td>
</tr>
<tr>
<td>Group B streptococcal polysaccharide type III</td>
<td>ScFv antibody (from anti-idiotype)</td>
<td>Elicits protective antibody when used as an immunogen</td>
<td>(256)</td>
</tr>
<tr>
<td>Group B streptococcal polysaccharide type III</td>
<td>Peptide from phage-displayed peptide library</td>
<td>Elicits polysaccharide cross-reactive antibody when used as an immunogen</td>
<td>(1)</td>
</tr>
<tr>
<td>Cryptococcal glucuronoxylomannan</td>
<td>Peptide from phage-displayed peptide library</td>
<td>Peptides are good antigenic mimetics but poor immunogenic mimetics</td>
<td>(252, 257-259)</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> lipooligosaccharide</td>
<td>Anti-idiotype</td>
<td>Elicits protective antibody when used as an immunogen</td>
<td>(260)</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em> group A lipooligosaccharide</td>
<td>Peptide from phage-displayed peptide library</td>
<td>Elicits protective antibody when used as an immunogen and it is preliminary evidence of immunogenic mimicry</td>
<td>(261)</td>
</tr>
<tr>
<td><em>Neisseria gonorrhoeae</em> lipooligosaccharide</td>
<td>Anti-idiotype</td>
<td>Elicits anti-lipooligosaccharide cross-reactive antibodies</td>
<td>(262)</td>
</tr>
<tr>
<td>Adenocarcinoma-associated carbohydrate antigens (Lewis Y)</td>
<td>Peptide from phage-displayed peptide library</td>
<td>Elicits antibody responses that cross-react with human tumor-associated carbohydrate antigen.</td>
<td>(263, 264)</td>
</tr>
<tr>
<td>HIV carbohydrate antigens</td>
<td>Peptide from phage-displayed peptide library</td>
<td>Elicits carbohydrate cross-reactive antibodies</td>
<td>(265, 266)</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>Anti-idiotype</td>
<td>Elicits protective antibody when used as an immunogen</td>
<td>(235)</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em> capsular polysaccharide</td>
<td>Peptide from phage-displayed peptide library</td>
<td>Elicits protective antibody when used as an immunogen</td>
<td>(267, 268)</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>Mimetic</td>
<td>Comment</td>
<td>Reference</td>
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<tr>
<td><em>Haemophilus influenza</em> lipooligsaccharide</td>
<td>Peptide from phage-displayed peptide library</td>
<td>Elicits protective antibody when used as an immunogen</td>
<td>(269)</td>
</tr>
<tr>
<td><em>Brucella</em> lipopolysaccharide</td>
<td>Peptide from phage-displayed peptide library</td>
<td>Elicits weak immune response</td>
<td>(270)</td>
</tr>
<tr>
<td>P-selectin</td>
<td>Peptide from phage-displayed peptide library</td>
<td>Peptide inhibits the binding of glycosid ligand for P-selectin.</td>
<td>(271)</td>
</tr>
<tr>
<td>Gal-α1,3-Gal</td>
<td>Peptide from phage-displayed peptide library</td>
<td>Melibiose was able to inhibit the binding of the human natural anti-αGal antibody to the peptide competitively</td>
<td>(272)</td>
</tr>
<tr>
<td>Glycosphingolipids</td>
<td>Peptide from phage-displayed peptide library</td>
<td>Peptide inhibits β–galactosidase activity towards lactotetraosylceramide.</td>
<td>(273)</td>
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<tr>
<td>Lipopolysaccharide</td>
<td>Peptide from phage-displayed peptide library</td>
<td>Biopanning on LPS-conjugated epoxy beads was used to identify peptides</td>
<td>(274)</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> serovar Typhi</td>
<td>Peptide from phage-displayed peptide library</td>
<td>Biding activity between mAb to the mimotope was blocked by CPS Vi antigen of the bacteria.</td>
<td>(275)</td>
</tr>
<tr>
<td><em>Shigella flexnerii</em> LPS</td>
<td>Peptide from phage-displayed peptide library</td>
<td>Peptide can block the binding activity between the bacterial LPS and mAb</td>
<td>(276)</td>
</tr>
</tbody>
</table>

In conclusion, phage-displayed technology has proven to be a valuable multipurpose tool with which to probe protein-ligand interactions. Phage libraries are a rich source of unexpected functional diversity. Because of the very large library sizes that can be achieved, it is tempting to maximize the number of varied positions and then carry out many rounds of selection in order to increase the chance of identifying a rare, highly functional clone. It also provides an economical alternative way to identify mimic peptides. The technology requires only basic understanding of genetics to carry out
experiments. These reasons make the phage technology grow explosively. It has become an indispensable tool for protein engineering. There will be further improvements in library construction such as vector design and selection methodology that will further enhance phage display technology.

Peptide Mimics of the Capsular Polysaccharide of Group B Streptococcus

In the Pincus lab, we have used a highly protective mAb designated S9 to select a phage-displayed peptide library for peptides that mimic the GBS type III CPS (1). The S9 mAb is highly protective against experimental infection. With S9 treatment, animals survive an otherwise lethal bacterial infection. In isolating phage that bind S9, two peptides have been identified, depending on the desorption conditions. With acidic desorption, peptide WENWMMGNA was identified, and with the basic desorption, peptide, FDTGAFDPDWPA. ELISA results demonstrate that these peptides bind to S9 and no other antibodies tested. Phage blocks the binding of S9 to type III GBS, but does not block the binding of a group specific mab to GBS. Phage displaying peptide FDTGAFDPDWPA shows a greater inhibition of GBS binding than WENWMMGNA. Antibody S9, other type-specific mAbs, and polyclonal anti-GBS type III antisera bind the synthetic peptide FDTGAFDPDWPA. The FDTGAFDPDWPA is named peptide S9 after the antibody S9, which is used for its selection. The binding of S9 to GBS is inhibited by free S9 peptide with an IC50 of 30 ug/ml. The CPS and intact GBS could block the binding activity between S9 peptide and polyclonal anti-GBS antibodies. The result indicates that the S9 peptide bears antigenic properties of GBS type III capsular
polysaccharide. A conjugate S9 peptide with carrier proteins (KLH, BSA or OVA) induces significant antibody responses against both GBS and purified CPS after a single immunization. The results indicate that this peptide mimetic of the GBS capsular polysaccharide type III is both antigenic and immunogenic.

To investigate the origins of peptide-carbohydrate mimicry, the conformational preferences of peptides that mimic the GBS type III CPS have been investigated by NMR spectroscopy (277). The NMR spectra of the peptide FDTGAFDPDWPA reveal that the peptide exists in two isoforms due to cis-trans isomerization. The ratio of trans to cis isomer is estimated to be 7:3. For the trans-isomer, a type I $\beta$-turn is deleted from residues Asp-7 through Trp-10. The type I $\beta$-turn is not represented in the population of structures of the cis isoform. The S9 mAb also binds at - Asp-7 through Trp-10. Two new peptides were synthesized, the peptide 1A, FDTGAFDPDWPYD, and peptide 1B, FDTGAFDPDWPY. The Ala-12 was replaced by a tyrosine residue in both analogues. An extra Asp residue was incorporated at the C-terminus of the 1A sequence to test the possibility that the negative charge imparted by the Asp residue might mimic the negative charge of the sialic acid component of the original peptide. The peptide analogues were tested for biological activity and investigated by NMR. Peptide 1A is shown to be able to compete with the CPS approximately twice as effectively as either the original peptide or peptide 1B. Thus, the addition of an Asp residue at the C-terminus appears to increase the relative avidity of the antibody for the peptide. NMR study reveals that peptide 1A, like the original peptide, has the propensity to form type I $\beta$-turns in solution.
In this thesis I further investigate the structural basis of the peptide mimicry of the GBS type III CPS carbohydrate epitope. We designed a sublibrary, expressing a mutated version of the S9 peptide with 3% random nucleic acids at each position of the original S9 nucleotide sequence, creating approximately one mutation in each inserted sequence. Peptides with higher affinity to the S9 mAb were identified by affinity selection. From this sublibrary, we also explored effects of the amino acid changes in the antigenic S9 peptide. The relationship of antigenicity and amino acid in each position is discussed in the thesis.

**Conjugate Vaccines and Carriers**

**Vaccines to Microbial Polysaccharides**

Capsular polysaccharide (CPS) is both a microbial virulence factor and the target antigen of many protective antibodies. As a virulence factor, CPS functions by inhibiting phagocytosis by host immune cells (278). CPS is often a T-independent antigen that predominantly elicits low avidity IgM antibodies and fails to induce immunological memory. The lack of immunogenicity is most apparent in infants less than 2 years of age. Although the polysaccharide is recognized by the B cell receptor (i.e. antibody), it usually cannot be presented to T cells in conjunction with major histocompatibility complex (MHC) class II molecules. The lack of specific T cell interactions in the immune response limits the immunogenicity of the polysaccharide, the development of memory B cells, and class-switched antibody. On subsequent antigen exposure, maturation of the immune response does not occur. However, anti-CPS antibodies are frequently protective and the induction of anti-CPS antibody to certain bacteria is believed to be the hallmark
of prevention (279-281). Thus, polysaccharide capsule is attractive as a vaccine antigen. Because the immune response to purified CPS vaccines is generally not satisfactory, further approaches to this problem must be taken.

_Hemophilus influenzae_ type b (Hib) is one of the leading causes of invasive bacterial infection in young children (282). It possesses a polyribosyl ribitol phosphate (PRP) capsule (283). Hib is a significant cause of bacterial infection including meningitis, septicemia, epiglottitis, pneumonia and septic arthritis, especially in children under 5 years of age. Studies have been shown that antipolysaccharide antibody is protective against invasive Hib disease (284). Thus PRP is attractive as vaccine antigen. However, purified PRP induces low titers of serum antibodies that are usually insufficient to protect against invasive disease (285-287), particularly in the target age groups. Hib conjugate vaccines, which contain PRP conjugated to peptide carriers (Diphtheria toxoid, CRM 197 mutant _C. diphtheriae_ toxin protein, _N. meningitidis_ protein outer membrane complex and tetanus toxoid) are shown to be more immunogenic and to show boosted responses characteristic of T-dependent memory (288, 289). Estimates of efficacy for prevention of invasive disease range from 90-100% for up to 1 year following vaccination with Hib conjugates.

The success of Hib conjugate vaccine has led to the improvement of vaccines to bacteria such as _Streptococcus pneumoniae_ and _Neisseria meningitidis_. Recent results of efficacy trails of heptavalent pneumococcal conjugate vaccine are promising. _Streptococcus pneumoniae_ is a common bacterial agent causing a variety of infections including mucosal infections, pneumonia, arthritis, pericarditis, peritonitis and severe
invasive infections such as meningitis and septicemia (290). Pneumococcal polysaccharide vaccines are not effective in children under 2 years old because they are T cell-independent immunogens. The seven-valent pneumococcal conjugate vaccine includes seven purified CPS of *S. pneumoniae*, each coupled to a CRM 197 (291). The seven-valent pneumococcal conjugate vaccine demonstrates remarkable efficacy against invasive pneumococcal disease (292). *Neisseria meningitidis* is a major cause of bacterial meningitis and sepsis. Effective conjugate vaccines for the prevention of meningococcal disease caused by group A, C and Y strains have been licensed (293). Immunogenicity of these meningococcal polysaccharides is markedly improved by conjugation to carrier proteins.

The protein carriers in conventional vaccine development induce T cell responses. T cell memory produced by conjugate vaccines is specific for peptide sequences derived from the carrier proteins of these vaccines. However, carrier protein specific T cells are unable to contribute to a secondary response to an invasive disease because they are not specific for polysaccharide antigen. This phenomenon may explain some cases of the existence of immunological memory without protective immunity.

In an effort to induce immune responses of greater magnitude, or of different quality, different forms of carriers are being investigated. For example, our own studies use a novel carrier, an RNA plant virus. Such carriers present antigen in repeated forms, and in association with virus structures.

We will now describe the development of virus carriers for presenting antigens. Carrier structures may be used to present antigens other than bacterial polysaccharides.
The immune response to any material considered a hapten, because of its molecular size or other properties, may be enhanced by conjugation to a carrier. Examples include peptides and low molecular weight organic compounds. There are a variety of different carrier approaches. The most popular method is chemical coupling to large proteins such as keyhole limpet hemocyanin (KLH) or in the vaccine conjugates described in the section above. An alternative method exists for haptenic peptide antigens, fusing the DNA encoding the desired peptide to the gene encoding the carrier protein and producing the protein in a genetic expression system or using it directly as a DNA vaccine.

**Virus-Based Carrier Systems**

Conventional carrier proteins when administered systemically may induce a strong antibody response, but often they can be inefficient in inducing T helper (Th) or cytotoxic T lymphocyte (CTL) responses. They are also inefficient in the induction of mucosal responses to the immunogens. The expression of antigen on viral coat proteins by genetically fusing to viral DNA or chemically fusing to viral capsid protein, results in a particulate structure providing a more effective stimulus for induction of local and systemic immune responses. Virus particles are examples of highly organized and complex structures (294). The degree of complexity of antigens is crucial to the efficiency and quality of B cell response. Moreover, repetitiveness of antigen is used as a self/nonself discrimination by B cells as well as by innate defenses such as Toll receptors (295).

There are two strategies in using viruses as delivery systems or vaccine vectors. The first strategy is making use of viruses that replicate in the host for induction of
immune responses, while the second strategy immunizes with preassembled virions or pseudovirions expressed in heterologous expression systems such as bacteria \((E. \ coli)\), yeast, mammalian cells, or the natural host (e.g. plant and insect viruses). Examples of viral vectors suggested for the first approach include modified poliovirus (296) and human rhinovirus (297). In these cases, live viruses are proposed for use as vaccine vectors. Alternatively in the second approach, whole viruses or virus-like particles (VLPs) self-assembled in heterologous systems, may be used. Intact viruses that have been proposed for vaccines include plant viruses, such as plum pox virus (298), cowpea mosaic virus (CPMV) (299), and others. The first VLP used was hepatitis virus surface antigen containing particles expressed in yeast (300). Examples of VLPs obtained using this approach include rotavirus (301), picornavirus (302), orbivirus (303), calicivirus (304), herpesvirus (305) and parvovirus (306).

The strategy to create efficient delivery systems from virus or VLP consists of three steps. The first step is identification of permissive sites in the virus particle that do not interfere with virus structure and assembly and maintain the full immunogenicity of the foreign epitope. The second step is purification and characterization of chimeric viruses or chimeric VLPs. The third step is investigation of the immunogenic properties of the chimeras.

**Plant Virus-Based Vaccine**

Recent advances in the molecular biology of plant viruses have enabled the development of expression systems that use viral vectors to produce large amounts of protein in a short period of time. The general approach is to insert the foreign gene into
the viral genome under the control of a strong subgenomic promoter. The resulting recombinant viruses can then be introduced into the appropriate host plant by mechanical inoculation, where the virus can spread systemically throughout the plant. If the construct allows viable virus particles to be produced, the recombinant virus has the potential to spread from plant to plant. Expression of recombinant protein using viral vectors is a fast and relatively simple method for examining proteins. The infected plants can produce high amounts of protein within 1-4 weeks of inoculation. These systems can be divided according to the architecture of the virus capsid into helical (rod-shaped) viruses or icosahedral viruses. Helical viruses consist of a single strand of RNA around which is wound the capsid protein. Icosahedral viruses encapsulate their genome and associated proteins in a 20-sided particle of a defined size. An example of a helical virus system is tobacco mosaic virus (TMV). TMV was used as a virus vector to express epitope from Hepatitis C virus (307). Another example is plum pox potyvirus (PPV). It has been developed into antigen presentation system (298). One or two copies of the 15 amino acid epitope from canine parvovirus (CPV) are inserted into the N-terminus of the capsid coat protein. Neutralizing antibodies are detected against CPV. An example of icosahedral viruses is tomato bushy stunt virus (TBSV) (308). A 13 amino acid peptide from HIV-1 p120 with 3 amino acid linker sequence is expressed at the C terminus of the coat protein from TBSV in tobacco plants. The resulting virions resembled wild-type particles. The HIV epitope is recognized by monoclonal antibodies and the immunization of mice results in high antibody response to both the HIV peptide and the virus vector. Cowpea
mosaic virus (CPMV) and cowpea chlorotic mottle virus (CCMV) are other examples of icosahedral viruses and are the viruses used in our studies.

**Cowpea Mosaic Virus (CPMV)** CPMV is a positive-strand RNA virus of the family comoviridae. It has a narrow host range. Most experimental work has been carried out in its natural host, the legume cowpea. It is a nonenveloped virus of approximately 300 Å diameter that can be isolated from infected plants in yields of 1-2 grams per kilogram of leaves. The genome consists of two RNA molecules, RNA-1 and RNA-2. RNA-2 encodes the capsid proteins. A systemic infection can be achieved by mechanical abrasion of the leaf and application of both viral RNAs. An icosahedral capsid consists of 60 copies each of the large (L) and small (S) subunits. Two capsid proteins form three distinct β-barrel domains which are arranged in the capsid in a pseudo T=3 lattice. The crystal structure of the virus particle has been solved (309). One of the loops on the S protein was chosen for insertion of an epitope from the VP1 protein of foot and mouth disease (FMDV) (310). The result indicates that the insertion of this epitope interferes with CPMV infectivity and spread.

Johnson et al. have used a surface exposed reactive lysine on CPMV as the site for chemical modification (311-313). CPMV possess a single uniquely reactive lysine residue per asymmetric unit (60 per virus particle). CPMV reacts with fluorescein N-hydroxysuccinimide (NHS) ester and isothiocyanate, which selectively react with available lysine residues. At neutral pH, wild-type CPMV is found to carry up to 60 dye molecules per virion, in a dose-dependent manner (with a maximal yield at dye:viral protein = 200:1 ). At pH 8.3, 1.5 dye molecules per asymmetric unit (90 per virion) are
bound at a 200:1 ratio, whereas when the ratio was 4000:1 a maximum loading of 240 dye molecules per virion is observed. In conclusion, under maximal conditions, up to 4 lysine residues per asymmetric unit could be labeled.

CPMV has a stable structure. The half-life of particles in 80% DMSO is approximately 30 minutes. This allows chemistry to be performed on the viruses even with relatively hydrophobic organic compounds. The study further reveals that CPMV can be conjugated to biotin. In this application, in this thesis, lysine residues on CPMV were conjugated to a peptide that mimics the type III GBS epitope. We studied the ability of this chimera to induce an immune response in mice.

**Cowpea Chlorotic Mottle Virus (CCMV)** CCMV is a member of the *Bromovirus* genus and in the *Bromoviridae* family. The bromovirus group has five members: CCMV, brome mosaic virus, broad bean mottle virus, cassia yellow blotch virus and spring beauty latent virus. These viruses have a 28 nm icosahedral capsid. The genome consists of four positive-sense single stranded RNA molecules encapsulated in three virions, each with similar or identical capsid structures. RNA 1 and 2 are monocistronic and encode nonstructural proteins involved in RNA-dependent RNA replication. The dicistronic RNA 3 contains open reading frames for movement protein and the coat protein. The coat protein is translated from RNA 4, a subgenomic RNA transcribed from minus-strand RNA 3. CCMV induces an extensive systemic chlorosis in cowpea. Continuous propagation of CCMV in cowpea results in an attenuated variant, CCMV, which induces mild green mottle symptoms (314). Movement protein plays a crucial role in determining host specificity (315) and also has been shown to modulate symptom expression (316).
The structure of CCMV was determined to 3.2 Å resolution by X-ray crystallography (317). The capsid is composed of 180 chemically identical protein subunits. The quaternary structure of CCMV displays a $T = 3$, quasi-symmetry with 32 prominent capsomers. The capsomers occur as 12 pentamers and 20 hexamers assembled from the coat protein. Each protein subunit is comprised of an eight-stranded, antiparallel, $\beta$-barrel core (from amino acids 52-176). The capsomers are formed from these $\beta$ barrels. The C-terminal (amino acids 176-190) and N-terminal (amino acids 1-51) of the coat protein extend in opposite directions away from the $\beta$ barrel core. The pentameric and hexameric capsomers of the virion are linked through C-terminal extensions originating from two coat proteins. This linkage stabilizes the noncovalent dimer. The C-terminal extension from one coat protein subunit extends across two-fold or quasi two-fold axes to interact with subunits in adjacent capsomers. The N-terminus from a nearby coat protein subunit clamps the invading C-terminal arm. The N-terminal extensions of the threefold adjacent coat proteins converge at the quasi-sixfold vertices of the virion and form a hexameric tubular structure ($\beta$-hexamer). The $\beta$-hexamer is proposed to stabilize virion structure. An eight-stranded, antiparallel, $\beta$-barrel core (from amino acids 52-176) results in the outward projection of five loops. In the Mark Young lab, the DNA encoding each loop was engineered to have BamHI restriction enzyme sites to allow for the insertion of DNA encoding epitopes. In our study, we inserted DNA encoding mimetic peptide of GBS type III capsular polysaccharide in the BamHI site located on each loop of CCMV coat protein. Analysis of these chimeras was studied in this project.
Wild-type CCMV is stable around pH 5.0 and sediments at 88 S. At pH 7.0 and low ionic strength, the particle undergoes a transition state to a swollen form, where the average size of particle increases by roughly 10%. Swelling is the result of a radial expansion of the virion capsid around the three-fold axes, where there are divalent calcium ion binding sites (318). Young et al. analyzed a salt stable mutant of CCMV (ssCCMV) (319). The ssCCMV resists disassembly in 1.0M NaCl, pH 7.5, whereas the wild-type virions completely disassemble into RNA and capsid protein under these conditions. Sequence analysis of the ssCCMV revealed a single A to G nucleotide change at position 1484 of RNA 3 (position 134 of RNA 4). The nucleotide change results in a lysine (K) to arginine (R) change at position 42 of the coat protein. Expression of the K42R mutant coat protein in E. coli and then in vitro assembly produces virions with the salt stable phenotype. The ssCCMV was used in our study to couple with a mimetic peptide of type III GBS epitope. Another mutant, designated SubE mutant (discussed later), was also conjugated to the peptide. We studied the ability of these chimeras to induce an immune response in mice.

To study CCMV as a virus-based protein carrier, Young et al. developed methods to produce CCMV in high yield in a Pichia pastoris heterologous expression system. Expression of the coat protein in this system results in the assembly of particles as virus-like particles (VLP). The study of VLP from this expression system indicates that the VLP are visibly indistinguishable from virus particles produced in the natural host. This system has the advantage of providing a method for expressing a range of viral protein mutants using large-scale fermentation technology. They also have investigated the
ability of a wide variety of CCMV coat protein mutants to be expressed and assembled into VLPs using this system. The SubE mutant, in which eight basic amino acid residues (K and R) of the N-terminus are replaced with acidic residue glutamic acids, was modified to express in the yeast expression system. The wild-type virus and the Sub E mutant results in equivalent level of protein accumulation, as determined by immunoblot assays, of about 0.05 to 0.5 mg (from a gram of wet yeast) (320). Transmission electron microscopic analysis of CCMV VLP demonstrates intact protein cages for the wild-type and the SubE mutant. The same system was also applied in our study to produce VLP expressing the mimetic peptide epitope of GBS type III.

Both CCMV and CPMV were used in our study to present the peptide mimic epitope of GBS type III CPS. CCMV were either genetically engineered or chemically conjugated with the peptide to produce the chimera. Mice were immunized with all chimeras and the immune response against the chimera was studied in this thesis work.

Research Goals

GBS is a major causative agent of neonatal sepsis and meningitis. Our laboratory previously identified a peptide, S9, which is an antigenic mimic of the epitope that is the target of protective antibody. The goals of this study were to enhance the S9 peptide immunogenicity, for the purpose of GBS vaccine development, and to study the structural basis of antigenic mimicry by the S9 peptide. In chapter two, we conjugated the S9 peptide to CCMV as a platform to enhance the immune response. Subsequently, in chapter 3, the CCMV-S9 constructs were injected in to mice and the immunogenicity of
the constructs was characterized. Finally in chapter four, we created mutants of the S9 sequence to study the structural basis of antibody binding to the S9 peptide and to search for better mimetic peptides.
Introduction

We chose to express the GBS mimetic peptide in the plant virus Cowpea Chlorotic Mottle Virus (CCMV). The virus serves as a carrier for the mimetic peptide. CCMV was chosen for this purpose because of its repetitive structure allowing multiple epitopes to be expressed, because the Young lab had invested time and energy in developing virus mutants that could be used for epitope expression, and because large amounts of virus could be obtained in both natural and recombinant expression systems. CCMV is a member of the Bromoviridae family of viruses. CCMV has a 28 nm icosahedral capsid. The genome consists of four positive-sense single stranded RNA molecules encapsulated in three virions, each with similar or identical capsid structures. RNA 1 and 2 are monocistronic and encode nonstructural proteins involved in RNA-dependent RNA replication. The dicistronic RNA 3 contains open reading frames for movement protein and the coat protein. The coat protein is translated from RNA 4, a subgenomic RNA transcribed from minus-strand RNA 3. CCMV induces an extensive systemic chlorosis in cowpea. The structure of CCMV determined by X-ray crystallography demonstrates that the virion is comprised of 180 copies of the coat protein subunit arranged on a $T = 3$ icosahedral structure with 12 pentamer and 20 hexamer capsomeres (317). The Young laboratory has studied expression and assembly of virions and virus-like particles (VLPs) based on CCMV. These were utilized in these studies.
Initially, genetic approaches were taken to express the S9 mimetic peptide FDTGAFDPDWPA, first in infectious virus, then as VLPs made in a yeast expression system. These viruses failed to assemble and function, and so the final constructs, used later in this thesis research were made by chemical conjugation.

Materials and Methods

Engineering CCMV-Peptide S9 Chimera (Plant Expression)

Five plasmids each encoded loop-specific BamHI cloning sites where peptides could be expressed on the virus coat, at amino acid positions 63, 102, 114, 129 or 161 in the wild-type coat protein, were kindly provided by the Young lab. We inserted DNA to encode the S9-peptide (FDTGAFDPDWPA) within the loop sequence. Plasmid vectors for each specific loop are referred to as pCC3-63, pCC3-102, pCC3-114, pCC3-129 and pCC3-161. Plasmids were digested with BamHI for 2 h at 37°C. Then the digested plasmids were dephosphorylated with a calf intestinal alkaline phosphatase (CIP). The sense encoding the S9 peptide, 5’GATC [TTC GAT ACG GGC GCG TTC GAT CCA GAT TGG CCA GCG] GG 3’ (IP[FDTGAFDPDWPA]) with phosphate at 5’ end and the complementary strand (antisense strand), were purchased from Integrated DNA Technologies. The red highlights indicate the BamHI overhang, the letters in the parentheses indicate the S9 coding region, and blue letters indicate S9 peptide. Each strand was heated at 95°C for 3 min and rapidly cooled down for 5 min. Sense and antisense strands 25 ul of 50 ng/ul were mixed together, giving 1:1 molar ratio reaction. The mixture was incubated 15 min at 50°C and then allowed to cool down to room temperature. The S9 peptide genes were ligated into the compatible ends of restriction
digested *BamH*I sites. The ligation reaction was performed with a ratio of 1:3, vectors: inserts with ligation reaction composed of 1 ul T4 DNA ligase (Promega, Madison, WI) and 1 ul of 10x ligation buffer (containing 100mM mgCl2 and 10 mM ATP, Promega) in 10 ul total volume. The reaction was incubated at 4°C overnight to facilitate ligation. The digested plasmids alone were used as a control. The ligation reactions were then transformed into XL2-Blue ultracompetent *E. coli* cells (Stratagene, Cedar Creek, TX) by mixing 2 ul of the reaction with 100 ul of the competent cells. The transformation reactions were incubated in an ice tub for 30 min and then briefly in 67°C for 42 sec. LB broth was added to the transformation reactions and the mixtures were incubated at 37°C for 1 h. The transformation reactions were spread on LB media agar with 100 ug/ul ampicillin supplement and incubated at 37°C overnight. Single colonies were picked and subcultured in LB broth. Clones were tested for the presence of the S9 insert by PCR and further, by sequencing.

**Verifying Positive Clones**

Clones containing inserts were identified by two steps: PCR and then sequencing analysis. Selected clones were cultured in 3 ml LB broth medium. Plasmids were extracted from cells by alkaline lysis method (321). 3 mls of overnight culture in LB broth with complement of ampicillin 100 ug/ml were spun down to collect a cell pellet. Cells were resuspended in 100 ul of 20 mM Tris buffer pH 8 and then incubated at room temperature for 5 min. 200 ul of lysis buffer were added and the mixture was mixed by inversion. The mixture was incubated for 10 min and then 150 ul of KAC/AC (5 M potassium acetate, 10 M (57%) acetic acid) were added. The mixture was mixed
vigorously and placed in ice for 30 min. Cell debris was removed by centrifugation at 12 krpm 10 min twice. Supernatant was collected and was then mixed with an equal volume of phenol/chloroform/isopropanol. The upper phase was collected and transferred to another eppendorf tube. DNA was precipitated by mixing the mixture well with 2X volume of 95% ethanol and 1/10 volume of 3 M sodium acetate pH 5. The mixture was incubated at –20°C overnight. DNA was collected by centrifuging 12 krpm 10 min. The DNA pellet was washed with 70% ethanol and resuspended in 10 ul water. 2 ul of DNA suspension was loaded in 0.8% agarose gel. Purified plasmids were amplified by PCR using primers 20 and 21, kindly provided by the Young lab. The PCR products were run in 1% agarose electrophoresis gels. Positives clones were sent for DNA sequencing analysis (Davis Sequencing, Inc, Davis, CA).

In Vitro Transcription of CCMV cDNAs for Plant Inoculation

Plasmids pCC1, pCC2, pCC3-wt and pCC3-S9 constructs were prepared for in vitro transcription by linearization with XbaI followed by proteinase K (Sigma, St. Louis, MO) digestion for 30 min at 65°C, phenol-chloroform extraction and ethanol DNA precipitation. Chimeric CCMV-S9 RNAs were synthesized by in vitro transcription from approximately 1.0 ug of linearized DNA with T7 Megascript (Ambion, Austin, TX) in a 20 ul reaction, containing RNA polymerase enzyme and its buffer and ribonucleotides, supplied by the company. RNA quantitation and integrity were determined by UV spectrophotometry (1OD_{260} = 44 ug/ml) and visualization on denatured agarose gel electrophoresis.
Plant Inoculation

The 20 ul mixtures containing 1 ug of each RNA 1, 2 and wt RNA 3 or chimeric RNA 3-S9 were spotted on carbon dusted leaf of cowpea plants (Vigna unguiculata (L.) var California Blackeye) and tobacco plants (Nicotiana benthamiana) at the primary stage (two leaves). The inoculation was performed on 2-4 leaves. Four weeks after inoculation, leaves were collected and kept at –80°C. Virus from collected samples was extracted. Collected leaves were immersed in liquid nitrogen and crushed in homogenization buffer (0.2 M sodium acetate, 0.2 M acetic acid, 0.01 M disodium EDTA, pH4.8). The crude extract was tested for the presence of virus by ELISA and observation under electron microscope.

ELISA

ELISA was performed as follows. Briefly, 100 ul of 1:5000 dilution of 1 mg/ml purified rabbit anti-CCMV polyclonal antibody with phosphate buffer was absorbed on the surface of ELISA plates at 4°C overnight. Plates then were blocked with blocking buffer (containing BSA) 200 ul at 4°C at least 6 h. Plates were washed with ELISA washing buffer, 10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20. Plant crude extracts were loaded in prepared plates and then incubated at 4°C overnight. Plates then were washed again with washing buffer. A 1:500 dilution of alkaline phosphatase (AP)-conjugated rabbit anti-CCMV polyclonal antibody was loaded in the plates and incubated at room temperature for 4 h. After washing with ELISA washing buffer, 100 ul
of substrate, p-nitrophenyl phosphate, were added (Sigma, St. Louis, MO). The resulting color reaction was measured with ELISA plate reader at absorbance 405 nm.

**In Vitro Transcription and Translation**

The CCMV-S9 plasmid constructs, pCC3-63, pCC3-102, pCC3-114, pCC3-129 and pCC3-161, encoding coat protein gene were PCR amplified using upstream primer, which contained a T7 RNA polymerase promoter element and a start codon, ATG. The PCR reaction resulted in a PCR product, which contained T7 RNA polymerase promoter element and a start codon at the 5’ and stop codon at 3’ end. DNA quantitation and integrity was determined by UV spectrophotometry and visualized by agarose gel electrophoresis. Chimeric CCMV-S9 RNA was *in vitro* transcribed from PCR product by using RNA transcription kit (mMessage mMACHINE@T7 kit, Ambion, Austin, TX). RNA quantitation and integrity were determined by UV spectrophotometry and visualized by denatured agarose gel electrophoresis. Resulting 5’ capped mRNAs were then *in vitro* translated using cell free wheat germ extracts translation kit (Promega, Madison, WI). Radioactive (S\(^{35}\)) methionine was incorporated into the reaction. The proteins then were visualized by using gel electrophoresis and autoradiography.

**Engineering CCMV-S9 Chimeras in Pichia pastoris**

CCMV wt and CCMV-S9 chimeric genes were subcloned from their original plasmid vector pCC3-wt and pCC3-S9s into the *P. pastoris* expression plasmid pPicZa (Invitrogen) to construct pPicZa 63-S9, pPicZa 102-S9, pPicZa 114-S9, pPicZa 129-S9 and pPicZa 161-S9. PCR was used to amplify the DNA cassette using primers: 5’ GAT
AGT AAG AAT TCA TGT CTA CAG TCT T 3’ as upstream primer, and 5’ GTA ACG GTC GAC AGC GGG C 3’ as downstream primer. The upstream primer was complementary to a region encoding the coat protein start codon ATG (underlined sequence), and was created to introduce an EcoRI endonuclease restriction site (red block sequence) into the 5’ end of the amplified fragment. The downstream primer was complementary to the region encoding the coat protein stop codon, TAG and was also designed to contain SalI endonuclease restriction enzyme site. Thus the PCR products contained both EcoRI and SalI sites. Agarose gel electrophoresis was used to follow the PCR products. The products were purified from gels using QIAquick DNA/gel purification kits (Qiagen, Valencia, CA).

The pPicZa plasmid vectors were digested with EcoRI and Xhol. The Xhol digestion created SalI compatible ends. Then plasmids were dephosphorylated by CIP as described above. The 5 digested PCR products from pCC3-S9s were ligated into digested pPicZa plasmids. The ligation reactions were then transformed to XL-2 Blue ultracompetent E. coli cells as described. Transformed E. coli were spread on low salt LB agar plates containing 25 µg/ml Zeocin. Single colonies from each ligation reaction were randomly selected and cultured overnight in low salt LB media under Zeocin selection (25 µg/ml). Recombinant plasmids were extracted from clones by the alkaline lysis method (321). Recombinant clones were verified by PCR and DNA sequencing.

Recombinant plasmids containing pCC3-S9 were extracted by the alkaline lysis method (321) and then linearized with SacI. The linearized plasmids were then electroporated into competent P. pastoris X-33 mut+ yeast (Invitrogen) using a T800L
Electro Cell Manipulator (BTX, San Diego, CA) electroporation unit according to the manufacturers’ protocol. The pPicZa does not contain a yeast origin of replication. Transformants can be obtained only if the recombination occurs between plasmid and Pichia genome. Zeocin resistant transformants were selected after 2-3 days growth on YPDS agar plates (1% w/v yeast extract, 2% w/v dextrose, 1M sorbitol, 2% w/v agar) containing Zeocin (100 ug/ml). Single colonies were subcultured into 3 ml of YPDS broth containing Zeocin (100 ug/ml) and incubated at 30 °C overnight. Direct PCR-based screening of clones was performed (322). Briefly, 10 ul of P. pastoris culture (1:10 diluted with water) was disrupted using 25 U lytiase (Sigma). Primers for CCMV coat protein were used to amplify DNA. The PCR products were observed by gel electrophoresis and sent for DNA sequencing analysis (Devis Sequencing, Inc., Davis, CA).

Protein Expression and Small Scale Time Course Experiment

Positive yeast clones were cultured in 25 ml MGY (1.34% w/v yeast nitrogen base without amino acids (YNB), 1% v/v glycerol, 0.00004% w/v biotin) 30°C for 18 h 250 rpm. For induction of the coat protein expression, the cells were harvested by centrifugation, washed once with MM medium (1.34% w/v YNB, 0.00004% biotin, 0.5% methanol) and resuspended in 150 ml MM medium. During the incubation, methanol was added to the culture at 24 h intervals, each time to give a final concentration of 0.5%. Samples of 1 ml were collected every day for 7 days. Cells were stored at –80°C.
Medium Scale Expression of CCMV-S9 Chimeras in *P. Pastoris*

Five positive recombinant *Pichia* clones (pCC3-63, pCC3-102, pCC3-114, pCC3-129 and pCC3-161) were cultured in 50 ml MGY for 24-48 h at 30 °C on a 250 rpm incubation shaker. Cells were harvested by centrifugation and washed with MM medium. The washed harvested cells were then inoculated into 500 ml of MM medium. 100 % methanol 2.5 ml (0.5% final concentration) was added to the culture at 24 h intervals. The culture was incubated for five days at 30°C in 250 rpm incubation shaker. Cells were harvested by centrifugation and kept in -80°C for further analysis.

Large Scale Protein Expression

Improved overall recombinant protein yields in *Pichia* have been achieved by large scale *Pichia* fermentation process. The fermentation method followed the Invitrogen *Pichia* fermentation process guidelines. A Bioflo 3000 bench-top fermenter (New Brunswick Scientific) with a 5L water-jacketed glass fermentation vessel was used in the project. The fermentation procedure was separated into three phases, glycerol batch phase, glycerol fed-batch phase and methanol fed-batch phase. Dissolved oxygen (DO) measurement was used as the tool to observe and manipulate the metabolic rate of the culture during all 3 phases of fermentation process.

The positive yeast clone producing the largest amount of protein was selected based on time course data. This recombinant clone was cultured in 300 ml MGY medium (10% of initial fermentation volume) at 30°C, 250-300 rpm, 16-24 h as an inoculum seed for the fermentation. The glycerol batch phase began with adding the inoculum seed into
the fermentor vessel containing 3L of fermentation basal salts medium (85% v/v phosphoric acid, 0.93 g/L CaSO₄, 18.2 g/L K₂SO₄, 14.9 g/L MgSO₄7H₂O, 4.13 g/L KOH and 4% v/v glycerol). The batch culture was allowed to grow until the glycerol was completely consumed (18-24 h). The DO was set to 100% before the culture started to grow. As the culture grew, oxygen was consumed resulting in a decrease of DO. DO was maintained at 20% by adding oxygen and providing agitation. When glycerol in the medium was completely utilized by culture (18-24 h), yeast stopped growing. The oxygen in medium was not consumed. Thus the DO was close to 100%. Therefore, we measured the DO when the agitation tool and the oxygen pump stopped. If the DO measurement was close to 100%, the next phase was started. Next, the glycerol-fed batch was started by pumping in a culture medium containing 50% w/v glycerol at a feed rate of 18.15 ml/h/L initial fermentation volume. This phase improved the cellular yield from 180 to 220 g/L wet cells. Glycerol feeding was carried out for 4 h and stopped 30 min before starting the next fermentation phase to assure that cells used up all glycerol in the medium. The DO measurement was again used as an indicator of when to start the next phase. DO close to 100% indicated the complete consumption of glycerol. The methanol-fed batch phase was initiated by gradually feeding 100% methanol into the vessel to allow cells to adapt to growth on methanol. The DO measurement was used as the indicator to tell how cells adapted to the methanol feeding. A reduction of the DO indicated that the culture was able to adapt to methanol. However if the DO was lower than 20%, it indicated that the yeasts had died and the methanol feeding needed to be stopped. After full adaptation, methanol was fed with the rate of approximately 7.3-10.9
ml/h/L feed rate. The entire methanol fed-batch phase lasted approximately 70-100 h with a total of approximately 750-1000 ml methanol fed per liter of initial volume. Temperature during all fermentation phases was maintained at 30°C. The DO was kept above 20% through out the fermentation process by agitating and adding oxygen. Foam was controlled by manually adding an antifoam agent, Silwel L-7602 (Witco Corporation, Michigan). The cellular yield could reach a final level of 350-450 g/L after the full fermentation cycle. Cells were harvested by centrifugation and wet cells were preserved in -80 °C for further analysis.

Chimeric Virus Purification

Frozen cells from medium scale culture were thawed and lysed using acid-washed glass beads in an equal volume of virus buffer (0.1 M sodium acetate/acetic acid, 1 mM sodium azide, 1 mM disodium EDTA, pH 4.8, 0.22 um filtered). The mixture was vortexed vigorously 4 times for 5 min, with 2 min on ice in between. Cell debris and glass beads were separated by centrifugation at low speed. Chimeric viruses in supernatant were precipitated by adding 10% (w/v) polyethylene glycol (PEG) MW 8000. The mixture was then slowly stirred overnight at 4°C. The mixtures were centrifuged and the pellets were resuspended in 1/10 initial supernatant volume virus buffer. The mixture was then centrifuged at low speed. Supernatants containing virus were kept in -80°C for further analysis.

For the fermentation experiment, 100 g of collected cells were lysed by grinding with 235 g acid-washed glass beads in a bead-beater (BioSpec Products-BSP, model 1107900) in the presence of 200 ml homogenization buffer. The grinding cycle process
was to grind for 5 min and rest on ice for 3 min. To completely break cells, 5 cycles of grinding were performed. Beads and cell debris were separated by centrifugation and approximately 0.2 g of cell debris was kept at -80°C for further analysis. Supernatant was added with the same buffer to a final volume of 500 ml and impurities eliminated by using filter paper (Water/bushman) 0.6 um. 200 ul of filtrate was kept at -80°C for further analysis and the remainder was centrifuged using a Beckman Type 30 Rotor at 25 krpm for 2 h. Supernatant was kept at -80°C for further analysis and the pellet was resuspended in 1 ml virus buffer. The virus mixture was centrifuged at low speed to pellet out debris and aggregates.

To improve viral purification from the fermentation culture, diethylamino ethanol (DEAE) was applied in the process. 100 g of collected cells were lysed by grinding in the presence of 200 ml 50mMTris-HCl buffer pH 6.5. In one experiment the grinding process was performed in the presence of 2 M urea to increase recombinant viral protein solubility and disrupt noncovalent bonds between the viral coat protein and yeast cell debris. Beads and cell debris were separated by centrifugation and approximately 0.2 g of cell debris was kept at -80°C for further analysis. Supernatant was added to 50 mM Tris-HCl buffer pH 6.5 to have final volume of 500 ml. DEAE 2 g was added to the supernatant. The mixture was stirred slowly at 4°C for 2 h. The mixture was then filtered (Buchner funnel and filter paper (Water/bushman 90 mm) and washed with 50 ml of the same buffer twice. The filter cake was resuspended in 50 ml virus buffer supplement with 0.5 M NaCl. The suspension was filtered and the filtrate was collected. To elute all chimeric constructs from the filter cake, the filter cake was resuspended one more time
with 50 ml of the same virus buffer supplement with 0.5 M NaCl. The suspension was filtered. The filtrate was collected and pooled with the filtrate from the previous step and then centrifuged using a Beckman Type 30 Rotor at 25 krpm for 2 h. The virus-containing pellet was resuspended in 1 ml virus buffer. The virus suspension was briefly centrifuged at low speed to pellet out debris and aggregates.

SDS PAGE, Western Blot, and Dot Blot Analysis

For \([S^{35}]\)-labeled in vitro protein translation products, samples were boiled with gel loading buffer (0.5 M Tris buffer pH 6.8: 10% SDS: Glycerol (1:1:1), bromophenol blue, 0.036% betamercaptoethanol) and then loaded onto pre-cast 12.5% SDS polyacrylamide gel according to the manufacturer guidelines (PhastSystem, Pharmacia Biotech, Uppsala, Sweden). For other purposes, samples were boiled in the gel loading buffer and then loaded onto 12.5% SDS polyacrylamide gels. Gels were visualized by silver staining or proteins in gel were transferred to polyvinylidene fluoride (PDVF) membranes (Sigma), by using western blot apparatus (Bio-Rad, Laboratories, Hercules, CA). For dot blot analysis, prepared boiled samples were directly dotted into membranes and air-dried for 10 min before proceeding. Membranes from both western and dot blot were then blocked of any nonspecific binding overnight at 4°C with blocking buffer (5% non fat dry milk, 0.2% tween 20 in PBS buffer pH7.4). Membranes were washed briefly with ELISA washing buffer 3 times for 1 min each. Membranes were incubated overnight at 4°C with blocking buffer containing primary antibody: either 5 ug/ml S9 monoclonal antibody or 1/5000 anti-ccmv antibody. After 3 washes with the ELISA washing buffer, membranes were incubated with blocking buffer containing secondary antibody either
1/1000 alkaline phosphatase-conjugated anti-mouse IgM antibody or 1/1000 Horseradish peroxidase-conjugated anti-rabbit antibody. The colorimetric reaction was then
developed (Bio-Rad, Laboratories, Hercules, CA).

CCMV Wild-Type Plant Virus Purification

Frozen CCMV infected leaves 100 g were blended for approximately 3 min in
100 ml homogenization buffer. The homogenate was filtered through 2 layers of
cheesecloth. The filtrate was allowed to sit on ice for 1 h before low speed centrifugation
at 20,000 g for 20 min. The supernatant was precipitated by adding 10% (w/v) PEG MW
8000 followed by overnight stirring at 4ºC. The pellet containing CCMV virus was
collected by centrifugation and resuspended in virus buffer. A second round of viral
precipitation was carried out with 15% (w/v) PEG MW 8000 and resuspended in virus
buffer. Next the suspension containing CCMV virus was purified by centrifuging in 38%
(w/v) CsCl at 38000 rpm for 20 h (Beckman, SW41). The virus band was collected and
dialyzed against virus buffer. Viral concentration and impurity were determined by
measuring UV absorbance ratio Abs$_{260/280}$ and observing the presence of virus under
transmission electron microscope (TEM). The Abs$_{260/280}$ =1.5-1.7 is considered good. The
viral concentration was calculated based on absorbance at 260 nm (1Abs$_{260}$ = 0.17
ug/ml).

Chemical Linking between CCMV Virus and S9 Peptide

Two approaches were used to chemically conjugate S9 peptide to CCMV. The
first was to conjugate through a thiol group by forming a disulfide bond between S9 and
CCMV. S9 peptide with an added cysteine at the amino terminal was synthesized for this purpose. The CCMV mutant, CCMV A163C with cysteine substituted at position 163, was used in the experiment. The reaction was performed in the presence of 10 mM CuSO₄, 1 mg of virus, and 1 mg of S9 peptide at room temperature for 2.5 h. For analysis, 10 ul of the coupling reaction were loaded on SDS nonreducing PAGE gel. Gels were silver stained to visualize the conjugation product.

The second approach used the chemical linkers N-succinimidyl 3-(2-pyridyldithio) propionate (spdp) and Sulfosuccinimidyl 4-(N-maleimidomethy)cyclohexane-1-carboxylate (sulfo-smcc), which were purchased from Pierce (Rockford, IL).

The spdp is a heterobifunctional cross-linker. It is composed of two functional groups, NHS ester and 2-pyridyldithio group. The NHS ester reacts with the primary amine on the viral coat protein. The 2-pyridyldithio group reacts with sulfhydryl at the amino terminal of S9 peptide, forming disulfide bond between the linker and S9 peptide and releasing pyridine 2-thione (Figure 2.1).

To prepare for the cross-linking reaction, virus ssCCMV, originally kept in the virus buffer pH 4, was dialyzed against 20 mM NaH₂PO₄ pH 7.5. After dialysis, the pH of virus was measured again and adjusted with 1M NaH₂PO₄ pH 9 to pH 7.5. The freshly prepared spdp linker in dimethyl sulfoxide (dmsö) was used in 5-fold molar excess to CCMV capsid protein (MW of CCMV capsid protein = 22,000). The pH of the reaction was adjusted to pH 7.5 and the reaction was incubated at room temperature for 1 h in a shaker. The reaction was stopped by incubating 1 h at room temperature with 50 ul of
hydroxylamine 1.5 M pH 8.5. The pH of the reaction was adjusted to 8.5 with 5 M sodium acetate pH 4.5. The S9 peptide in a 50-fold molar excess to CCMV was added. The pH of the reaction was adjusted to pH 6.5 with 5 M sodium acetate buffer pH 4.5. The reaction was performed at room temperature for 1.5 h. Then 5 ul of reaction sample was mixed with 5 ul of SDS loading buffer without β-mercapto ethanol and boiled for 10 min. The samples were loaded onto a 12% SDS-PAGE gel and visualized by silver staining or transfer to a PVDF membrane for western blot analysis. The conjugation product was also characterized by sucrose gradient velocity analysis.

![Chemical Reaction diagram](image)

Figure 2. 1 Chemical Reaction between SPDP, CCMV and S9

Sulfo-smcc consists of an NHS-ester and a maleimide group connected with a spacer arm. NHS-esters react with primary amines on CCMV, and maleimides react with the thiols on the cysteine-containing S9 peptide. Primary amines are the principal targets for the NHS-ester. Thus the e-amine of lysine available on the capsid protein will react
with the NHS-ester. An amide bond is formed when the NHS ester conjugation reagent reacts with primary amines releasing N-hydroxysuccinimide (Figure 1. 2).

![Figure 2. 2 Chemical Reaction between Sulfo-SMCC, CCMV and S9](image)

To begin the coupling process, ssCCMV in the virus buffer pH 4.8 was dialyzed against 20 mM NaH$_2$PO$_4$ pH 7.5. The pH of the virus was measured again before the coupling reaction, and adjusted to pH 7.5 with 1M pH 9 NaH$_2$PO$_4$. Tris (2-carboxyethyl) phosphine (TCEP) 0.5 M was added to the virus, to a final concentration of 50 mM, to reduce protein disulfide bonds in viral coat protein. The reaction was allowed to continue at room temperature for 1 h. An alkylation sulfhydryl reagent 2-iodoacetamide (IA) 0.5 M was added to the virus to 50 mM final concentration and incubated at room temperature for 30 min. The virus solution was purified to get rid of excess TCEP and the IA by using microcon 100 filters (Millipore, Billerica, MA). The virus was washed extensively 3 times with 20 mM NaH$_2$PO$_4$ pH 7.5 and eluted with the same buffer. Freshly prepared sulfo-smcc linker was used in 25, 50, 100 or 200-fold molar excess to
the virus. After adding the linker, the pH of the reaction was remeasured and adjusted to 7 with 1 M NaH$_2$PO$_4$ pH 9. The reaction continued for 12 h at 4°C. To stop the amide reaction between virus and the linker, lysine was added to the reaction to a final concentration of 1 mM and kept for 15 min at room temperature. The excess linker and lysine were removed by passing through a size-exclusion filter microcon 100, washed extensively 3 times and eluted with the 20 mM NaH$_2$PO$_4$ pH 6. The S9 peptide was pretreated with 50 mM TCEP for 1 h at room temperature prior to coupling with the derivatized virus. The pretreated S9 peptide was used in 12.5, 25, 50, 100 and 200 molar excess to the ssCCMV. The pH of the reaction was adjusted to pH 6 with 1 M NaH$_2$PO$_4$ pH 9. The reaction of maleimides with thiols was incubated for 6 h at 4°C. 5 ul of reaction sample was mixed with 5 ul of SDS loading buffer containing $\beta$-mercaptoethanol and boiled for 10 min. The samples were loaded into 12% SDS-PAGE gel and visualized by silver staining technique or transfer to a nitrocellulose membrane for western blot analysis. The conjugation product was also characterized by sucrose gradient velocity analysis.

Sucrose Gradient Analysis

Samples were separated on 10-40% or 5-25% sucrose (w/v virus buffer) sedimentation velocity gradients. Gradients were poured in Beckman SW41 centrifuge tubes (10 ml/tube) using a Gradient Master (BioComp Instruments, New Brunswick, Canada). 500 ul samples were overlaid and then centrifuged at 37,000 rpm for 3 h. The gradients were manually carefully fractionated obtaining 1 ml/fraction. Abs280 of gradient fractions was measured for presence of virus. 10 ul of each fraction were mixed
with loading dye to load into SDS-PAGE gel analysis. Gels were silver stained or/and blotted to PVDF membranes for western blot analysis.

Results

Plant Expression System

Plasmids containing cDNA of RNA3 were kindly provided by the Young lab. Plasmids of RNA3 were designated pCC3-63, pCC3-102, pCC3-114, pCC3-129 and pCC3-161 according to the position of loop-specific BamHI cloning sites. A synthetic oligonucleotide encoding the S9 peptide sequence (FDTGAFDPDWPA) flanked with BamHI restriction sites was inserted into the loop sequence BamHI site of each plasmid. The positive recombinant clones were verified by PCR and DNA sequencing analysis. PCR products from positive clones are 677 bp compared to vectors, which all are 632 bps resulting in the slight mobility shift for 45 bp differences (Figure 2.3).

All positive clones were sent for DNA sequencing analysis to confirm the result. Only one positive recombinant clone from each construct was kept for further study and were named pCC3-63-S9, pCC3-102-S9, pCC3-114-S9, pCC3-129-S9 and pCC3-161-S9. Each construct needed to be transcribed in vitro to RNA. For in vitro transcription, recombinant clones and cDNA of CCMV-RNA1 and CCMV-RNA2 (kindly provided from Young lab) were linearized with XbaI, followed by proteinase K treatment, phenol/chloroform extraction and ethanol precipitation. RNA1, RNA2, RNA3-wt and chimeric CCMV-S9 RNAs were synthesized by in vitro transcription from linearized DNA with T7 Megascipt (Ambion, Austin, TX) as described in the Materials and
Methods section. RNA quantitation and integrity were determined by UV spectrophotometry and visualization on denaturing agarose gel (Figure 2.4). We obtained approximately 100 ug of RNA1 and RNA2 and approximately 12 ug of wt RNA3 and all chimeric RNA3-S9s from \textit{in vitro} transcription process.

Figure 2. 3 PCR Analysis Recombinant Constructs
S9 DNA sequences, 45 bp, was inserted into pCC3-63, pCC3-102, pCC3-114, pCC3-129, and pCC3-161 plasmid. PCR analysis using primer 20 and 21 was applied to identify positive recombinant clones. The first lane in each panel is 1 Kb standard DNA marker. The second lane is the pCC3-63, pCC3-102, pCC3-114, pCC3-129 or pCC3-161 plasmid vector. The remainders are recombinant clones. The mobility shifts indicated the positive recombinant clones.
From left to right are RNA products from \textit{in vitro} transcription pCC1 (cDNA of RNA1), pCC2 (cDNA of RNA2), pCC3-wt (cDNA of wt-RNA3), the recombinant clone pCC3-63 and the recombinant clone pCC3-102 respectively.

From left to right are RNA products from \textit{in vitro} transcription pCC1 (cDNA of RNA1), pCC2 (cDNA of RNA2), pCC3-wt (cDNA of wt-RNA3), the recombinant clone pCC3-114 and the recombinant clone pCC3-129 respectively.

From left to right are RNA products from \textit{in vitro} transcription pCC1 (cDNA of RNA1), pCC2 (cDNA of RNA2), pCC3-wt (cDNA of wt-RNA3) and the recombinant clone pCC3-161 respectively.

Figure 2. 4 RNA Products from \textit{In Vitro} Transcription of pCC1, pCC2, pCC3-wt and Recombinant pCC3-63-S9, pCC3-102-S9, pCC3-114-S9, pCC3-129-S9, and pCC3-161-S9

Positive recombinant pCC3-S9 clones, pCC1 encoding coat protein1 and pCC2 encoding protein 2 were \textit{in vitro} transcribed to obtain RNAs. The RNA products were visualized by gel electrophoresis.

For plant inoculation, 1 ug of RNA1, RNA2, and RNA3-wt or chimeric RNA3-S9 from \textit{in vitro} transcription reaction were mixed together and 20 ul/leaf administered.

Leaves of cow pea plant (\textit{Vigna unguiculata} (L.) var. California Blackeye) and tobacco
plants (Nicotiana benthamiana) were carbonrundum dusted at the primary stage (two leaves) to create abrasion. The RNA cocktails were manually inoculated. Three weeks following inoculation, infected leaves were collected. The leaf crude extract was subjected to ELISA to detect the presence of wt CCMV and the chimeric viral particles (Table 2.1).

Table 2.1 Elisa Result of Leaf Crude Extract

<table>
<thead>
<tr>
<th>Construct</th>
<th>Tobacco Plant</th>
<th>Cowpea Plant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Repetition1</td>
<td>Repetition2</td>
</tr>
<tr>
<td>Wild-type</td>
<td>Mean± SD</td>
<td>Mean± SD</td>
</tr>
<tr>
<td>63-S9 chimera</td>
<td>0.05±0.003</td>
<td>0.071±0.010</td>
</tr>
<tr>
<td>102-S9 chimera</td>
<td>0.055±0.005</td>
<td>0.056±0.005</td>
</tr>
<tr>
<td>114-S9 chimera</td>
<td>0.061±0.007</td>
<td>0.091±0.004</td>
</tr>
<tr>
<td>129-S9 chimera</td>
<td>0.089±0.006</td>
<td>0.051±0.007</td>
</tr>
<tr>
<td>161-S9 chimera</td>
<td>0.095±0.007</td>
<td>0.082±0.008</td>
</tr>
<tr>
<td>PBS</td>
<td>0.087±0.008</td>
<td>0.080±0.004</td>
</tr>
</tbody>
</table>

*OD405 detected in ELISA well, mean of triplicate samples ± SD

Inoculated leaves were collected after 3 weeks of infection. Leaves were frozen by immersing in liquid nitrogen. Then, 20 g of frozen leaves were crushed by placing in a blender with 20 ml of homogenization buffer. Supernatants were collected after centrifugation. Each crude extract was diluted 1:3 with PBS buffer and 100 ul of the dilution were loaded onto anti-CCMV coated wells. Elisa was performed as described in the materials and methods section.
The ELISA result indicated the absence of chimeric viral particles in the crude extract when compared with wild type inoculation. The crude extracts were then studied by transmission electron microscope for the presence of viral particles. Virus particles were seen following inoculation with constructs encoding wild-type virus, but not with the chimeric constructs (not shown). The RNA from each construct was in vitro translated to assure that each construct was capable of expressing a translated protein. The CCMV-S9 plasmid constructs, pCC3-63-S9, pCC3-102-S9, pCC3-114-S9, pCC3-129-S9 and pCC3-161-S9, encoding recombinant S9-coat protein gene were PCR amplified using primer, which contained T7 RNA polymerase promoter element. The PCR amplified fragments were quantified and visualized by gel electrophoresis (Figure 2.5).

Chimeric CCMV-S9 RNA was in vitro transcribed from PCR products by using T7 mMessage mMachine (Ambion, Austin, TX) resulting in 5’ capped mRNAs. The chimeric CCMV-S9 RNAs were then in vitro translated using cell free wheat germ
extract translation kit (Promega, Madison, WI). Radioactive (S\textsuperscript{35})methionine was incorporated into the reaction. The proteins then were visualized by using gel electrophoresis and autoradiography (Figure 2.6).

![Figure 2.6 In vitro Translation of RNA Construction.](image)

The RNA from each construct was able to be \textit{in vitro} translated resulting in CCMV coat protein-S9 product. This data assured that each construct is capable of expressing a translated protein. All these data indicated that the insertion of the S9 peptide into extended loops of the CCMV viral capsid protein may inhibit viral production in its natural host, the cow pea plant.

Yeast Expression System

Due to our failure to express infectious CCMV-S9 chimeras in plants, which are the natural host, we chose a yeast expression system as an alternative method to create chimeric CCMV-S9 capsids. The \textit{Pichia} expression system has been used widely to express high levels of heterologous proteins and intact virions (320, 323-325). \textit{Pichia}
*P. pastoris* is a methylotrophic yeast capable of using methanol as a sole carbon source. It contains the AOX1 gene responsible for alcohol oxygenase activity in the cell. We subcloned cDNA of RNA3-wt and RNA3-S9 from each loop construct to the *Pichia* expression vector, pPicZA. The insertion of recombinant and wt cassettes was accomplished in two steps. First, the genes for the RNA3s encoding wt and recombinant constructs were PCR amplified from the pCC3 recombinant clones, and EcoR1 and Sal1 restriction enzyme sites added to the ends of the sequences during the PCR. The PCR products were verified by gel electrophoresis (Figure 2.7) and DNA sequencing analysis. The slight band shifts indicated the different size between wt and CCMV-S9 construct. Then the PCR products were digested with enzymes EcoR1 and Sal1.

![Figure 2.7 Introduction EcoR1 and Sal1 Restriction Enzyme Sites to the Ends of pCC3-wt, pCC3-63-S9, pCC3-102-S9, pCC3-114-S9, pCC3-129-S9 and pCC3-161-S9 by PCR. From left to right are 1 kb DNA marker, pCC3-wt, pCC3-63-S9, pCC3-102-S9, pCC3-114-S9, pCC3-129-S9 and pCC3-161-S9.](image)
Second, the digested products were ligated into prepared pPicZA vector, which was cut with EcoR1 and Xho1 and dephosphorylated with CIP. The constructs were linearized by using Kpn1 restriction endonuclease to reduce negative clones. The Kpn1 cut only the plasmid vector not recombinant plasmids. The recombinant plasmids were transformed into E. coli competent cells. Positive recombinant clones were verified by PCR (Figure 2.8). Clones, which have same size PCR products as positive control pCC3-161-S9 were selected and sent for DNA sequencing analysis. The positive recombinant clones were named pPicZa 63-S9, pPicZa 102-S9, pPicZa 114-S9, pPicZa 129-S9, and pPicZa 161-S9.

Figure 2. 8 PCR Products from Positive Recombinant E. coli Clones
From left to right are 1 kb DNA marker, positive control from pCC3-161-S9, pPicZa 63-S9, pPicZa 102-S9, pPicZa 114-S9, pPicZa 129-S9, pPicZa 161-S9 and negative control (water)

Next, the positive recombinant plasmids were extracted and linerized with Sac1 and then electroporated into Pichia pastoris competent cells. The Zeo^r transformants were
selected after 2-3 days growth on YPDS agar plates containing Zeocin 0.1 mg/ml. Direct PCR-based screening of clones was performed as described in the Materials and Methods section. PCR products were visualized by agarose gel electrophoresis (Figure 2.9) and then sent for DNA sequencing analysis to confirm positive clones.

Figure 2. 9 PCR Products from Recombinant Yeast *Pichia pastoris* Chromosomal DNA Transformants from YPDS plates containing Zeocin were selected and PCR amplified by using primers 20 and 21. From left to right are DNA marker 1kb, PCR product from pPicZa wt transformant (kindly provided by Young lab), recombinant pPicZa 63-S9, pPicZa 102-S9, pPicZa 114-S9, pPicZa 129-S9, pPicZa 161-S9 transformants and pPicZa wt transformant.

To show that cloning of CCMV-S9 DNA inserts into pPicZa plasmid was in frame and able to express CCMV-S9 coat protein, *in vitro* translation was performed. The yeast positive clones containing pPicZa 63-S9, pPicZa 102-S9, pPicZa 114-S9, pPicZa 129-S9, and pPicZa 161-S9 respectively, were PCR amplified using upstream primer, which contained a bacterial phage T7 RNA polymerase promoter element and a start codon. Chimeric CCMV-S9 RNA was *in vitro* transcribed from PCR products by using T7 mMessage mMachine (Ambion, Austin, TX). Resulting 5’ capped mRNAs were then
in vitro translated using cell free wheat germ extracts translation kit (Promega, Madison, WI). The proteins then were visualized by using gel electrophoresis and autoradiography (Figure 2.10). The result showed that the cloning of CCMV-wt and recombinant CCMV-S9 into Pichia plasmids were in frame and capable of being translated in vitro.

![Figure 2.10 In vitro Translation from pPicZa-pCC3-S9 Constructs](image)

The ability of the positive yeast clones to express CCMV coat protein was investigated. The expression of CCMV-S9 coat proteins in transformed yeast was analyzed by adding methanol as inducer. Methanol induces the alcohol oxidase (AOX) promoter, which controls expression of integrated coat protein construct. Small scale time course experiments were created to observe the ability of recombinant yeasts to express chimeric CCMV-S9 coat protein. As described in the Materials and Methods section, the positive recombinant yeast clones were cultured in 25 ml MGY medium at 30°C for 18 h 250 rpm as inoculums for small scale time course experiments. Then, the cells were harvested by centrifugation and washed with MM medium and resuspended in 150 ml MM medium. The inducer of gene expression, 100% methanol, was added to the culture
at 24 h intervals. Samples were collected every day for 7 days. Dot blot analysis was applied to evaluate protein expression (Figure 2.11).

**Figure 2.11 Small Scale Time Course Protein Expression by Dot Blot Analysis**

Recombinant yeast expressing chimeric CCMV coat protein 63-S9, 102-S9, 114-S9, 129-S9 and 161-S9 were cultured for 7 days in methanol inducing medium. Culture samples were collected each day. Samples were centrifuged and resuspended in 100 ul SDS gel loading buffer. The cell suspensions were boiled for 10 min and cooled down in an ice tub. Samples were centrifuged. 3 ul of supernatants were dotted onto the nitrocellulose membrane. The samples from first horizontal lane to last horizontal lane are nontransformed Pichia wt, transformed yeast with CCMV 63-S9, 102-S9, 114-S9, 129-S9 and 161-S9 respectively. From left to right, they are transformed yeasts or yeast-wt on day 1 to day 7 respectively and the last lane is the positive control 0.5 ug/ml of CCMV-wt purified from yeast, kindly provided from Young lab. The membrane was blocked with blocking buffer at 4°C over night. After washing with washing buffer, membrane was incubated with anti-CCMV antibody 1/5000 dilution and finally 1/1000 Horseradish peroxidase-conjugated anti-rabbit antibody. Colorimetric detection was developed.

The dot blot result indicated that there was no CCMV protein detected from any CCMV-S9 construct, but we easily detected product from the CCMV wild-type
construct. The undetectable product in the dot blot may result from insufficient CCMV protein in samples. Western blot analysis with anti-CCMV antibody was performed using the same samples from day 7 (Figure 2.12).

Figure 2.12 Silver Staining and Western Blot Analysis of Small Scale Expression Experiment in CCMV-S9 Transformed Yeasts

Recombinant yeasts expressing chimeric CCMV-S9 coat proteins, 63-S9, 102-S9, 114-S9, 129-S9 and 161-S9 and CCMV-wt coat protein were cultured in methanol-inducing media for 7 days as described in the Materials and Methods section. Samples were collected at day 7. Cell pellets from 1.5 ml cultures were resuspended in SDS loading buffer and boiled for 10 min and placed on ice. Samples were centrifuged and 5 ul of CCMV-wt and 20 ul of CCMV-S9 were loaded into 2 of 10% SDS PAGE gels. From left to right, samples are CCMV-wt day 6, CCMV-wt day 7, high molecular weight marker, 63-S9 day 7, 102-S9 day 7, 114-S9 day 7, 129-S9 day 7, 161-S9 day 7 and 161-S9 day 6. One gel was visualized by silver staining and the other was analyzed by western blot analysis using anti-CCMV antibody 1/5000 as primary antibody and mouse anti-rabbit IgG-HRP as secondary antibody. Colorimetric detection was developed.

The western blot result indicated that recombinant CCMV proteins were expressed in each construct. However the expression yield of the S9 constructs might have been too low to be detected by dot blot analysis. CCMV wt cassette was expressed in higher yields, compared to the chimeric constructs. To prepare sufficient CCMV-S9
chimeric protein, a medium scale culture of 500 ml for 7 days was performed. Five positive recombinant *Pichia* clones (pCC3-63, pCC3-102, pCC3-114, pCC3-129 and pCC3-161) were cultured in non-methanol media, MGY for 24-48hs at 30 °C with 250 rpm incubation shaker, and then transferred to methanol-induced medium for 5 days. Yeast cells were harvested by centrifugation and CCMV-S9 coat proteins were purified from yeast cells. Samples were collected from each step of purification process for dot blot analysis (Figure 2.13). Dot blot analysis showed that most of the CCMV-S9 construct was associated with the cell pellet. However, with the yeast transformed with CCMV 161-S9, a detectable amount of CCMV-S9 can be visualized in the final purification step. Samples from each purification step were observed for the presence of viral particles by transmission electron microscopy. We could not detect any chimeric virus particles at any step of the purification but we could detect CCMV wt viral particle from the final purification step. The dot blot and EM data suggest two explanations. The first is that the purification protocol was not optimized resulting in less sensitive detection of proteins. The second is that the low production of protein may be the result of inappropriate conditions in cell growth or protein production resulting in less biomass or desired protein at the start of the purification process. A high density fermentation culture was introduced to circumvent this obstruction.
Figure 2. 13 Medium Scale CCMV-S9 Coat Protein Expression Experiment
Recombinant yeasts expressing CCMV-S9 coat protein were cultured in non-methanol medium for 2 days and transferred to methanol-inducing medium for 5 days. Cells were harvested and kept in -80 °C for viral purification. Samples of each purification step were collected for dot blot analysis. Frozen cells were lysed by adding acid-washed glass beads in equal volume virus buffer. The mixture was vortexed vigorously. Cell debris and glass beads were separated by centrifugation at low speed. Cell debris (sample 1) was kept in -80 °C. The supernatant (sample 2) was mixed with PEG 8000. The mixtures were stirred slowly overnight at 4 °C, centrifuged and the supernatant (sample 3) stored at -80 °C. The pellets were resuspended in virus buffer. Mixtures were briefly centrifuged with low speed. The pellets (sample 4) were stored at -80 °C and the supernatants (sample 5) containing virus were kept in -80°C for further analysis. Collected samples (number 1 to 5) were mixed with SDS loading buffer and boiled for 10 min and immediately put in an ice tub. Then 10 ul of each sample were loaded onto nitrocellulose membranes. The membranes were blocked with blocking buffer. Then the sample dots were determined by anti-CCMV antibody dilution 1/5000 as a primary antibody and anti-rabbit IgG-HRP dilution 1/1000 as a secondary antibody. Colorimetric detection was developed. The symbol + represents a CCMV-wt positive control purified from yeast cells.
Yeasts transformed with pcc3-161-S9 were picked to culture in the fermenter because they showed the highest protein expression in the small scale time course experiment and also the medium scale experiment. The fermentation method followed the Invitrogen *Pichia* fermentation process guidelines and the details are described in the Materials and Methods section.

Cells from the fermenter were harvested by centrifugation and divided into 100 g aliquots and kept at -80 ºC. Samples from each viral purification step were loaded onto nitrocellulose membranes for dot blot analysis with anti-CCMV antibody (Figure 2.14). Dot blot analysis indicated that 161-S9 chimeric protein was mostly maintained in the yeast cell debris (Figure 2.14, sample 1). The chimeric construct can be detected in later purification steps. However, it was still associated with pellet as seen in the dot blot analysis. The construct was found to associate with the DEAE particles in the filter cake, (Figure 2.14: sample 3 and sample 8). Even at the final step of viral purification, the dot blot analysis indicated that construct was associated with the pellet, (Figure 2.14, sample 11). This data shows that the association between the chimeric CCMV-S9 construct and the pellet or yeast cell debris was strong. A smaller amount of the chimeric construct was found in later purification step.

In order to dissociate chimeric CCMV-S9 construct from cell debris and pellet, urea was applied at the first step of virus purification. The presence of 2 M urea during the breaking of yeast cell was expected to increase recombinant viral protein solubility and disrupt noncovalent association between the viral coat protein and yeast cell debris. As shown in figure 2.15, most of the chimeric protein was still in the cell pellet.
Figure 2. 14 Large Scale CCMV-S9 Chimeric Construct Expression Experiment

The recombinant yeast clone, CCMV 161-S9, was cultured in the fermenter. Cells were harvested by centrifugation and lysed by grinding vigorously with acid-washed glass beads in a bead-beater in the presence of 50mM Tris-HCl buffer pH 6. Beads and cell debris (sample 1) were separated by centrifugation. Supernatant (sample 2) was used for further steps. DEAE was added to the supernatant. The mixture was stirred slowly at 4°C for 2 h. The mixture was then filtered and the filtrate (sample 4) was kept at -80°C for the dot blot analysis. The filter cake was washed twice with the Tris buffer. The filtrate after the first wash and the second wash were mixed together (sample 5) and kept at -80°C for the dot blot analysis. The filter cake (sample 3) was resuspended in virus buffer supplement with 0.5 M NaCl. The suspension was filtered and the filtrate (sample 6) was collected. The filter cake was resuspended again with the virus buffer. The suspension was again filtered. The filtrate (sample 7) was collected and pooled with the filtrate from earlier step (sample 6). The filter cake (sample 8) was kept at -80°C for the dot blot analysis. The pooled filtrate was centrifuged at 25 krpm for 2 h. The supernatant (sample 9) was kept at -80°C for the dot blot analysis and the pellet was resuspended in the virus buffer. The mixture was centrifuged briefly at low speed to remove impurities. The supernatant (sample 10), which was supposed to contain virus and the pellet (sample 11) was kept at -80°C for the dot blot analysis. Collected samples (number 1 to 11) were mixed with SDS loading buffer and boiled for 10 min and immediately put in an ice tub. Then 10 ul of each sample were loaded onto a nitrocellulose membrane. The membranes were blocked with blocking buffer. Then the sample dots were detected by anti-CCMV antibody and anti-Rabbit IgG-HRP. Colorimetric detection was developed. The symbol + represents a CCMV-wt positive control purified from yeast cells. The symbol – represents a non transformed yeast wt as negative control.
Figure 2. 15 Virus Purification with or without the Presence of Urea

Cells were lysed by grinding vigorously with acid-washed glass beads in a bead-beater in the presence 50mM Tris-HCl buffer pH 6 without 2 M Urea (upper panel) or with 2 M urea (lower panel). Beads and cell debris (sample 1) were separated by centrifugation. Supernatant (sample 2) was used in further steps. DEAE was added to the supernatant. The mixture was stirred slowly at 4°C for 2 h. The mixture was then filtered and the filtrate is sample 4. The filter was washed with the Tris buffer twice. The filtrate after the first wash and the second wash were mixed together (sample 5). The filter cake (sample 3) was resuspended in virus buffer supplemented with 0.5 M NaCl. The suspension was filtered and the filtrate (sample 6) was collected. The filter cake was resuspended again with the virus buffer. The suspension was again filtered. The filtrate (sample 7) was collected and pooled with the filtrate from earlier step (sample 6). The filter cake was sample 8. The pooled filtrate was centrifuged at 25 krpm for 2 h. The supernatant was saved (sample 9) and the pellet was resuspended in the virus buffer. The mixture was centrifuged briefly at low speed to remove impurities. The supernatant (sample 10), which was supposed to contain virus and the pellet (sample 11) was kept at -80°C for the dot blot analysis. Collected samples (number 1 to 11) were mixed with SDS loading buffer and boiled for 10 min and immediately put in an ice tub. Then 10 ul of each sample were loaded onto a nitrocellulose. The membranes were blocked with blocking buffer. Then the sample dots were determined with anti-CCMV antibody and anti-rabbit IgG-HRP or 5 ug/ml S9 antibody and anti-mouse IgM-AP (right panel). Colorimetric detection was developed. The symbol + represents a CCMV-wt purified from yeast cells using the same protocol with the absence of urea. The CCMV-wt sample at final step was collected for the dot blot analysis with anti-CCMV antibody. The symbol – represents crude extract from a non transformed yeast wt as a negative control. S9 peptide concentration 0.2 ug/ml and 0.02 ug/ml were loaded onto the membrane as positive controls for the dot blot analysis with S9 antibody.
The presence of 2 M urea during the breaking of yeast cells was expected to increase recombinant viral protein solubility and disrupt noncovalent association between the viral coat protein and yeast cell debris. As shown in figure 15, most of the chimeric protein was still in the cell pellet. The presence of urea did not affect the interaction between the chimeric construct and cell debris. CCMV-S9 viral particles could not be obtained from the purification with or without urea application. Dot blot analysis with S9 mAb indicated that CCMV-S9 construct can be expressed but was found in the pellet with cellular debris. On the other hand, the CCMV wt construct could be obtained easily with the same protocol even without urea. These data indicated that the chimeric CCMV-S9 construct can be expressed but cannot be separated from yeast cell debris or pellet particles, probably because it failed to form intact virus-like particles, but rather aggregates and precipitates.

Samples from each purification step were observed for the presence of viral particles by transmission electron microscopy. We could not detect any chimeric virus particles at any purification steps, whereas wt CCMV viral particle was observed in final step purification (figure 2.16)

These results demonstrated that chimeric coat proteins fail to assemble virus-like particles, just as they failed to assemble infectious virions during plant expression. Because we are able to produce both infectious virions, and virus-like particles with wild-type constructs, we believe that these results clearly demonstrate the inability of the CCMV carrying the S9 epitope to assemble a functional capsid.
To present the S9 peptide on a viral particle, chemical linkage of S9 peptides to CCMV viral particles was pursued in our next experiment.

Chemical Linkage

To present peptide S9 on the surface of the CCMV virion, we first applied a chemical method to conjugate S9 peptide to CCMV through disulfide formation. Thus we introduced an additional C-terminal cysteine residue to the S9 peptide, creating a thiol group for disulfide linkage. Mutant CCMV, A163C, which has Cys instead of original Ala at position 163, was chosen for use. The coupling reaction was performed as described in the Materials and Methods section. The coupling products were loaded onto SDS non-reducing gels and visualized by silver staining (Figure 2.17). CCMV A163C mutant strain virus can conjugate with S9 peptide through disulfide bond formation. The
oxidizing agent CuSO₄ can cause a minor improvement of the chemical reaction. The problem experienced with this cross-linking method is that the conjugation product was aggregated. The aggregation occurred when the product was left at 4°C in the virus buffer. The aggregation reaction may be the result of continuous covalent disulfide bonds forming between viruses. The insoluble conjugation product could not be utilized in the vaccination, which is our purpose. Heterobifunctional linkers, sulfo-smcc and spdp were then introduced in the experiment to crosslink CCMV to the S9 peptide and circumvent the aggregation problem.

Figure 2. 17 Cross-Linking between Cys of S9 and Cys of Mutant A163C
CCMV mutant, A163C, 0.5 mg/ml, 2 ml was mixed with S9 peptide 0.5 mg/ml, 2 ml in the presence or absence of 40 ul 1 M CuSO₄. The mixture was incubated at room temperature for 2 h. 10 ul of the mixture were mixed with 10 ul SDS PAGE gel buffer without mercaptoethanol and boiled for 10 min and then placed in the ice tub immediately. 10 ul of the sample were loaded into 12% nonreducing SDS PAGE gel. From left to right are mutant A163C, the cross-linked A163C-S9 without CuSO₄, the cross-linkedA163C without S9 peptide, and the A163C-S9 with CuSO₄.
The spdp linker is composed of a N-hydroxysuccinimide (NHS-ester) group and 2-pyridyldithil group (Figure 2.1). The NHS-ester will react with lysine associated amines of the CCMV coat protein leading to a conjugation product via amide bond formation. The reaction occurs at pH 7-8. Thus CCMV wt is not suitable for conjugation because it is only stable at a low pH (pH4) (317). The salt stable CCMV mutant (ssCCMV), which can tolerate a high pH and high ionic strength (319) was used in the experiment instead of CCMV wt.

The ssCCMV virus was first conjugated to S9 by using the spdp linker. ssCCMV was mixed with 3-fold molar excess to CCMV at pH 7.5 and the S9 peptide with 50-fold molar excess of spdp. The pH of the reaction was adjusted to be pH6.5 and was performed at room temperature for 1.5 h. The conjugation product was visualized by silver staining and characterized by western blot analysis (Figure 2.18).

Figure 2. 18 Product of ssCCMV and S9 Peptide Cross-Linked via the spdp Linker. ssCCMV and S9 were conjugated via the linker spdp. The conjugation products were loaded onto 12% SDS-PAGE and visualized by silver staining or transferred to nitrocellulose membranes for western blot analysis with CCMV antibody.
The silver staining and western blot result indicated that the ssCCMV was conjugated to S9 peptides via the spdp linker. The extra bands seen in the western blot analysis reflects the conjugation products.

Since primary amine reacts with NHS-ester at pH 7-9, to get the best coupling reaction, we performed an experiment with two different pH conditions, 7 and 7.5. We also varied the concentration of spdp linkers to be 1, and 2-fold molar excess to CCMV virus. The conjugation products were loaded into SDS-PAGE gel and visualized by silver staining (Figure 2.19). A greater degree of coupling was observed at pH 7.5 and more cross-linked products were obtained when the spdp linker was equimolar to the virus.

Figure 2.19 The Conjugation Product of ssCCMv-S9 via spdp Linkers at Different pH Conditions and Different spdp Concentrations.

The spdp linkers were used in 1 or 2-fold molar excess to CCMV. The reaction between the linkers and viruses was performed at pH 7 or 7.5 (pH of reactions was adjusted by adding NaH₂PO₄ 1 M pH 9 for 1 hr at room temperature in the shaker. The conjugation products were loaded onto 12% SDS-PAGE and visualized by silver staining.

We next performed coupling experiments with different S9 peptide concentrations to analyze if concentration of peptide affects the coupling reaction resulting in more, or less, CCMV-S9 conjugation product (Figure 2.20). Different concentrations of S9
peptide, 0.5, 1, 10, 20, 30, 40 and 50-fold molar excess to CCMV were separately added to the spdp-CCMV derivatized and the pH of reaction was controlled at 6.5. Adding peptides at less than 10-fold molar excess to CCMV reduced the cross linking reaction. Using a 10-fold molar excess of peptide to CCMV seemed optimal since a 50-fold molar excess of peptide to CCMV did not provide a better yield of cross-linked material.

Figure 2. 20 ssCCMV-S9 Conjugation Products via spdp Linkers at Different Peptide Concentrations
The ssCCMV was incubated with the fresh prepared spdp linker at 1:1 molar ratio. The reaction was allowed to continue at room temperature pH 7.5. After 1 h, the reaction was stop by incubating with 1.5 M hydroxylamine pH 8.5 for 1 h. Different concentrations of S9 peptide were used in 0.5, 1, 10, 20, 30, 40 and 50-fold molar excess to CCMV. The reactions were allowed to continue at room temperature for 1.5 h. 5 ul of reaction sample was mixed with 5 ul of SDS loading buffer without β-mercapto ethanol and boiled for 10 min and placed on ice. The samples were loaded onto 12% SDS-PAGE and visualized by silver staining.

The CCMV-S9 conjugates were next characterized by sucrose gradient sedimentation. The ssCCMV-spdp-S9 conjugates were overlaid on the top of a 5-25% sucrose density gradient and sedimented. The gradients were manually fractionated and test samples loaded onto SDS-PAGE (Figure 2.21).
Figure 2. Characterization of CCMV-S9 by Sucrose Gradient Sedimentation

ssCCMV was incubated with freshly prepared spdp linker at a 1:1 molar ratio. The reaction was allowed to continue at room temperature pH 7.5. After 1 h, the reaction was stopped by incubating with 1.5 M hydroxylamine pH 8.5 for 1 h. S9 peptides were used in 10-fold molar excess to CCMV. The reactions were allowed to continue at room temperature for 1.5 h. 500 ul of the conjugation product, and unconjugated ssCCMV virions were loaded 5-25% sucrose gradients and centrifuged at 37,000 rpm for 3 h. The gradients were fractionated 1 ml/fraction (21 fractions/tube). 10 ul of odd numbered fractions were mixed with SDS loading buffer without β-mercapto ethanol and boiled for 10 min, loaded onto 12% SDS-PAGE, and visualized by silver staining. The odd number fractions, 1-15, from ssCCMV were loaded onto gel A. The odd numbered fractions, 1-15, from ssCCMV-spdp-S9 conjugate were loaded onto gel B. The remaining odd number fractions, 17-21, from ssCCMV were loaded into the first half of gel C and the remaining odd number fractions, 17-21, from ssCCMV-spdp-S9 were loaded into the second half of gel C (C figure). The ss indicates ssCCMV mutant and ss-S9 indicates ssCCMV-spdp-S9 conjugates. M indicates protein molecular weight marker.
The ssCCMV nonconjugate virion fell in the middle of the gradient (fraction 9-11), whereas the CCMV-S9 conjugate fell in the bottom of the gradient (fraction 21). The result indicated that the conjugation product exhibited higher sedimentation velocities compared to the unconjugated ssCCMV particles. These higher sedimentation velocities of the conjugated particles probably indicate that the conjugated particles aggregated together. This may result from unexpected disulfide bond exchanging between derivatized CCMV (CCMV-spdp) (Figure 2.1), thus causing aggregation that results in different sedimentation velocities.

To attain coupling of S9 to CCMV without aggregation, the mutant CCMV, subE, was used. SubE is a CCMV mutant stain, in which eight basic amino acid residues (K and R) of the N-terminus were replaced with the acidic residue glutamic acid (E) (320). Conjugation to SubE virions leads to efficient viral disassembly (unpublished data). The SubE-S9 construct serves as a control for the effect of fully assemble virus, since it represents the peptide conjugated to a monomer form of virus coat protein. Thus we conjugated SubE to S9 via spdp linkers at 1:1 molar ratio of spdp:CCMV. The reaction was allowed to continue at room temperature pH 7.5, 1 h, and stopped by incubating with 1.5 M hydroxylamine pH 8.5 for 1 h. The S9 peptides were used in 10-fold molar excess to SubE. The reactions were allowed to continue at room temperature for 1.5 h. The subE-S9 conjugates were visualized by SDS-PAGE analysis and analyzed by western blot analysis (Figure 2.22). The conjugated SubE-S9 products were able to be detected by western blot analysis against anti-CCMV antibody.
SubE CCMV and S9 were conjugated via the linker spdp. The conjugation products were loaded onto the 12% SDS-PAGE and visualized by silver staining or transferred to the nitrocellulose membrane for western blot analysis. Anti CCMV antibody and enzyme conjugated mouse anti rabbit IgG antibody were used in the western blot.

SubE-S9 conjugates were next characterized by sucrose gradient sedimentation. The SubE-spdp-S9 conjugates were overlaid on the top of 5-25% sucrose gradients and sedimented. The gradients were manually fractionated and loaded onto SDS-PAGE (Figure 2.23). The unconjugated SubE virions fell in the middle of the gradient (fraction 5-7). The SubE-S9 conjugate fell in the first fraction of the gradient (fraction 1). This result indicated that cross linking SubE with S9 peptide via the spdp linker caused the virus to fall apart. Thus the conjugation product from SubE is capsid protein subunit conjugated to the S9 peptide. The result was confirmed by observation of virus under EM. We could not see any particles from SubE-S9 conjugate.
Figure 2. 23 Conjugation Product SubE-spdp-S9 in Sucrose Gradient Velocity

The SubE was incubated with the fresh prepared spdp linker at a 1:1 molar ratio. The reaction was allowed to continue at room temperature pH 7.5. After 1 h, the reaction was stopped by incubating with 1.5 M hydroxylamine pH 8.5 for 1 h. The S9 peptides were used in 10-fold molar excess to CCMV. The reactions were allowed to continue at room temperature for 1.5 h. 500 ul of the conjugation product and or unconjugated SubE were loaded on 5-25% sucrose gradients and then centrifuged at 37,000 rpm for 3 h. The gradients were fractionated 1 ml/fraction (21 fractions/tube). 10 ul of odd numbered fraction were mixed with SDS loading buffer without β-mercapto ethanol and boiled for 10 min, loaded onto 12% SDS-PAGE and visualized by silver staining. The odd numbered fractions, 1-13, from SubE were loaded into gel A from the third lane (one lane apart from the protein molecular weight marker, to prevent contamination from reducing buffer β-mercapto ethanol contained in the markers). The odd numbered fractions, 1-13, from SubE-spdp-S9 conjugate were loaded into gel B from the third lane (one lane apart from the protein molecular weight marker).

Another linker sulfo-smcc was introduced to replace the spdp linker to create non-aggregated ssCCMV-S9 particles. The sufo-smcc linker is composed of an NHS ester
group and maleimide group. As with the spd-p linker, the NHS ester group reacts with a primary amine in the CCMV coat protein, likely lysine. The amide bond is formed, releasing N-hydroxysuccinimide (Figure 2.2). The maleimide group reacts with thiol group of the peptide when the pH of the reaction mixture is maintained between 6.5 and 7.5. To preclude the aggregation between virus particles through the disulfide linkage that might occur during the amide bonding, the ssCCMV was treated with tris (2-carboxyethyl) phosphine (TCEP) to a final concentration of 50 mM. The TCEP reduces the disulfide formation without affecting the other protein functional groups in the virus, especially free amines. To assure that all disulfide bonds were reduced the ssCCMV was treated with 2-iodoacetamide (IA), an alkylating sulfhydryl reagent. The IA binds covalently with cysteine on the virus. Thus there was no available cys from the virus to react with the maleimide group. Cross-linking between ssCCMV and S9 was performed with or without the IA treatment (Figure 2.24).

Figure 2.24 shows that treatment with 50 mM IA did improve the conjugation result. With or without IA treatment, the virus can cross-link to the peptide via the sulfo-smcc linker, resulting in the conjugated product. With IA treatment, a dimer aggregation band did not occur. The result showed that pretreating ssCCMV with 2-iodoacetamide reduced disulfide formation between the viruses resulting in less dimer product formation.
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Figure 2. The Conjugation Products Between ssCCMV and S9 Peptide via Sulfo-smcc Linker with and without Iodoacetamide Treatment (IA treatment).

ssCCMV (ss) in 20 mM NaH₂PO₄ pH 7.5 was treated with TCEP to a final concentration of 50 mM at room temperature for 1 h. The 2-iodoacetamide (IA) 0.5 M was added to the virus to 50 mM final concentration, and incubated at room temperature for 30 min. The virus solution was purified to eliminate excess TCEP and the IA by using the microcon 100 (m). Virus was washed extensively with 20 mM NaH₂PO₄ pH 7.5 and eluted with the same buffer. Fresh prepared sulfo-smcc (sc) linker was used in 50-fold molar excess to the virus. After adding the linker, the pH of the reaction was remeasured and adjusted to be 7.5 with 1 M NaH₂PO₄ pH 9. The reaction was for 1 h in room temperature. Lysine was added to the reaction to have 1 mM final concentration and kept for 15 min at room temperature to stop the reaction. The excess linker and lysine were removed by using m100 and washed and eluted with the 20 mM NaH₂PO₄ pH 7.5. The S9 peptide was pretreated with TCEP to a final concentration of 50 mM for 1 h at room temperature prior to coupling with the derivatized virus. The pretreated S9 peptide was used in 50 molar excess to the ssCCMV. The pH was controlled at pH 7 with 1 M NaH₂PO₄ pH 9. The reaction of maleimides with thiols was for 1.5 h in room temperature. 5 ul of each step in the cross-linking process was mixed with 5 ul of SDS loading buffer containing β-mercapto ethanol and boiled for 10 min, loaded into 12% SDS-PAGE, and visualized by silver staining.
We next asked if the incubating temperature and time period of the reaction between the ssCCMV virus and the linker affects the coupling reaction. To answer the question, the ssCCMV was treated with TCEP and IA before adding the linker. The sulfo-smcc linker was used in 50-fold molar excess to the ssCCMV. The virus was incubated with the linker in 2 conditions, at room temperature or at 4°C. The length of incubation was 2, 6 or 24 h for the room temperature condition and 6, 12, 18 or 24 for the 4°C temperature condition. After removing excess linkers out of the solution by using m100, pretreated S9 peptide with TCEP was added in 50-fold molar excess to the ssCCMV. The conjugation product was analyzed by SDS-PAGE gel (Figure 2.25). The silver staining data indicated that conjugation can occur in both condition. NHS reactions with primary amine can occur in 6 h and reached the maximum in 12 h. Incubating the virus with the linker for 24 h at room temperature pushed the NHS reaction, resulting in multimer conjugation between the viruses. The reaction at 4°C slows the amide forming reaction compared to the reaction at room temperature. The slower reaction rate allowed the virus to conjugate as amonomer, since multimer coupling products cannot be detected when the reaction was performed at 4°C. Performing the reaction at 4°C allowed more selective amide formation than did performing the reaction at room temperature, and thus prevented the formation of multimers.
Figure 2. 25 Time Course Experiment and Temperature Effect on Coupling Reaction

The ssCCMV (ss) was treated with TCEP and IA before adding the linker. The sulfo-smcc linker was used in 50-fold molar excess to the ssCCMV. The virus was incubated with the linker in 2 conditions, at room temperature (upper panel) or at 4°C (lower panel). The length of incubation was 2, 6 or 24 h (sc2, sc6 and sc24) for the room temperature condition and 6, 12, 18 or 24 h (sc6, sc12, sc18 and sc24) for the 4°C temperature condition and the reaction was stopped by adding lysine to final concentration 1mM.

After removing excess linkers out of the solution by using m100 (m), TCEP-treated S9 peptide was added at 50X molar excess to the ssCCMV. 5 ul of samples from each step of cross-linking process were mixed with SDS loading gel buffer containing β-mercapto ethanol and analyzed by SDS-PAGE gel. M is the protein molecular weight marker, ss stands for ssCCMV, ft is the flow through after passing reaction solution through microcon 100. The sc represents the linker sulfo-smcc and the number after sc represents the incubation time length. For example, sc6 means incubating with the sulfo-smcc linker for 6 h.
To ask if the linker concentration affected the cross-linking reactivity, different concentrations of sulfo-smcc, (25, 50, 100 or 200-fold molar excess to the ssCCMV virus), was added in the cross-linking reaction. The conjugation products were analyzed by SDS-PAGE gel analysis (Figure 2.26).

The primary lysines on the ssCCMV are shown to react with NHS-ester from linker in a dose dependent manner causing different amounts of ssCCMV-sufo smcc-S9 conjugation products. Using the linker in 100-fold molar excess to the virus pushed the reaction to the plateau. There was not much difference between using the linker in 100 and 200-fold molar excess to the virus. The derivatized ssCCMVs with a higher concentration of sulfo-smcc linker (200-fold molar excess) were found in the flow through in the step of passing the derivatized virus through the size-exclusion filter microcon 100. However, with lower concentrations of linker, 25, 50 and 100-fold molar excess, the derivatized ssCCMV-linker cannot be detected in the flow through. Based on this result, the next experiment was performed by using the linker sulfo-smcc in 100-fold molar excess to the ssCCMV virus.

To investigate if the S9 peptide concentration affected the cross-linking, different concentrations of S9 peptide: 12.5, 25, 50, 100 or 200-fold molar excess to the ssCCMV virus, were tested in the cross-linking reaction. The conjugation products were analyzed by SDS-PAGE gel analysis (Figure 2.27).
Figure 2. 26 Effect of Different Concentration of Sufo-smcc: 25, 50, 100 and 200 Molar Excess to the ssCCMV Virus in Cross-Linking Reaction

The ssCCMV (ss) was treated with TCEP and IA before adding the linker. The sulfo-smcc linker was used in 25, 50, 100 and 200-fold molar excess to the ssCCMV. The pH of reaction is 7. The virus was incubated with the linker at 4°C for 12 h and the reaction was stopped by adding lysine to final concentration 1 mM. After removing excess linkers out of the solution by using m100 (m), TCEP-treated S9 peptide was added at 50 molar excess to the ssCCMV. 5 ul of samples from each step of cross-linking process were mixed with SDS loading gel buffer containing β-mercapto ethanol and analyzed by SDS-PAGE gel. M is the protein molecular weight marker. The ss stands for ssCCMV. The ft is the flow through after passing reaction solution through microcon 100. The sc represents the linker sulfo-smcc and the number before sc represents the concentration of the linker in term of molar ratio excess to the ssCCMV. For example, 25sc means that the linker was used in 25 molar excess to the ssCCMV virus.
Figure 2.27 indicated that using peptide in 12.5-fold molar excess to the virus provided the least conjugation product. Using peptide in 50-fold molar excess to the virus increased the reaction between the maleimide group of the linker and thiol group of S9 leading to high conjugation productivity. Adding more peptide than 50-fold molar excess to the virus did not improve the conjugation.

Figure 2. 27 Effect of Peptide Concentration on Cross-Linking
The ssCCMV (ss) was treated with TCEP and IA before adding the linker. The sulfo-smcc linker was used in 100-fold molar excess to the ssCCMV. The pH of the reaction was 7. The virus was incubated with the linker at 4°C for 12h and the reaction was stopped by adding lysine to final concentration 1 mM. After removing excess linkers out of the solution by using microcon100, TCEP-treated S9 peptide was used in 12.5, 25, 50, 100 or 200-fold molar excess to the ssCCMV. The reaction was performed at 4°C for 6 h. 5 ul of samples from each step of cross-linking process were mixed with SDS loading gel buffer containing β-mercapto ethanol and analyzed by SDS-PAGE gel. From left to right are ssCCMV (ss), coupling product with 200, 100, 50, 25, 12.5-fold molar excess to the ssCCMV and the last lane is protein molecular weight marker (M).
The ssCCMV-sulfo smcc-S9 particle was characterized by sucrose gradient sedimentation velocity. The ssCCMV was treated with TCEP and IA before adding the linker. The sulfo-smcc linker was used in 100-fold molar excess to the ssCCMV, pH 7. The virus was incubated with the linker at 4°C for 12h and the reaction was stopped by adding lysine to final concentration 1 mM. After removing excess linkers out of the solution by using microcon100, TCEP-treated S9 peptide was used in 50-fold molar excess to the ssCCMV. The reaction was performed at 4°C for 6 h. The ssCCMV-sulfo smcc-S9 conjugation products were loaded on to 5-25% sucrose gradient and sedimented. The gradients were manually fractionated and loaded into SDS-PAGE gel (Figure 2.28).

The sucrose gradient data showed that ssCCMV-sulf smcc-S9 particles remained intact and stayed as monomer virions, since they exhibited the same sedimentation velocities as underivatized ssCCMV particles. The ssCCMV-sulf smcc-S9 particles were observed under TEM, and particles were found.

Both conjugation products, SubE-spdp-S9 (Figure 2.22) and ssCCMV-sufo smcc-S9 (Figure 2.27), were purified by passing through size exclusion microcon 100 filters. The conjugation products were washed several times with sterilized 20 mM NaH2PO4 pH 6. The SubE-S9 coat protein-peptide conjugation product and ssCCMV-S9 particle were ready for the immunization experiment, which will be discussed in later chapters
Figure 2. 28 Conjugation Product ssCCMV-sulfo-smcc-S9 in Sucrose Gradient
500 ul of the conjugation product and unconjugated ssCCMV virions were loaded on the
top of 5-25% sucrose gradients and then centrifuged at 37,000 rpm for 3 h. The gradients
were fractionated 1 ml/fraction (total 21 fractions/tube). 10 ul of odd numbered fractions
were mixed with SDS loading buffer without β-mercapto ethanol and boiled for 10 min,
loaded onto 12% SDS-PAGE and visualized by silver staining. The odd number
fractions, 1-15, from ssCCMV (ss) were loaded into gel A. The odd numbered fractions,
1-15, from ssCCMV-sulfo smcc-S9 conjugate (ss-S9) were loaded into gel B. The
remaining odd number fractions, 17-21, from ssCCMV were loaded into the first half of
gel C and the remaining odd number fractions, 17-21, from ssCCMV-sulfo smcc-S9 were
loaded into the second half of gel C. The ss indicates ssCCMV mutant, and the ss-S9
indicates ssCCMV-sulfo smcc-S9 conjugates. M indicates protein molecular weight
marker.
Discussion

The use of plant viruses for recombinant protein expression has been studied extensively, including for vaccine development. In our study, we used CCMV as the model for the design of a new vector for protein expression. We describe in detail the techniques that we used to prepare chimeric CCMV and also CCMV virus-like particles (VLP). The X-ray crystal structure of CCMV reveals that viral coat protein has a core structure consisting of an eight stranded, antiparallel $\beta$-barrel motif, with exposed loops (326-328). The N-terminus and the C-terminus of the coat protein subunit extends in opposite directions from the $\beta$-barrel motif and plays an important role in virion assembly and stability (329-331). The CCMV coat protein can be expressed in *E. coli* and can be assembled *in vitro* into either empty virions or in combination with viral RNA (329). Based on three-dimension EM and X-ray crystal analyses, the Young lab identified permissive sites on exposed loops of coat protein subunit for foreign DNA insertion. Loop-specific *BamH1* cloning sites, where peptides could be expressed on the virus coat protein subunit, were created.

We initially inserted DNA to encode the S9-peptide (FDTGAFDPDWPA) within the loop sequence. The S9 peptide is a mimotope of type III Streptococcus group B capsular polysaccharide, which was shown to be both antigenic and immunogenic (1). Recombinant RNA3 encoding the S9 peptide sequence was *in vitro* transcribed for plant inoculation. Chimeric recombinant virus particles were expected after inoculating on cowpea and tobacco plants. The ELISA results revealed that no intact chimeric particles were obtained, whereas the wild-type CCMV particles did produce virus following plant
inoculation. *In vitro* translation demonstrated that inserts were in frame and could be translated. Results of inserting other peptides: peptide 44, HIV mimetic peptide (unpublished data) and peptide 11, cell-targeting peptide (332, 333), into CCMV exposed loops were consistent with our results (unpublished data). However, insertion of peptide 11 in loop position 129 did yield recombinant virus, but with loss of infectivity. The productive yield of CCMV-peptide 11 was low, and the plant infection was localized not systemic. It has been shown that movement protein of CCMV depends on the coat protein to mediate the cell-to-cell movement (334, 335). All data indicates that insertion of a peptide sequence into CCMV exposed coat protein loops leads to disruption of the coat protein structure and its ability to assemble. Insertion of peptides into viral coat proteins to create antigenic chimeras has been demonstrated in icosahedral virus such as poliovirus (296), human rhinovirus (297) and also cowpea mosaic virus (CPMV) (299). With these viruses the difficulty arises with a limitation of the size of peptide that can be inserted and the loss of insert after serial passaging; whereas for CCMV, viral assembly appears to be blocked.

The second attempt to present the S9 peptide incorporated into CCMV coat protein was by creating self-assembling recombinant S9-viral coat protein in heterologous systems resulting in procapsids called virus-like particles (VLP). We chose *P. pastoris* as our heterologous expression system. Yeast requires methanol for protein production and has been used to produce other VLPs such as bacteriophage Qbeta (324) Norovirus (336), Hepatitis B virus (337) and Hepatitis C virus (338). For this, we subcloned the DNA cassette encoding S9-CCMV recombinant coat protein into a *Pichia*
pastoris, expecting intracellular recombinant VLP production. DNA sequencing analysis and \textit{in vitro} translation results indicated that the cassettes were in frame and able to be translated. The dot blot results indicated that recombinant VLP were obtained after methanol induction. However, the recombinant protein yield was low, even when we used the fermentor batch to produce the recombinant yeasts. In the fermentor batches, we produced recombinant proteins but could not purify the recombinant chimeric VLP from the yeast cell debris, whereas wt CCMV VLPs can be obtained easily from the same purification. Urea was applied to disrupt noncovalent associations between the viral coat protein and yeast cell debris, but we were still unable to gain CCMV-S9 VLP. This data indicated that the insertion of S9 into the CCMV virus coat protein also inhibits the formation of intact chimeric VLP, just as it does infectious virions. However, other chimeric plant viruses have been successfully made as VLPs (299, 307, 308). It has been shown that success in chimeric VLP production is dependent upon the insertion sites into the coat protein. In the development of epitope expression in CPMV, the insertion of foot-and-mouth disease virus (FMDV) epitope into the S protein results in genetic instability resulting from homologous recombination (299, 310). Repositioning of the epitope insertion site results in the successful assembly of viral particles (310, 339, 340). However, some chimeric CPMV constructs are incapable of systemic infection, probably because the coat protein modifications interferes with viral movement (299). In our study, it is likely that coat protein modification in CCMV interfered with viral assembly.

Our third attempt to produce S9-CCMV particle was by using chemical methods to conjugate the CCMV to the S9 peptide. We first conjugated dS9 and CCMV through
disulfide bond formation between thiol groups on the virus coat protein and the C-terminal of peptide. The CCMV mutant, CCMV A163C with cysteine substituted at position 163, was used in the experiment because position 163 is on the exposed loops and considered to be suitable as B-cell epitope. The result indicated that conjugation products could be obtained, but they were aggregated resulting in precipitated particles at the bottom of the tube when the product was stored at 4°C. The aggregation reaction may have resulted from continuous covalent disulfide bonding between viruses.

We next used the chemical linkers, spdp and sulfo-smcc to conjugate the virus and peptide. The NHS ester group on spdp or sulfo-smcc reacts with primary amines on the viral coat protein. The 2-pyridyldithio group of the spdp linker reacts with sulfhydryl at the amino terminal of S9 peptide. A maleimide group of sufo-smcc reacts with the thiols on the S9 peptide. The coupling reaction requires pH 6-7.5. Wild-type CCMV is stable at pH 5 and low ionic strength. Therefore, we used the salt stable mutant of CCMV (ssCCMV), which resists disassembly in 1.0M NaCl, pH 7.5 (319, 341). The result indicated that the ssCCMV can conjugate to the S9 via spdp linker but the conjugation product again aggregated. According to the sucrose gradient analysis, the conjugation product was found in the bottom of the gradient whereas the unconjugated ssCCMV was found in the middle of gradient. The data indicated aggregation of the conjugation product, which may result from unexpected disulfide bond exchanging between derivatized CCMV (CCMV-spdp). Then we changed the linker to sulfo-smcc to create nonaggregated S9-CCMV particles. We could obtain the conjugation product with the help of 2-iodoacetamide (IA), an alkylating sulfhydryl reagent. The IA binds covalently
with cysteine on the virus, resulting in no available cys from virus to react with the maleimide group. The sucrose gradient analysis revealed that the ssCCMV-S9 particles remained intact and stayed as monomers, since they exhibited the same sedimentation velocities as underivatized ssCCMV particles. To create the coat protein subunit-S9 conjugate, the SubE mutant in which eight basic amino acid residues (K and R) of the N-terminus were replaced with acidic residue glutamic acid was used (320). The sucrose gradient analysis indicated that conjugation of SubE virions to S9 results in efficient viral disassembly, since the coupling product was found at the top of the gradient. The western blot analyses showed that the yield of conjugation products was low and less virus seemed to be conjugated. One possible explanation is that there might be few accessible lysines on the coat protein, resulting in less conjugation product. A second explanation is that the coupling reaction might not be suitable, disrupting natural coat protein folding of viral particle. Another virus, Cowpea Mosaic Virus (CPMV), was used to conjugate to fluorescein N-hydroxysuccinimide (NHS) and biotin (311, 312). At neutral pH, wild-type CPMV was found to carry up to 60 dye molecules per virion, in a dose-dependent manner. At pH 8.3, 1.5 dye molecules per asymmetric unit (90 per virion). Under maximal conditions, up to 4 lysine residues per asymmetric unit could be labeled (311, 312). The conjugation between CPMV and S9 was kindly performed by the Johnson Lab, (Scripps Institute, La Jolla CA). The conjugation product yield was higher than CCMV-S9 conjugation (discussed more in next chapter). In conclusion, the CCMV-S9 particles and CCMV coat protein subunit-S9 conjugates (made with the SubE mutant, which fails
to assemble) were obtained from the experiment and were ready to use in immunization experiments, which will be discussed in the next chapter.
MURINE IMMUNE RESPONSE TO CCMV-CONJUGATED PEPTIDE MIMICS OF GBS CAPSULAR POLYSACCHARIDE

Introduction

Group B streptococci (GBS) *Streptococcus agalactiae* is a commensal of the genitourinary tract in 15-40% of healthy adult women (342). It is reported as a major cause of neonatal sepsis and meningitis in developed countries (99), with type III GBS predominate (100, 343). Neonatal GBS infection is acquired during delivery by vertical transmission. Neonates are exposed to GBS either via GBS ascending through ruptured amniotic membranes, aspiration of contaminated amniotic fluid, or through exposure to colonized tissues while passing through the birth canal (344). Immunity to GBS infection in humans (168), mice (345) and rats (346) is due to type-specific capsular polysaccharide antibody. GBS capsular polysaccharide (CPS) is both a virulence factor and the target of a protective humoral immune response (143, 168, 191, 345-348). GBS vaccine development has been based on immunization with CP or CP conjugated to a protein carrier, leading to protective levels of maternal IgG type specific anti-GBS antibodies, which could be transplacentally passed to the newborn. Vaccines containing CPS of group B streptococcus induced protective IgG antibodies in only 60% of vaccinees (191, 346). Conjugation of CPS to protein carriers, including tetanus toxoid (TT) (192, 346) and choleratoxin B subunit (199) induce much better protective IgG antibodies. GBS CPS is a poor immunogen and acts as a T-independent antigen resulting in IgM production instead of IgG, and a lack of immunological memory.
An alternate approach is to design antigenic mimics, and relevant to our approach is using peptide-display phage libraries to search for peptides that mimic carbohydrate epitopes. The technique was developed by G. P. Smith (238) and is a powerful tool to identify mimotopes for vaccine development. Several carbohydrate mimetic epitopes have been reported by using this technique (1, 273, 276, 349, 350). In our lab, previously, we identified peptide mimics of a protective epitope of GBS by the means of a phage display library (1). The S9 mAb demonstrating in vivo protection against GBS type III (172, 174) was used to select displayed peptides. The S9 peptide, FDTGAFDPDWPA was isolated. The S9 peptide is shown to be both antigenic and immunogenic. Immunization of mice with peptide-carriers results in the production of GBS antibodies (1).

Several systems have been developed to improve vaccines for small peptides epitopes. Conjugation to carrier protein such as TT or keyhole limpet hemocyanin (KLH) has been extensively used in experimental studies to test candidates for vaccine development. An alternative scheme to enhance the immunogenicity of the peptides is to fuse the peptide sequences to the genes coding for virus capsid proteins, which have ability in self-assembly to be virus-like particles (VLP), or chemically conjugate peptides to virion or to VLP. Several plant viruses have been successfully used as expression vectors to present epitopes for vaccine development (308, 310, 340, 351, 352). Our studies have focused on the use of the plant virus Cowpea Chlorotic Mottle Virus (CCMV) to present the S9 peptide on viral coat proteins. The structure of CCMV, determined by X-ray crystallography, demonstrates that the virion is comprised of 180
copies of the coat protein subunit (317). The attempt to genetically modify CCMV coat protein to express the S9 peptide is discussed in the previous chapter. We failed to genetically express S9 fused to the viral coat protein, due to failure of virus assembly. We then chemically conjugated the S9 peptide to the CCMV virus and to the VLP of CCMV. In this study, we investigated antibody responses of mice immunized with the virus-S9 conjugate compared with KLH-S9 conjugate and evaluated their immunogenicity.

**Materials and Methods**

**Preparation of Antigens**

The S9 peptide, which mimics the type III GBS CPS, was chemically conjugated to CCMV ss and SubE mutants, as described in the previous chapter. CCMV wild type was obtained from the Young lab (Montana State University). Cowpea Mosaic Virus (CPMV) wild type and conjugated to S9 peptide were kindly provided by Dr. John E. Johnson (Scripps Institute, La Jolla CA). Synthetic peptides (Sigma Laboratories (USA)) containing Cys at the COOH peptide terminals were conjugated to maleimide derivatized KLH (Pierce Chemical Co., Rockford, IL). Maleimides react with sulfhydryls at pH 6.5-7.5 to form stable thioether bonds. Peptides (300 ug) were mixed with KLH (300 ug) (1:1 ratio) in phosphate buffer pH 7 in a reaction volume of 300 ul. The reaction was allowed to continue at room temperature for 3 h. The unconjugated peptides were removed by using microcon 100 (Millipore, Billerica, MA) filtration. The conjugated KLH-peptides were quantified by using BCA protein assay reagent kit.
We used ELISA to determine the amount of S9 peptide and virus in each preparation. Protein concentrations were measured using BCA Protein Assay Reagent Kit from Pierce (Rockford, IL). The first titration was performed to compare CCMV concentration in each preparation. 96 well microtiter plates were coated with the 0.2, 0.1, 0.05, 0.025 and 0.0125 ug/ml (based upon BCA concentration) of CCMV, CCMV-S9, SubE-S9 or KLH-S9. Then the coated plates were blocked with 1% BSA in phosphate-buffered saline (PBS). Primary antibody, rabbit anti-CCMV (1:3000 dilution) was incubated in microtiter wells at 4°C for 18 h. Plates were washed with ELISA washing buffer, 10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20 and incubated with alkaline phosphatase-conjugated anti-rabbit Ig (Zymed Laboratories, South San Francisco CA) at a 1/10,000 dilution for 6 h, followed by washing and the addition of colorimetric substrate p-nitrophenyl phosphate (Sigma Chemical) 1 mg/mL in 10% diethanolamine buffer, pH 9.6, with 0.5mM MgCl2. Absorbance at 405 nm was determined using a microplate reader (EL-320, Bio-Tek Instruments, Winooski, VT). A second assay was performed to measure the amount of S9 peptide in each antigen preparation. Plates were coated with the 0.4, 0.2, 0.1, 0.05, 0.025 and 0.0125 ug/ml of ccmv-S9, subE-S9, cpmv-S9, cpmv or KLH-S9. S9 mAb at 5ug/ml in PBS buffer was used as the primary antibody and alkaline phosphatase-conjugated anti-mouse Iggs (H+L chain specific, Zymed) at 1/10,000 in PBS was used as the secondary antibody.

Immunization

Two sets of immunizations were performed. Female BALB/c mice 8-10 weeks were used. We compared the antibody responses to immunization with different antigen
preparations, with and without adjuvant, and the ability to prime for secondary immune responses to GBS.

In the first experiment, mice were separated into 2 main groups (20 mice in each group): adjuvant and no adjuvant. In the adjuvant group, mice were immunized with antigens, which were emulsified in a 1:1 ratio with complete Freund’s adjuvant (CFA, Difco, Detroit, MI) for the primary immunization, and with incomplete Freund’s adjuvant (IFA) for booster injections. The non-adjuvant groups were immunized with antigens which were diluted with PBS for primary and booster immunizations. Each group was separated into 5 subgroups (4 mice in each subgroup) each receiving a different antigen: CCMV, CCMV-S9, SubE-S9, CPMV-S9 and KLH-S9. Mice were immunized with following amount of antigens: CCMV 2 ug, CCMV-S9 20 ug, SubE-S9 20 ug, CPMV-S9 10 ug, KLH-S9 10 ug. The concentration of antigen was based upon ELISA results characterizing the amount of S9 peptide coupled to each antigen, as determined by ELISA. Mice were bled to collect sera before immunization (Bleed 1). Primary immunization was given subcutaneously (sc) and boosters intraperitonealy (ip). Booster immunization followed the primary immunization by four weeks and one week after boosting, all mice were bled to collect sera (Bleed 2) to measure antibody. Sera were diluted to appropriate concentrations in PBS to measure different antibody responses: 1/200 to test the antibody response to GBS; 1/1000 to test the antibody response to other mimetic peptides: S9-2, S7 and S11; and 1/5000 to test the antibody response to CCMV and S9 peptide. ELISA was used to measure total Ig and specific subclasses of antibodies.
Six subgroups of mice from this experiment were chosen for boosting with GBS: CCMV, CCMV-S9 and KLH-S9 from both the adjuvant and non-adjuvant groups. Five weeks after boosting with peptide, these mice were injected with heat-killed GBS. Overnight cultures of GBS type III strain 125 in Todd Hewitt broth was spun down and washed three times with sterilized PBS and then resuspended into sterilized PBS. Absorbance at 600 nm of suspended GBS was adjusted to OD 0.9. The cells were killed by heating at 60 °C for 30 min. Mice were injected subcutaneously with 0.1 ml of heat-inactivated culture. The heat-inactivated culture was checked for viability by plating on Todd Hewitt agar plates. Sera were collected at 3 and 7 days after challenge (Bleed 3 and Bleed 4). Antibody to GBS, S9 peptide and CCMV was measured.

In the second experiment, 24 mice were separated into 4 groups (6 mice in each group). Each group received a different antigen: CCMV, CCMV-S9, CPMV-S9 and KLH-S9. Groups of mice were immunized subcutaneously, without adjuvant, with following amount of antigens; CCMV 2 ug, CCMV-S9 20 ug, CPMV-S9 10 ug, and KLH-S9 10 ug. Mice were bled to collect sera before immunization (Bleed 1). Four weeks later, mice were reimmunized ip with the same amount of antigen. One week after boosting, all mice were bled to collect sera (Bleed 2) and antibody to GBS, S9 and CCMV was measured. Mice with high anti-S9 and anti-GBS antibody titers were chosen to be investigated for cytokine production.

Cell Purification and Culture

Mice from the second experiment with high anti-S9 and anti-GBS antibody titers were bled (Bleed 3) and immunized ip with the same antigen and dose as in the prime
and boost. Immunized and age-matched naive mice were bled and sacrificed 4 days after
the second booster immunization. Spleens and mesenteric lymph nodes were removed
aseptically. The spleens and mesenteric nodes were pooled, single-cell suspensions
prepared by aspiration into syringes, washed in culture medium (RPMI 1640), and
incubated on ice for 30 min in RPMI 1640. After one wash in RPMI 1640 medium, the
cells were resuspended in 10 ml of the same medium and layered onto 10 ml of
Lymphocyte M separation medium (Cedarlane Laboratories limited, Ontario, Canada).
The gradient was spun at 1750 rpm for 30 min at room temperature. Cells at the interface
of the gradient were collected, washed twice, and resuspended in cold culture media
(RPMI 1640 containing 2% fetal calf serum (FCS)), 100 U/ml penicillin, 100 g/ml
streptomycin, 50 mM 2-mercaptoethanol and 2mM l-glutamine (Gibco BRL,
Gaithersburg, MD). Cells were counted and used as splenocytes in some experiments, in
others the cells were passed through a column (Mouse T cell Recovery Column Kit from
Cedarlane Laboratories Limited, Ontario, Canada) to prepare CD4 T cells. Cells were
plated 100 ul per well (2×10^5 cell per well) into sterile 96-well microplates (Costar,
Corning Inc., NY) and stimulated either with medium, CCMV, CCMV-S9, S9, KLH-S9,
KLH, all at 20 ug/ml, or PHA-M (Sigma Chemical) at 10 ug/ml. Cultures were
incubated for 7 days at 37 °C in 5% CO2. The supernatants were collected at day 3, 5 and
7 and frozen at −20 °C until analyzed for cytokine production.
Collection and Preparation of Blood

Blood samples were taken from the lateral saphenous vein. Serum was prepared by centrifugation and stored at \(-20\, ^\circ C\). Sera were diluted to appropriate concentration with PBS/1\% BSA/0.01\% Na Azide before ELISA.

Enzyme-Linked Immunosorbent Assays (ELISA) of Peptide-Specific Antibodies, GBS-Specific Antibodies, and CCMV-Specific Antibody

CCMV or the peptide Ags: S9, S9-2, S7 and S11 were coated onto microtiter wells (Immulon, Costar) at 5 µg/ml for at least 18 h. Amino acid sequences of each peptide are indicated in Table 3.1.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>S9</td>
<td>FDTGAFDPDWPA</td>
<td>Mimic epitope of type III, GBS group B</td>
<td>(1)</td>
</tr>
<tr>
<td>S9-2</td>
<td>WENWMMGNAGC</td>
<td>Mimic epitope of type III, GBS group B</td>
<td>UO</td>
</tr>
<tr>
<td>S7</td>
<td>WGNWQDRLKC</td>
<td>Mimic epitope of GBS group B, C, G</td>
<td>UO</td>
</tr>
<tr>
<td>S11</td>
<td>EEYPHGPEPC</td>
<td>Mimic epitope of type I, GBS group B</td>
<td>UO</td>
</tr>
</tbody>
</table>

Note; UO : unpublished observation by Dr. Seth Pincus

GBS were coated onto microtiter wells using poly-L-lysine and glutaraldehyde, as described elsewhere (353). Plates were blocked with 1\% BSA and used within 1 week. Plates were washed with ELISA washing buffer (10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05\% Tween 20) prior to use. Test sera at appropriate dilutions were added to antigen-coated wells in triplicate and incubated at 4\(^\circ\)C for 18 h. The plates were washed
and incubated with alkaline phosphatase-conjugated anti mouse-Ig (heavy and light chain specific, Zymed laboratories, South San Francisco, CA) for 4 h at room temperature. Following incubation with the secondary antibody, plates were washed and the colorimetric substrate $p$-nitrophenyl phosphate (Sigma Chemical) was added. Absorbance at 405nm was determined using a microplate reader (EL-320, Bio-Tek Instruments, Winooski, VT).

**ELISA of S9-Specific Antibodies Subclasses in Serum**

S9-peptide was coated onto microtiter wells (Immulon, Costar) at 5 µg/ml. Mouse sera at a dilution of 1/2000 were added in triplicate to the plates and incubated at 4°C for 18 h. The plates were washed with ELISA washing buffer and incubated with rabbit antisera specific for -mouse IgG1, IgG2a, IgG2b or IgG3 (Zymed laboratories, South San Francisco, CA) diluted 1/8000 at 4°C for 18 h. Following a washing step, goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (Zymed Laboratories) was incubated in the wells for 6 h, followed by washing and addition of colorimetric substrate and $A_{405}$ was determined.

**ELISA of S9-Specific and GBS-Specific IgM Antibodies in Serum**

S9-peptide and GBS plates were prepared as described in section 4. Sera diluted 1/1000 for S9 plates and 1/200 for GBS plates were added in triplicate to the plates and incubated at 4°C for 18 h. The plates were washed and incubated with rabbit anti-mouse IgM μ chain specific antibody (Zymed laboratories, South San Francisco, CA diluted 1/1000). Goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (Zymed
Laboratories), diluted 1/10,000 was incubated in wells for 6 h, followed by washing and addition of colorimetric substrate and A405 was determined.

Assessment of Cytokines in Supernatants from Cultured Splenocytes and CD4 T Cells

A sandwich ELISA was used to determine the amount of gamma interferon (IFN-γ), interleukin 4 (IL-4) and IL-10 produced by the splenocytes and CD4 T cells during antigen activation. Antibody pairs, recombinant standards and ELISA reagent for the assay were purchased from eBioscience, Inc. Corning Costar 9018 96-well ELISA plates were coated in 100 µl/well of capture antibody in Coating Buffer. Plates were sealed and incubated overnight at 4°C. Wells were blocked with 200 µl/well of assay diluent (provided with reagents) and incubated at room temperature for 1 hour. The standards (IFN-γ, IL-4 or IL-10) were diluted by 2-fold serial dilutions to create appropriate standard curves and were added in duplicate to the plates. The supernatants from cell media were added in duplicate to the plates in a series of two-fold dilutions. We then added 100 ul/well of the diluted biotin-conjugated detection antibody. Plates were sealed and incubated at room temperature for 1 hour. After 5 washes, we added 100 ul/well avidin-peroxidase and the plates were incubated at room temperature for 30 min. After 7 washes, 100ul/well substrate, 2,2-azinobis-3-ethylbenzthiazoline-sulfonic acid with 0.3% H₂O₂ were added and incubated at room temperature for 15 min. The reaction was stopped with a stop solution (0.7M SDS in DMF and purified water, 1:1). The absorbance was measured at 450 nm. In each sample, the final concentration of IFN-γ, IL-4 or IL-10
was determined from the standard curves by calculating the mean concentration from two dilutions.

**Results**

**Antigen Titration**

To immunize with the same amount of the relevant antigen (S9 peptide) in preparations having different carrier proteins, methods of coupling, and coupling efficiencies; CCMV-S9, SubE-S9, CCMV, KLH-S9 and cpmv-S9 were titrated by ELISA. Antigens were diluted in PBS. The graphs in figure 3.1 and 3.2 display the results.

![Graph showing optical density vs. protein concentration](image)

**Figure 3.1 Comparison of CCMV Virus Antigen in Different Antigen Preparations**

96 well microtiter plates were coated with the 0.2, 0.1, 0.05, 0.025 and 0.0125 ug/ml of CCMV, CCMV-S9, SubE, SubE-S9 or KLH-S9. The coated plates were then blocked with 1% BSA in PBS. Anti-CCMV serum (1:3000 dilution) was incubated in the microtiter wells at 4°C for 18 h. Plates were washed and incubated with alkaline phosphatase-conjugated anti-rabbit Ig for 4 h, followed by washing and addition of colorimetric substrate p-nitrophenyl phosphate. Absorbance at 405 nm was determined. Results are means and standard errors.
Figure 3.2 Comparison of S9 Peptide in CCMV-S9, SubE-S9, CPMV-S9, CPMV and KLH-S9 Antigens

Plates were coated with 0.4, 0.2, 0.1, 0.05, 0.025 and 0.0125 ug/ml of CCMV-S9, SubE-S9, CPMV-S9, CPMV and KLH-S9. Plates were blocked with 1% BSA and washed with ELISA washing buffer. S9 mAb 5ug/ml in PBS buffer was added and incubated at 4°C for 18 h. After washing with ELISA washing buffer, alkaline phosphatase-conjugated anti-mouse Igs 1/10,000 in PBS were added into the wells and incubated at room temperature for 4 hours, followed by washing and addition of colorimetric substrate p-nitrophenyl phosphate. Absorbance at 405 nm was determined.
At the same nominal protein concentrations, the amount of CCMV antigen in SubE-S9 and CCMV-S9 was 10 fold less than the amount of CCMV antigen in unconjugated CCMV (Figure 3.1). This may have resulted from a degree of denaturation of the S9-conjugated viruses, and loss of antigenicity of the virus coat protein.

The amount of S9 peptide in the KLH-S9 is approximately equal to the amount of S9 antigen in the CPMV-S9 (Figure 3.2). The amount of S9 in the KLH-S9 is two-fold higher than the amount of S9 protein antigen in the CCMV-S9 and SubE-S9 (Figure 3.2). According to these titration results, we decided to choose concentrations of CCMV 2 ug, CCMV-S9 20 ug, SubE-S9 20 ug, CPMV-S9 10 ug, KLH-S9 10 ug in immunization experiments.

**Immune Response after Immunization**

In the first experiment, we immunized 2 groups of mice, with and without Freund’s adjuvant. Mice in the adjuvant group were immunized with antigens which were emulsified in complete Freund’s adjuvant and then 4 weeks later were reimmunized with the same amount of each antigen emulsified in incomplete Freund’s. Mice in the non-adjuvant group were immunized with antigens diluted in PBS. Each group was separated into 5 subgroups (4 mice in each subgroup), each receiving a different antigen: CCMV, CCMV-S9, SubE-S9, CPMV-S9 and KLH-S9. Groups of mice were immunized with the amount of antigen determined above. Mice received a primary, and 4 weeks later, a booster immunization. One week after the booster, sera were collected and tested for antibody response to S9 peptide, CCMV and GBS (Figure 3.3).
Figure 3. Antibody Response of Individual Mice to S9 Peptide, CCMV and GBS

Plates were coated with S9 peptide, CCMV or GBS. Prebleed (Bleed 1) and post-booster sera, (Bleed 2) at dilutions of 1/5000 for S9, 1/1000 for CCMV and 1/200 for GBS, were added into triplicate wells. Plates then were incubated at 4°C for 18 h. After washing with ELISA washing buffer, the anti-mouse IgG dilution 1/1000 was added into wells and incubated for 4 hours at room temperature. Colorimetric substrate p-nitrophenyl phosphate was added. Absorbance at 405 nm was measured. Results are means and standard errors.
ELISA results showed that sera from mice immunized with CCMV-S9, CPMV-S9 and KLH-S9, with or without adjuvant, made antibody response to the S9 peptide and that immunizing with Freund’s adjuvant increased the antibody response to the S9 peptide. Mice immunized with SubE-S9 antigen, with or without adjuvant were able to induce anti-CCMV response but not an anti-S9 response. Mice immunized with unconjugated CCMV, with or without adjuvant, made antibody responses to CCMV but not the S9 peptide. Adjuvant had little effect on the anti-CCMV response in mice immunized with CCMV, which was different than the effect of adjuvant on the anti-S9 response. It is difficult to judge the development of anti-GBS antibody response because prebleeds had high levels of “natural” anti-GBS antibody, and in controls not immunized with S9, the anti-GBS antibody increased with time. To confirm the results of the first experiment, we repeated the immunizations with a subset of these antigens (Figure 3.4). We immunized 4 groups of mice (6 mice per group) with the following antigens using no adjuvant: CCMV, CCMV-S9, CPMV-S9 and KLH-S9. All mice were bled to collect sera before immunization (Bleed 1). Mice received the primary immunization sc. Four weeks later, mice were boosted with the same antigen, given ip. Sera were collected 1 week after the booster immunization. Sera collected from CCMV-S9, KLH-S9 and CPMV-S9 bound to plates coated with S9 antigen. CPMV-S9 induced higher anti-S9 antibody levels than CCMV-S9 and KLH-S9. Control mice, immunized with unconjugated CCMV, did not produce anti-S9. Similar problems as described above (Figure 3.3) made analysis of the anti-GBS difficult, but it appears that a small number of mice in CCMV-S9 and CPMV-S9 groups produced anti-GBS antibody.
Figure 3.4 Antibody Response of Individual Mice to S9 Peptide CCMV and GBS Plates were coated with S9 peptide, CCMV or GBS. Prebleed (Bleed 1) and post-booster sera, (Bleed 2) at dilutions of 1/5000 for S9, 1/1000 for CCMV and 1/200 for GBS, were added into triplicate wells. Plates then were incubated at 4°C for 18 h. After washing with ELISA washing buffer, the anti-mouse IgG dilution 1/1000 was added into wells and incubated for 4 hours at room temperature. Colorimetric substrate p-nitrophenyl phosphate was added. Absorbance at 405 nm was measured. Results are means and standard errors.
To test whether CCMV-S9, KLH-S9 and CCMV were able to induce cross reactivity to other phage selected peptides, collected sera from these mice were added to ELISA plates coated with S7, S9-2 and S11. These peptides were selected with the indicated monoclonal antibody (S7, S9, or S11). The S9-2 peptide has a completely different sequence than the S9 peptide used here. The ELISA results showed no cross reactivity between anti-S9 antibody and any of these other peptides representing group B streptococcal carbohydrate antigens (Figure 3.5, Figure 3.6 and Figure 3.7).

Figure 3.5 Antibody Response of Individual Mice to S7 Peptide
S7 plates were prepared as described in Materials and Methods. Bleed 2 from the first sequence of immunizations was tested. S7 mAb was used as a positive control and PBS was used as a negative control. Plates were incubated at 4°C for 18 h. After washing with ELISA washing buffer, enzyme conjugated anti-mouse IgG was added and incubated for 4 hours at room temperature. Colorimetric substrate p-nitrophenyl phosphate was added. Absorbance at 405 nm was measured and shown as mean and SEM.
Figure 3.6 Antibody Response of Individual Mice to S9-2 Peptide
S9-2 plates were prepared as described in Materials and Methods. Bleed 2 from the first sequence of immunizations was tested. S9-2 mAb was used as a positive control and PBS was used as a negative control. Plates were incubated at 4°C for 18 h. After washing with ELISA washing buffer, enzyme conjugated anti-mouse IgG was added and incubated for 4 hours at room temperature. Colorimetric substrate $p$-nitrophenyl phosphate was added. Absorbance at 405 nm was measured and shown as mean and SEM.

Figure 3.7 Antibody Response of Individual Mice to S11 Peptide
S11 plates were prepared as described in Materials and Methods. Bleed 2 from the first sequence of immunizations was tested. S11 mAb was used as a positive control and PBS was used as a negative control. Plates were incubated at 4°C for 18 h. After washing with ELISA washing buffer, enzyme conjugated anti-mouse IgG was added and incubated for 4 hours at room temperature. Colorimetric substrate $p$-nitrophenyl phosphate was added. Absorbance at 405 nm was measured and shown as mean and SEM.
The Th1/Th2 Response Is Influenced by Antigen Conjugate Carriers

To determine the Th1/Th2 profile of the antibody response, the subclass profile of the humoral IgG response, antigen-specific IgGl, IgG2a and IgG2b, and IgG3, was analyzed using ELISA as described in Materials and Methods (Figure 3.8 and Figure 3.9).

Figure 3. 8 IgG Subclass Identification from the First Immunization Experiment
Bleed 2 sera from the first immunization were added to ELISA plates coated with S9 peptide. Following this, rabbit anti mouse IgG1, IgG2a, IgG2b or IgG3 antisera were added and incubated at 4°C for 18 h. Goat anti rabbit IgG antibody conjugated with alkaline phosphatase was then added to wells for 6 h, followed by washing and addition of colorimetric substrate. A405 was determined using a microplate reader. Results are means and SEM of triplicate samples.

Antibody subclass was determined on Bleed 2 sera from mice in the first set of immunizations (Figure 3.8), which had been immunized with KLH-S9, CPMV-S9, and CCMV-S9, with or without adjuvant. In the non-adjuvant group, CCMV-S9 and CPMV-
S9 predominantly induced antigen-specific IgG2a whereas KLH-S9 predominantly induced antigen-specific IgG1 (Figure 3.8). When adjuvant was used, these differences disappeared, all subgroups showed a mixed Th1/Th2 response as reflected by equivalent levels of antigen specific IgG1, IgG2a and IgG2b (Figure 3.8).

The result was confirmed using sera from the second immunization experiment. Immunization with the virus carrier conjugate induced predominantly antigen-specific IgG2a for a Th1 type of response, whereas KLH-S9 predominantly induced antigen-specific IgG1, a Th2 pattern (Figure 3.9).

Figure 3.9 IgG Subclass Identification from the Second Immunization
Bleed 2 sera from the second immunization were added to ELISA plates coated with S9 peptide. Following this, rabbit anti mouse IgG1, IgG2a, IgG2b or IgG3 antisera were added and incubated at 4°C for 18 h. Goat anti rabbit IgG antibody conjugated with alkaline phosphatase was then added to wells for 6 h, followed by washing and addition of colorimetric substrate. A405 was determined using a microplate reader. Results are means and SEM of triplicate samples.
Immune Response after Injection with Heat-Killed Group B Streptococcus

We next asked if mice primed with the S9 peptide were able to produce rapid GBS-specific antibody response after exposure to intact GBS. From the first immunization, six groups of mice (CCMV, CCMV-S9, KLH-S9), with and without adjuvant, were injected with heat-killed GBS. Sera were collected 3 and 7 days after injection (Bleed 3 and Bleed 4) to test for the induction of S9 and GBS-specific antibodies. All groups of mice produced small amounts of GBS-specific antibodies (Figure 3.10) and S9-specific IgG antibodies (Figure 3.11) at day 7, but not day 3 after immunization.

![Figure 3.10 GBS-Specific IgG Antibodies after Injection of GBS](image_url)

Figure 3. 10 GBS-Specific IgG Antibodies after Injection of GBS
Mice were injected with heat-killed GBS. Sera were collected 3 and 7 days after injection (Bleed 3 and Bleed 4) to test for GBS-specific IgG antibodies and compared to Bleed 2 sera (obtained prior to exposure to GBS). Sera were diluted to 1/200 and added to GBS coated plates in triplicate and incubated. The plates were washed and incubated with alkaline phosphatase-conjugated anti mouse-Ig, followed by washing and addition of colorimetric substrate. A405 was determined using a microplate reader. Results are mean and SEM.
Figure 3. 11 S9-Specific IgG Antibody after Injection of GBS
Mice were injected with heat-killed GBS. Sera were collected 3 and 7 days after injection (Bleed 3 and Bleed 4) to test for S9-specific IgG antibodies, and compared to Bleed 2 sera (obtained prior to exposure to GBS). Sera were diluted to 1/5000 and added to S9 coated plates in triplicate and incubated. The plates were washed and incubated with alkaline phosphatase-conjugated anti mouse-Ig, followed by washing and addition of colorimetric substrate. A405 was determined using a microplate reader. Results are mean and SEM.

All groups of mice produced more GBS-specific and S9-specific IgM antibodies at day 7, but not day 3, after immunization (Figure 3.12). The results confirmed that GBS immunization results in the production of anti-S9 antibody. But the results did not provide any evidence that immunizing with the S9 peptide resulted in priming for a challenge with GBS.
Figure 3. 12 GBS-Specific IgM and S9-Specific IgM Antibodies after Injection of GBS
Mice were injected with heat-killed GBS. Sera were collected at 3 and 7 days after
injection (Bleed 3 and Bleed 4) to test for GBS-specific IgM antibodies, and compare to
Bleed 2 sera (obtained prior to exposure to GBS). Sera were diluted to 1/200 for GBS-
specific IgG antibody response or diluted to 1/5000 for S9-specific IgG antibody
response. The diluted sera were added to GBS or S9 coated plates coated plates in
triplelicate and incubated. The plates were washed and incubated with rabbit anti-mouse
IgM, and then alkaline phosphatase-conjugated anti rabbit-IgG. A405 was determined
using a microplate reader. Results are mean and SEM.
Cytokine Production in Primed Splenocytes after Antigen Stimulation

Because the subclass analysis of the antibody response suggested that immunizing with antigen in association with virus induces a Th1 type of response, whereas antigen conjugated to KLH induced a Th2 response, we examined cytokine expression in helper T cells following antigen stimulation. Selected groups of mice from the second immunization were boosted a second time ip with the same antigen. Immunized and naive mice were sacrificed 4 days after the last booster. The spleens and mesenteric lymph nodes were removed aseptically. Splenocytes were purified and were plated 100 ul per well (2×10^5 cell per well) into sterile 96-well microplates (Costar, Corning Inc., NY) and stimulated either with CCMV, CCMV-S9, S9 peptide alone, KLH-S9, or KLH, at concentration 20 ug/ml, PHA at concentration 10 ug/ml (positive control), or with sterile RPMI-medium supplement with 10% FCS (negative control). Cytokine production on days 3, 5 and 7 was measured and is represented in figures 3.13. The data indicated that splenocytes primed with CCMV or CCMV-S9 and then stimulated with CCMV or CCMV-S9 produced large amounts of IFN-γ, with variable amounts of IL10 and no IL4. On the other hand, splenocytes primed with KLH-S9 and then stimulated with KLH-S9 or KLH produced IL4 and IL10, and little IFN-γ. There appeared to be a greater overall cytokine response when the immunogen is associated with virions. These data showed that immunizing with virus particles expressing S9 provide a Th1-biased response whereas immunization with protein, KLH-S9 induces a Th2-biased response. These results are consistent with the Th1 vs Th2 bias indicated by the subclass of antibody produced.
Figure 3. Cytokine production in primed splenocytes 3, 5 and 7 days after antigen stimulation
Mice immunized with CCMV, CCMV-S9 or KLH-S9 were sacrificed. Splenocytes were purified, cultured *in vitro*, and stimulated either with antigen, PHA, or medium. The supernatants were collected on day 3, 5 and 7 and analyzed for cytokine production. Results are from individual mice immunized with the antigen indicated in the legend.
Discussion

Immunization with capsular polysaccharide (CPS) of GBS has been known to induce protective antibodies (85). Vaccines using CPS (191) or CPS conjugated to protein carriers (193, 346) have been developed. In our lab, we used an alternative approach to GBS vaccine development. A CPS mimetic peptide, S9, was previously identified by the means of phage display (1). The S9 peptide consists of 12 amino acids and is immunogenic when coupled to large proteins. Conjugating peptide epitope to large proteins such as KLH is a common method to enhance immunogenicity of the peptide. An alternative way, using viruses to present epitopes by forming chimeric viruses and virus-like particles has been developed (310, 340, 352, 354). CPMV, an icosahedral virus comprised of 60 capsomeres, has been used as a viral vector to express an epitope on the virus coat protein (299, 313).

In the present study, we aimed to improve the immunogenicity of the GBS mimotope, S9 peptide, by chemically coupling it to the plant virus particle, CCMV. We choose the CCMV as virus vector to deliver the S9 peptide to the immune system because the X-ray crystal structure of CCMV has been resolved (326-328) and it has been developed as a new expression vector in the Young lab (Montana State University). CCMV is a member of Bromoviridae virus family. It is an icosahedral virus 28 nm in diameter. The structure of CCMV has been determined to 3.2 Å resolution (317). The virus particle is composed of 180 copies of the coat protein. The S9 peptide was chemically conjugated to four different carriers: CCMV, CPMV, SubE (CCMV mutant) and KLH. The CPMV-S9 conjugate is a gift from Dr. John E. Johnson (Scripps Institute,
La Jolla CA). CPMV is a picorna-like icosahedral virus with size of 30 nm with 60 copies of coat protein subunit (355). The crystal structure of CPMV has been refined (356). CPMV has been used as a virus vector to present many epitopes in chimeric form and as chimeric VLPs (299, 310, 356-358). The amount of S9 peptide coupled to CPMV-S9 was two-fold higher than the amount of S9 protein antigen in the CCMV-S9 and SubE-S9. The reactive lysine side chains on the virus coat protein were used in the conjugation procedure for both CCMV-S9 and CPMV-S9. This may indicate that lysines are less accessible on the CCMV coat protein than on CPMV.

Mice immunized with CCMV-S9 can produce anti-S9 antibody even without adjuvant. With the help of adjuvant, the antibody response to S9 peptide increased. The anti-S9 antibody response from mice immunized with CPMV-S9 and KLH-S9 conjugate seemed slightly higher than the response from mice immunized with CCMV-S9. The degree of organization of antigens has been shown to be important and to have effects on the B cell response (295, 352, 359). The S9 antigen in the CCMV-S9 conjugate might not be optimal for antibody production. Even though the amount of S9 peptide in each antigen was equal in each sample, the amount of S9 per virus particle was less in the CCMV-S9 conjugate. Thus, more repetition of antigen in CPMV-S9 enhanced antibody response. This explanation was supported by the result of SubE-S9 conjugation immunization. SubE-S9 conjugate is a monomeric form of mutant CCMV-S9 conjugate. The SubE is the CCMV mutant in which eight basic amino acid residues (K and R) of the N-terminus were replaced with acidic residue glutamic acid(320). Conjugating S9 to the SubE led to a virus that falls apart (monomeric form). Mice immunized with SubE-S9
conjugate without adjuvant resulted in less anti-S9 antibody production. When adjuvant was used, the response increased but the level was still lower than response from other conjugates. Experiments using papaya mosaic virus-like particles fused to a hepatitis C virus epitope also demonstrate that the immunogenicity is highly dependent upon antigen organization. Immunization with the chimeric VLP induces long-lasting higher antibody response compared to immunization with the monomeric form (352).

In the second immunization experiment, the result indicated a better anti-S9 response in mice immunized with CPMV-S9 when compared with the KLH-S9. However this observation cannot be obviously seen in the first immunization experiment, but is consistent with our other observations that virion-associated antigen may be more immunogenic than antigen attached to soluble proteins (352).

Sera from all mice immunized with CCMV-S9, CPMV-S9, and KLH-S9 were tested for cross reactivity to other peptides (peptide S9-2, S7 and S11) representing group B streptococcal carbohydrate antigens. S7 and S11 were detected using Mabs specific for other GBS antigens, whereas S9-2 was from the same set of experiments that produced the S9 peptide, but eluted at acid, rather than alkaline pH (1). The result indicated no cross reactivity between antibody elicited by immunization with any of the S9-antigen preparations, and any peptides. This shows that the S9 peptide elicits antibodies specifically to this peptide, and there is little if any antigenic mimicry among the peptides.

The anti-GBS antibody response was tested in sera from all mice. The result is difficult to interpret. Control mice showed an increase in anti-GBS antibody with time,
suggesting possible cross-reactions with the normal flora of the mice (mice were tested and negative for GBS). Also results were weak and inconsistent. In the first immunization experiment, an anti-GBS response seemed to be induced in mice immunized with or without adjuvant with CCMV-S9, CPMV-S9, SubE-S9 and KLH-S9. However, when the sera from the second immunization experiment were tested for anti-GBS antibody, an anti-GBS antibody response was only seen in CPMV-S9, when compared to the prebleed sera.

Immunized mice were injected with heat-killed GBS to test the ability of the S9 conjugates to prime for a secondary response to the microbial antigen expressed on the killed GBS. The results indicate that immunization with the S9 peptide was unable to do so. Interestingly, the injection of the GBS elicits a strong anti-peptide IgM response at day 7.

The goal of vaccination is to provide protection from infection. Effective stimulation of the immune system depends upon stimulation of strong T-cell response. In general, Th1 cells stimulate cell-mediated immunity (CMI), while Th2 induce an antibody-mediated response. In vaccine design, the type of immune response is important because different immune responses have different effects on different pathogens. In general, Th1 cells are required for the elimination of intracellular pathogens, whereas the Th2 cells are required for inhibition of attachment or entry of microbes during extracellular stages of the infectious cycle. The route of immunization and the form of the antigen and delivery method can have effects on the immune response. The antibody subclass profile (IgG1, IgG2a, IgG2b and IgG3 in mice), can suggest a Th1 or Th2
response. In our study, mice immunized without adjuvant with the CCMV-S9 and CPMV-S9 conjugate predominantly produce anti-S9 specific IgG2a, whereas KLH-S9 predominantly induced anti-S9 specific IgG1. The antibody profile result suggested that S9 conjugated to virus particle induced Th1 response, whereas conjugation S9 peptide to soluble protein (KLH) induced a Th2 response. Notably, the Th1/Th2 bias was obscured when adjuvant was used in the immunization. To substantiate that the antigen, in the form of virus particles affects the Th1/Th2 response, we examined cytokine expression in helper T cells following antigen stimulation. The data indicated that splenocytes primed with CCMV or CCMV-S9 and then stimulated with CCMV or CCMV-S9 produced the Th1 cytokine, IFN-γ. On the other hand, splenocytes primed with KLH-S9 and then stimulated with KLH-S9 or KLH produced the Th2 cytokines, IL4 and IL10. The ability of multimeric VLP vaccines to induce a Th1 biased response has been shown before (360-363). This may result from the multimeric form of the antigen, or it may be the effect of RNA within the VLP or virion contributing to adjuvanticity. CCMV virion and pseudovirion assembly has been shown to be dependent upon the inclusion of RNA within the capsid (364-368). Whatever the mechanism, our study clearly indicated that immunizing with virus particles expressing S9 induces a Th1 response, whereas immunization with protein, KLH-S9, induced a Th2 response.
Introduction

Group B streptococci (GBS) can cause neonatal sepsis and meningitis (369). Neonates are exposed to GBS either via GBS ascension through ruptured amniotic membranes, aspiration of contaminated amniotic fluid, or through exposure to colonized tissues while passing through the birth canal (344). GBS possess a polysaccharide capsule which allows the GBS to avoid opsonophagocytosis (145). Capsular polysaccharides (CPS) are poorly immunogenic and induce a T-independent response without immunologic memory. GBS vaccines using conjugates of the carbohydrate to a protein carrier have been developed and are able to generate a T-dependent response (370).

In our lab, we have followed an alternative strategy, using peptides that mimic the structure of microbial polysaccharides. Phage display technique was used to seek peptides that mimic carbohydrate epitopes. The S9 mAb, which demonstrates \textit{in vivo} protection against GBS type III (172, 174) was used to select displayed peptides. The S9 peptide, FDTGAFDPDWPA was isolated. The S9 peptide was shown to be both antigenic and immunogenic (1), specifically blocking the binding of antibody to type III CPS and eliciting antibody that cross-reacts with GBS.

In this thesis we further investigated the structural basis of the peptide mimicry of the GBS type III CPS carbohydrate epitope. We designed a sublibrary, expressing a mutated version of the S9 peptide with 3\% random nucleic acids at each position of the
original S9 nucleotide sequence, creating approximately one mutation in each inserted sequence. Peptides with higher affinity to the S9 mAb were identified by affinity selection. From this sublibrary, we also explored effects of the amino acid changes in the antigenic S9 peptide. Phages displaying modified S9 peptides therefore were randomly selected. Peptides from both affinity selection and random selection were characterized. The results allow us to map the antigenic portion of the S9 peptide, and suggest ways to generate better mimotopes of GBS.

Materials and Methods

Peptide Library Construction

To create a library expressing mutated versions of the S9 peptide, we inserted oligonucleotide sequences between the *Bgl*II sites of the filamentous phage vector fUSE2 (237). The inserted sequence is expressed at the amino terminus of the pIII coat protein, giving the final amino acid sequence H2N-AEDPG[FDTGAFDPDWPA]GGP TD. The modified S9 oligonucleotide sense strands (New England Biolabs) were designed so that the nucleotide sequence, indicated within the brackets, consisted of 97% of the appropriate nucleotide with the other 3% consisting of the other three nucleotides at each position, thus creating approximately one mutation in each inserted sequence:

5’CAAA G GATCC AGGA [TTC GAT ACG GGC GCG TTC GAT CCA GAT TGG CCA GCG] GGT GGG CCC ACG G 3’. The antisense strands, which were used to prime the fill-in reaction, were 5’GAT GGA TCC GTG GGC CCA CC 3’. The red highlight indicates *BamHI* sites. Each strand was heated at 95°C for 3 min and rapidly
cooled down for 5 min. Sense S9 library strands 25 ul of 40 ng/ul were mixed with 2 ul of 40 ng/ul antisense strands, giving 1:1 molar ratio reaction. The mixture was incubated 15 min at 50 °C and 15 min at 37 °C and then allowed to cool down to room temperature. Klenow Fragment (Takara), its buffer, and dNTP mix were added to the mixture to have total volume of 250 ul. The reaction was incubated at 37°C 3 h and at 65°C for 5 min. The oligonucleotide library was then digested with BamHI (Promega). The double stranded mutated S9 oligonucleotide DNA library was then purified by phenol-chloroform extraction, followed by ethanol precipitation. The library was ligated into Bgl II digested, and dephosphorylated fUSE2. The ligation reaction was performed at a vector to insert ratio of 1:2 with T4 DNA ligase (Promega, Madison, WI) and 1 ul of 10x ligation buffer (containing 100mM mgCl2 and 10 mM ATP, Promega) in 10 ul total volume. The reactions were incubated at 4°C overnight to facilitate ligation. Digested and dephosphorylated fUSE2 plasmids were used as a control. The ligation reactions were precipitated by mixing the mixture well with two volumes of 95% ethanol and 1/10 volume of 3 M sodium acetate pH 5. The mixture was incubated at –20°C overnight. DNA was collected by centrifuging12 krpm 30 min. The DNA pellet was washed with 70% ethanol and air dried. DNA was resuspended in 10 ul water. Two transfections were performed by electroporating 4 µl DNA into MC1061 E. coli (371, 372). SOC medium with 0.2 ug/ml tetracycline was added to the transformation reactions and the mixtures were incubated in an incubated shaker at 37°C for 1 h. The transfection reactions were spread on LB media agar plates supplemented with 40 ug/ml tetracycline and incubated at 37°C overnight, or added to 200 ml LB broth media supplemented with 40 ug/ml
tetracycline and incubated with shaking at 37°C overnight. Phages were purified from the culture by using large scale phage purification methods described below.

**Plasmid Extraction**

A modified alkaline lysis method was used to purify plasmid DNA (321). 3 ml of overnight culture in LB broth with tetracycline 20 µg/ml were spun to collect the cell pellet. Cells were resuspended in 100 µl of 20 mM Tris buffer pH 8 and then incubated at room temperature for 5 min. 200 µl of lysis buffer (fresh prepared 0.2 N NaOH, 1% SDS) were added and the mixture was mixed. The mixture was incubated at RT for 10 min and then 150 µl of KAC/AC (5 M potassium acetate, 10 M (57%) acetic acid) were added. The mixture was mixed vigorously and sat in ice for 30 min. Cell debris was removed by centrifugation at 12 krpm 10 min twice. Supernatant was collected and was mixed with equal volume of phenol/chloroform/isopropanol. The upper phase was collected. DNA was precipitated with 95% ethanol and sodium acetate, and washed with 70% ethanol. DNA was resuspended in 10 µl water. DNA was characterized by running on 0.8-1.5% agarose gels.

**PCR**

Double stranded plasmid DNA (RF form of phage) was studied to assure the presence of DNA inserts. DNA from individual transfectant colonies was mixed with PCR buffer with MgCl, dNTP mix, primers (fUSE2 primer1 (TCAAGATCCGTGGGC-C CACC) and fUSE2 primer2 (GCTTCTCTTATGATTGACCCTCTGCG)), Taq
polymerase, and the DNA template. PCR cycles were 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. 5 ul of PCR product were loaded onto 1% agarose gels.

**Single Stranded DNA (ssDNA) Extraction (373)**

Phage clones containing inserts, as verified by PCR, were cultured in 1.5 ml LB containing 20 ug/ml tetracycline and incubated overnight at 37°C with shaking. Cultures were centrifuged and 1 ml of supernatant was mixed with 150 ul PEG/NaCl solution (16.7%/3.3M). The tube was inverted several times and kept at 4°C overnight. The tube was centrifuged at 12 krpm for 10 min and all supernatant was removed. The pellet, containing precipitated phage virions, was dissolved in 200 ul TE buffer (10 mM Tris·HCL 1mM EDTA pH8). An equal volume of phenol/chloroform solution (phenol:chloroform = 1:1) was added to the phage solution and mixed well. After centrifugation, 200 ul of the aqueous phase was transferred to another tube. The ssDNA was precipitated by adding 20 ul of 3M sodium acetate pH6 and 440 ul 95% ethanol and kept at 4°C overnight. DNA was obtained by high speed centrifugation at 12 krpm for 30 min. The pellet was washed with 500 ul 70% ethanol and allowed to air-dry. The pellet containing ssDNA was resuspended in 20 ul of sterilized deionized water. The ssDNA was kept in -20°C until sent for sequencing analysis (Davis Sequencing, Inc, Davis, CA), using fUSE2 primer, CCCTCATAAGCGTAACG. Peptide sequences were inferred from the DNA sequences using MacVector 6.5.3 and EditView 1.0.1.
**Phage Amplification**

*E. coli* strain K91Kan was cultured in a 10 ml LB broth containing 100 ug/ml kanamycin in an incubation shaker 37°C until an OD 600 of a 1/10 dilution reached 0.2. Purified phages or the eluate from the phage affinity selection were added to the culture and incubated for 15 min at 37°C without shaking. A fresh 10 ml of LB media containing 0.2 ug/ml tetracycline was added and incubated at 37°C with high speed shaking (250 rpm) for 30 min. Tetracycline was added to the culture to a final concentration of 20 ug/ml. The total culture was spread on an LB agar plate containing 20 ug/ml tetracycline and 100 ug/ml kanamycin in a 9x24 inch glass baking dish. The plate was incubated at 37°C overnight. On the next day, 25 ml of TBS (50mM Tris-Cl, 150 mM NaCl pH7.5) with 0.5% Tween 20 was flooded over the culture plate. An alcohol-flamed sterilized glass spreader was used to gently remove colonies from the plate. Phage particles were purified and quantified by phage titration method as described below. The phages were further reamplified using 100 ml of *E. coli* stain K91Kan. Purified phage particles from the previous amplification were added to the culture, incubated, and purified as described above.

**Phage Purification**

Phage were purified as follows (374). The overnight culture of phage-infected K91Kan was centrifuged at 8 krpm for 10 min. The supernatant was transferred and mixed with 0.15 volume of PEG/NaCl. The phage particles were allowed to precipitate overnight at 4°C. The pellet containing phages was collected by centrifugation at 10
krpm for 30 min. The pellet was resuspended with 15 ml TBS buffer. The phages were reprecipitated 3 times with PEG/NaCl. After the final round of precipitation, the pellet was resuspended in 1 ml TBS phage buffer. Phage were quantified by titration, preserved by adding 50% glycerol, and stored at 4°C. Phage protein concentration was measured by using BCA protein assay reagent kit (Pierce, Rockford, IL).

Phage Titration

Phage were titrated as follows (375) (376). Starved *E. coli* K91Kan were used as host cells for phage titration. K91Kan cells were cultured in 20 ml LB media supplemented with 100 ug/ml kanamycin at 37°C with shaking until reaching OD$_{600}$ 0.4-0.5. Cells were centrifuged at 2.5 krpm for 10 min and resuspended in 20 ml 80 mM NaCl. The cell suspension was incubated in the 37°C shaker incubator for 45 min and centrifuged 2.5 krpm for 10 min. Cells were resuspended in 1 ml cold NAP buffer (80 mM NaCl and 50 mM NH$_4$H$_2$PO$_4$, adjusted pH to 7 with NH$_4$OH) and kept at 4°C until use. Purified phages were 10-fold serially diluted with TBS phage buffer with 100 ul final volume. Phages were allowed to infect the cells at room temperature for 10 min and the mixture was added with 1 ml LB supplemented with 0.2 ug/ml tetracycline. The infection was allowed to continue in the 37°C shaking incubator for 40 min. 100 ul/plate of infected cells were spread on LB agar supplemented with 40 ug/ml tetracycline and 100 ug/ml kanamycin. Culture plates were incubated overnight in the 37°C incubator. Infected colonies able to grow on the tetracycline/kanamycin plates were counted and calculated as transducing units (TU titer).
Affinity Selection of Phage-Displayed Peptides

S9 monoclonal antibody (mAb) was diluted with coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl, pH8.3-8.5) to 1.5 mg/ml. Cyanogen bromide-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO) was washed and swollen in 200 ml cold 1mM HCl per gram of dry gel for 30 min at 4°C. The resin was washed with 200 ml cold distilled water 5 times. The resin was then mixed with S9 mAb at 3 mg of Ab/ml of beads in the coupling buffer overnight at 4°C rotating end-over-end. The S9 mAb-resin was spun down at low speed for 1 min and washed 3X with the coupling buffer. Supernatants were collected for protein assay by using BCA protein assay reagent kit (Pierce, Rockford, IL). The quantity of S9 mAb conjugated to the matrices was determined by subtracting total S9 mAb protein released from each coupling step from the input S9 mAb protein. Unreacted groups on resin were blocked with 0.2 M glycine pH 8 overnight at 4°C. The mAb-conjugated resin was separated into three aliquots and kept at 4°C in 0.02% azide until ready to use. The S9 mAb-conjugated resins were mixed with 1x10^12-13 phage (1 ml) from the phage library at 4°C overnight. The mixture was loaded into a 5 ml syringe plastic barrel (Becton, Dickinson and company, Franklin Lakes, NJ). The unbound phages were removed by washing 10 times with phage buffer. Bound phages were eluted with 0.5 M NH₄OH pH 11 and the pH of the eluate was neutralized immediately with 1 M acetic acid. The titer of phage in each wash and in each eluate was determined by the phage titration method as described above. The eluted phage were then amplified in *E. coli* strain K91Kan, as described above, to a TU titer of 10^12 and reapplied to the column for a second round of affinity selection. The same incubation, washing, and
elution procedures were used. The yield was determined from the second round. The eluted phages were reamplified in K91Kan for the third round of affinity selection. After the last round of selection, individual phages were rescued by infecting starved *E. coli* strain K91Kan and plated on the LB agar media supplemented with 40 ug/ml tetracycline and 100 ug/ml kanamycin. The yield was determined. Phage ssDNA were purified and sent for sequencing analysis. Phages were purified and characterized for binding to selecting, and other, Abs using ELISA.

**ELISA and Antibodies**

Direct binding ELISA was used to measure the binding of phages, peptides, and KLH-peptide conjugates to select Abs. 100 ul of the appropriate concentration of antigen were loaded onto microtiter wells (Immuron 2, Dynatech, McLean, VA). Plates were blocked with ELISA blocking buffer (1% BSA in phosphate-buffered saline (PBS)) 4°C overnight. 100 ul of appropriate concentrations of the following antibodies: S9, S7, S11, or anti-M13 were incubated in microtiter wells at 4°C for 18 h. Unbound antibody was removed by washing with ELISA washing buffer (10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20). After several washings, 100 ul of 1/1000 alkaline phosphatase-conjugated anti-Ig was added and incubated at room temperature for 6 h. After washing, 100 ul of colorimetric substrate *p*-nitrophenyl phosphate (Sigma Chemical) (1 mg/mL in 10% diethanolamine buffer, pH 9.6, with 0.5mM MgCl2) was added. A405 was determined using a microplate reader (EL-320, Bio-Tek Instruments, Winooski, VT).
Antigen capture ELISA was also used. Plates were coated with 3 ug/ml of mAbs S9, S11, or B6.1. After blocking with ELISA blocking buffer, 100 ul of each phage dilution was added. After incubating at 4°C overnight, anti-M13 antisera, at a 1/10,000 dilution was added and incubated at 4°C overnight. Plates were washed and incubated with alkaline phosphatase-conjugated anti-rabbit IgG, followed by washing and the addition of substrate, p-nitrophenyl phosphate. Absorbance at 405 was determined.

ELISA assays for phage or peptide inhibition of binding activity of mAb to Group B streptococcus (GBS) were performed by coating microtiter wells with GBS (353). Plates were blocked with ELISA blocking buffer overnight at 4°C. The inhibition assay was performed by preincubation of the mAb with different concentrations of phages or peptides. Small scale purified phage clones were diluted with phage buffer to have concentration of 3 ug/ml. Phages were two-fold serially diluted ranged from 3 ug/ml to 0.006 ug/ml. Peptides were diluted to be 2.5, 0.5, 0.1, 0.2 and 0.004 ug/ml. The KLH-peptide conjugates were two-fold serially diluted, and ranged from 0.5 ug/ml to 0.001 ug/ml. The concentration of mAb used for the inhibition assay was in the middle of the linear portion of the binding curve between mAb and GBS (S9 mAb ascites was diluted to 1/25,000, SIIIIV18.C2 was diluted to 1/250,000, SIIIIS8.C3 was diluted to 1/3,000). The mixture was allowed to incubate at room temperature for 1 h before placing 100 ul of the mixture in wells and incubating 4°C overnight. The plates were washed with ELISA washing buffer, and incubated with alkaline phosphatase-conjugated anti-mouse Ig, followed by washing and addition of colorimetric substrate p-nitrophenyl phosphate (Sigma Chemical). Absorbance at 405 nm was measured.
ELISA assays for binding activity of sera from immunized mice or mabs to GBS were performed by coating microtiter wells with GBS (353). Information regarding the mAbs used in this experiment are listed in table 4.1.

Table 4.1 Mabs Used in This Study

<table>
<thead>
<tr>
<th>Name</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>S9</td>
<td>Type III capsular polysaccharide</td>
</tr>
<tr>
<td>S11</td>
<td>Beta-C protein of type I and II GBS</td>
</tr>
<tr>
<td>SIII S8.C3</td>
<td>Complete type III</td>
</tr>
<tr>
<td>S3.1A6</td>
<td>Type III capsular polysaccharide</td>
</tr>
<tr>
<td>S3.2A6</td>
<td>Type III capsular polysaccharide</td>
</tr>
<tr>
<td>S3.1B1</td>
<td>Type III capsular polysaccharide</td>
</tr>
<tr>
<td>SIII IV18.C2</td>
<td>Complete type III</td>
</tr>
<tr>
<td>B6.1</td>
<td>Candida albicans</td>
</tr>
</tbody>
</table>

Plates were blocked with ELISA blocking buffer overnight at 4°C. Diluted antibody was loaded onto the GBS coated plates and incubated at 4°C overnight. After washing, alkaline phosphatase-conjugated anti-mouse Ig was added and incubated. After another wash, colorimetric substrate \( p \)-nitrophenyl phosphate was added. \( A_{405} \) was determined using a microplate reader.

Conjugation of Peptides to Keyhole Limpet Hemocyanin (KLH)

Synthetic peptides (Sigma Laboratories (USA)) containing Cys at the COOH peptide terminals were conjugated to maleimide derivatized KLH (Pierce Chemical Co., Rockford, IL). Maleimides react with sulfhydryls at pH 6.5-7.5 to form stable thioether bonds. Peptides (300 ug) were mixed with KLH (300 ug) in phosphate buffer pH 7 in a reaction volume of 300 ul. The reaction was allowed to continue at room temperature for 3 h. The unconjugated peptides were removed by using microcon 100 (Millipore, Billerica, MA) filtration. The conjugated KLH-peptides were quantified by using the
The specificity of conjugated peptides binding to mAb and ability to inhibit mAb to GBS were characterized by ELISA.

Results

Library Constructions

To identify peptides that would engage the S9 mAb more effectively and to map the binding motif within the S9 peptide, we created a mutated phage library based on the S9 peptide sequence having approximately one mutation per phage. The library was constructed by inserting a randomly modified version of the S9 DNA sequence between the BglII sites of the filamentous phage vector fUSE2. To make the oligomers that would form the library, the sense strands were designed to have 97% correct nucleotide and 3% consisting of the other three nucleotides in each position of the S9 coding sequence. Klenow fragment DNA polymerase was used to extend the antisense strands complementary to the sense strands resulting in the oligomer of modified S9 DNA fragments (Figure 4.1).

![Figure 4.1 Construction of Randomly Modified S9 DNA ds Oligomer](image)

Red, white and blue areas indicate BamHI site, the modified S9 library and bases encoding unmodified AA’s, respectively.
The modified S9 DNA oligomers were digested with *BamH1* restriction enzyme. The digested products were purified and loaded onto the 12% polyacrylamide gels (Figure 4.2).

![Figure 4.2 Modified S9 DNA Fragment Library](image)

**Figure 4.2 Modified S9 DNA Fragment Library**

Sense S9 library strands 25 ul of 40 ng/ul were mixed with 2 ul of 40 ng/ul antisense strands. Klenow fragment (Takara), its buffer and dNTP mix were added to the mixture to have total volume of 25 ul. The reaction was incubated at 37 °C at least 3 h and at 65 °C for 5 min. The complete set of double stranded S9 DNA oligomers was then digested with *BamH1* and purified by using phenol-chloroform extraction followed by ethanol precipitation.

The library was ligated into the ends of restriction digested *Bgl* II sites of fUSE2 vector located at the amino terminal part of the pIII coat protein giving the final amino acid sequence H2N-A EDPG [FDTGAFDPDWPA] GGPT D. The blue letters indicate the S9 peptide sequence that was modified. Two batches of electroporation were performed, which gave a transfection efficiency of $4.7 \times 10^4$ and $5 \times 10^4$ colonies, respectively. Only 36 colonies derived from transfection with control dephosphorylated fUSE2 plasmid.
Random and Affinity Selection of Phage

Phages were selected using two different screening protocols, random selection and affinity selection. In random selection, the entire first electroporated reaction was spread on the LB supplemented with 40 ug/ml tetracycline with appropriate dilution to provide isolated colonies on plates. 1,224 isolated colonies were randomly selected for PCR analysis to identify positive clones containing inserts in the correct orientation. A 642 bp PCR product was expected from the positive clones (Figure 4.3). From 1224 random-selecting clones, 493 clones (40.3%) contained foreign DNA inserts in the correct orientation.

![PCR Analysis of Transfected Clones](image)

Figure 4.3 PCR Analysis of Transfected Clones
Recombinant plasmids (RF form) were isolated from each clone and then PCR amplified. 5 ul of PCR products were analyzed by 2% agarose gel electrophoresis. The first lane was 1 kb DNA marker. The last lane is negative.

Single stranded phage DNAs from the total 493 positive clones were analyzed by DNA sequencing. Table 4.2 displays a summary of the DNA sequencing analysis.
Table 4.2 Summarized DNA Sequencing Analysis

<table>
<thead>
<tr>
<th>Description</th>
<th>Number of clones</th>
<th>Number of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Random selection</td>
<td>Affinity selection</td>
</tr>
<tr>
<td></td>
<td>Second round</td>
<td>Third round</td>
</tr>
<tr>
<td>Base change, resulting in amino acid change</td>
<td>120</td>
<td>5</td>
</tr>
<tr>
<td>Base change without amino acid change</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>Original S9 identical</td>
<td>356</td>
<td>9</td>
</tr>
<tr>
<td>Subtotal</td>
<td>493</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>532</td>
<td></td>
</tr>
</tbody>
</table>

Single stranded DNA from 532 recombinant clones from random selection and affinity selection were sent for sequencing analysis (Davis Sequencing, Inc, Davis, CA).

The sequencing result indicated that 72.2% of positive phage clones encoded the S9 identical sequence, FDTGAFDPDWPA, without any DNA base alteration. Only 27.8% of the positive phage clones contained modified S9 DNA sublibrary inserts. From 120 phage clones contained the modified S9, 41 clones encoded redundant amino acid sequences. In conclusion only 79 clones encoded modified S9 sequences. All 79 clones were cultured and the phages were purified for further characterization.

In the affinity selection, the second electroporation batch was added to 200 ml LB broth media supplemented with 40 μg/ml tetracycline and cultured overnight in a shaking incubator at 37 °C overnight. Phages were purified from the culture by using the large scale phage purification method described above. After amplification, $1.2 \times 10^{13}$ phages were obtained for phage displayed peptide selection with S9 monoclonal antibody. S9 mAb was conjugated to cyanogen bromide-activated sepharose, containing 0.7 mg of S9 mAb conjugated to the resin. The S9-conjugated matrices were separated into three aliquots, resulting in approximately 0.2 mg of S9 mAb for each affinity selection round. After the first affinity selection round, the yield of affinity selected phage was $5 \times 10^5$. The
output phages were amplified to have $8 \times 10^{12}$ TU for the second round of affinity selection. The yield in the second round was $1 \times 10^8$. The output phages were amplified to $4 \times 10^{12}$ TU for the last round of affinity selection, yielding $4.8 \times 10^9$ phage. 15 and 24 clones from the second and the third round were sequenced, respectively. Single stranded phage DNAs were purified and sent for sequencing analysis. A summary of the sequencing data is displayed in the table 3.2. Four clones isolated from the second round and three clones isolated from the final round displayed a modified sequence. Three (2 from the second round, one from the final) displayed FDTGAFDPDWRA (designated RP1.8).

**Specificity and Avidity of Phage-Displayed Peptides**

In order to determine the binding between the selected phages and mAbs, ELISAs using different antibodies were performed. A total of 86 phages, from both random and affinity selection, were tested. Phage binding to S9 mAb and anti-M13 antibody is shown in Figure 4.4 and 4.5. With the same phage concentration, binding by anti-M13 was more or less equivalent for all phage preparations. In contrast, binding of the mutant phages by the S9 mab was different, with some having increased binding, some equivalent, some less, and some lacking binding altogether; when compared to phage displaying the original S9 peptide. Six out of seven phages selected by affinity selection showed increased S9 binding activity. A phage displaying the wrong orientation of S9 surprisingly showed some degree of binding by S9 mAb.
Figure 4. Direct binding of phage by S9 and anti-M13 antibodies (set I)
ELISA plates were coated with phages at 50 μg/ml and 1 μg/ml, for S9 and antiM13 binding activity respectively. For S9 binding activity (A) 100 ul of S9 mAb 15 μg/ml was added and the binding activity was detected by adding anti-mouse IgG-AP conjugate. For antiM13 binding activity (B), 100 ul of antiM13 1/10,000 was added and the binding activity was detected by adding anti-rabbit IgG. Absorbance at 405 nm was measured. The blue bar represents a phage displaying the original S9 peptide. The pink bar represents a phage displaying the wrong orientation of the S9 coding sequence. The green bar represents a fUSE2 original phage vector. The purple bars represent phage displaying modified S9 sequences.
Figure 4. 5 Direct binding of phage by S9 and anti-M13 antibodies (set II)
ELISA plates were coated with phages at 50 ug/ml and 1 ug/ml, for S9 and antiM13 binding activity respectively. For S9 binding activity (A) 100 ul of S9 mAb 15 ug/ml was added and the binding activity was detected by adding anti-mouse IgG-AP conjugate. For antiM13 binding activity (B), 100 ul of antiM13 1/10,000 was added and the binding activity was detected by adding anti-rabbit IgG. Absorbance at 405 nm was measured. The blue bar represents a phage displaying the original S9 peptide. The pink bar represents a phage displaying the wrong orientation of the S9 coding sequence. The green bar represents a fUSE2 original phage vector. The purple bars represent phage displaying modified S9 sequences.
To confirm these results, phages with increases in binding activity to the S9 mAb and some phages with reduction in binding activity were recultured and retested (Figure 4.6). The data were consistent with the previous data.

Figure 4.6 Direct Binding of Phage by S9 and Anti-M13 Antibodies
To measure binding by S9 and M13 antibodies ELISA plates were coated with phages at 50 μg/ml and 1 μg/ml, respectively. For S9 binding activity (A and B) 100 ul of S9 mAb 15 μg/ml was added and the binding activity was detected by adding anti-mouse IgG-AP conjugate. For antiM13 binding activity (C and D), 100 ul of antiM13 1/10,000 was added and the binding activity was detected by adding anti-rabbit IgG. Absorbance at 405 nm was measured. The blue bar represents a phage displaying the original S9 peptide. The pink bar represents a phage displaying the wrong orientation of the S9 coding sequence. The green bar represents a fuse2 original phage vector. The purple bars represent phage displaying modified S9 sequences.
In order to determine the specificity and avidity of binding of phage-displayed peptides by antibody, a capture ELISA was designed, in which purified phages were captured by S9 mAb and detected with anti-M13 antibody. ELISA plates were coated with S9 mAb or irrelevant antibodies, B6.1 (antibody against *Candida albicans*) and S11 (mAb IgM against Beta-C protein of type I and II GBS). The immobilized antibodies were incubated with different concentrations of the phage clones. Phages were detected with antiM13 antibody and the binding activity was measured (Figure 4.7).

![Figure 4.7 Capture ELISA](image)

Figure 4.7 Capture ELISA
Plates were coated with S9, S11 or B6.1 mAbs. Phage clones 172, 1.2, 2.16, parental S9, original fuse2, and phages with wrong orientation insert at different concentrations were added. After incubating at 4 °C overnight, anti-M13 antibody was added and incubated. The reactions were detected with alkaline phosphatase conjugated anti-rabbit IgG and addition of colorimetric substrate. Absorbance at 405 nm was observed.
The ELISA results show that phage-displayed peptides bound to S9 mAb in a concentration-dependent manner. Phage clone 1.2 from the second round affinity selection and 2.16 from the third round affinity selection gave stronger reactions than the parental S9 phage, suggesting higher binding avidity by the S9 mAb. fUSE2 vector phage was not recognized by S9 mAb, whereas the phage-displayed wrong orientation S9 insert showed some degree of binding activity. No phage clones were recognized by the irrelevant antibodies B6.1 and S11.

To determine whether peptides displayed as phage fusion protein are mimetic of a group B streptococcus (GBS) determinant, we tested the ability of phage-displayed peptides to inhibit the binding of GBS by S9 mAb. We chose those phages with the greatest direct binding activity for testing. To perform the inhibition experiment, different concentrations of phages were premixed with mAb and allowed to incubate for 1 h at room temperature and then loaded onto the plates coated with GBS. The concentration of S9 mAb used for the inhibition assay was in the middle of the linear portion of the binding curve (Figure 4.8), which was 1/25,000. The inhibition assay (Figure 4.9) indicated that phage clone 10 showed highest activity in blocking the binding activity of S9 mAb to GBS. Phage clone 1.2 was also a good inhibitor. However, inhibition obtained from both clones was not much different from that obtained from phage displaying the original S9 peptide. The original fUSE2 phage did not show any inhibition.
Figure 4. 8 Titration of mAb binding to GBS
ELISA assays for mAb interaction with Group B streptococcus (GBS) were performed by coating microtiter wells with GBS. MAbs were two-fold serially diluted and binding was detected with alkaline phosphatase-conjugated anti-Ig and colorimetric substrate p-nitrophenyl phosphate. A405 was determined.
ELISA assays for phage inhibition of mAb interaction with Group B streptococcus (GBS) were performed by coating microtiter wells with GBS. The inhibition assay was performed by preincubation of S9 with different phage concentrations and adding to GBS coated wells. Binding was detected with alkaline phosphatase-conjugated anti-mouse Ig followed by washing and addition of colorimetric substrate p-nitrophenyl phosphate (Sigma Chemical). Absorbance at 405 nm was determined.

Specificity and Avidity of Peptides

In order to ensure that the interaction between the selected phages and S9 mAb is caused by insert sequences, peptides representing sequences from phage clones, which showed higher binding activity to S9 mAb and the ability to compete with GBS for S9 binding, were synthesized. Binding of S9 mAb to the synthetic peptides was demonstrated by ELISA (Figure 4.10).
Figure 4.10 Binding activities of peptides to S9 mAb
ELISA plates were coated with peptides at 1, 3 and 10 ug/ml or with PBS as negative control. S9 mAb was added and the binding was detected by adding anti-mouse IgG-AP conjugate. Absorbance at 405 nm was observed. Note that WO peptide represents the peptide from the wrong orientation of original S9 DNA sequence.
While there is a concentration dependent effect of the S9 peptide binding by S9 mAb, several of the peptides show very good binding even at the lowest concentration tested, indicating a higher avidity interaction with S9 mab. The peptide representing the wrong orientation of S9 DNA did not show binding activity to S9 mAb. Other peptides, which showed strong binding activity when they were displayed on phage particles (Figure 4.5, 4.6 and 4.7) showed low binding values.

To determine whether peptides are mimetic of the group B streptococcus (GBS) determinant, we tested the ability of peptides to compete with S9 mAb binding to GBS. Different concentrations of peptides were premixed with S9, SIIIIS8.C3 or IIIIV18.C2 mAbs and allowed to incubate for 1 h at room temperature and then loaded onto plates coated with GBS. The concentration of mAb used for the inhibition assay was in the middle of the linear portion of the binding curve (Figure 4.8), which was 1/25,000 for S9, 1/250,000 for IIIIV18.C2 and 1/3,000 for SIIIIS8.C3. The inhibition assay (Figure 4.11) indicated that peptides which had stronger binding activity with S9 mAb when compared to the original S9 peptide also were better inhibitors of binding of S9 mAb to GBS. There was no inhibition when peptides, which had lower binding activity with S9 mAb than the parental S9 peptide, were used as inhibitors. All peptides were better inhibitors of the binding reaction between GBS and IIIIV18.C2 mAb IgM when compared with the original S9 peptide (Figure 4.12). None of the peptides showed inhibition between GBS and SIIIIS8.C3 mAb IgG2a (Figure 4.13). The inhibition assay indicated that certain modified S9 peptides can be better mimetic peptides than the original S9 peptide.
Figure 4. 11 Inhibition Assay by Peptides of S9 mAb Binding to GBS

ELISA assays for peptide inhibition of mAb interaction with GBS were performed by coating microtiter wells with GBS. The inhibition assay was performed by preincubation of the S9 mAb with different peptide concentrations. S9 mAb ascites was diluted to 1/25,000. The mixture was allowed to incubate at room temperature for 1 h before placing in wells. Red lines represent inhibition curves when peptides were used. The blue line represents inhibition curve when S9 original peptide was used. The green line represents inhibition curve when peptide from the wrong orientation S9 DNA sequence was used.
Figure 4. 12 Inhibition Assay by Peptides of SIIIIV18.C2 mAb Binding to GBS ELISA assays for peptide inhibition of mAb interaction with GBS were performed by coating microtiter wells with GBS. The inhibition assay was performed by preincubation of the mAb with different peptide concentrations. SIIIIV18.C2 mAb was diluted to 1/250,000. The mixture was allowed to incubate at room temperature for 1 h before placing in wells. Red lines represent inhibition curves when peptides were used. The blue line represents inhibition curve when S9 original peptide was used. The green line represents inhibition curve when peptide from the wrong orientation S9 DNA sequence was used.
Figure 4. Inhibition Assay by Peptides of SIIIS8.C3 mAb Binding to GBS

ELISA assays for peptide inhibition of mAb interaction with GBS were performed by coating microtiter wells with GBS. The inhibition assay was performed by preincubation of the mAb with different peptide concentrations. SIIIS8.C3 mAb was diluted to 1/3,000. The mixture was allowed to incubate at room temperature for 1 h before placing in wells. Red lines represent inhibition curves when peptides were used. The blue line represents inhibition curve when S9 original peptide was used. The green line represents inhibition curve when peptide from the wrong orientation S9 DNA sequence was used.
Specificity of KLH-Peptides Conjugates

To determine whether multivalent peptides were more effective at inhibiting GBS mab interactions, peptides were conjugated to the carrier protein KLH. We compared the binding of several closely related anti-GBS mAb to different KLH-peptide conjugates by ELISA (Figure 4.14). At the same protein concentration, KLH-S9 bound to mAbs more strongly than the S9 peptide alone. Compared with KLH-S9 conjugate, KLH-219 bound more strongly to S9 mAb. None of the KLH-peptides bound to S3.1A6, S3.2A6 and S3.1B1, which detect a conformational carbohydrate determinant.

Figure 4. 14  Binding Activity of KLH-Peptide Conjugates by a Panel of Anti-GBS mAb ELISA plates were coated with KLH-peptide conjugates, S9 peptide, KLH or GBS. Anti-GBS mabs, anti-KLH, or PBS as negative control, were added and the binding activity was detected by adding anti-mouse IgG-AP conjugate. Absorbance at 405 nm was observed.
Competitive ELISA s between the KLH-peptides and the S9 mAb was then performed (Figure 4.15).

Figure 4.15 Inhibition Assay by KLH-Conjugates of S9 mAb Binding to GBS
GBS coated plates were blocked with ELISA blocking buffer overnight at 4°C. The inhibition assay was performed by preincubation of the S9 mAb with different KLH-peptide concentrations. The mixture was allowed to incubate at room temperature for 1 h before placing 100 ul of the mixture in wells and incubating 4°C overnight. The plates were washed with ELISA washing buffer, and incubated with 100 ul of alkaline phosphatase-conjugated anti-mouse Ig, followed by washing and addition of colorimetric substrate p-nitrophenyl phosphate. Absorbance at 405 nm was read.

The competitive ELISA indicated that all KLH-peptides and S9 inhibit the binding of S9 mAb to GBS. KLH-S9 gave greater inhibition of binding than did the unconjugated peptide at higher concentrations, indicating that the multivalent ligand had greater avidity, even though the free peptide gave greater inhibition at lower
concentrations, where there were more antigenic molecules and binding was not yet saturated. Other peptide-KLH conjugates behaved as expected compared to the S9-KLH conjugate.

**Sequence Analysis of Peptides**

Binding activity and sequence of the peptides described in this chapter are summarized in table 4.3 and table 4.4. Table 4.3 displays peptides, which have one amino acid different from the original S9 peptide. The data indicate that changes amino acid at positions 3, 4, 5, 7, 8, 9, 10 resulted in a decrease in binding activity to S9 mAb compared to the original S9 peptide. Changing amino acid at positions 1, 2, 6, 11 and 12 led to increased or unchanged binding activity compared to the original S9 peptide. Table 4.4 shows the information for peptides containing more than one amino acid different from the original S9 peptide. ELISA data analysis was separated into three groups. Group I represents a population of peptides with changes at positions 7, 8, 9 and 10. Group II represents peptides with changes of positions 7, 8, 9 and 10 combined with changes at other positions. Group III represents peptides with changes outside positions 7, 8, 9 and 10. Changing amino acids in positions 7, 8, 9 and 10 led to a reduction in binding by S9 mAb even though other changes are at positions 1, 2, 6, 11 and or 12, which alone may have increased avidity. Group III indicates that amino acid combination changes in positions outside 7, 8, 9 and 10 reduced or slightly reduced the binding activity by the S9 mAb.
Table 4.3 Summary of Binding Activity and Sequence of Peptides Which Have One Amino Acid Different from the Original S9 Peptide

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Selection</th>
<th>Amino Acid Position</th>
<th>Binding Activity</th>
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</thead>
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<td>Original</td>
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<td>S/</td>
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<td>G</td>
<td>S</td>
</tr>
<tr>
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<td>S/+</td>
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<td>Rp2.16</td>
<td>Affinity</td>
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### Table 4.3-Continued Summary of Binding Activity and Sequence of Peptides Which Have One Amino Acid Different from the Original S9 Peptide

<table>
<thead>
<tr>
<th>Peptide Selection</th>
<th>Amino Acid Position</th>
<th>Binding Activity</th>
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<tbody>
<tr>
<td>S9 Original</td>
<td>F D T G A F D P D W P A</td>
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<tr>
<td>10 Random</td>
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<tr>
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</tr>
<tr>
<td>820 Random</td>
<td>R S</td>
<td></td>
</tr>
</tbody>
</table>

172 M1 and M2 are peptides modified from peptide 172. S, S/+, +, S/- and - represent same, slightly increased, increased, slightly decreased, and decreased binding by S9 mAb compared to the parental peptide.

### Table 4.4 Summary of Binding Activity and Sequence of Peptides Which Have More than One Amino Acid Different from the Original S9 Peptide

<table>
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<th>Peptide Selection</th>
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<tr>
<td>S9 Origin</td>
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<tr>
<td>74 Random</td>
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<td>146 Random</td>
<td>G A</td>
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<tr>
<td>545 Random</td>
<td>E O S</td>
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<tr>
<td>261 Random</td>
<td>R R</td>
<td></td>
</tr>
<tr>
<td>850 Random</td>
<td>V E L</td>
<td></td>
</tr>
</tbody>
</table>

**Group I**

| 74 Random         | A L T               |                 |
| 146 Random        | G A                 |                 |
| 545 Random        | E O S               |                 |
| 261 Random        | R R                 |                 |
| 850 Random        | V E L               |                 |

**Group II**

| 113 Random        | C G                 |                 |
| 475 Random        | G K E               |                 |
| 51 Random         | G L                 |                 |
| 61 Random         | C K T S             |                 |
| 208 Random        | I G R S             |                 |
| 172 Random        | Y T                 |                 |
| 238 Random        | G A                 |                 |
| 319 Random        | H T                 |                 |
| 390 Random        | S A                 |                 |
| 457 Random        | H L                 |                 |
| 74 Random         | A L T               |                 |
| 90 Random         | A C                 |                 |
| 156 Random        | S G                 |                 |
| 237 Random        | A Y                 |                 |
| 378 Random        | M A                 |                 |
| 608 Random        | L N L               |                 |
| 850 Random        | V E L               |                 |
| 214 Random        | P G                 |                 |
| Rp2.13 Affinity   | G R                 |                 |
Table 4.4-Continued Summary of Binding Activity and Sequence of Peptides Which Have More than One Amino Acid Different from the Original S9 Peptide

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Discussion

The peptide-display phage library technique has served as a powerful system to identify mimetic peptides. In our lab, this technique has been used successfully to define a mimotope of type III *Streptococcus* group B capsular polysaccharide epitope identified by the protective antibody S9. This is designated the S9 peptide (1). Here in this study, the technique was used to clarify the nature of the portion of the peptide bound by antibody and to identify better antigen mimetic peptides. Phage fUSE2 vector, which has been used in our study, originally was developed by Smith lab (237, 373, 375, 377-379) and proved to be successful in identifying epitopes identified by antibodies to African horsesickness virus (380), Hepatitis C virus (381), HIV (382), Bluetongue virus (383), and the rickettsia, *Cowdria ruminantium* (384). The fUSE2 vector is a type 3 vector, which accepts DNA inserts and encodes fusion protein of the pIII molecule. The foreign
peptides are displayed on all five pIII molecules on a virion (377). We decided to choose the type 3 vectors because the type III vector can display long foreign peptides, whereas the type 8 vector, which allows insertion of foreign peptides in pVIII molecules, can only display short foreign peptides (242, 385). To achieve our aims, we created the modified S9 sublibrary to have approximately one mutation in each inserted original S9 sequence. Transforming the recombinant DNA into competent cells gave a low efficiency \(4.85 \times 10^4\) compared to the Puc19 vector control \(10^{11}\). This resulted in less diversity in the library. However, the need for library diversity was not a priority in this study since the library need only cover mutations in a 12-mer peptide. The diversity of our library was further diminished according to the sequencing analysis. Most clones with inserts had an unmutated version of the peptide encoding gene, indicating that our mutagenesis was less efficient than hoped. Cloning the library into a single cloning site of the fUSE2 vector resulted in two possible orientations giving 40.3% of transformant with foreign DNA inserts in the correct orientation. The library in this study was small in size, but it was large enough to analyze effects of amino acid changes in the 12 amino acid S9 peptide and search for better mimetic peptides based on the original S9 sequence.

Affinity selection with the S9 mAb was used to seek out peptides with higher binding activity. It has been shown that the yield in the first round of selection is very important (375, 376). If the yield is not high enough in the first round (<1% of the input phage), clones have a good chance of being lost (373). Even though, the yield in the first round of the selection was low \(5 \times 10^5\), the increasing yield in the second round of our experiment indicated the success of affinity selection. In the third round of affinity
selection, the yield increased by 1 log suggesting that the phage population was saturated with phages displaying high binding activity to S9 mAb. Sequencing analysis of clones from the second and the third round of affinity selection revealed that the S9 original sequence was found most commonly among the phage clones. Only six unique peptide sequences were identified either in the second or the third round of affinity selection. The peptides are RP1.2, RP1.12 and RP1.15 from the first round of selection and RP2.10, RP2.13 and RP2.16 from the second round of selection. One unique deduced peptide, RP1.8, was detected from both rounds of the selection (from 2 clones in the second round and one clone in the final). Six out of seven phages selected by affinity selection showed increased or equivalent binding by the S9 mab. One clone showed reduced S9 binding. It has been shown that there is always a background of non-specifically bound phages, especially when low stringency phage elution conditions are used, as we did (373, 386). Even so, the affinity selection protocol appeared to select for phage with enhanced binding by the mab, i.e. enhanced immunogenicity.

The relationship between peptide sequence and antibody binding is summarized in tables 3.3 and 3.4. The data show that amino acid positions 7, 8, 9, 10 are most important for binding activity by the S9 mAb. Changes in these positions resulted in reductions in binding by the S9 mAb. In position 7 and 9, the original S9 residue is D, which is an acidic amino acid. Even changing the residue from D to other acidic amino acids, E and H, still led to reduced binding activity by the S9 mAb. In position 10, the original residue is W, an aromatic amino acid. Changing this residue also led to reduced binding. We do not know if a change to another aromatic amino acid would have
preserved binding. These results are consistent with the data reported by Johnson et al. 
(277). These NMR studies of the S9 peptide, as bound by the S9 mAb, indicated that the 
mAb bound to peptide residues 7 to 10, DPDW (Figure 4.16).

![Figure 4. 16 Views of S9 mAb Bound Structure of the Peptide FDTGAFDPDWPA (Peptide S9) (277)](image)

Epitope mapping techniques and sequence analysis from anti-Id and phage-
displayed libraries have demonstrated that antibodies bind to an epitope of three to five 
amino acids within a sequence. The fine specificity of some carbohydrate-binding 
proteins maps to peptides containing central W/YXY residues (387-392). A protective 
antibody against *Cryptococcus neoformans* glucuronoxylomannan selected phage 
displaying a binding motif of TPXW(M/L)(M/L) (393). Peptide mimicry of the 
carbohydrate epitope of *Mycobacterium leprae* is W(T/R)LGPY(V/M) (349). The peptide 
motif, DPDW from the S9 is different but there are some similarities. There are aromatic 
(W) and hydrophobic (W) residues. The acidic residue, D, may also imitate properties of 
the GBS capsular saccharide epitope, in which sialic acid plays a key role.
Our study also found that AA positions 3 to 5, TGA, play an important role in peptide binding. The mutation in this motif led to a reduction in binding activity by the S9 mAb. In position 3, the original amino acid residue is T, a non-aromatic amino acid with a hydroxyl group. Changing to S, which is also a non-aromatic amino acid with hydroxyl groups, still led to a reduction in binding avidity. In position 4, the original amino acid residue is G, which is an aliphatic amino acid. Changing to A still led to the reduction in peptide avidity. In position 5, the original amino acid residue is A, another aliphatic amino acid. Changing to V (peptide Rp12) or to G (peptide 189), also aliphatic amino acids, led to reductions in peptide avidity, although less so with G than V. The hydrophobicity of each amino acid residue may also play an important role: V is very highly hydrophobic whereas the A and G possess less hydrophobicity. Thus the TGA motif may possess hydrophobic properties that are important in antibody binding. This result indicated that the peptide may imitate the hydrophobic properties of the native bacterial capsule.

All data indicated that amino acid residues at positions 1, 11, and 12 are less important in binding to the S9 mAb. But altering positions 2 and 6 can lead to increased antibody binding. No single amino acid alteration in position 1 (F), 11 (P) and 12 (A) had an effect on peptide binding by the S9 mAb. A combination of amino acid alterations in these positions (peptide 332 and 624) led to slightly decreased peptide binding. Amino acid alterations at position 2 led to unchanged or increased binding to the S9 mAb, which depended upon the amino acid properties. In peptide 219, changing the amino acid from D to H, which is more hydrophobic, resulted in increased binding activity to the S9 mAb.
Alteration of position 6 (F), which is aromatic and highly hydrophobic, to non-aromatic but still highly hydrophobic amino acids (I,C,L) led to an increase in the binding activity to the S9 mAb. It would be interesting to investigate the result of the amino acid alteration from a hydrophobic to a non-hydrophobic amino acid in this position.

The results of sequence analysis of peptides obtained randomly, and by avidity binding to the S9 mAb, were consistent. Five of seven phages from the affinity selection, have amino acid alterations at positions 2, 6, 11 and/or 12 which resulted in increased binding to the S9 mAb. Several phages with increased binding to the S9 Ab randomly selected were not picked by the affinity selection. Several clones were lost during the affinity selection process, and we did not fully explore the effects of different elution methods.

Data from our study indicated that peptides with stronger binding by S9 mAb also were better inhibitors of the binding of S9 mAb to GBS. When peptides which had lower binding than the parental peptide were used as inhibitors, no inhibition of the S9 mAb-GBS interaction was seen. The result implied that peptides with higher avidity for the S9 mAb are also better inhibitors of GBS binding to the S9 mAb. Peptides did not bind to irrelevant antibodies, B6 (antibody against Candida albicans) and S11 (antibody against Beta-C protein of type I and II GBS), showing that the peptides we isolated were specific for the S9 mAb.

We next asked whether the peptides were bound by other mabs that react with GBS type III polysaccharide. Antibody SIIIIV18.C2 mAb (specific for the complete type III CPS) bound a similar array of peptides as S9, whereas an antibody with similar
specificity, SIIIS8.C3, binds to none. Antibodies to the conformational type III CPS epitope do not bind the peptides either.

The results presented here indicated that phage peptide sublibraries can be powerful tools for isolating high affinity peptides and mapping the original peptide mimotope. Immunization with phages displaying these peptides (E. Moran, S.H. Pincus, unpublished) did not lead to significantly higher anti-GBS titers than immunization with the original S9 phage. At best, only a small fraction of the anti-peptide antibody is anti-GBS. These results have implications for the design of peptide mimotope vaccines to carbohydrate antigens.
CONCLUSION

Streptococcus group B, (GBS) is a major cause of neonatal sepsis and meningitis. Even though the pathogen can be successfully treated with antibiotics, deaths due to infection still occur (100, 342). The main virulence factor of GBS is capsular polysaccharide (CPS), which is anti-phagocytic (94, 394). The antibody against CPS confers protection against GBS infection (170, 194, 346). GBS vaccines using CPS alone were of limited effectiveness. This is likely because CPS of GBS, as a carbohydrate antigen, is a T-independent antigen resulting in low IgG induction and lack of immunologic memory. Conjugation with protein carriers has improved its immunogenicity and conferred protection against GBS infection (192, 195, 199).

To develop a vaccine against GBS, an alternative approach using a phage displayed library was applied to search for a peptide that mimics the CPS of GBS. The S9 peptide, FDTGAFDPDWP, was identified and shown to be both antigenic and immunogenic. S9-KLH conjugate conferred antibody against S9 peptide and GBS (1).

In this study we continued the focus on GBS vaccine design. The goal of this thesis research is first to enhance immunogenicity of the S9 peptide, second to investigate the structural basis of the peptide mimicry of the GBS type III CPS carbohydrate epitope, and third to identify better mimic peptides.

The standard method to improve immunogenicity of small peptides is by conjugating them to protein carriers such as KLH and tetanus toxoid. In our study, we used an alternative method by presenting the peptide on plant virus coat protein. Plant viruses have been used as vaccine vectors to deliver peptides or small proteins to the
immune system (reviewed in reference (395)). There are two approaches to accomplish these using recombinant techniques. The first strategy is to create replicative chimeric virus expressing peptide by fusing the foreign protein to the virus coat protein. The second approach is based on the property of virus coat proteins to self-assemble on expression in bacteria, such as *Escherichia coli* or in yeast, such as *Saccharomyces cerevisiae* or *Pichia pastoris*. In our study, Cowpea Chlorotic Mottle Virus (CCMV) was chosen to carry the S9 peptide. The capsid of CCMV is composed of 180 copies of coat protein subunits. The CCMV structure has been revealed by X-ray crystallography(317). Based on the structure and X-ray crystallography, the Young lab has modified coat protein gene at permissive sites for peptide insertion. Each site was located on the exposed loop of CCMV coat protein. Nucleic acids encoding the peptide S9 were cloned into each site in a separate construct. The RNA of the recombinant constructs was *in vitro* transcribed and manually inoculated on the natural host, cowpea plant and tobacco. No infection with chimeric virus was obtained. The result suggested that inserting peptide into coat protein disrupted virus assembly process. The coat protein of CCMV can self-assemble in *P. pastoris*, a heterologous expression system, resulting in the formation of virus-like particles (VLP). The cassettes of chimeric S9-coat protein genes for each construct were subcloned into the *P. pastoris* expression plasmid pPicZa. The S9-CCMV coat protein from each construct can be expressed inside the yeast cells. However, intact recombinant VLP could not be purified from yeast cell debris, while the wild-type VLP can be obtained easily. This data indicates that the insertion of the S9 into the CCMV coat protein might cause virus to fall apart or disrupt viral assembly. We therefore
presented the S9 peptide on the CCMV coat protein via chemically conjugating CCMV and S9 through chemical linkers. Two CCMV mutants were used in the coupling reaction, salt stable mutant (ssCCMV) and SubE mutant. Because the ssCCMV (K42R) can resist disassembly in 1.0M NaCl, pH 7.5 (319, 341) whereas the wild type CCMV is only stable at pH4 and lower ionic strength, and the coupling reaction is done at the higher pH and ionic strength, the salt stable mutant is the appropriate virion for conjugation. The ssCCMV-S9 conjugation resulted in monomeric intact virions. SubE is a mutant that cannot assemble virions, and thus serves as a control with the S9 peptide conjugated to the monomeric coat protein. We next tested if the CCMV-S9 conjugate can enhance S9 peptide immunogenicity. Mice were immunized, with or without adjuvant, with different antigens, CCMV, CCMV-S9, SubE-S9, CPMV-S9 (kindly provided from Dr. John E. Johnson), and KLH-S9. Mice immunized with CPMV-S9, CCMV-S9 and KLH-S9 produced an antibody response to S9 and GBS without the help of adjuvant. When adjuvant was used in the immunization, the antibody response was augmented. SubE-S9 conjugate only induced an antibody response with the help of adjuvant. The data suggested that organization of antigen is important in immunogenicity. In fact the anti-S9 antibody response from mice immunized with CPMV-S9 conjugate was higher than the response from mice immunized with KLH-S9 conjugate. The data suggested that conjugation of peptide to virus coat protein created repetitive structures expressing the peptide, leading to enhanced in immunogenicity.

The antigen-specific IgG subclass profile was characterized. Mice immunized with both virus-S9 conjugates expressed predominant IgG2a, whereas mice immunized
with KLH-S9 conjugate produced predominant IgG1, suggesting Th1/Th2 bias with the different carriers. The cytokine profile analysis confirmed this, showing that splenocytes primed with CCMV or CCMV-S9 and then stimulated with CCMV or CCMV-S9 produced predominantly IFN-\(\gamma\), whereas splenocytes primed with KLH-S9 and then stimulated with KLH-S9 or KLH produced predominantly IL4 and IL10. These data suggested that immunizing with virus particle expressing S9 provides a Th1 biased response whereas immunization with protein, KLH-S9 induces a Th2 biased response.

Even though, the data indicates that CCMV is not suitable for use as vaccine vector because insertion of peptides into the virus coat protein disrupts virus assembly, this study elucidated immunological issues. The study indicated that organization and the composition of the carrier for a peptide antigen is important to its immunogenicity. Virions are particulate antigens, with repetition of the basic epitope, and may also have accompanying nucleic acids. These differences appear to make them more immunogenic than carrier proteins, and may also influence the Th1/Th2 orientation of the resulting immune response. This knowledge could benefit vaccine design.

The second goal of the study was to characterize the structural basis of S9 mimetic antigen. To accomplish this goal, we designed a DNA sublibrary, expressing a mutated version of the S9 peptide with 3% random nucleic acids at each position of the original S9 nucleotide sequence, creating approximately one mutation in each inserted sequence. By affinity selection, peptides with better binding activity to the S9 mAb were obtained. By random selection, it was shown that phage clones with amino acid alterations in positions 3-5 and 7-10 had reduction in S9 antibody binding activity. This
data indicates that these residues are involved in antibody binding of the peptide. These findings are consistent with those of an NMR study of antibody binding to the S9 peptide (277). The characterization of peptides with higher affinity for antibody showed that changing certain amino acids to more hydrophobic amino acid residues increased binding. This suggests that the S9 peptide mimics the hydrophobicity of the native capsular polysaccharide.

In summary, this thesis examined an alternative approach toward GBS vaccine development. Phage display libraries have been used in our study and proven to be a powerful tool to search for peptides that mimic carbohydrate epitopes. The technique also allows us to explore the structural basis of the mimetic peptide providing a better understanding of the structural basis for mimicry epitope. By using CCMV plant virus as a vaccine vector we also show that using particulate antigens influences the immunogenicity of peptide antigens.

Our original goals in this study were to enhance the immunogenicity of S9 as a GBS mimetic and to study the structural basis of the peptide. Our results indicated that we were not able to improve upon the original S9 in terms of eliciting an anti GBS response. However, our studies have provided answers to the structural questions of S9 mimetic structure.
REFERENCES CITED


design of peptide presentation on a viral surface: the crystal structure of a

357. Liu, L., M. C. Canizares, W. Monger, Y. Perrin, E. Tsakiris, C. Porta, N. Shariat,

particles as novel, stable vaccines. *Developments in Biological Standardization*
87:201-205.

Bachmann. 2002. Regulation of IgG antibody responses by epitope density and

bone marrow-derived dendritic cells to produce alpha interferon and Th1 immune

2007. Vesicular stomatitis virus glycoprotein displaying retrovirus-like particles
induce a type I IFN receptor-dependent switch to neutralizing IgG antibodies.
*Journal of Immunology* 178:5839-5847.

K. Estes. 1999. Recombinant Norwalk virus-like particles given orally to

Compsans, and C. Yang. 2006. Ebola virus-like particles produced in insect cells
exhibit dendritic cell stimulating activity and induce neutralizing antibodies.
*Virology* 351:260-270.

2001. Neutralization of human papillomavirus type 11 (HPV-11) by serum from
women vaccinated with yeast-derived HPV-11 L1 virus-like particles: correlation
with competitive radioimmunoassay titer. *Journal of Infectious Diseases*
184:1183-1186.


