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UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

Cell Surface Exposure and Immunogenicity of Foreign Epitopes in Outer Membrane Proteins

A Dissertation

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

Doctor of Philosophy

By

Zengbiao Qi
Norman, Oklahoma
2001

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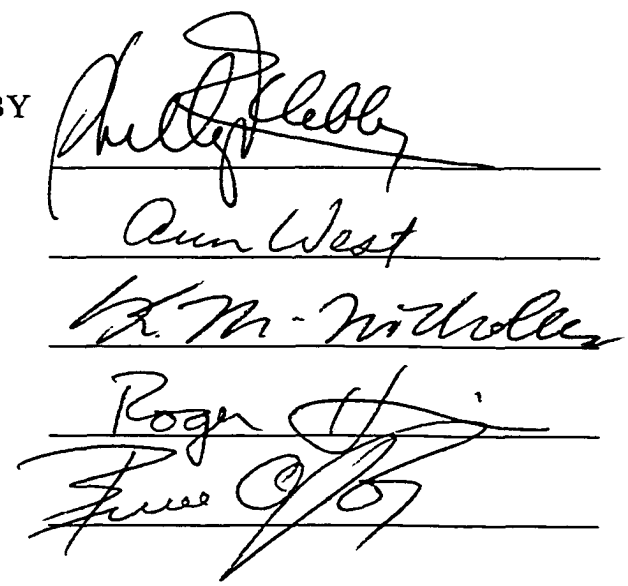
Cell Surface Exposure and Immunogenicity of Foreign Epitopes in Outer Membrane Proteins

A Dissertation APPROVED FOR THE
DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY

BY



To Honggui and Min

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ABSTRACT

To display antigenic epitopes on the bacterial surface, LamB and OmpA, two outer membrane proteins from *E. coli*, were used as carriers for two heterologous epitopes: the V3 loop (residues 293 to 334) of gp120 from HIV-1 and the TOP epitope, a smaller part of the V3 loop (residues 309 to 320). In order to optimize the exposure of the heterologous epitopes on the cell surface, three amino acids were introduced into the immediate upstream and downstream junction regions flanking the epitopes. PCR mutagenesis was utilized to randomly mutate these amino acids, creating chimeric protein libraries: each member of the libraries had unique flanking sequences. Because bends and turns in proteins derive from four sequential amino acids, the individual chimeric proteins projected the epitope in different ways.

To evaluate the accessibility of epitopes to the extracellular environment, bacteria expressing LamB:V3 or OmpA:TOP chimeric protein were fluorescently labeled with mouse anti-gp120 (V3), and analyzed by cytofluorimetry. Clones with the highest fluorescence intensity were selected for further studies.

To investigate how the exposure of an epitope on the bacterial surface influences the immunogenicity of the epitope, mice were immunized with live attenuated *Salmonella typhimurium* expressing the selected fusion proteins with various degrees of epitope exposure. The humoral response to *Salmonella typhimurium*/chimeric proteins was analyzed by ELISA and Western immunoblot. The data showed strong correlation between the exposure of the epitopes to the extracellular environment, and the immunogenicity in mice.

CHAPTER I

INTRODUCTION

Vertebrate animals live in an environment which is abundant in pathogenic microorganisms, such as bacteria, fungi, viruses and parasites. These microorganisms present a serious threat to the well being of mammals in their daily life. To cope with such a hostile environment, vertebrates have a defense mechanism to protect themselves from these disease causing agents. This self defense system of vertebrate animals involves two components: an innate defense system and an adaptive immune system.

Innate Defense Against Infectious Agents

The innate defense system of vertebrates provides the first line of defense for vertebrates against the microorganisms' invasion. Two important features of the innate protective system are lack of specificity and lack of adaptation. The innate defense mechanism exists even before exposure to foreign microorganisms

or macromolecules. The ability of innate defense to eliminate microbes and macromolecule is not enhanced by repeated exposure and is independent of the kind of pathogens involved (viruses, bacteria or parasites). Since the innate immune response does not involve adaptation, the response to a pathogenic invasion is prompt. These features of the innate defense system are critical to the vertebrates' survival in surroundings that are filled with opportunistic pathogens. Thanks to the innate defense system, many infectious agents are unable to invade vertebrates, or even if they are successful in getting into the organism, they are eliminated by the innate defense before seriously damaging to the host.

The innate immune system is composed of the outermost barrier, the external skin, the mucous membranes, internal defense cells and circulating proteins. Most microorganisms are unable to penetrate the barrier natural of the external skin and the mucous membrane. Most that do cross the external barrier are digested and eliminated by internal defense cells such as phagocytes and natural killer cells. Although phagocytes have the ability to recognize and digest the majority of foreign invaders, interactions with the products of the humoral response greatly enhances their activity. The circulation proteins, or effectors in non-specific defense, include the complement system and lysozyme.

Adaptive Immune Response

Unlike the innate defense system, the immune response in vertebrates is specific, adaptive and long lasting. Once the immune system detects a foreign invader, it takes time to develop and respond. Foreign invaders are specifically recognized by the cells of the immune system. Also, the elimination of the invading pathogens is the result of a well orchestrated interaction among cells and proteins of the immune system. Amazingly, the elimination of a foreign invader is not the only result of immune response. The immune system also produces memory cells, which live for decades and circulate in the lymphatic system of vertebrates (Kishimoto and Hirano, 1988). As a result, the host produces a much faster and stronger immune response upon subsequent encounters with the same pathogen, usually resulting in its rapid elimination. Thus, the adaptive immune protection mechanism minimizes the damage to the host caused by subsequent invasions of the same microbes. It is the ability of the adaptive or specific immune system to remember the previous encounter with a foreign invader that lays the foundation of vaccination against infectious diseases.

Two Arms of Immune System of Vertebrates

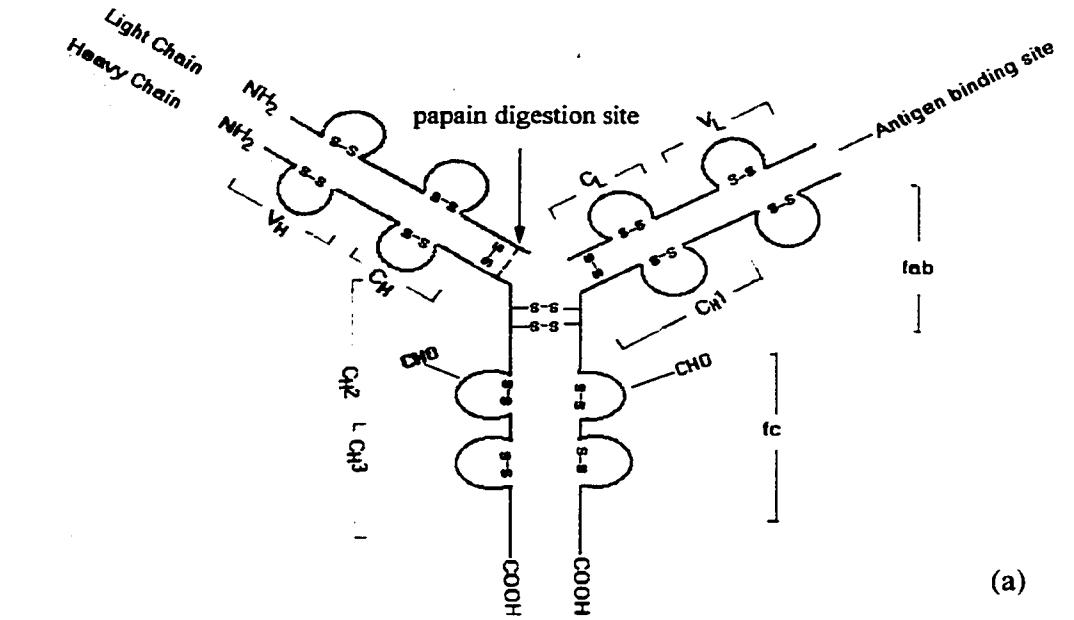
Immune protection in vertebrate species consists of humoral and cellular immunity. Both of these arms are specific and adaptive. Although both may

respond specifically to a foreign invader, the humoral immune response is more prominent towards extracellular bacteria, viral infections and secreted toxins (Scherle et al., 1992), whereas the cellular immune response fights more against fungi, parasites, intracellular viral infections, cancer cells and foreign tissues (Murphy, 1990). The humoral and cellular immune mechanisms use different effectors to mediate the immune response. Humoral immunity involves secreted proteins in serum, called "antibodies", to mediate immune response, and these can be transferred to an unimmunized individual by plasma or serum transfusions. Cellular immunity involves white blood cells, and can only be transferred to a naive individual through introduction of cells from an immune host.

Humoral Immune Response

A. Antibody Structure and Function

The humoral immune response is mediated by antibodies (Figure 1.1A, 1.1B), which are either membrane bound or circulating, soluble proteins produced by B cells or plasma cells. An antibody consists of two identical heavy chains (H) and two light chains (L), having a formula of H_2L_2 (Alzari et al., 1988). Each



(b)

Figure 1.1 Immunoglobulin structure. Each immunoglobulin consists of two heavy chains (H) and two light chains (L). Each heavy and light chain has repeating domains. C - constant region; V - variable region; Fc - crystalline fragment; Fab - antigen binding site; CHO- carbohydrates. (a) illustration of IgG. (b) stereo view of IgG.

chain contains two parts, a constant region (C) and a variable region (V), based on their amino acid composition among different antibody molecules. For each antibody molecule, one light chain is attached to one heavy chain by an inter-chain disulfide bond; and two heavy chains are connected together by two inter-chain disulfide bonds. The resulting tetramer contains two identical antigen binding sites, formed by the variable regions of the heavy and light chains. Based on the amino acid sequence of the constant region of heavy chains, immunoglobulins (Ig) are classified into five classes (isotypes): IgG, IgA, IgM, IgE and IgD, which contain the heavy chains γ, α, μ, ε and δ, respectively. In human, IgA can be further subdivided into two subtypes: IgA1 and IgA2 (Kunkel and Prendergast, 1966). IgG has 4 subclasses in human: IgG1, IgG2, IgG3, IgG4 (Abbas, 1991). In mice, IgG immunoglobulin are classified as IgG1, IgG2a, IgG2b and IgG3. The heavy chain amino acid sequences of antibodies from the same isotype or subtype are for the most part identical. However, the sequences of antibodies from different isotypes or subtypes are different. Upon partial proteolytic digestion by papain, an immunoglobulin dissociates into three fragments: two Fab fragments (the fragment containing antigen binding sites) and one Fc fragment (the crystalline fragment) (Figure 1.B). The heavy chain constant region (Fc) of an antibody mediates the effector functions of immunoglobulins after they bind to antigens (Unkeless, 1989). Therefore, antibodies from the same isotype or subtype carry out the same effector functions,

such as binding to cell surface receptors or activating the complement system.

The specificity of an antibody molecule arises from the variable regions of light chains and heavy chains. Different antibody molecules have unique amino acids in their antigen binding sites; therefore, they recognize different antigens. Vertebrates have enormous antibody repertoire with levels up to 10^8 different antibodies (Alt et al., 1987). This huge diversity of antibodies originates from somatic recombination and hyper mutation (Leder, 1982; French et al., 1989; Rjewsky et al., 1987; Tonegawa, 1988;Lieber, 1991).

Secreted antibodies or serum immunoglobulins exist either as single tetramers or as aggregate of different numbers of tetramers. IgM usually forms aggregates of five tetramers and IgA may occur as monomers or dimers. In the serum, IgG is the dominant immunoglobulin; IgA is the major immunoglobulin in exocrine secretions.

Antibodies neutralize and eliminate the antigen that induced their formation. The elimination of different antigens requires different effector mechanisms, which depends on the isotype or subtype of the antibodies. Secreted antibodies can bind toxic antigens and neutralize them. Also, by binding to antigens, antibodies may hinder the interaction of the antigen with cell surface receptors. As a result, they guard host cells from infection by microorganisms bearing the antigens. Antibodies also enhance the phagocytosis of the antigen bearing microorganisms by natural killer cells and macrophages. These

professional phagocytes express the Fc receptors on their surface. Antibody coated microorganisms are more efficiently ingested by phagocytes. The Fc portion of antigen-bound IgG or IgM activates the complement system and induces the lysis of certain target cells (Unkeless, 1989).

Initially, antibodies are expressed as membrane proteins on the surface of B cells. These membrane-bound antibodies are mostly IgM and IgD, both existing as single tetramers. A membrane bound antibody acts as a receptor on B cells. Upon encountering an antigen, B cell clones with high affinity for the specific antigen are activated, and then undergo antigen-dependent proliferation and differentiation. During the process of proliferation, B cell clones that specifically recognize the antigen expand and the immune response to the antigen is amplified. These B cells change from cells, whose function is to recognize a specific antigen, into effector cells which eliminate the antigen. Effector cells can either be plasma cells, that produce antibodies with the same specificity as the original B cells but with a higher affinity, or memory cells, which circulate in the immune system for a long period of time and are ready for a subsequent encounter with the same antigen.

B. Primary and Secondary Immune Response

When the immune system first encounters a foreign invader, the antibody concentration in blood reaches a peak after about a week, with most of the

circulating antibodies being IgM, which is the characteristic of the primary immune response (Figure 1.2). During the primary response, antibodies are secreted by the previously unstimulated B cells, which only express membrane bound IgM and IgD immune globulins and IgD is rarely secreted. When the immune system encounters the same antigen again, the immune response to this antigen, the secondary immune response is different in several aspects from the primary response. For example, the secondary immune response is both faster and stronger than the primary response. The concentration of antibodies in the serum reaches its maximum in a shorter period of time, and the resulting concentration of antibodies is much greater in the secondary immune response. This is the result of the activation of memory B cells. Furthermore, the secreted antibody classes in the secondary immune response include IgA, IgG and IgE, with IgG being dominant. So the immune response changes from only being capable of producing IgM and IgD class antibody, to being able to produce other classes of antibodies with the same specificity. This phenomenon is called Ig class switching (Esser and Radbruch, 1990). Finally, antibody affinity in the secondary immune response is higher than in the primary response. This is called affinity maturation of antibodies, which is achieved through somatic mutations in immune globulin genes (French et al., 1989).

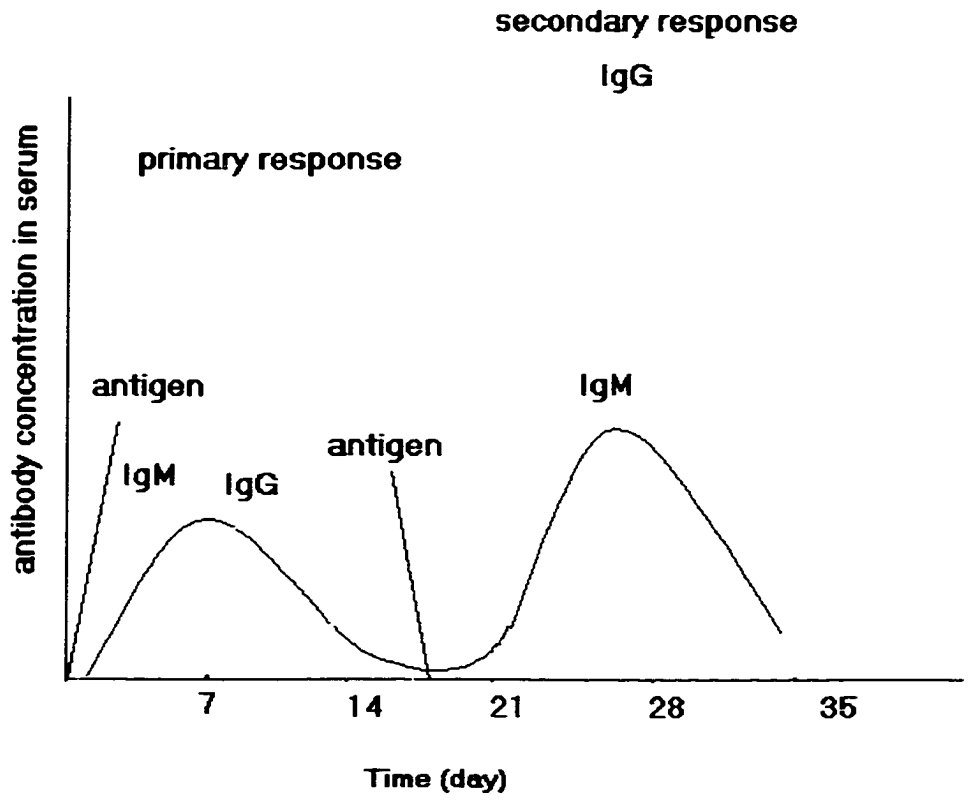


Figure 1.2 Primary and secondary immune response. After first antigen exposure, antibody production starts after several days. IgM appears before IgG. When exposed to the same antigen second time, immune system responds faster and the maximum level of IgG is much higher in the secondary immune response than in the primary immune response.

C. Activation of B cells

Immunoglobulins, as B cell receptors, recognize soluble protein antigens. polyssacharides, lipids, nucleic acids and small chemicals. However, small molecules (<500D) must be covalently linked to macromolecules, such as proteins to generate antibodies against them. Although B cell receptors can recognize protein antigens and initiate the immune response, the secretion of the antibodies and the immune memory are not accomplished by B cells alone. B-cell activation, antibody production, affinity maturation, antibody class switching, and B-cell differentiation into memory cells are achieved by the interaction between B cells and another class of lymphocytes, the antigen-specific T helper cells. Therefore, protein antigens are called T cell-dependent antigens. Antigen specific T helper cells and their secreted products provide antigen specific B cells with a second signal for their activation, that occurs after the interaction of membrane bound antibodies with antigens (Defrano, 1987; Jelinek and Lipsky, 1987; Abbas, 1988; Kishimoto and Hirano, 1988).

Unlike protein antigens, polyssacharides and lipids activate B cells in the absence of T helper cells. They are therefore called T cell-independent antigens. T cell-independent antigens, cannot induce immune memory, and the humoral response against polysaccharides and lipids is not boosted by repeated immunization of host animals (Andersson and Blomgren, 1971). In other words, they do not induce a secondary immune response, and the reason is that immune

memory cannot be induced without the antigen specific T helper cells and their secreted products.

Cellular Immunity

T-cells mediate cellular immune response. T cells only respond to protein antigens, not lipids, polyssacharides, or nucleic acids, whereas B cell receptor immune globulins respond to all these antigens. Protein antigens are not directly recognized by antigen-specific T cell receptors (TCR). In fact, TCR do not recognize soluble protein antigens. Rather, they recognize fragments of a protein (Guillet et al., 1987). Protein antigens are processed and hydrolyzed into peptides before they can be recognized by TCR. As a result, TCR recognize only linear peptides or linear epitopes, contrary to B cells, which recognize conformational epitopes. Moreover, TCR do not recognize foreign peptides unless these peptides are bound to Major Histocompatibility Complex (MHC) and presented on the surface of other cells (Schwartz, 1985), called antigen presenting cells (APC). To fully describe the antigen recognition by TCR and T cell activation, it is necessary to understand the MHC.

A. Major Histocompatibility Complex

MHC gene products are responsible for graft rejection (Lafferty et al.,

1983; Krensky et al., 1990). If MHC gene products identify a transplant as foreign, it will be rejected, but if they perceive the transplant as itself, then the transplant is accepted. The ability of MHC gene products to distinguish self from non-self occurs by genetic polymorphism among different MHC alleles. Generally, if a transplant recipient and a donor possess different MHC alleles, the graft will be rejected. There are two types of MHC gene products, MHC class I (MHC I) and MHC class II (MHC II). Both MHC I and MHC II are crucial in foreign antigen recognition by T cells. MHC II is expressed on B lymphocytes, dendritic cells, macorphages, and endothelial cells. MHC I is expressed in almost all nucleated cells.

B. Structure of MHC Class I and MHC Class II Complexes

The structures of complexes of MHC I and MHC II with peptides were crystallographically solved. The complexes show a lot of similarity in their structures. They possess an extracellular peptide binding region, which can bind a peptide 8-20 amino acids long (Rammensee et al., 1993), an extracellular nonpolymorphic immunoglobulin like region, a transmembrane region and a cytoplasmic region. The MHC I complex is composed of a heavy chain, polymorphic protein α, noncovalently associated with a nonpolymorphic serum protein β_2 microgobulinb, and an endogenously derived antigenic peptide [Figure 1-3, (Bjorkman et al., 1987)]. MHC II complex is composed of two

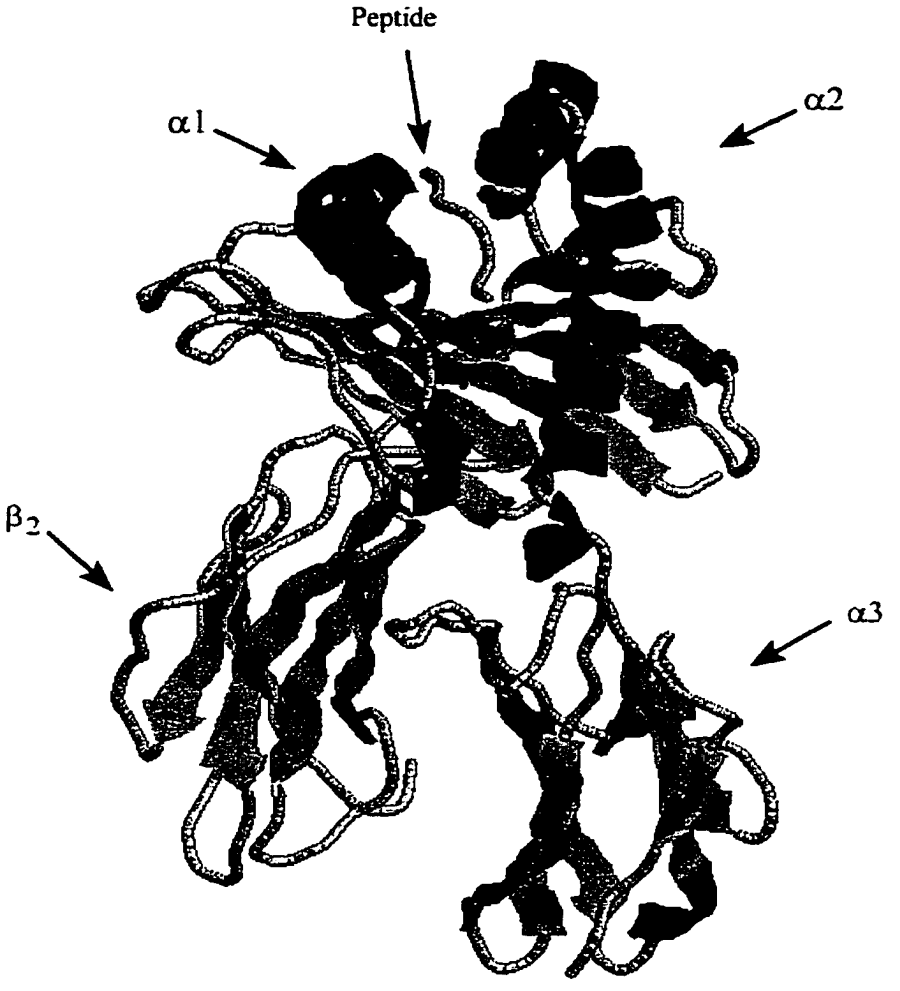


Figure 1.3 Crystal structure of the human class I histocompatibility antigen HLA-A2 The Figure shows the complex of Class I molecule with an endogenous peptide. Class I molecule consists of two chains, α chain and β_2 microglobulin as shown in the Figure. The Figure also depicts three extracellular domains of α chain, α1, α2 and α3.

polymorphic transmembrane proteins, α (22kd) and β (33kd), and an exogenously obtained peptide [Figure 1.4, (Fremont et al., 1996)].

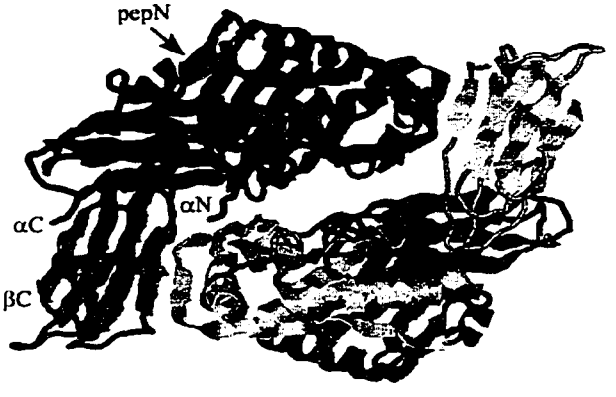


Figure 1.4 Crystal structure of mouse MHC II. The Figure shows the crystal structure of a dimer of mouse MHC II (I-E^k). Each heterodimer consists of two chains, α and β. A peptide convalently attached to N-terminus of β chain binds to peptide binding clef between two α helix, each one from a different chain.

C. Antigen Presentation and Antigen Recognition

In the T cell-mediated cellular immune response, TCR do not recognize soluble proteins or free peptides. TCR only recognize the MHC-bound foreign peptides displayed on the surface of other lymphocytes (Schwartz, 1985). This is called antigen presentation, and the cells that display MHC-peptide complexes on their surface are called antigen presenting cells (APC). MHC I molecules are associated with foreign protein peptides synthesized intracellularly (such as viral proteins) and present the foreign peptides to cytotoxic T cells (CTLs), which express the T cell surface marker CD_8 (Boon and Van Pel, 1989; Harding et al., 1991). MHC II molecules, on the other hand, present exogenously derived foreign protein peptides (such as soluble proteins or proteins from extracellular microorganisms) to helper T cells, which express the T cell surface marker CD_4 (Nuchtern et al., 1990; Harding et al., 1991; Long, 1992; Germain and Margulies, 1993). Therefore, CTLs are MHC class I restricted and helper T cells are MHC class II restricted.

Antigen presentation begins with the binding of foreign proteins by APCs. Dendritic cells and macrophages bind proteins with varying specificity and efficiency (Unanue and Allen, 1987; Steinman and Inaba, 1989; King and Katz, 1990; Rosenbusch, 1990). B cells efficiently bind proteins, due to the high affinity of membrane Ig for protein antigens, and then internalizing them for presentation (Chesnut et al., 1982a; Chesnut et al., 1982b; Kappler et al., 1982;

Ashwell et al., 1984). Macrophages express surface receptors for the immunoglobulins constant region (Fc), and the complement protein C3b. As a result, macrophages can very efficiently bind antibody-coated microorganisms or foreign proteins. After the binding of extracellular microorganisms or foreign proteins by APCs, then antigens are internalized by receptor-mediated endocytosis or phagocytosis. Soluble protein antigens may also be internalized by fluid-phase pinocytosis, without actually binding to the cell surface. The internalized protein antigens are sequestered in acidic endosomes and partially degraded by into peptides, which are then loaded into the MHC II molecules (Nuchtern et al., 1990; Davidson et al., 1991).

T cell antigen receptors recognize foreign antigens that are associated with MHC molecules and displayed on another cell surface. T cell receptors recognize both MHC-associated foreign peptides and the MHC molecule itself. To activate the T cell, the peptide-containing MHC molecule has to be identified as a self-MHC molecule. In other words, T cell receptors recognize only self-MHC-bound foreign peptides. If peptides are associated with the MHC class I molecules, then the TCRs of CTLs recognize the complex and perceive that the cells displaying the foreign peptides are infected by virus, CTLs lyse the cell (Nabholz and MacDonald, 1983; Kupfer and Singer, 1989). In this case, the antigen presenting cell is called a target cell. If peptides bind MHC II molecules on APC, on the other hand, TCRs on helper T cells bind to MHC II-peptide

complexes, providing the first signal for helper T cell activation. However, the binding of MHC II-peptide complexes to TCRs is not enough for helper T cell activation. Some undefined co-stimulator molecules serve as a second signal for the activation of T cells. Those co-stimulator molecules are secreted by accessory cells, such as macrophages. Certain cytokines, such as a tumor necrosis factor, interleukin-1 and interleukin-6 have co-stimulator effects on T cell populations (Balkwill and Burke, 1989; Arai et al., 1990).

D. The Consequence of T Cell Activation

Activated T cells carry out the effector functions of the immune response against foreign antigens. Activated T helper cells secrete cytokines (Balkwill and Burke, 1989; Arai et al., 1990), which act on the same T cell, or B cells, or macrophages or inflammation leukocyte. These cytokines regulate and promote humoral and cell- mediated immune responses. The effector function of CTLs is to lyse cells displaying foreign peptides and secrete cytokines. T cell proliferation, clonal expansion, and differentiation into antigen-specific memory T cells are the major events in response to antigen recognition. These events are mostly regulated by cytokines. T cells that secrete cytokines also expresses the receptors for the cytokines.

Vaccination

The most striking feature of the humoral and cell mediated immunity response is memory. Once the immune system responds to a foreign antigen, it remembers the encounter with the antigen. Some of the antigen activated B cells and T cells become memory cells, which circulate for a very long time. Immune memory provides the host with a means to defend itself more efficiently and more rigorously in subsequent encounters with the same antigen. As a result, virulent microorganisms are eliminated quickly with less damage and destruction to the host.

Vaccination is based on the memory of immune system of vertebrates. Vaccination induces immune memory without harming the host, by inoculation of the host with avirulent or attenuated forms of microorganism, or with a part of a microorganism. Later, when the immunized host encounters microorganisms related to the previous inoculation, the host quickly mounts a vigorous immune response to the invaders and quickly eliminates them.

The earliest systematic vaccination is attributed to Edward Jenner's work with cowpox (Jenner, 1798). At the time, there was a notion that people in certain villages in England who had contracted cowpox from their herds were not infected by widespread smallpox. Because cowpox was not a wide spread disease

at the time, not many people knew this. Jenner observed the phenomenon and took the big step to inoculate people with cowpox to prevent them from being infected by the more deadly virus, smallpox.

Since Edward Jenner's cowpox first vaccination, vaccinology has made tremendous progress. It is one of the major weapons against pathogens. Today, vaccination has controlled the following 10 major diseases, at least in parts of the world: smallpox, diphtheria, tetanus, yellow fever, pertussis, *Haemophilus influenzae* type b disease, poliomyelitis, measles, mumps, and rubella. In fact, smallpox as totally eradicated, worldwide. Vaccinations against influenza, Hepatitis B, and pneumococcal infections have also made great progresses. The effects of vaccination on vertebrates health should not be underestimated. Vaccination has greatly reduced mortality, and had profound inpact on population growth in the world. The role of vaccination in fighting against infectious disease and protecting mankind, perhaps, can only be matched by antibiotics.

The strategies of vaccination vary, depending on the unique biological properties of each microorganism, and the mechanisms by which a specific microorganism infects its host and causes damage. It also depends on the feasibility of routine of immunization and the cost for producing and handling vaccination products.

A. DNA Vaccines

In the past decade, the fields of DNA vaccines emerged. Although it has only been a short period of time since the introduction of DNA vaccines, they have already been applied to a wide range of infectious and malignant diseases. In this method of vaccination, plasmid DNA encoding specific antigens is delivered into a host by intramuscular injection, called gene gun inoculation [Boyle and Robinson, 2000; for a complete review on DNA vaccination, see (Encke et al., 1999; Spiegelberg and Raz, 1999; Gurunathan et al., 2000)], or by using attenuated intracellular bacteria (Dietrich, 2000). These plasmid-encoded antigens induce both humoral and cellular immune responses. This strategy is more effective for vaccination against intracellular organisms that require cell-mediated immunity, such as the agents of tuberculosis, malaria, and human immunodeficiency virus infection. So far, vaccination against the above infectious agents either has not been available or totally ineffective.

A plasmid containing full-length cDNA encoding paramyosin of Chinese *Shistasoma japonicum*, intramuscularly introduced into mice, induced a Th1-type immune response and conferred protective immunity in c57BL/6 mice against *Shistosoma cercariae* (Zhou et al., 2000). Similarly, expression of ubiquitin-conjugated tuberculosis protein in mice resulted in significant resistance to tuberculosis challenge (Delogu et al., 2000). Next, a single DNA vaccine encoding the MPT-63 and MPT-83 tuberculosis antigens evoked partial protection

against an aerogenic challenge with M. tuberculosis Erdman in the mouse model of pulmonary tuberculosis (Morris et al., 2000). Finally, the administration of a combination of tuberculosis DNA vaccines (containing the EAST-6, MPT-64, MPT-63, and KatG constructs) elicited a strong protective response relative to the protection evoked by live BCG vaccine (Morris et al., 2000).

B. Inactivated Vaccines

Vaccines consisting of inactivated virulent microorganisms are inexpensive and easy to prepare. Another advantage is that by administering whole inactivated virulent microbes, multiple potential protective antigens can be presented to immune system. However, inactivated bacterial or viral vaccines are not as effective as live ones, because inactivated vaccines only induce antibody responses and are unable to elicit cellular immunity (Hormaeche, 1993). Inactivated whole *S. typhi* bacteria administered orally elicited meager serum O, H or Vi antibody responses (Dupont, et al., 1971; Kantele et al., 1991). The intestinal SIgA antibody response and gut-derived IgA antibody secreting cell response after oral inactivated whole-cell vaccine were also quite minimal (Landy, 1953; Forrest and LaBrooy, 1991; Kantele, 1991), despite injection of a large number of inactivated typhoid bacilli. The most effective whole cell inactivated vaccine is the pertussis vaccine. Parenterally administered, killed whole cell pertussis vaccine renders protection against the mucosal pathogen *Bordetella*

pertussis (Brennan, 1992). In general, inactivated viral vaccines do not induce long lasting immunity in the host and mucosal antibody responses are very weak following parenteral immunization (Murphy, 1990).

C. Oral Vaccines Based on Live Attenuated Salmonella

Live attenuated microorganisms are much more effective vaccines than inactivated microorganisms. They induce long lasting humoral and cellular immune responses, and are especially more effective in eliciting cell-mediated immune responses (Mackaness, 1971; Collins, 1974; Hormaeche et al., 1990). They can confer protection for hosts with only a single dose. Presumably, it is because the attenuated strains establish limited infections in the host which mimic the early stages of natural infection and they express most of the natural target immunogenes of the natural infection. These target genes are processed and presented in a way that is most similar to the natural infection (Chatfield et al., 1994).

The majority of pathogenic microorganisms first achieves infection through mucosal surfaces. Therefore, the specific and nonspecific defense on mucosal surfaces play a very important role as the first line of defense. An effective vaccine must have the ability to elicit a mucosal immune response, which can only be achieved by priming the mucosal associated lymphoid tissue. Parenteral vaccination does not evoke an effective mucosal immune response

because the primary requirement for such a response is contact of the antigen with the mucosal surface (Waldman and Ganguly, 1974; Holmgren and Czerkinsky, 1992; McGhee et al., 1992). Live attenuated *Salmonella* strains, when administered orally, the natural route of infection, elicit potent humoral, cell mediated and secretory anti-*Salmonella* immune responses (Curtiss et al., 1988; Chatfield et al., 1989; Dougan, 1989; Hackett, 1990; Schodel, 1992). The ability of live attenuated *Salmonella* strains to elicit long lasting immunity is probably related to the way *Salmonella* infect hosts and how it persists in hosts. They gain entry into mucosal tissue by direct interaction with the follicular lymphoid tissues of the gut. They can remain localized or disseminate systemically, involving the cells and organs of the mononuclear phagocytic system, such as the spleen, liver, lymph node (Finlay and Falkow, 1989; Buchmeier and Heffron, 1990; Buchmeier and Heffron, 1991; Nnalue et al., 1992).

The use of live attenuated *Salmonella* strains to deliver heterologous antigens to the human immune system has been extensively studied. The results from these studies demonstrated that live attenuated *Salmonella* strains are capable of inducing a broad spectrum of immune responses against heterologous antigens. Many heterologous antigens from bacteria, viruses and parasites have been expressed in attenuated *Salmonella* strains using a variety of expression systems (Chatfield et al., 1989; Dougan, 1989; Chatfield et al., 1993; Schodel, 1992). The transfer of these systems to a carrier strain that can be practically used

in humans holds great promise for the development of a new generation of vaccines that may offer important advances in immunization against infectious diseases

Salmonella strains carrying mutations in genes of the aromatic pathway [Aro mutants, (Stocker, 1990)] were evaluated as human typhoid vaccines (Tacket et al., 1997). *aro*A, *aro*C and *aro*D mutants are all attenuated in mice. They are avirulent, due to their auxotrophy for certain aromatic compounds, such as the three aromatic amino acids (typtophan, tyrosin, and phenylalanine), as well as *para*-aminobenzoic acid and 2,3-dihydroxybenzoate. Some of these compounds are not available at sufficient levels in mammalian tissues to sustain growth of *Salmonella aro* mutants, leading to attenuation. Aro *Salmonella* vaccines are effective in mice (Stocker, 1990), cattle (Robertsson et al., 1983; Smith et al., 1984; Jones et al., 1991; Segall et al., 1994), sheep (Mukkur et al., 1987), and chickens (Cooper et al., 1990; Cooper et al., 1993; Cooper et al., 1994). Live *aroA* mutant *Salmonella* were used to present foreign antigens to the immune system by expressing them as intracellular proteins, or as peptides exposed at the surface of flagella filaments or at the surface of the outer membrane protein LamB or within MalE protein in the periplasm. (Newton et al., 1989; Charbit et al., 1993; Stocker and Newton, 1994). The antigen-specific systemic IgG, T helper responses, CD8+ class I-restricted or CD+ class II-restricted CTL responses, and mucosal antibody responses are induced by antigens expressed by attenuated

Salmonella typhimurium strains (Hormaeche et al., 1991). Mice immunized with a *Salmonella* vector expressing fragment C of tetanus toxoid were protected from parenteral challenge with tetanus toxin (Fairweather et al., 1990; Chatfield et al., 1992).

D. Expression of Heterologous Antigenic Epitopes in Attenuated Salmonella Strain

Foreign epitopes may be easily fused to a carrier protein. Although the insertion of an immunogenic heterologous epitope into a chosen protein is a trival molecular biological operation, care must be taken to preserve the functionality of the carrier protein, in order to avoid the degradation of the resulting hybrid protein. Thus, the insertion of an epitope should not interfere with normal functions that the chosen carrier performs under normal growth conditions of the bacteria. The over-expression of the hybrid protein should not cause toxic effects on the host cells. Therefore, it is always desirable that the structure of a carrier protein is known or that enough information regarding its structure has been obtained to facilitate the task of choosing insertion sites for an epitope.

a. Outer Membrane Proteins as Foreign Epitope Carriers

LamB. Maltoporin (Wandersman, et al, 1979; Luckey and Nikaido, 1980, Ferenci, 1989), also known as LamB, is an outer membrane protein in gram-

negative bacteria. It is a trimer and forms an aqueous channel of diameter 5-6 angstroms within each monomer. The gene for maltoporin, *lamB,* is located on the maltoporin regulon ((Desaymard et al., 1986), which also encodes other proteins involved in transporting and metabolizing maltose and other linear maltooligosaccharides. Maltoporin facilitates transport of maltose and other maltooligosaccharides through the outer membrane of Gram-negative bacteria, and also functions as a receptor for bacteriophage λ and other bacteriophages (Randall-Hazelbauer and Schwartz, 1973).

The crystal structure of LamB was solved with a resolution of 3.1 angstroms [Figure 1.5, (Schirmer et al., 1995)]. For each monomer, 18 antiparallel β-strands constitute the frame of an aqueous channel within the outer membrane. Nine loops and nine turns connect neighbor strands. The external loops which join the nearest neighbor strands on the cell surface are long. On the other hand, the turns in periplasmic space are short. Three of the external loops (L1, L3 and L6) do no stretch into the bacterial surface. Instead, they fold inward towards the barrel, with loop L2 from another neighboring subunit. The surface loops also form an umbrella-like structure, which shields the true pore of maltoporin. Because of the residues from loop 3, the size of the channel is narrowed about halfway through the membrane. The channel opening at the other end of the barrel toward periplasmic space is also restricted by the residues from

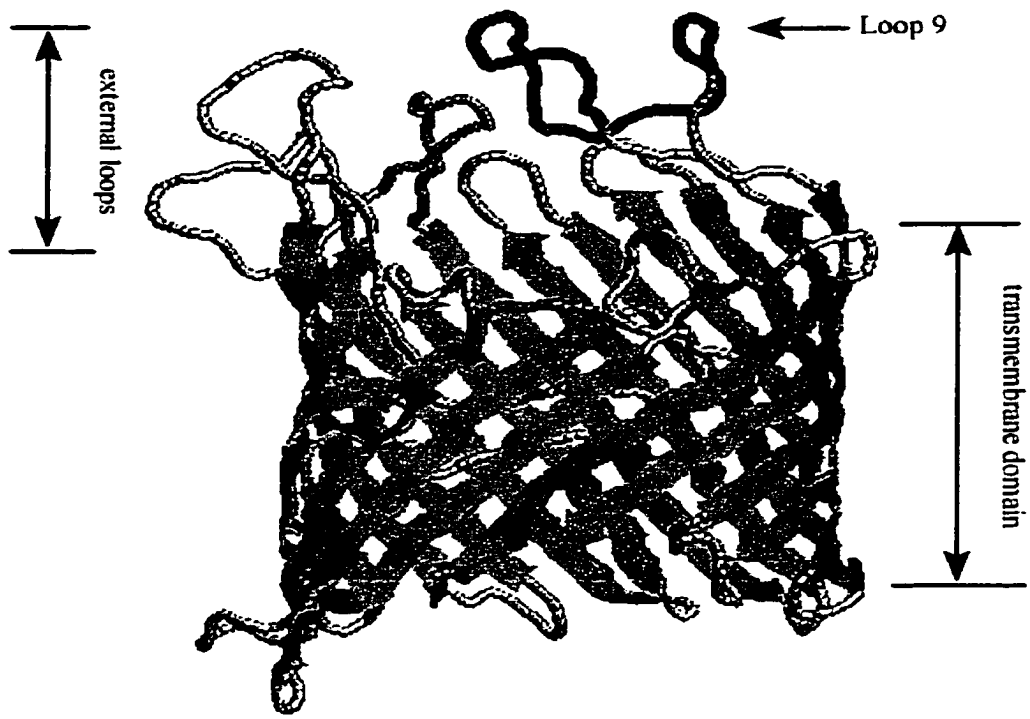


Figure 1.5 Crystal structure of *E.coli* LamB. Colored in green are residues 379 to 402 which were replaced with the third variable loop (V3 loop, residues 293-334) of gp120 from Human Immunodeficiency Virus Type-1 in LamB:V3 chimeric protein.

Loop 6. As a result, the channel has a diameter of 5 to 6 angstroms.

Before its crystal structure was published, the permissive sites for epitope insertion in LamB were investigated by using a reporter epitope, the C3 epitope from polio virus (Boulain et al., 1986; Charbit et al., 1987; Charbit et al., 1991). The epitope was fused to maltoporin at different sites, and the expression of the hybrid proteins was detected by a monoclonal antibody against C3 epitope. A total of 11 permissive sites were identified, namely, after residues 123,146, 153, 183, 253, 219,236, 352, 368, 374, 399 (Charbit et al., 1991). Among them, based on the published crystal structure of LamB, the epitope was exposed to the periplasmic after residues 183, 219 and 352; whereas it was cell surface exposed after residues 123,146, 152, 253, 236, 368, 374, and 399.

The use of maltoporin as a carrier for heterologous epitopes was extensively studied. C3 and preSB epitopes were fused into the site after residues 386 and the hybrid was expressed in a LamB negative derivative of the *aroA* attenuated strain of *Salmonella typhimurium*, SL3261, under an anaerobically inducible promoter, *pniR* (Wang et al., 1999). Simultaneous insertion of C3 after residue 386 and preSB after residue 153 was also carried out. It was found that insertion after residue 386 was more favorable for the induction of anti-epitope antibodies than after residue 153. The antibodies against both poliovirus and hepatitis B were induced in the mice immunized with LamB hybrid containing both epitopes.

Live recombinant bacteria harboring LamB-C3 hybrid proteins (insertion after residues 153 and 374) were intravenously injected into rabbits (Charbit et al., 1988). The high titer antibody against C3 epitope ($>5*10^3$) was raised against bacteria harboring recombinant protein containing the epitope after residue 153. The neutralizing antibody activity was also detected in the serum of rabbits. Using the same method, two epitopes of pre-S2 region (residues from 132-145, 120-145) from the envelope proteins of hepatitis-B virus(HBV) , were fused into LamB after residue 153 and live bacteria were injected into rabbits and mice. The sera from rabbits and mice were able to react with synthetic peptides corresponding to residues 132-145 and 120-145, respectively. The high titers (10^4) of anti-surface antigen antibodies were also induced in both rabbits and mice (Charbit et al., 1988).

LamB and MalE, a periplasmic protein in *E. coli*, were both used as carriers for the V3 epitope of the envelope protein gp120 from the human immunodeficiency virus type 1(HIV-1) (Charbit et al., 1993). The hybrid proteins were expressed in *Salmonella typhimurium* and live bacteria were injected into mice. MalE-V3 hybrid was stably expressed in the periplasm of S. *typhimurium*. However, the LamB-V3 (insertion after residue 153) hybrid was degraded both in vivo and vitro in S. *typhimurium*. MalE-V3 hybrid induced anti-HIV-1 envelope antibodies, which were detected by both ELISA and Western blot. On the other hand, LamB-V3 induced anti-HIV-1 antibodies which were detected

only by Western blot.

Shiga toxin B subunit was inserted into LamB and mice were immunized orally and intraperitoneally with an *aroA* mutant of *S. typhimurium* expressing the LamB-shiga toxin B subunit. The hybrid proteins developed specific mucosal and humoral antibody responses (Su et al., 1992).

Other outer membrane proteins from *E.coli*, *Shigella*, *Neisseria*, and *Salmonella* and other bacteria were also explored as carriers for expression of heterologous epitopes on cell surface (Georgiou et al., 1997). Antibodies to cholera toxin were induced by intraperitoneal immunization with cells expressing *Salmonella* flagellin-cholera toxin B subunit (Newton et al., 1989).

OmpA. The outer membrane protein A of *Escherichia coli* (OmpA) has 325 residues, and is one of the most abundant proteins in Gram-negative bacteria. It is believed that OmpA plays a role in bacterial conjugation (Ried and Henning, 1987) and serves as a receptor for bacteriophages (Morona et al., 1985) and colicines (Foulds and Barrett, 1973). The protein consists of two domains: N-terminal outer membrane embedded domain, and C-terminal periplasmic domain. The N-terminal domain, residues 1-171, forms a β-barrier with eight antiparallel strands. Residues 172-325 localize in the periplasmic space and presumably provide a binding site to peptidoglycan (Vogel and Jahnig, 1986; Klose et al., 1988; Ried et al., 1994; Koebnik and Kramer, 1995; Rodionova et al., 1995)

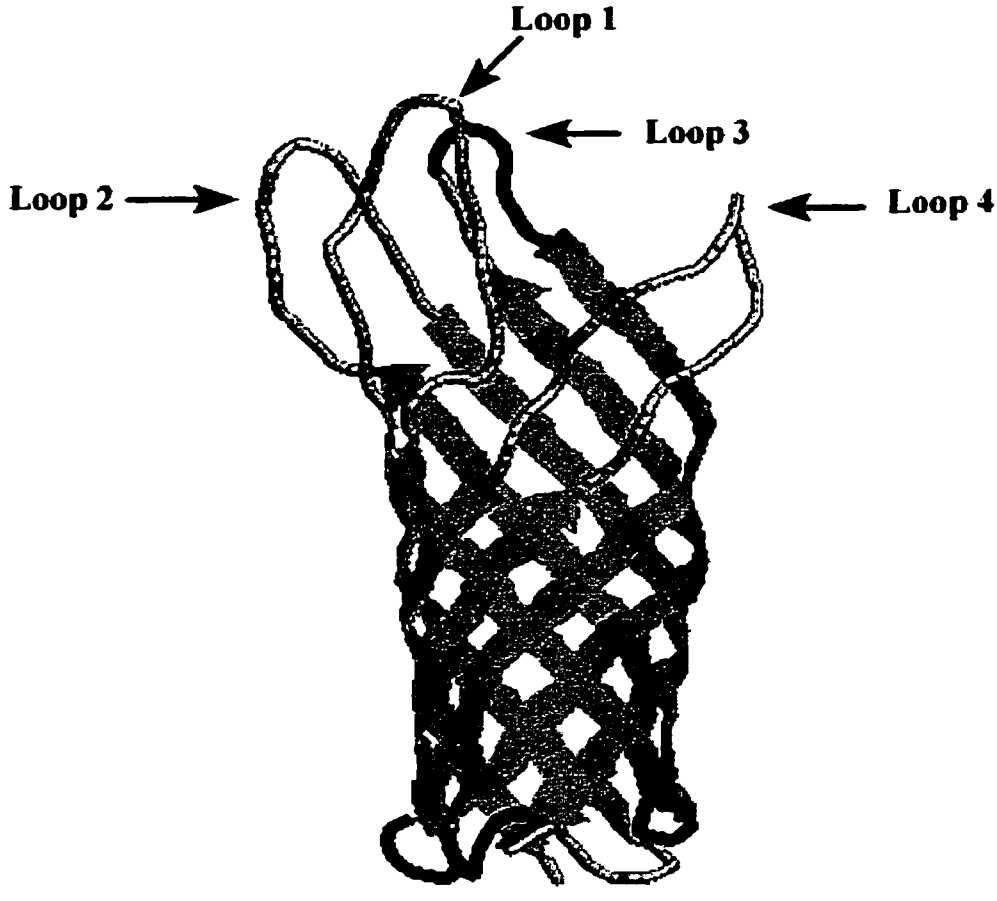


Figure 1.6 Crystal structure of *E. coli* OmpA. Four external loops of OmpA were labeled as such. Colored in red are residues 109 to 113 which were replaced with a part of the V3 loop (residues 309-320) in OmpA:TOP chimeric protein.

(Koebnik, 1995; Sugawara et al., 1996). All β strands are connected by four long surface loops at the exterior of the cell and small turns in the periplasmic space (Figure 1.6). The function of long external loops is not clear. Conceivably, the surface loops mediate some of OmpA functions, such as bacterial conjugation, and perhaps, inadvertently, contribute to the binding sites for phages and colicins. The pore formed by the β-barrel provides a channel for hydrophilic compounds (such as nutrients and wastes) with a molecular weigh cut-off of about 600 daltons.

Using OmpA as an expression system, several passenger proteins were fused to OmpA and hybrid proteins were displayed on the cell surface of *E. coli* (Francisco et al., 1992; Francisco et al., 1993a; Francisco et al., 1993b; Georgiou et al., 1996). Malarial antigens carried by OmpA in *Salmonella typhimurium* administered orally into mice induced serum antibody responses (Schorr et al., 1991). An Lpp-OmpA system was used to express HIV antigens. When orally administered, *Salmonella* SL3261 (aroA) harboring the recombinant plasmid elicited a fecal IgA responses specific to HIV reverse transcriptase, as well as a reverse transcriptase-specific helper T cell response in mice (Burnett et al., 2000). Additionally, tests with the synthetic peptide showed a selective cytotoxic CD8 T cell response (Burnett et al., 2000).

b. Presentation of Antigens on the Surface of Bacteria

It is known that heterologous antigens from viruses, bacteria, parasites may be efficiently displayed on the surface of either *E.coli* or attenuated *Salmonella typhimurium.* This technique may play a significant role in new vaccine design for the following reasons: (1) polypeptide antigens exposed to the outside surface of the cell may be more easily recognized by the immune system, and (2) bacterial outer membrane proteins, lipopolysaccharides (LPS), and secreted toxins are strongly immunogenic and have been exploited to develop a number of non-recombinant vaccines. Therefore, cell surface components may mediate an immuno-adjuvant effect to surface displayed heterologous antigens. Proteins of *Listeria monocytogenes* secreted from *Salmonella* using a hemolysin A-derived vector had greater protective efficacy than intracellularly expressed antigens (Hess et al., 1996). This result supported the hypothesis that extracellularly accessible antigens are better recognized than antigens localized in the interior of the bacterial cell. The presentation of heterologous proteins on live bacterial cells may also be a useful tool in other areas, such as microbiology, molecular biology and biotechnology. For example, random peptide libraries expressed on the surface of bacteria may be screened for ligands and antigens (Smith et al., 1997). Enzymes may also be immobilized by expressing them on the bacterial surface (Francisco et al., 1992).

c. β-turns

Three dimensional structure of a protein usually consists of α helices, β-strands, loops and other undefined structural features. Loops connecting neighbor antiparallel β-strands are called β-turns (Chou and Fasman, 1977; Smith and Pease, 1980). The classic definition of β-turns is "continuous four residues,

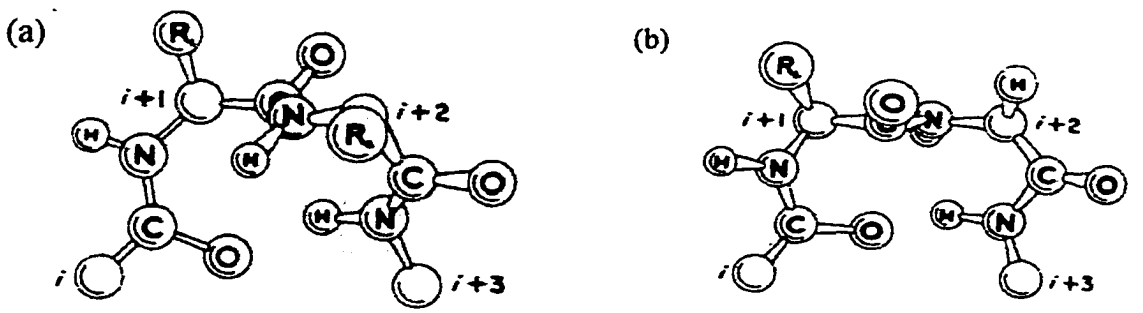


Figure 1.7 Type I β-turns. Two classical types of β-turns are shown. (a) type I; (b) type II. R is side chain.

where the interatomic distance of C_i^{α} and C^{α}_{i+3} is less than 7Å, but the substructure is not part of an α-helix (Chou and Fasman, 1977; Smith and Pease, 1980) (Figure 1.7). Based on the torsion angles, β-turns are classified into nine types (Wilmot and Thornton, 1988). Type I and type II occur most frequently than any other types (Perczel A., et al., 1995). β-turns are common feature in protein structure. About 25 % residues in all proteins are in β-turns (Shepherd et al.,

1999). Residues in β-turns are more likely hydrophilic because β-turns mostly occur near β-strands, at the solvent-exposed surface (Perczel et al., 1996; Shepherd et al., 1999). Wilmot and Thornton (1988) analyzed the structure of 59 proteins and found 735 β-turns. They discovered that almost half the turns did not fit in any of classical β-turn types. Therefore, there are many structural variations that may allow a polypeptide chain to reverse its direction. From their analysis, they concluded that it was reasonable to consider only four residues at a time when developing a method to predict β-turns. However, β-turns are not isolated from each other, instead, they share one or more residues with each other to form multiple β-turns (Hutchinson and Thornton, 1994).

β-turns not only occur in a protein structure, they also occur in liner peptides to form β-hairpin structure (Blanco et al., 1994; de Alba et al., 1995; Ramirez-Alvarado et al., 1996). De Alba et al. (1999) synthesized several pentadecapeptides with variations in turn sequence and length of hairpin structure. They used ^{1}H NMR to analyze the conformation of these peptides. They found that turn sequence was more important than side-chain interactions in determining the type of β-hairpin. They concluded that turns dictated paring of amino acids in a hairpin structure and the orientation of side chains. β turns also formed in a cyclic pentapeptide (Perczel, et al., 1996).

d. Optimization of Heterologous Antigen Displaying on Cell Surface

In many applications of surface display of proteins or peptides on the bacterial surface, it is desirable that the displayed proteins or peptides be maximally exposed to the extracellular environment. Evidence exists that the degree of exposition of a foreign epitope at the cell's surface correlates with the immunogenicity of the epitope, when live bacteria are used as immunogens.

The orientation of a protein or peptide antigen on the cell surface may depend on many factors, such as the structure of the carrier protein, the location of the fusion heterologous protein or peptide in the carrier protein, the amino acid composition of the junction regions that flank the inserted protein or peptide and the structure of the fusing protein or peptide itself. Under certain circumstances, the factors affecting the orientation of a passenger protein or epitope may be optimized. After a carrier protein and a passenger epitope are defined, and the fusion site chosen, the orientation of the passenger protein or epitope may be influenced by the flanking region sequences because β-turns may form in these regions. Optimization of the exposure of a foreign epitope can be achieved through creating the possibility of forming various β-turns in flanking regions.

The factors that influence the immunogenicity of a foreign epitope have not been well defined, even though the immunogenicity of a foreign epitope in its native environment is a decisive factor in regard to vaccination. For a heterologous epitope displayed on the bacterial surface, the immunogenicity of

the host bacterial strain and the carrier protein, the structure of carrier protein, and the location of the insertion are all important considerations. In this research, I focus on one of the possible factors, exposition of the foreign epitope on cell surface. My ultimate goal is to investigate how the immunogenicity of a foreign epitope is affected by its exposition, by virtue of the flanking region amino acid sequences. I expected that optimization or the changing of flanking region amino acid sequences would not have any effect on the cell mediated immune response. The cell mediated immune response to passenger epitopes certainly depends on the host strain and the epitope itself. There may be other factors which could influence the cellular response. We are confident that optimization of a passenger epitope has minimal influence on the cellular immunity of the passenger epitope. Therefore, we are not concerned with the cell mediated immune response in our research. LamB and OmpA were chosen as the carriers for passenger epitopes. Three passenger epitopes were used in our study: V3 loop (residues from 293 to 334) from human immunodeficiency virus type-1 (HIV-1); TOP epitope, a part of the V3 loop (residues from 309 to 320); and the streptococcal type 5 M protein epitope, Strep, corresponding to the first 15 amino acids of the protein from *Streptococcus pyogenes*. The envelope protein gp120 of HIV-1 involves the viral membrane fusion with human $CD4^+$ T cells. The third variable loop (V3) of gp120 is the most antigenic determinant involved in the neutralization of the laboratory adopted virus strains (Rusche et al., 1988; Backstrom et al., 1994;

Ahlers et al, 1997; Parren et al., 1998). The Strep epitope was immunogenic when fused to carrier proteins, and conferred protection in mouse experiments with attenuated *Salmonella* (Newton et al., 1991).

To change the flanking amino acids and vary the exposition of the epitope on the cell surface, we used the PCR amplification to randomly introduce mutations into the flanking regions. The expression of the epitope was analyzed by Western blot. The degree of exposition of the epitope was evaluated by fluorescent labeling of bacteria and measuring the florescence intensity of the cells by a cytofluorimetry. The mutants with different fluorescence intensities were transferred into *Salmonella* SL3262 and live bacteria displaying foreign epitope were administered into mice intraperitoneally. The humoral immune response was analyzed by enzyme linked immune assay (ELISA) and Western blot.

CHAPTER II

EXPRIMENTAL PROCEDURES

1. Bacterial Strains and Plasmids

1.1 Bacterial Strains

All *E.coli* strains are K-12 derivatives. *E. coli* pop6510 (*thr leu tonB thi lacY1 recA dex5 metA supE*) was used as a host for the plasmids used for epitope coupling with LamB. JM101 (*supE thi Δ(lac-proAB)F' [traD36 proAB$^+$ lacI^q lacZ ΔM15*), and UH203 (*lac supF ompA recA proA* or B *rpsL F' lacI^Q lacZ ΔM15 proAB+*) were used as the host for the recombinant plasmids involving in epitope insertion into OmpA. *E.coli* JM101, CJ236(*lac supF ompA recA proA or B rpsL F' lacI^q lacZ ΔM15*) and bacteriophage M13 were used for the site-directed mutagenesis. *Salmonella typhimurium* strain SL3261 [*aroA his*, (Hoiseth and Stocker, 1981)] was used as the host for the recombinant plasmids and subsequent mice immunization.

1.2 Plasmids and Oligonucleotides

A. <u>Plasmids and Oligonucleotides used for Constructing LamB-Foreign Epitope Hybrids</u>

Plasmid pAC1 (Boulain et al., 1986) is a pBR322 derivative which contains wild type *lamb* under the *ptac* promoter. Plasmid pDV3 was constructed from pAC1 by deleting the nucleotides specifying the hypervariable, top portion of loop 9 of LamB and replaced by a single BamHI site. The oligonucleotide used for constructing pDV3 from pAC1 is as follows: 5'GAT AAC AAG CGA ACG CGG ATC CCT TCG GCA AAG CCG TT 3'. Plasmid pDVQ4, which contains nucleotides encoding six histidine residues and an XhoI site immediately downstream of BamHI restriction site, was constructed from pDV3 by using the following oligonucleotide: 5' GAG AAA TGG GGT TAC TCG GAT CCT CAT CAC CAT CAC CAT CAC CTC GAG AGC TTC GGT CGT GGC GAC AGC 3'. Plasmid pX1V3 was constructed from pDV3 by insertion of an oligonucleotide encoding the residues 293-334 of gp120, third variable loop (V3) from HIV-1 into the BamHI site of pDV3. The inserted nucleotides also encode three additional residues (SDP) immediately upstream of the V3 loop sequence and three additional residues (QDP) immediately downstream of the V3 loop sequence. pX1V3 was used as the template for randomly mutagenizing the V3 upstream and downstream flanking regions and amplification of the V3 fragment with the following primers: EXP right: 5' GTC GCC ACG ACC GAA GCT CTC GAG XXX XXX XXX TGC TTG TCT CAT ATT TCC TAT 3'), EXP left: 5' AAA TGG GGT TAC TCG GAT CCG GCG XXX XXX XXX ACA AGA CCC AAC AAC AAT ACA 3'. X stands for a random nucleotide.

B. Plasmids and Oligonucleotides Used for Constructing OmpA-Foreign Epitope Hybrids

Plasmid pRD87 (Freudl et al., 1985) contains *ompA* gene cloned into pUC8 (Vieira and Messing, 1982) by PstI and SmaI restriction sites. To perform site-directed mutagenesis, pRD87 was digested with two restriction enzymes, HindIII and EcoRI. The 1.3 kb EcoRI/HindIII fragment containing *ompA* gene was ligated into M13 vector (Messing, 1983; Messing and Vieira, 1992). To facilitate the cloning process, a restriction site for BamHI located in *ompA* gene was eliminated by site-directed mutagenesis using the following primer: 5' ACG CAG CTG AGC AAC TTG GAC CCG AAA GAC GGT TCC GTA GTT 3'. Oligonucleotide 5' CGT GCA GAC ACT AAA GAG GAT CCA CCC GGG CTC GAG AAA AAC CAC GAC ACC 3' was used to introduce BamHI and XhoI restriction sites into the region of *ompA* gene encoding the third external loop (L3), precisely after residue 108. The residues 109 to 113 in L3 were deleted. After the mutations were confirmed in M13 by sequencing, double stranded M13 DNA was purified and digested with HindIII and EcoRI. The segment containing *ompA* gene was ligated back to pUC18. The resulting plasmid was named pL3.

The following oligonucleotides were used to insert "TOP" epitope into pL3: Top1, 5' GAT CCG ATT CAA CGT GGT CCC GGG CGT GCT TTT GTT ACT ACC C 3' and Top2, 5' TCG AGG ATA GTA ACA AAA GCA CGC CCG GGA CCA CG TGA ATG G 3'.

"Strep" epitope was introduced into pL3 by using the following oligonucleotides: Strep1, 5' GAT CCG GCC GTG ACT AGG GGT ACC ATA AAT GAC CCG CAA AGA GCA AAA GAA C 3'; Strep2, 5' TCGAG TTC TTT TGC TCT TTG CGG GTC ATT TAT GGT ACC CCT AGT CAC GGC CG 3'.

Plasmid pL3Strep was constructed by the following method: 1.2 μmol of oligonucleotides Strep1 and Strep2 were mixed and the final volume of oligonucleotides was adjusted to 20 μl with distilled H_2O. To anneal the oligonucleotides, the mixture was incubated at 80°C for 5 minutes and allowed to slowly cool down to room temperature in a beaker containing 80°C water. The annealed Strep1 and Strep2 oligonucleotides were ligated back into pL3 vector using BamHI and XhoI restriction sites (see the illustration in Figure 2.1).

Plasmid pL3TOP was similarly constructed (see the illustration in Figure 2.2).

The following oligonucleotides were used to introduce mutations into the regions flanking TOP epitope in OmpA by PCR random mutagenesis (illustrated in Figure 2.3): Omp L3 TOP exp-right, 5' AAA GAG GAT CCG XXX XXX XXX ATT CAA CGT GGT 3'; Omp L3 TOP exp-left, 5' GTT TTT CTC GAG XXX XXX XXX GAT AGT AAC AAA 3'.

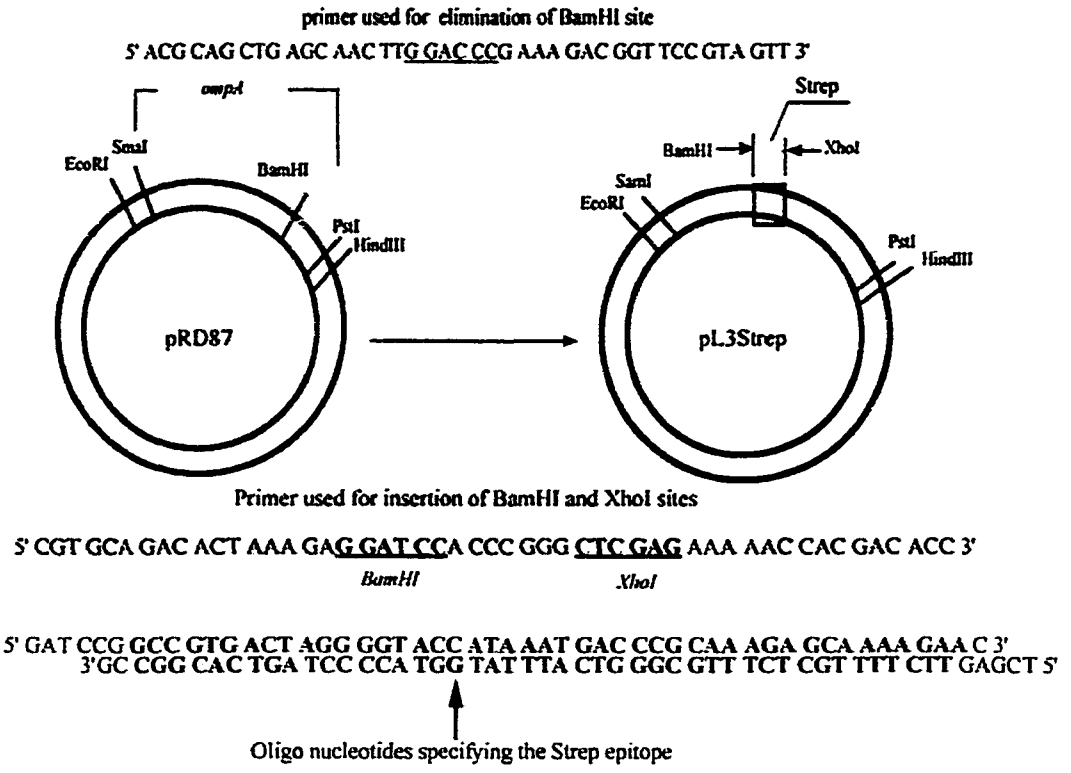


Figure 2.1 Construction of plasmid pL3Strep. pL3Strep was constructed from pRD87 by several steps. In the first step, a restriction site for BamHI on *ompA* gene was eliminated by site-directed mutagenesis. In the second step, restriction sites for BamHI and XhoI were introduced into ompA gene by site-directed mutagenesis. This new plasmid was named "pL3". In the third step, oligonucleotides specifying the "Strep" epitope were inserted into pL3 through BamHI and XhoI restriction sites.

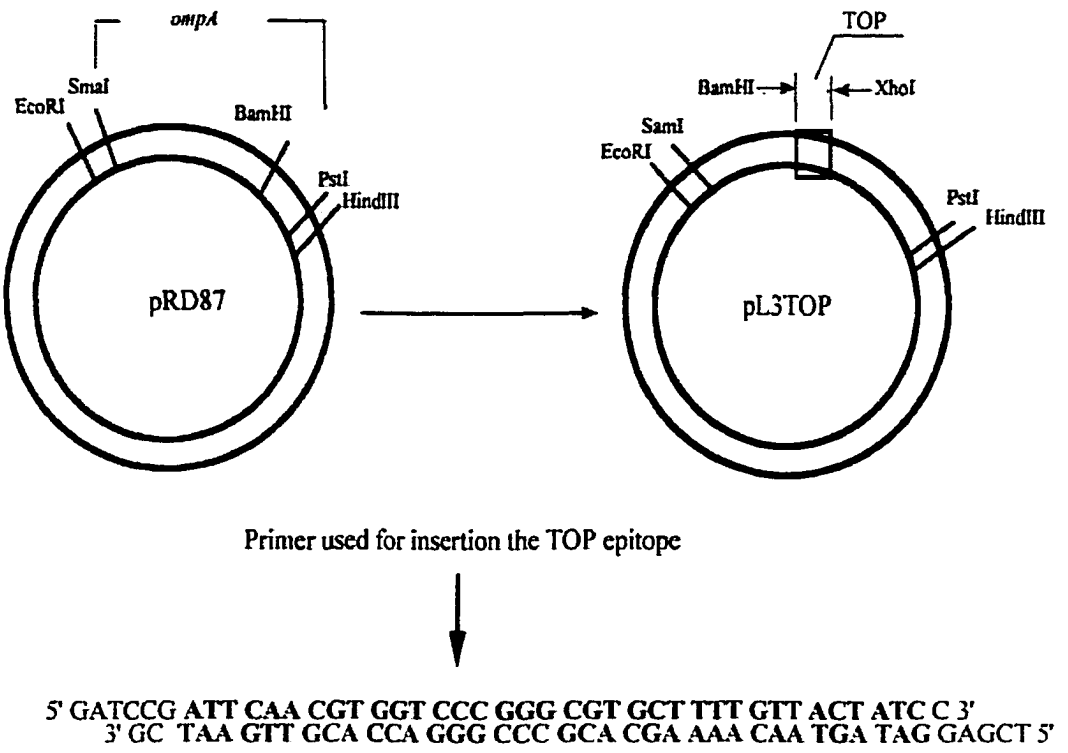
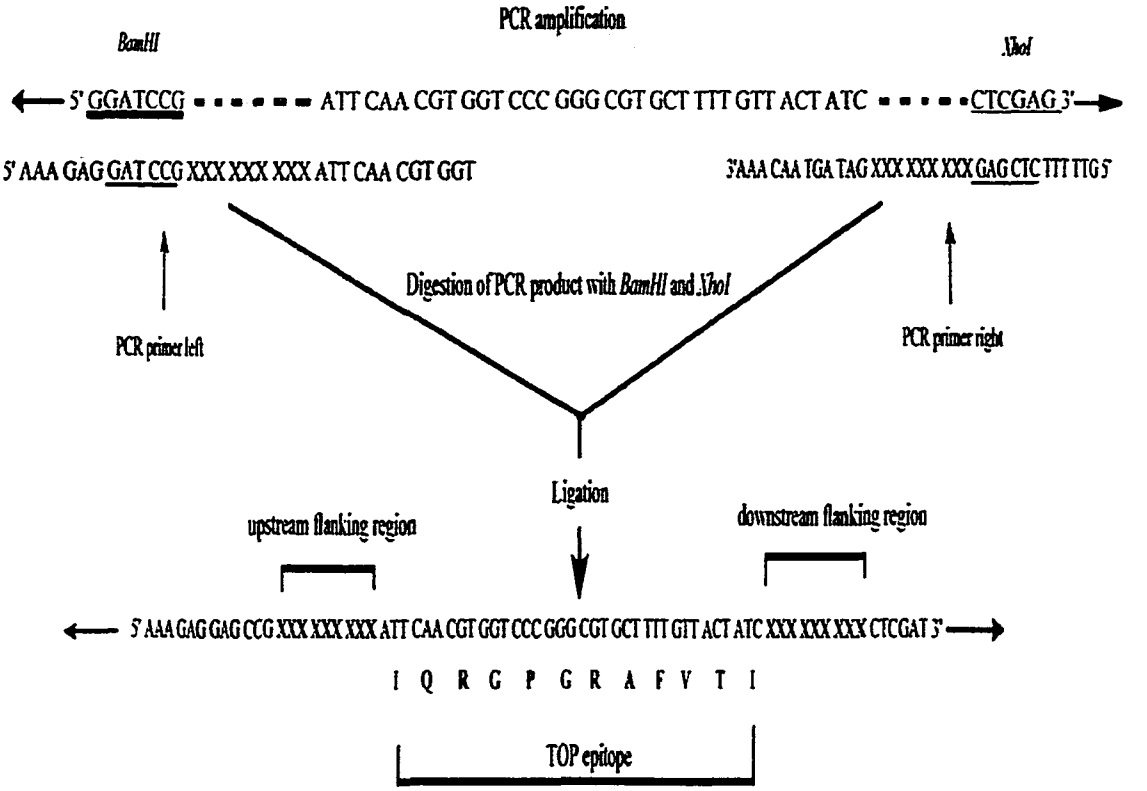


Figure 2.2 Constructing plasmid pL3TOP. Restriction sites for BamHI and XhoI were incorporated into *ompA* gene by site-directed mutagenesis. TOP epitope was inserted into *ompA* gene through BamHI and XhoI sites.



X stands for a random nucleotide

Figure 2.3 Strategy for generation of random sequences. Two oligonucleotides were used to incorporate random sequences into the regions flanking the TOP epitope by PCR mutagenesis. The PCR product was digested by BamHI and XhoI and ligated into pL3TOP plasmid.

The oligonucleotides used for randomly mutagenizing the regions flanking the Strep epitope in OmpA were: Omp L3 strep exp-left: 5' GTT TTT CTC GAG XXX XXX XXX TTC TTT TGC TCT 3'; Omp L3 strep exp-right: 5' AAA GAG GAT CCG XXX XXX XXX GCC GTG ACT AGG 3'.

2. Site-Directed Mutagenesis

2.1 Uracylation of M13 DNA

A replicative form of M13 DNA was electroporated into JM101 competent cells. After incubation for 15 minutes at 37°C, 50 µl of the electroporation mixture were mixed with 50 µl of overnight JM101 culture. The mixture was plated on a tryptone plate. The plate was incubated at 37°C overnight. A phage plaque was picked and transferred into an eppendorf tube containing 1 ml of 2x YT medium. The tube was placed into a 60°C water bath for 5 minutes to kill the bacteria, spun for 4 minutes in a table top centrifuge, and 50 µl of the supernatant containing phage particle were transferred into a 250 ml flask with 2 ml of overnight CJ236 culture, 50 ml of 2x YT medium, 50 µl of uridine (0.25 mg/ml). The culture was grown for 6 hours at 37°C. M13 particles and cells were separated by centrifugation at 10,000g. The supernatant was used to purify the template DNA for the site-directed mutagenesis.

2.2 Single Stranded DNA Template Purification

A phage plaque was picked and transferred into 5 ml LB media with 100 μl of overnight JM101 culture. The phages were separated from bacteria after 6 hours of incubation at 37^{0}C by centrifugation for 4 minutes in an eppendorf centrifuge tube at 10,000g. The supernatant containing phage particles was transferred into a new eppendorf tube and mixed with 200 μl of 20% polyethylene glycol-2.5M NaCl (J.T. Baker, NJ) by inverting the tube. The mixture was incubated at room temperature for 30 minutes to precipitate phage particles. After 15 minutes of centrifugation, the supernatant was discarded and phages were resuspended in 100 μl of 10 mM Tris-HCl, pH 7.6. To extract the single stranded M13 DNA, 100 μl of buffered phenol (USB, OH) were added into the tube. After vortexing vigorously, the mixture was centrifuged for 5 minutes at room temperature to separate the aqueous and organic phases. The aqueous phase containing single stranded M13 DNA was transferred into a new eppendorf tube and mixed with 50 μl phenol and 50 μl chloroform(Fisher, NJ). After mixing by vortexing, the mixture was centrifuged for 5 min. The aqueous phase was transferred into a new tube and DNA was precipitated by adding 5M NaCl (1μl for every 50 μl of aqueous phase), and two volumes of 100% ethanol(AAPER). After being mixed well, the mixture was incubated at -80^{0}C for 2 hours and followed by 25 minutes centrifugation. The pellet was washed with 200 μl of

75% ethanol and centrifuged. After discarding the supernatant, the pellet was dissolved in 30 μl of distilled H_20.

2.3 Phosphorylation of Primers

Customer-designed primers were purchased from Gibco. Twenty microliters of primer (10 pmol/μl) were mixed with 3 μl of 10X kinase buffer, 1.5 μl of ATP(Gibco), 5μl of distilled H_20 and 0.5 μl of polynucleotide kinase(Gibco). The mixture was kept at 37°C for 45 minutes and then placed into a 65°C water bath for 10 minutes to deactivate the kinase.

2.4 Hybridization of Template with Primers

Three microliters (200 ng) of single stranded, uracylated DNA were mixed with 1μl of phosphorylated oligonucleotides (6 pmol/μl), 1μl of 10x hybridization buffer (Bio-RAD), and 5 μl of dH_20. The hybridization mixture was incubated at 37°C for 2 minutes and then allowed to cool in a dish of 70°C water on the bench until the temperature of the water was below 30°C.

2.5 Mutagenesis Reactions

All reagents used in the mutagenesis reactions were from the Bio-RAD M13 mutagenesis kit. One microliter of the 10X synthesis buffer, 1μl of T4 DNA ligase (2-3 units), and 1μl of T7 DNA polymerase (1 unit) were add to 10 μl of

the hybridization mixture. The reaction mixture was incubated at 0^0C for 5 minutes, then 37^0C for 45 minutes. The reaction was stopped by adding 90 µl of stopping solution (10 mM Tris, 10 mM EDTA, pH 8.0). One microliter of the reaction solution was used to transform JM101 cells.

2.6 Double Stranded DNA Purification

Double stranded M13 DNA was purified from the cells infected with M13 phage by using Concert™ Rapid Plasmid Miniprep System (Gibco).

2.7 Sequencing

A. Preparation of Sequence Gel

21.8g of urea (Pharmacia), 6ml of 10X TBS(1M Tris, 0.82M boric acid, 1mM EDTA) , and 7.5 ml of gel solution (Amresco) were placed in a 100 ml cylinder and the final volume was adjusted to 60 ml with distilled water. After urea was completely dissolved, the solution was passed through a 0.45µm filter. Thirty µl of TEMED (Pharmacia) and 300 µl of newly prepared 10% ammonium persulphate were added to the solution. The solution was poured into a gel cast (Pharmacia) and allowed to polymerize for at least 2 hours at room temperature before use.

B. Sequencing Reactions

The sequencing reactions were done with Thermo Sequenase fluorescence labeled primer cycle sequencing kit (Pharmacia). Briefly, 10 μl of double stranded DNA, and 14 μl of H_2O were mixed with 2 μl of fluorescence dye labeled primers (2 pmol/μl). Six microliters of the mixture were transferred into 4 PCR tubes containing 2 μl of A, C, G and T reagent, respectively. Finally, the PCR tubes were placed on a thermal cycle machine (GeneAmp PCR System 9700, Perkin-Elmer) and subjected to the following conditions: 94^0C for 2 minutes, then 25 cycles of 55^0C for 30 seconds, 72^0C for 1 minutes, and 94^0C for 30 seconds. After the thermal cycles were over, 5 μl of loading dye were added into each tube and the solution was incubated at 95^0C for 3 minutes to denature DNA. Five microliters of the solution were loaded into each lane on the gel. The gel was run on the AFL express automatic sequencing machine.

2.8 Preparation of Ligation Vectors and Bands

A. Digestion of Plasmid

Fifty microliters of plasmid DNA purified as previously described were double-digested with 1 μl of BamHI, and 1 μl of XhoI, 6 μl of 10X BSA, 6 μl of 10X reaction buffer and 6 μl of H_2O. The digestion reaction went for 3 hours at 37^0C. The digestion was stopped by adding 7 μl of loading buffer (50% glycerol, trace amount of bromophenol blue) to the reaction mixture.

B. Polyacrylamide Gel

Five milliliters of TBE (0.09 M Tris, 0.09 M boric acid, 2 mM EDTA, pH 8.0) and 8.5 ml of 30% Acrylamide-0.8% bisacrylamide (Protogel) were mixed in a 100 ml cylinder and the final volume was adjusted to 50 ml with distilled water. To induce the polymerization, 94 µl of TEMED, 188 µl of newly prepared 10% ammonium persulphate were added into the gel mixture. The gel solution was poured into a gel cast with 3 mm spacers. Digestion mixture was loaded on the gel and run overnight at 65 V. The gel was soaked with 50 ml of the staining solution (0.5 µg/ml ethidium bromide in 1xTBE) for 30 minutes.

C. Elution of DNA from Acrylamide

The gel was placed on an UV box and the right size band was cut out from the gel and placed the inside of a dialysis tube with 12-14,000 daltons of molecular weight cutting off size (Spectrum) in 300 µl of TBE. The tube was placed in TBE and subjected to electrophoresis for 3 hours at 70 V. DNA adhered to the tube was released into the solution by gently massaging the dialysis tube. The solution was passed through a P1000 pipette tip containing a little glass wool. The dialysis tube and the pipette tip were washed twice with 100 µl of TBS and both aliquots were combined in a 1.5 ml eppendorf tube. To precipitate DNA, 1µl of dextran (10mg/ml), 1 µl of 5M NaCl for every 50 µl of solution and 2 volume of 100 % ethanol were added into the solution. After the tube was

placed at -80°C for 2 hours it was centrifuged at 10,000g for 20 minutes at 4°C. The supernatant was discarded and the pellet was washed with 200 µl of 75% ethanol. The tube was centrifuged again for 15 minutes at 4°C. After discarding the supernatant, the tube was left on the bench with the lid open for about 10 minutes to get rid of the residues of ethanol. Finally, the pellet was dissolved in 20 µl of dH_2O.

2.9 Ligation

Three microliters of vectors and bands were loaded on 1% agarose gel. The amount of vectors and bands used in the ligation reaction were adjusted to about 50 ng of vectors and 10-fold excess of ends in the final 15 µl of the ligation reaction, which contained 1µl of T4 ligase (Gibco) and 3 µl of 5X T4 ligase buffer. The ligation reaction was kept at 16°C overnight. The ligation mixture was precipitated by adding 35 µl of dH_2O, 1 µl of dextran (10 mg/ml), 1 µl of 5M NaCl and 100 µl of 100% ethanol. In the end, the pellet was dissolved in 20 µl of dH_2O. Four microliters of the solution were used to transform cells.

3. Fluorescence Staining of Cells

Cells expressing LamB:V3 or OmpA:TOP hybrid protein were grown in 5 mL LB medium with 50 µl of ampicillin (10mg/ml) overnight at 37°C. 250 µl of

the overnight culture were subcultured to a flask containing 25 mL LB medium, and 250 μl of ampicillin (10mg/ml). The cells were allowed to grow until optical density OD_{600} reached about 0.5. At that point, Isopropyl-β-D-thiogalacoside (IPTG) was added to the cell culture to a final concentration of $5*10^{-5}$ M to induce the expression of hybrid proteins. The cells were harvested when optical density of the culture reached 1.0. 10^8 cells were harvested in 1.5 mL eppendoff tubes by centrifuging for 1 minute at 14000rpm. The supernatant was discarded and cells were resuspended in 50 μl mouse anti-gp120 ascites fluid [1:100 dilution in TBS-1% gelatin (TBSG)] and incubated for 45 minutes at room temperature. After adding 1 mL of TBS, cells were centrifuged and resuspended in 50 μl of fluorescein (FITC) conjugated goat anti-mouse IgG (1:50 dilution, in TBSG). After 40 minutes incubation, 1 mL of TBS was added to the cells. The cells were pelleted and resuspended in 0.5 mL of TBS, 0.5 mL of TBSG. The mean fluorescence intensity of FITC-labeled cells was measured with a Coulter EPICS ELITE flow cytometry.

4. Western blot

4.1 Analysis of the Expression of Hybrid Proteins

E. coli or *S. typhimurium* cells expressing LamB:V3 or OmpA:TOP hybrid or OmpA:Strep proteins were grown and induced as previously described. $2.5*10^8$ cells were resuspended in 100 μl of the sample buffer (0.17 M Tris-HCl,

pH 6.8, 33% glycerol, 3.3% SDS, 0.4 M 2-mercaptoethanol and trace amount of bromophenol blue tracking dye), boiled for 3 minutes and centrifuged for 3 minutes at 14000 rpm. Twenty five microliters of the solution were applied to 10% SDS-polyacrylamide gel. The gel was run under 30 mA until the dye front was out of the gel. The proteins were transferred to a piece of nitrocellulose paper to immobilize proteins. The nitrocellulose paper was incubated with TBSG for 1 hour at room temperature and followed by incubation with mice ascitic fluid against gp120 (1:100 dilution, in TBSG) for 2 hours. The nitrocellulose paper was washed five times with washing buffer (TBS, 0.05% tween-20) in a period of 30 minutes. After the wash, the paper was incubated with alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (1:1000 dilution in TBSG) for 1.5 hours. After another five times of washing, expression of hybrid proteins was detected by incubating the nitrocellulose paper with 17 mg bromochloroindolylphosphate (BCIP), 33 mg nitro blue tetrazolium (NBT) in 100 mL of the substrate buffer (0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5) for 5-10 minutes. The reaction was stopped by washing with water.

4.2 Analysis of Mouse Sera

Outer membranes were prepared from *E. coli* cells that expressed wild type LamB, LamB:V3 hybrid, OmpA and OmpA:TOP. The outer membrane preparation was loaded on 10% SDS gel and proteins were transferred to

nitrocellulose paper. The nitrocellulose paper was blocked by incubation with TBSG for 1 hour. In order to analyze the reactivity of various mice sera with LamB, LamB:V3, OmpA and OmpA:TOP, the nitrocellulose paper was cut into strips of approximately 0.5 mm wide. Each strip was placed into a tube containing mouse sera with a certain dilution in TBSG and incubated for 2 hours at room temperature, gently shaking. After the incubation, each strip was taken out and placed into a new tube containing the washing buffer and washed 5 times. Then, the strips were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (1:1000 dilution in TBSG) for 1.5 hours, washed and then developed.

5. PCR Amplification and Cloning Strategy

5.1 LamB:V3 Constructs

A Perkin-Elmer 2000 PCR machine was used to perform all the amplifications. The template DNA was purified from *E.coli* cells carrying pX1V3 plasmid. Ten ng of pX1V3 DNA, 100 pmol of each primer, 100 μM dNTPs and 2.5 mM $MgCl_2$ were mixed. *Taq* polymerase was added to the amplification mixture when the temperature of the mixture reached 95 ^{0}C. First, 4 circles were performed at 95 ^{0}C for 30 seconds, 60 ^{0}C for 1 minute and followed by 1 minute at 72 ^{0}C. Afterwards, 30 cycles of 30 seconds each at 95 ^{0}C , 30 seconds at 65 ^{0}C and 1 minute at 72 ^{0}C were carried. The reaction mixture was loaded on a

polyacrylamide gel. The band containing the amplified product was cut out from the gel and placed into a dialysis tube containing 200 μl of TBS. To elute DNA from the gel, the dialysis tube was subjected to electrophoresis for several hours at 75 V. The product was concentrated by ethanol precipitation and digested by BamHI and XhoI. The digested DNA was purified by polyacrylamide gel. pDVQ4, the vector for the PCR product, was digested with BamHI and XhoI, purified on an acrylamide gel. The PCR product and vector were ligated together at 16^{0}C overnight. The ligation mixtures were introduced into the host strain pop6510 by electroporation. Colonies were isolated in ampicillin-containing LB plates and were inoculated into 5 mL of LB with ampicillin (100 μg/ml) and grown at 37^{0}C overnight. Plasmid DNA was purified by using the Concert™ Rapid Plasmid Miniprep System (GIBCO, and analyzed by restriction digestion with FspI and BamHI, and by sequencing. The expression of hybrid protein was analyzed by Western blot.

5.2 OmpA:TOP Constructs

The template DNA for performing PCR mutagenesis was purified from JM101 carrying pL3TOP plasmid, using the Concert™ Rapid Plasmid Miniprep System (GIBCO). The PCR mutagenesis reaction was done in a Gene Amp PCR System 9700. The reaction mixture consisted of the following: 200 μM dNTP's, 20 ng of template DNA, 200 ng of the left PCR primer, 200 ng of the right PCR

primer, 10 μl of 10X pfu buffer, and 2.5 units of pfu DNA polymerase (Stratagene, CA), in a 100 μl of final volume. The amplification process consisted of 35 cycles of 94°C for 30', 50°C for 30' and 72°C for 30'. The amplified PCR product (2 PCR reactions) was precipitated with 100% ethanol and resuspended in 62 μl of H_2O. To digest the PCR product, 62 μl of the PCR product were mixed with 1μl of BamHI, 1μl of XhoI, 8 μl of 10X reaction buffer, and 8 μl of 10X BSA. The digestion reaction was allowed to continue for 3 hours at 37°C. The digested PCR product was purified on an acrylamide gel.

The purified PCR band was ligated into the pL3TOP vector and electroporated into JM101 competent cells. The cells were plated on LB-ampicillin(100 μg/ml) plates and incubated at 37°C overnight. Ampicillin-resistant bacterial colonies were picked and inoculated into 5 ml of LB with ampicillin (100 μg/ml). The bacteria were incubated at 37°C for 14-18 hours and plasmid DNA was purified by the method described in section 2.6. The expression of hybrid proteins was analyzed by Western blot. Finally, the mutagenized flanking regions sequences were determined by sequencing.

6. Immunization of Mice

6.1 Immunization of Mice With Bacteria Expressing LamB:V3 Hybrid

S. typhimurium strain (SL3261 derivatives) harboring LamB:V3 hybrids was grown in LB with ampicillin (100 μg/ml). The expression of chimeric

protein was induced with IPTG. After two hours of the induction with IPTG, the culture (OD_{600}= 1.0) was diluted with sterile saline to a final concentration of $2.5x10^7$ cells/ml for mice immunization. Each mouse was immunized intraperitoneally with 200 µl of the diluted culture on days 1 and 21. The sera were collected on day 28.

E. coli (pop6510 derivatives) harboring LamB:V3 hybrids were grown and induced with IPTG. Cells were harvested by centrifugation. Cell pellets were resuspended in sterile saline and incubated in a 56^0 C water bath for 20 minutes to inactivate the bacteria. Finally, the concentration of bacteria was adjusted to $5x10^7$ cells/ml with sterile saline. Each mouse was injected intraperitoneally on days 1 and 21 with 200 µl of the resulting immunogen. Blood was collected on day 28. The blood from mice was rigorously vortexed and centrifuged. The sera was kept at -20°C.

6.2 Immunization of Mice with Bacteria Expressing OmpA:TOP Hybrid

S. typhimurium strains (SL3261 derivatives) were transformed with the plasmid pL3TOP series. Each pL3TOP plasmid of the pL3TOP series encoded an OmpA:TOP hybrid protein with a unique flanking sequence. Transformed *S. typhimurium* was grown in LB media with ampicillin (20 µg/ml) for 10 hours. The cells were diluted with sterile saline to a final concentration of $2.5x10^7$/mL.

$5x10^6$ cells were intraperitoneally administered into each mouse on days 1 and 21. Blood from immunized mice was collected on day 28 by tail bleeding.

7. ELISA Analysis of Mouse Sera

Purified gp120 (Protein Science) was diluted to 0.5 μg/ml in 50 mM $NaHCO_3$, pH 9.6, and 100 μl aliquots were added to each well of a 96 well plate (IMMULON). The plates were covered and kept at 4°C overnight. The plates were washed three times in a plate washer using ELISA washing buffer (0.14 M NaCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4, 3 mM KCl, 0.05% Tween20, pH 9.8) and blocked by addition of 235 μl of 2% BSA(USB), 1% Casein (USB) in 50mM NaHCO3, pH 9.6 to each well. After 90 minutes incubation in 37°C, plates were washed three times again with ELISA washing buffer. Five microliters of diluted mouse sera in the blocking buffer were transferred to each well of the plates. After 2 hours incubation at room temperature, the plates were washed three times with ELISA washing buffer. Then the plates were incubated with goat anti-mouse IgG (sigma) phosphatase conjugate for 1 hour at room temperature. The reaction was stopped by the addition of 100 μl of 1 M NaOH to each well. The absorbance at 405 nm was taken on an ELISA-plate reader (Titertek Multiskan Mcc).

8. Outer Membrane Preparation

8.1 Preparation of Outer Membrane Suspension

Cells were grown in 1.5 L of LB media to OD_{600} =.5 and induced by adding IPTG to 10^{-5}M. After 2 hours induction, cells were harvested by centrifugation at 4°C in a pre-cooled rotor, 5000xg, for 20 minutes. The pellet was resuspended in 200 ml of 10 mM HEPES, pH 7.4. After the centrifugation, the pellet was resuspended in 27 ml of 10 mM HEPES, pH 7.4, in a 30 ml Corex tube. To lyse cells, 1mg of DNase (Sigma) and 1mg of RNase (Sigma) were added to the bacterial suspension and the suspension was passed twice through the French Press, with the pressure set to 14000lb/in^2. The cell lysate was centrifuged at 3000xg for 5 minutes at 4°C to remove the cell debris. The supernatant was transferred into an ultra centrifuge tube and spun at 150,000xg for 1 hour (rotor 70 Ti, 45k rpm) at 4°C. The pellet was resuspended in 7.5 ml (final volume) of HEPES, pH 7.4, with syringe and needles. The membrane suspension was passed through # 18 and #23 needles consecutively. Finally, the membrane suspension was laid on the top of two sucrose gradients and spun in the SW27 rotor at 26,000 rpm for 16 hours, at 4°C.

8.2 Preparation of Sucrose Gradient

To prepare sucrose gradient for OM preparation, 2.02 M, 1.44 M and .77 M sucrose solutions were prepared in 10 mM HEPES, pH 7.4. 4.6 ml of 2.02 M

sucrose was transferred into a SW 27 ultra centrifuge tube. Sixteen milliliters of 1.44 M sucrose was laid gently on top of the 2.02 M sucrose solution. On the top of the 1.44 M sucrose solution, 12.4 ml of 0.77 M sucrose solution was gently laid. Care should be taken when laying the sucrose solutions to avoid the mixing of solutions with different concentrations.

9. Raising Polyclonal Antibodies in Rabbit

One hundred microgram of purified M5 protein (a generous gift from Dr. Cunningham, OU Health Science Center) in 200 μl of PBS, pH 7.4, were mixed with 200 μl of Freund's Adjuvant (complete, Sigma) and emulsified with a 1 mL syringe. A four month old New Zealand rabbit was injected subcutaneously with the emulsion. Two more injections were carried out in an one week interval with the exact same method except that Freund's incomplete adjuvant was used after the first injection. In the 4^{th} and 5^{th} weeks, the rabbit was injected with a mixture of 10 μg of M5 protein in 100 μl of saline-0.02% aluminum hydroxide gel adjuvant (Superfos Biosector) to boost the immune response. The blood was collected from the rabbit's ears on the 6^{th} week.

10. OmpA purification

Cells were grown in LB media overnight and subcultured into 15 L of LB by 1%. The cells were harvested when OD_{600} reached about 1.0. Cells were lysed

by two passages through the French Press. The total membranes were obtained by centrifuging cell lysate at 150,000xg for 1 hour. The supernatant was discarded and the membranes were washed twice by resuspending the pellet in 50 mL of 50 mM Tris, 10 mM $MgCl_2$, 10 mM benzamidine, pH 8.0 and centrifuging at 30000 RPM (Type 35 rotor) for 45 minutes. To separate the inner membranes from the outer membranes, the pellet was resuspended in 50 mL of 2% Triton X-100, 100 mM Tris, 10 mM benzamidine, pH 8.0. After 20 minutes shaking at room temperature, the suspension was centrifuged. The supernatant contained the inner membranes. Extraction of the inner membrane was repeated one more time and the outer membrane-containing pellet was washed by resuspending it in 50 ml of 10 mM Tris, 5 mM EDTA, 10 mM Benzamidine and centrifuging. Finally, to extract outer membrane proteins, the pellet was resuspended in 50 mL of 2% Triton X-100, 10 mM Tris, 5 mM EDTA, 10 mM benzamidine and kept shaking at room temperature for 30 minutes, followed by centrifugation for 40 minutes at 30000 RPM. The supernatant contained mostly outer membrane proteins. The supernatant was loaded on a DE-52 ion exchange column, and washing with 2% triton X-100, 10 mM Tris, 5 mM EDTA, pH 7.4. Proteins were eluted from the column by running a salt gradient, 0.0-.25 M NaCl. 200 µl of each fraction was taken and proteins were precipitated by adding 400 µl 100% ethanol. After being kept at -20^{0}C for 2 hours, the mixture was centrifuged and the pellet was

resuspended in 60 μl of the sample buffer. 20 μl of the solution was loaded on 10% SDS-PAGE gel. The fractions which contained OmpA were consolidated.

Chapter III. RESULTS

1. Randomization of Regions Flanking Vaccine Epitopes

In order to investigate the relationship between the exposure of foreign epitopes on the cell surface and the humoral immune response, we introduced the third variable loop from HIV-1 (V3 loop, 33 residues) into the 9th external loop of *E. coli* outer membrane protein LamB. We also took a part of the V3 loop (TOP epitope, 12 residues) and the Strep epitope from the M5 protein of *Streptococcus pyogenes*, and fused them into the third external loop of another *E. coli* outer membrane protein, OmpA. At the immediate upstream and downstream junctions of the inserted epitope and the carrier proteins, we inserted 3 amino acid residues and randomly mutagenized them by PCR mutagenesis. Our hypothesis was that the flanking amino acids influence the orientation and projection of the foreign epitopes on the bacterial surface, because bends and turns in a polypeptide derive from the juxtaposition of 3-5 sequential amino acids (Wilmot and Thornton, 1988, Wilmot and Thornton 1990; Hutchinson and Thornton, 1994; Shepherd et al.,

1999). The variations of amino acids in the flanking regions generated enormous amino acid combinations for various β-turn formation. We used synthetic oligonucleotides specifying the V3 loop, the TOP and the Strep epitope to insert corresponding epitopes into LamB and OmpA, respectively. Two populations of PCR primers for each carrier proteins were used to randomize the 9 nucleotides at the junction regions, and to incorporate the restriction sites for BamHI and XhoI, which facilitated the digestion and ligation of the PCR product into the expression vectors, pDVQ4, PL3TOP and pL3Strep. By this method, we created several libraries of hybrid proteins, LamB:V3, OmpA:TOP and OmpA:Strep. Each hybrid protein had unique junction region sequences, which, in turn projected and oriented the passenger epitope in different ways on the bacterial surface. For LamB:V3 hybrid, we isolated and sequenced 21 clones to verify the efficiency of the mutagenesis by PCR (Table 3.1). For the OmpA:TOP hybrid, we isolated and sequenced 23 clones (Table 3.2), and for the OmpA:Strep hybrid, we isolated and sequenced 17 clones (Table 3.3). The results confirmed that each junction site contained 9 different nucleotides, which encoded flanking sequences of varied composition. The PCR randomization was effective: among the twenty one clones that we analyzed for LamB:V3 hybrid, that contained 360 PCR-introduced bases, we found codons for all of the twenty common amino acids, and two stop codons. The junction DNA most frequently encoded A, T, P, S. I and R, and least frequently produced V, E, M and W.

Table 3.1. The library of LamB:V3 chimeric proteins originated by PCR-mutagenesis

Clones	Percent	Upstream junction	downstream junction	Mean Fluorescence
pDV3	94.0	N/A	N/A	0.1
pX1V3	68.9	Ser Asp Pro	Gln Asp Pro	5.4
1	96.4	Pro Ile Asp	Cys Gln Asn	6.8
2	95.8	Arg His Ser	Pro Gly Ala	6.6
3	95.0	Trp Asn Thr	Lys His Lys	5.1
5	94.9	Ser Tyr Ile	Gln Ile Arg	5.4
6	95.7	Ile Phe Ser	His Thr Ala	7.0
7	95.3	Thr Ser Ile	Leu Ala Lys	6.9
8	82.4	Cys Ser Gly	Ala Phe Asn	3.2
9	94.7	Arg Asp Pro	Ala Thr Leu	7.1
10	51.6	Ser Ser Ala	Gln Ala His	6.3
11	97.3	Thr Pro Ala	Thr Pro Ala	13.8
12	97.5	Pro Asp Pro	Ala Leu Thr	15.0
13	89.4	Ile Leu Cyc	Ile Pro Asn	7.9
14	90.0	Cys Thr Val	Gln (Stop) Tyr	5.7
15		Thr (stop)Ala	Ile Ser Pro	
17	96.3	Arg Phe Arg	Arg His Asn	10.8
18	94.7	Pro Arg Tyr	Thr Ile Ser	9.0
20	59.3	Ser Glu Ala	Thr Ala Asp	2.5
21	79.5	Met Leu Phe	Thr Glu Asp	2.1
22	97.3	Arg Phe Pro	Thr Gly Ala	19.8
23	94.4	Arg Val Ser	Cys Ala His	13.9
32		Ala Pro Ala	Ser Gln Leu	

(1) pDV3 encodes a LamB mutant, in which loop 9 (L9) was deleted. pX1V3 is the starting vector for PCR mutagenesis, in which L9 was replaced with the V3 loop of gp120 from HIV-1. Clones 1-23, 32 were generated by PCR mutagenesis.

Table 3.2. The library of OmpA:TOP chimeric proteins generated by PCR mutagenesis

clone	upstream junction	down stream junction
1	Cys Ile Cys	Gln Ser Lys
2	Gly VaL Thr	Aln Pro Glu
3	Asp Asp Ile	Gln Pro Leu
4	Gln Leu Leu	Ala Ser Val
5	Cys Thr Gly	Thr Glu Lys
6	Ala Gln Tyr	Arg Gln Ser
7	Cys Thr Cys	Thr His Ser
8	Gly Arg Phe	Pro Cys Cys
9	Cys Arg Leu	Thr His Ser
10	Met Ala Ser	Gln Pro Leu
11	Gly Thr Gly	Thr Glu Lys
12	Ile Ala Gly	Gly Leu Gly
13	Val Ser Ser	Pro Arg Asn
14	Ile Thr Leu	Leu Leu Val
15	Trp Glu Tyr	Ser Pro Arg

Table 3-3. The library of OmpA:Strep chimeric proteins generated by PCR mutagenesis.

clone	upstream	downstream
1	Ile Ser Arg	Gln Leu Gln
2	Glu Asp Ser	Thr Pro Cys
3	Asp Ser Glu	His Arg Pro
4	Phe Phe Gln	Cys Gly Tyr
5	Gly Ser Cys	Leu Gln Ala
6	Leu Ala Val	Val Gln Ile
7	Gly Leu Pro	Ala Pro Ser
8	Thr Pro Ser	Gln Ser Thr
9	Val Trp Val	Pro Pro Ala
10	Gly Arg Ser	Leu Gln Pro
11	Ser Tyr Tyr	Pro stop Thr
12	Ile Tyr Ser	Pro Ala Pro
13	Gly Glu Thr	Val His Arg
14	Thr Leu Cys	Arg Thr Tyr
15	Cys Ser Thr	Pro Glu Ser
16	Val Leu Thr	Ser Ile (?)
17	Met Val Gln	Ile His Thr

For the OmpA:TOP chimeric protein library, we sequenced 23 clones and found codons for all twenty amino acids, although the frequency of codon occurrence for twenty amino acids varied from 1% for Y, W, N, M, and F to 11% for L, 10% and for S. We did not find stop codons among 23 clones, which was expected, because these clones were previously screened for expression of OmpA:TOP fusion proteins. Clones that showed faster mobility on SDS-PAGE were discarded. Although a slight mobility difference among the members of the chimeric protein library existed, clones with a stop codon in the junction regions migrated though SDS-PAGE much faster than clones without a stop codon.

We sequenced 17 clones from the OmpA:Strep chimeric protein library and no codons for lysine and asparagine were found (Table 3.3). Most frequently occurring codons are for T, S, P; and less occurring codons are for W, M, and F.

2. Expression of Hybrid Proteins

We selected LamB and OmpA, *E. coli* outer membrane proteins, as carriers for V3, TOP, and M5 epitopes because their structures had been solved, which made it easier to choose sites for inserting the passenger epitope in order to express them on the cell surface. With the information regarding the structure of proteins, we exactly controlled where fusion took place. We may fuse a passenger epitope in surface loops, transmembrane domains or even periplasmic loops. In

our study, we chose outer membrane proteins as carriers and their surface loops as the fusion sites for passenger epitopes for obvious reason: our goal was to express these epitopes on bacterial surface. The questions we asked in choosing the carrier proteins and the fusion sites for passenger epitopes were the following: 1. Does insertion of a foreign epitope interfere with the assembly of the carrier protein in the outer membrane? 2. Does the inserted epitope affect the functions of carrier proteins? 3. Is expression of the hybrid proteins toxic to bacteria? 4. Is the hybrid protein stably expressed and not degraded by bacteria? The answers to all these questions came from experiments.

A. LamB:V3 Expression

Expressions of wild type LamB, a LamB mutant protein, devoid of L9 (DV3), and the LamB:V3 hybrids in *E. coli* strain pop6510 were tested by Western blot analysis. The results are shown in Figure 3.1. In lane 1, wild type LamB was detected; in lane 2, LamB:V3 was detected; in lane 3, a LamB mutant with a deletion in L9 external loop was stably expressed; in lane 4, which contained the host strain pop6510, no LamB expression was detected. In all cases, no obvious degradation of LamB mutants was observed. The relative positions of corresponding wild type LamB, mutant or LamB:V3 hybrid on the Western blot were consistent with their corresponding molecular weights. Both *E. coli* and *S. typhimurium* harboring LamB:V3 hybrid grew normally in LB and

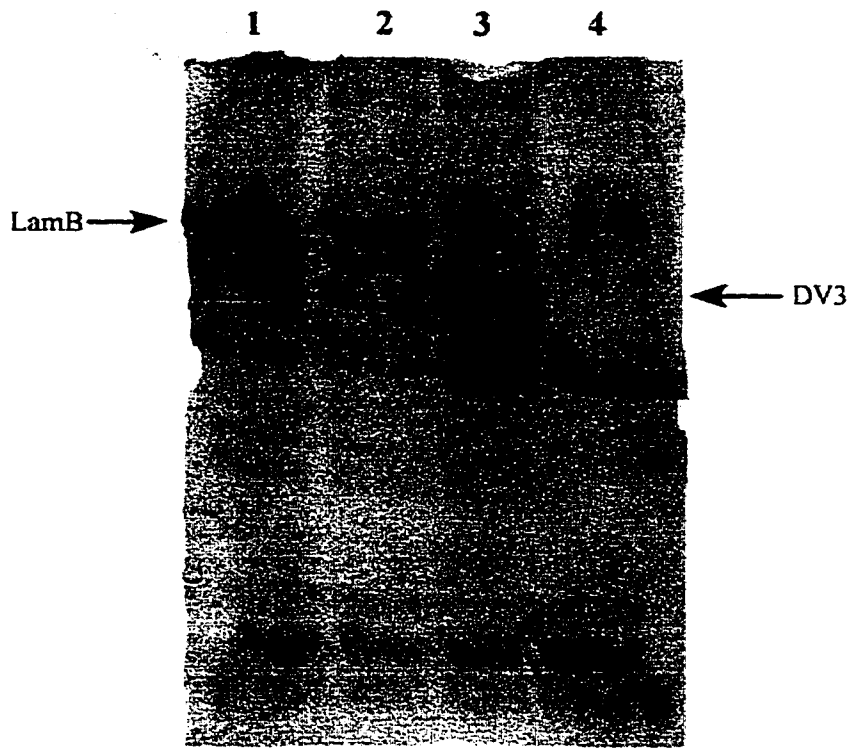


Figure 3.1 Expression of LamB:V3. Bacteria carrying various plasmids were grown and induced with IPTG. $7.5x10^7$ cells were loaded on SDS-PAGE gel and transferred to nitrocellulose paper. Wild type LamB and LamB mutants were detected by a rabbit anti-LamB antibody. 1- wild type LamB; 2 - LamB:V3; 3 - LamB mutant with deletion of L9 external loop.

no aberration was observed.

B. OmpA:TOP Expression

Expression of OmpA:TOP in *E. coli* UH2O3 strain proved to be problematic. The efficiency of transforming UH2O3 was extremely low and the bacteria harboring plasmids encoding OmpA:TOP grew very slowly, with the expression level of OmpA:TOP barely detectable by Western blot (data not shown). As a consequence, we used strain JM101 that expresses chromosomally encoded wild type OmpA, to harbor plasmid pL3TOP. Transformation of JM1O1 with the plasmid posed no problems at all. However, the resultant plasmid-containing bacteria grew slowly on LB-ampicillin plates. When inoculated into LB media with 100 μg /mL ampicillin, lysis was observed after overnight growth. When subcultured into LB media with 20 μg/mL ampicillin, the bacteria grew normally and no lysis was observed. The expression level of OmpA:TOP was IPTG-inducible and reached high levels, though a certain extent of degradation was also noticeable (Figure 3.2A, 3.2B).

Among seven bacterial clones selected, no OmpA:TOP expression was detected by Western blot for clone #6 (Figure 3.2A, lane 6), even with the IPTG induction. Without induction of the IPTG, the expression of the hybrid protein was detected only for clone #7 (Figure 3.2B, lane 7) and the expression level of

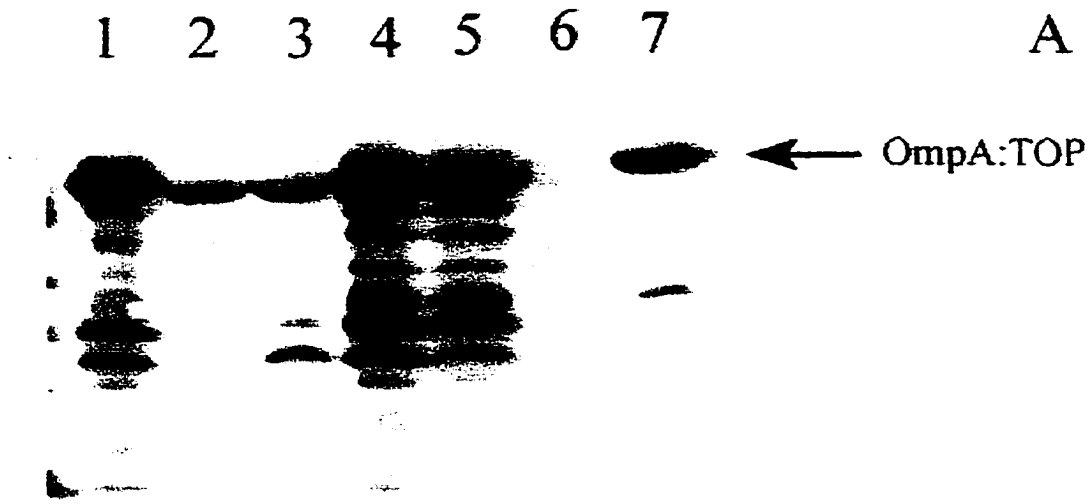
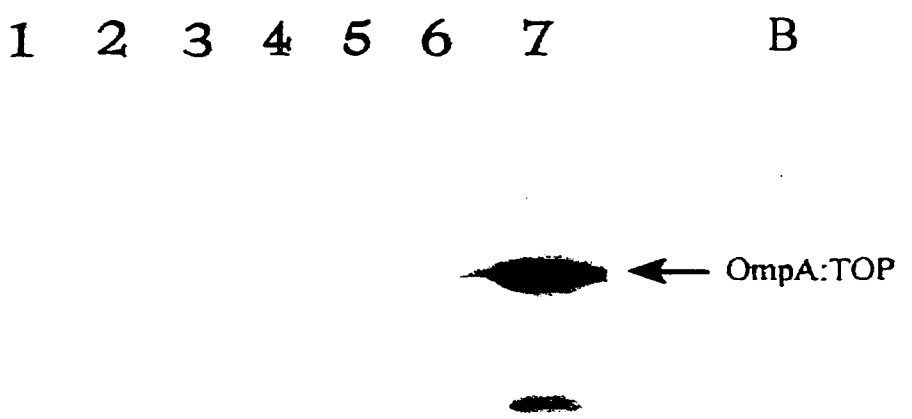


Figure 3.2 Expression of OmpA:TOP hybrid protein. *E. coli* carrying pL3TOP plasmid were grown and harvested. 2.5 x 10^8 cells were lyzed with 100 µl of the sample buffer and boiled. 25 µl of the solution were loaded on SDS-PAGE, subjected to electrophoresis and transferred to nitrocellulose paper. OmpA:TOP was detected by mouse anti-gp120. A. expression of OmpA:TOP was induced with IPTG; B. expression of OmpA:TOP was not induced with IPTG.



* see the legend on the previous page.

the hybrid for this clone had no obvious change with induction of IPTG (Figure 3.2A, lane 7). Degradation of OmpA:TOP was severe for clones 4 and 5 (Figure 3.2A, lane 4 and 5) and no significant degradation for clone #7 was observed (Figure 3.2A, lane 7; Figure 3.2B lane 7). It was also observed that if a frozen permanent was prepared from a culture grown for more than 12 hours, the

expression of OmpA:TOP hybrid would be substantially reduced (data not shown).

C. *Expression of LamB:V3 Hybrid Proteins with Different Flanking Region Sequences*

The expression of LamB:V3 fusion proteins with unique flanking region sequences is shown in Figure 3.3. The library of LamB:V3 hybrid proteins consisted of 26 clones. The expression of LamB:V3 was not detected for clone 26 (Figure 3.3A, 3.3B). Clones 15, 16, and 19 (Figure 3.3A, 3.3B) expressed a smaller protein than the LamB:V3 hybrid as detected by rabbit ploYclonal anti-LamB antibody, but not by sheep anti-gp120. Upon checking the amino acid sequence for these three clones, we found out that clone 15 contained a stop codon at the immediate upstream junction of the V3 epitope. Therefore, cells harboring pX1V15 plasmid expressed a truncated LamB that had smaller molecular weight than normal LamB:V3 hybrid; and was still recognized by rabbit polyclonal anti-LamB antibody. However, no clear explanation for the aberrations of clones 16 and 19 was found. Twenty three clones out of 26 expressed LamB:V3 fusion proteins that had the same mobility in SDS-PAGE as the starting vector X. The fusion proteins were also recognized by both rabbit polyclonal anti-LamB antibody and sheep anti-gp120. No significant differences in the expression levels of the fusion proteins were observed. Some apparent

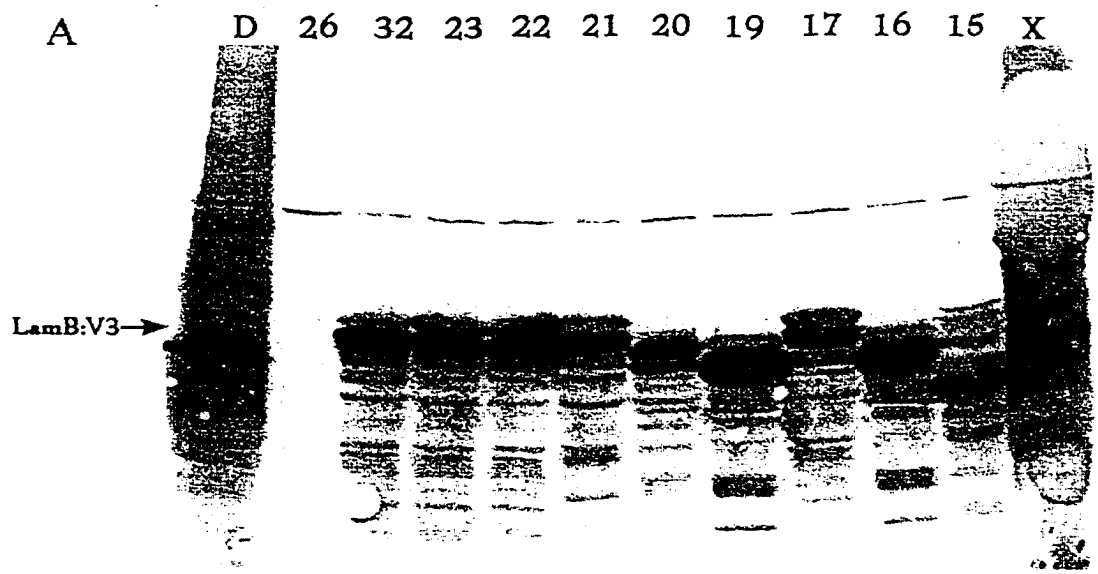
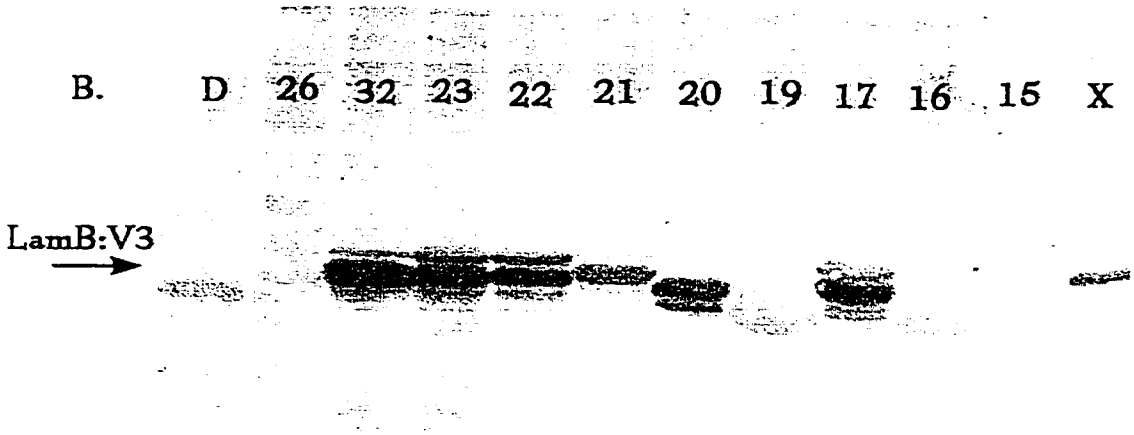
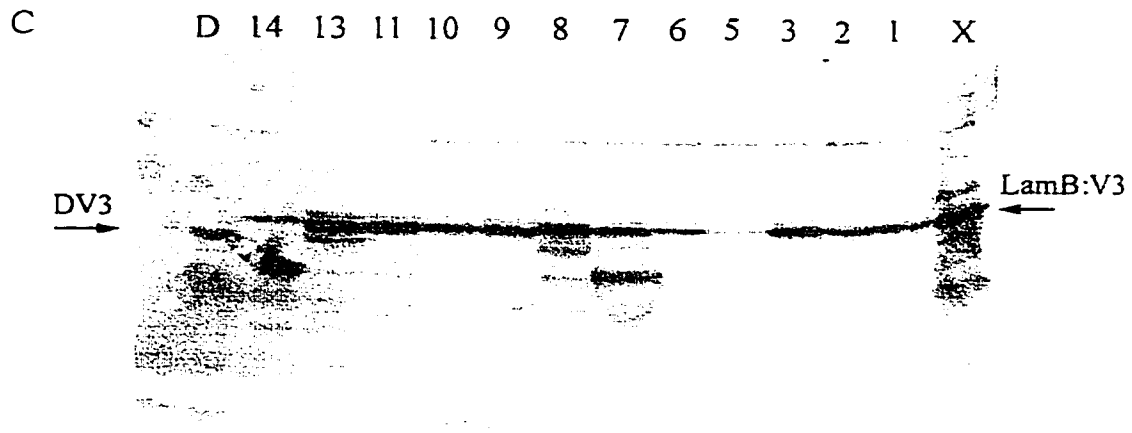


Figure 3.3 Expression of LamB:V3 hybrid protein. *E. coli* strain pop6510 was transformed with PCR mutagenized plasmid DNA. Colonies formed on LB-ampicillin plates were isolated, grown in LB broth, collected by centrifugation, lysed and subjected to Western immunoblot. The labels identify the clones that were analyzed in each of the lanes. LamB:V3 from the starting vector, pX1V3 (X) and a mutant of LamB, from pDV3 (D) were also analyzed. Duplicated gels were transferred to nitrocellulose papers, that were developed with sheep anti-gp120, and rabbit anti-LamB, respectively. **A** and **C**, LamB:V3 hybrids were detected by rabbit polyclonal anti-LamB; **B** and **D**, LamB:V3 hybrids were detected by sheep anti-gp120.





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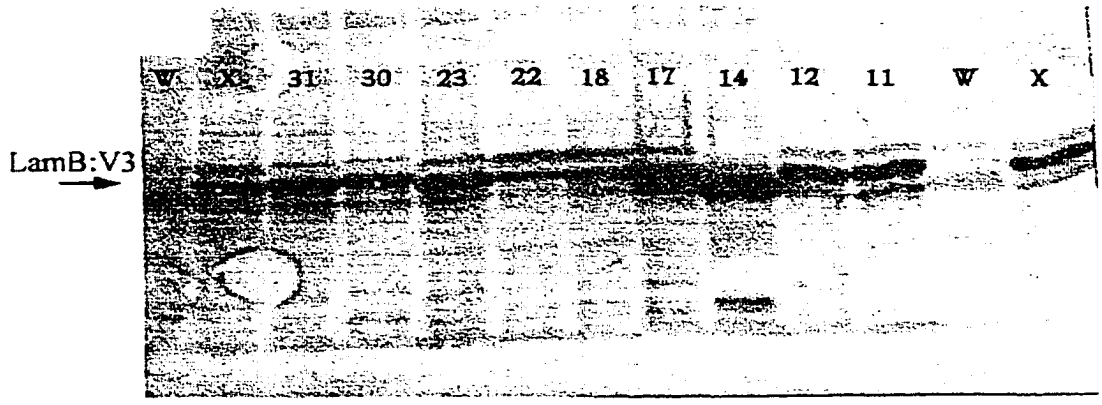
D



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differences of the intensity of the bands among different immunoblots were caused by variations in the experimental conditions. Degradation of the fusion proteins was not severe except for clone 7 and 13.

D. Expression of OmpA:TOP Hybrid Proteins with Different Flanking Region Sequence

The expression of OmpA:TOP chimeric proteins generated by PCR mutagenesis was examined by Western blot. The results in the host strain JM101

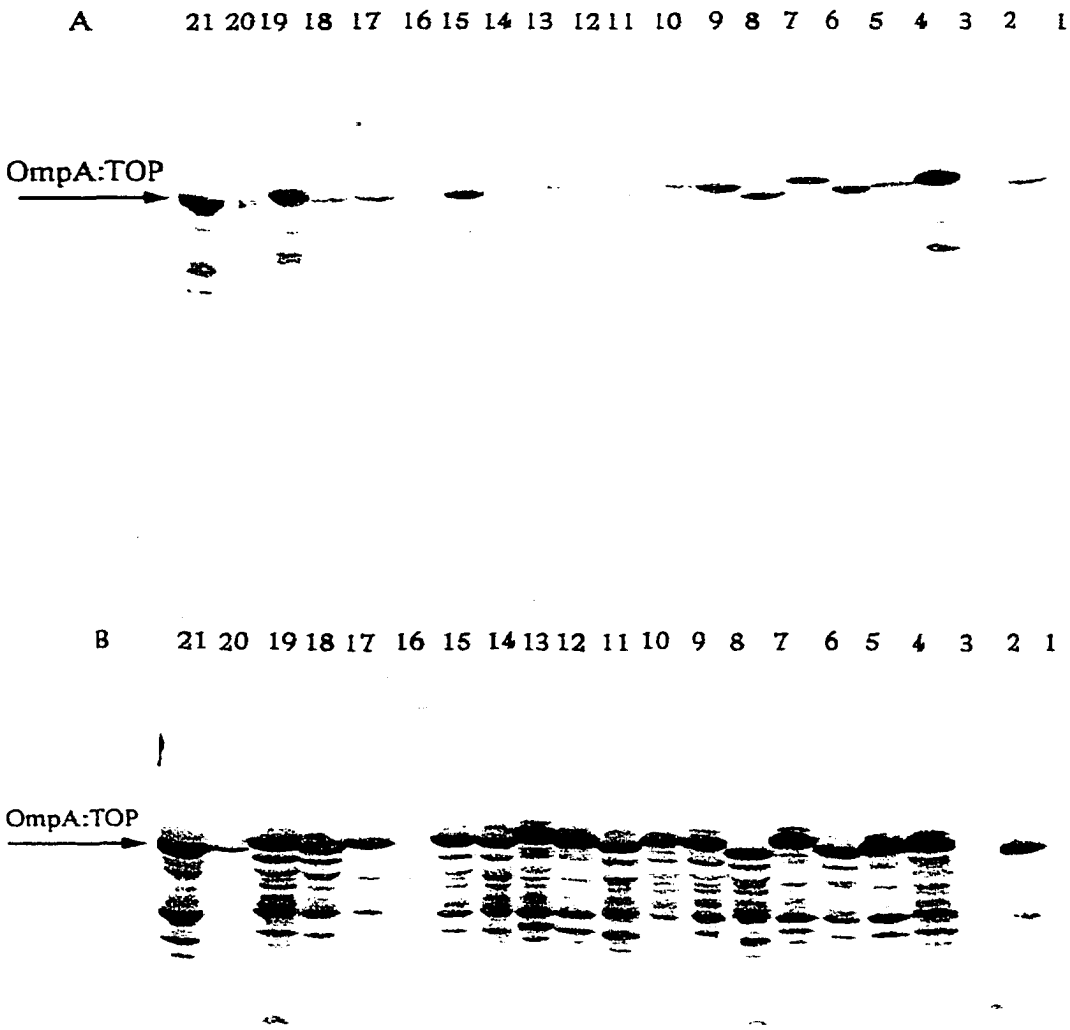

Figure 3.4 Expression of OmpA:TOP hybrid protein in JM101. *E. coli* strain JM101 was transformed with PCR mutagenized plasmid DNA. Colonies formed on LB-ampicillin plates were isolated, grown in LB broth, collected by centrifugation, lysed and subjected to Western immunoblot. The labels identify the clones that were analyzed in each of the lanes. OmpA:TOP hybrids were detected by mouse monoclonal anti-gp120. **A.** the expression of OmpA:TOP hybrids was not induced by IPTG. **B.** the expression of OmpA:TOP hybrids was induced by IPTG.

are shown in Figure 3.4A, 3.4B. No hybrid protein was detected by Western blot for clones 1, 3, and 16 in JM101 even with IPTG induction (Figure 3.4B). The level of chimeric protein expression responded to IPTG induction for all clones in the library, although in different degrees. Dramatic changes of expression levels under the presence and absence of IPTG occurred for clones 12 and 14, from undetectable without IPTG to the same level as other clones with IPTG induction. The OmpA:TOP fusion proteins displayed variable mobility in SDS-PAGE. Although the differences in mobility among the members of chimeric protein library was not significant, it was not exactly what we expected. The difference of mobility among clones was not observed for six OmpA:TOP clones (Figure 3.2) that lacked both upstream and downstream three amino acids, generated by PCR mutagenesis. It seemed that the mobility difference was caused by the variations of the junction region sequences. However, we did not observe this phenomenon for LamB:V3 fusion proteins. Some of the clones displayed degradation of the fusion proteins under IPTG induction and the degree of the degradation was proportional to the expression level of chimeric proteins.

The expression of OmpA:TOP chimeric proteins in *Salmonella* strain SL3261 is shown in Figure 3.5A, 3.5B. These results were obtained without IPTG induction. After comparing to the results of the expression in JM101, no discrepancy of the expression of OmpA:TOP chimeric proteins was found between *E.coli* and *S. typhimurium* strains, except for clone 23. For this clone, the

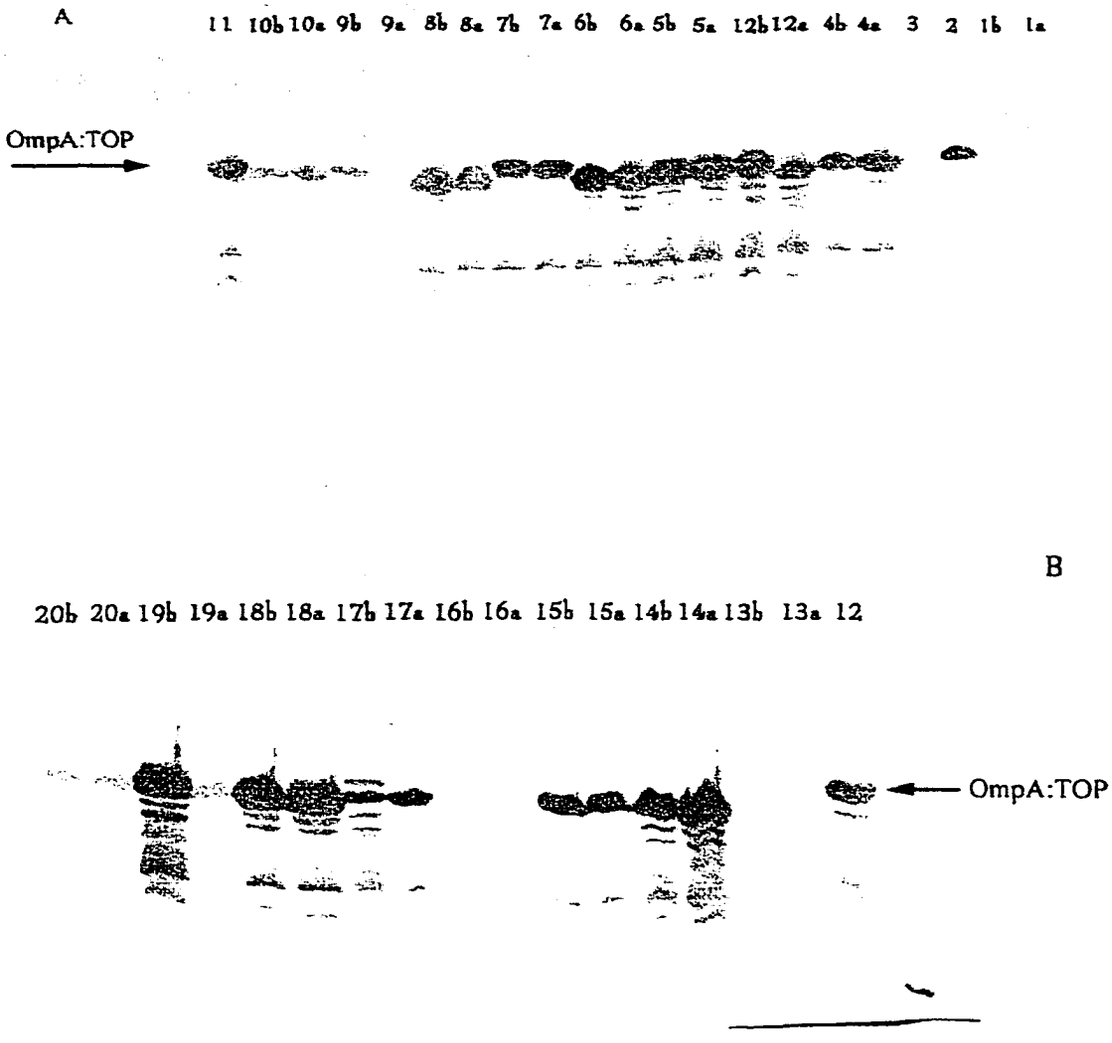

Figure 3.5 Expression of OmpA:TOP fusion protein in *Salmonella typhimurium.* SL3261 was transformed with PCR mutagenized plasmid. Colonies formed on LB-ampicillin plates were isolated, grown in LB broth, collected by centrifugation. 2.5×10^8 cells were lysed with 100 µl of the sample buffer. and subjected to Western immunoblot. The labels identify the clones that were analyzed in each of the lanes. OmpA:TOP hybrids were detected by mouse monoclonal anti-gp120. A. 25 µl of the solution was loaded on each lane for ; B. 30 µl of the solution was loaded on each lane.

OmpA:TOP fusion protein was expressed in *E.coli*, but not in *S. typhimurium*. Another noticeable difference for the expression of chimeric proteins in two strains was that there was less mobility variations among the members of the OmpA:TOP chimeric protein library when they were expressed in SL3261 than in JM101. The difference of band intensity between Figures 3.5A and 3.5B did not reflect the difference of expression level. These two Western blots were made under different conditions. For Figure 3.5B, gel had 20 wells and each lane was loaded with 30 μl of the sample, whereas for Figure 3.5A, gel had 25 wells, and each lane was loaded with 25 μl of the sample. Degradation of OmpA:TOP chimeric proteins was also observed in SL3261 strain and the degree of degradation was proportional to the expression level of the OmpA:TOP chimeric proteins.

E. Expression of OmpA:Strep Hybrid proteins with Different Flanking Region Sequence

In order to evaluate the expression of OmpA:Strep hybrids, we raised polyclonal antibodies against M5 protein in a rabbit. Reactivity of the sera against M5 is shown in Figures 3.6A, 3.6B.

Expression of OmpA:Strep hybrids was more problematic than that of OmpA:TOP. Although expression of OmpA:Strep was detected by Western blot

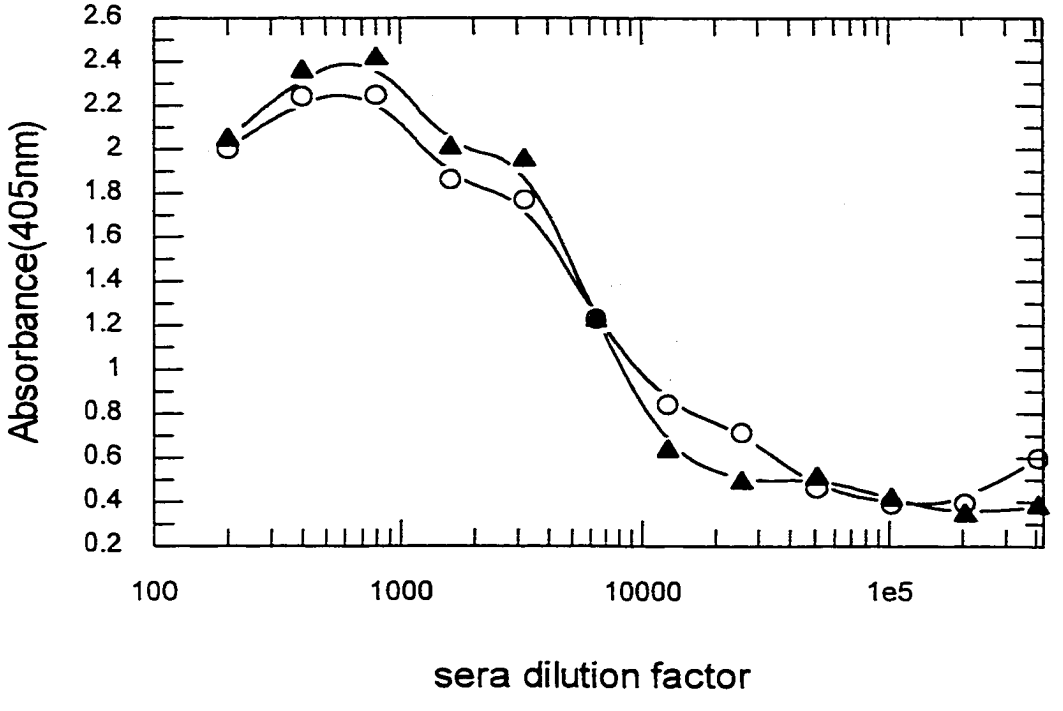

Figure 3.6A ELISA analysis of rabbit sera. Anti-M5 antibody was raised in a rabbit with purified M5 protein. ELISA plates were coated with purified M5 and incubated with the rabbit sera, followed by incubation with goat anti-rabbit IgG phosphatase conjugate. (O), first bleeding; (▲), 2nd bleeding.

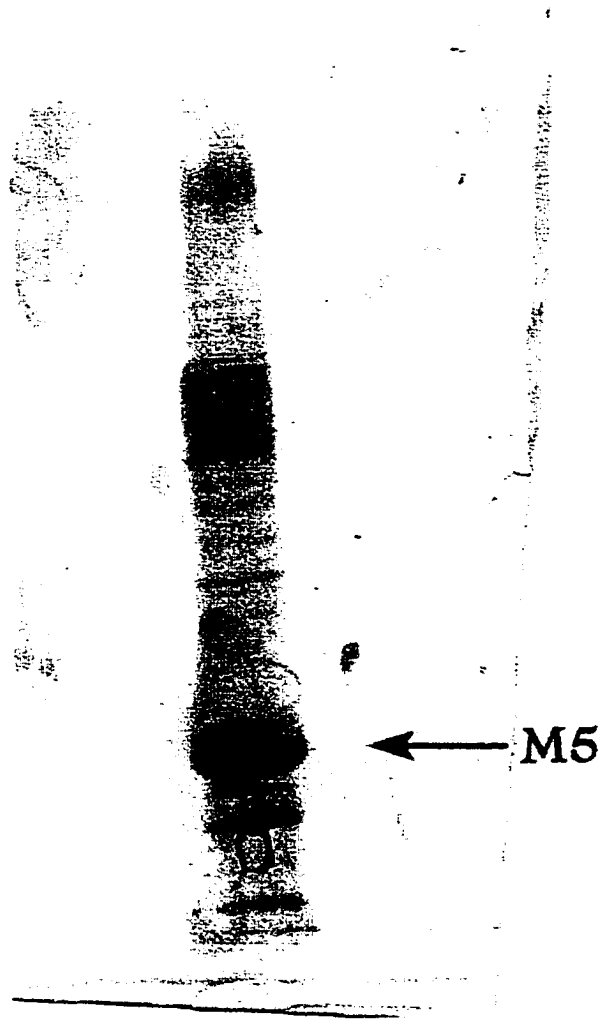

Figure 3.6B Western blot analysis of rabbit sera. Anti-M5 antibody was raised in a rabbit with purified M5 protein. 3 μg of M5 protein were loaded on a SDS-PAGE gel and transferred to nitrocellulose paper. The paper was incubated with the rabbit sera, followed by incubation with goat anti-rabbit IgG phosphatase conjugate.

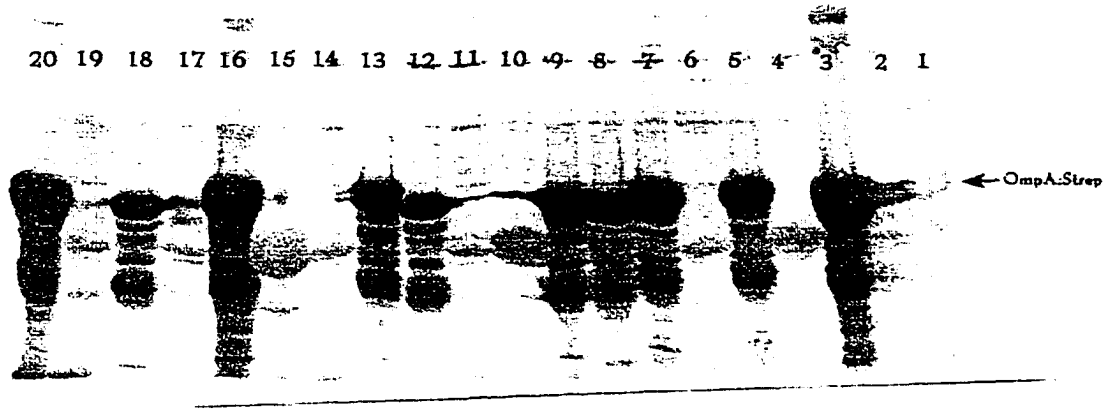

Figure 3.7 Expression of the OmpA:Strep chimeric proteins. JM101 was transformed with PCR mutagenized plasmid DNA. Colonies formed on LB-ampicillin plates were isolated and grown in LB broth. Expression of the OmpA:Strep fusion protein was induced by IPTG. Cells were harvested by centrifugation, lysed and subjected to Western immunoblot. The labels identify the clones that were analyzed in each of the lanes. The OmpA:Strep hybrids were detected by rabbit anti-M5.

(Figure 3.7), I was unable to obtain a consistent expression of OmpA:Strep. To make the matter worse, the Strep epitope on the cell surface was not well recognized by the rabbit anti-M5 antibody when bacteria expressing OmpA:Strep hybrid were fluorescently labeled. Therefore, no further data was collected for OmpA:Strep constructs in this study.

Overall, expression of the LamB:V3 hybrid was normal both in *E. coli* and *Salmonella* (data not shown), and no degradation of the chimeric protein was observed. The OmpA:TOP hybrid was expressed well both in *E. coli* and *Salmonella*, although a certain degree of degradation was observed.

3. Epitope Exposure on the Bacterial Cell Surface

Fluorescent probes have wide applications in cell biology and microbiology. Fluorescence spectroscopy has produced information about the structure, function and health of cells (Waggoner, 1986). This technique has enormously contributed to immunology. Classification of lymphocytes and identification of the surface antigens of lymphocytes were facilitated by using antigen-antibody interactions (Carleton, 2001). One of the tools for analyzing this interaction is flow cytometric analysis of the fluorescently labeled cells, by interaction between cell surface antigens and antibodies. We used flow cytometry to analyze the exposition of foreign epitopes on the cell surface, and the

correlation between the junction region sequences and the exposure of the epitope to the cell surface.

The V3 epitope that we inserted into LamB is immunogenic (Rusche et al., 1988), and a mouse monoclonal antibody (Mice NMO1, ATTC) against gp120 recognized this epitope. The TOP epitope that was fused into the third surface loop of OmpA was also recognized by the mouse anti-gp120. When cells expressing LamB:V3 or OmpA:TOP on their surface were incubated with the mouse antig-gp120, the antibody bound to the cell surface exposed hybrid proteins. In the next step, when incubated with FITC conjugated goat anti-mouse IgG, the cells were stained with the fluorescent probe FITC, due to the antibody and antigen interaction. The advantage of this probe is that it is insensitive to pH and the polarity of its local molecular environment (Waggoner, 1986). The intensity of fluorescence of stained cells depends on three factors: first, the affinity of the antibody to the epitope; second, the accessibility of the epitope to the antibody; third, the amount of epitope present on the cell surface. By changing growth conditions (with or without inducing chemicals), the amount of epitope present on each cell surface can be manipulated. Several factors may influence the access ability of the epitope to the antibody. Lipopolysaccharide (LPS) on the cell surface may hinder the interaction between the epitope on the cell surface and the antibody by acting as a physical barrier to antibody molecules. The orientation and projection of the epitope also influence the intensity of the

fluorescence of the cells, which carry foreign epitopes. Surface loops from a carrier protein for a passenger epitope can block or shield the epitope from the antibody molecules and hinder the interaction between them. By changing the orientation and projection of the epitope, the hindrance of the surface loops of the carrier protein and LPS of the host can be changed and thus lead to the maximization of epitope exposure.

We tried to optimize the exposure of foreign epitopes to the cell surface by introducing mutations in the immediate downstream and upstream regions of the epitope. We created two chimeric protein libraries, one for LamB:V3 and one for OmpA:TOP. Each member of the protein library had an unique junction regions. Therefore, the epitope was projected in a different manner in each hybrid protein. Variations of the intensities of the fluorescence of cells expressing a foreign epitope reflected the differentiation of the accessibility of the epitope to external environment among members of a chimeric protein library. A typical output of the measurement of fluorescence intensity is shown in Figure 3.8. X axis represents the mean fluorescence intensity of cells. Area under the curve is proportional to bacterial population. In Figure 3.8 (a), 95% of cells have a mean fluorescence intensity of 7. In Figure 3.8 (b), there are two distinct cell populations, one with mean fluorescence intensity 0.7, and another with mean fluorescence intensity 17.1.

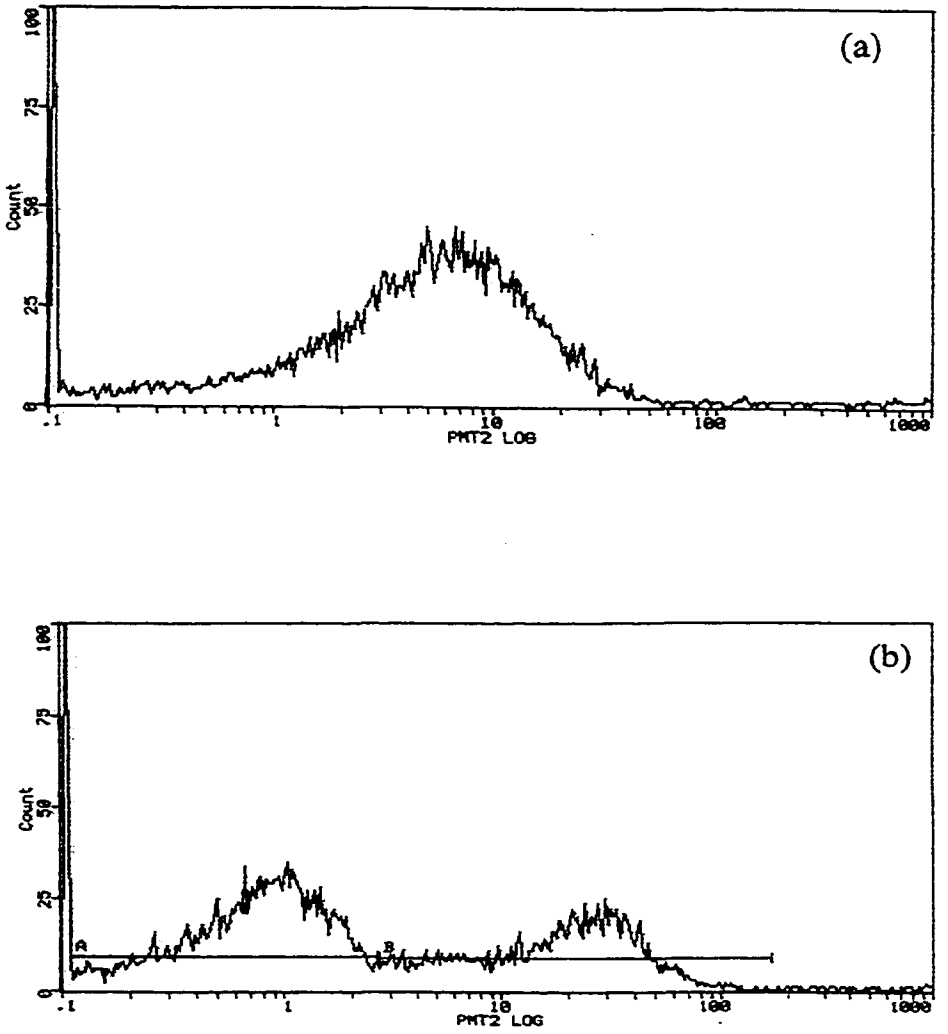

Figure 3.8 Flow cytometry measurements of fluorescence intensity of fluorescently labeled cells. Cells expressing foreign epitope were grown in LB media with ampicillin and induced with IPTG. 10^8 cells were harvested in an eppendorf tube. The cells were incubated with antibodies against the epitope and washed with TBS. Then, IFTC-conjugated goat anti-mouse IgG was added to the cells suspension. After 30 minutes incubation, cells were washed and centrifuged. Finally, the cells were resuspended in TBSG. In (a), 95% cells have a mean fluorescence intensity is 7. In (b), there are two distinct cell populations, one with mean fluorescence intensity 0.733, and another with mean fluorescence intensity 17.1.

The result of fluorescence intensity study of *E. coli* cells expressing LamB:V3 is given in table 1. *E. coli* strain used for the fluorescent experiment is a deep rough strain and does not have full LPS, which facilitated staining of cells. The variation of mean fluorescence intensities among members of the chimeric protein library was significant, from the high 19.8 (clone 22) to the low of 2.5 (clone 20). Clone with pDV3 plasmid which did not encode V3 loop was negative (0.1). Although the absolute value of mean fluorescence intensity for a specific clone might vary from day to day, their relative mean fluorescence intensities, in comparison to the controls and to each other, were always consistent.

Now the question is whether the difference of mean fluorescence intensities among the members of chimeric library are solely derived from the variation of the difference of the junction region sequences. The only other factor which may contribute to this variation is the level of expression of the chimeric proteins. From Western blot results, only a slight difference of expression level among different chimeric proteins existed. We also noticed that the highest expression level (Figure 3.3A, clone 19) did not give the highest fluorescence intensity. Expression level for the majority of chimeric proteins was very similar.

Table 3.4. Fluorescence intensities of *E.coli* cells expressing OmpA:TOP chimeric proteins

	not induced		IPTG induced	
clone	**Percent**	**intensity of fluoresce**	**percent**	**intensity of fluoresce**
JM101	99.5	0.2	99.7	0.2
1	97.8	0.2	99.7	0.2
2	80.8	10.1	82.7	13.9
3	99.2	0.2	99.6	0.2
4	80.0	10.9	80.7	13.3
5	60.1	13.7	82.9	14.1
6	77.6	11.5	84.8	13.8
7	62.6	15.3	81.2	14.1
8	73.2	11.3	78.4	15.5
9	59.6	17.8	56.0	20.2
10	76.6	11.9	80.7	15.4
11	60.8	15.7	77.2	16.1
12	37.8	5.34	75.2	15.7
13	35.1	11.7	81.9	14.5
14	93.1	0.2	77.6	17.5
15	73.5	11.6	82.0	15.0
16	99.4	.151	99.0	0.2
17	71.0	10.3	73.9	15.5
18	69.0	10.9	70.2	17.8
19	72.2	10.1	78.4	15.1
20	61.1	14.9	64.0	23.2
21	70.6	11.3	71.4	18.1

Table 3.5. Fluorescence intensities of SL32621 cells expressing OmpA:TOP chimeric proteins

clone	Percent	fluorescence intensity	percent	fluorescence intensity
SL3261	92.8	0.1	1.5	2.0
1	99.8	0.1		
2	96.4	0.1		
3	99.4	0.1		
4	78.5	0.1	19.4	7.0
5	37.2	0.1	52.7	8.4
6	44.2	0.1	48.3	6.1
7	97.9	0.1		
8	97.3	0.1		
9	63.6	6.4		
10	59.2	5.5		
11	84.4	0.1	14.8	1.9
12	95.3	0.1	4.1	1.7
13	98.1	0.1		
14	55.3	14.2		
15	56.1	12.7		
16	98.3	0.1		
17	97.8	0.1		
18	98.4	0.1		
19	95.5	0.1	3.0	5.1
20	93.4	0.2		
21	95.8	0.2		

For OmpA:TOP chimeric proteins, fluorescence intensity studies were carried out in *E. coli* and *S. typhimurium*. Result in *E. coli* strain pop6510 is given in Table 3-4. The result shows the correlation between expression levels and mean fluorescence intensities. Mean fluorescence intensities for the majority of clones responded to IPTG induction. The most significant increase of mean fluorescence intensity occurred for clone 14, whose intensity increased from 0.2 without IPTG to 17.5 under IPTG induction. The increase of mean fluorescence intensity correlated well with the increase of OmpA:TOP expression level. Without IPTG induction, OmpA:TOP expression for clone 14 was not detected by Western blot (Figure 3.4A), whereas with IPTG induction, the expression level of OmpA:TOP was similar to the other members of the library (Figure 3.4B). Clones 1, 3 and 16 were not fluorescently stained and their chimeric proteins were not detected even under IPTG induction. The expression levels for clones 4, 7, 13 and 21 were higher than others, but the highest mean fluorescence intensity came from clone 21, which had the lowest expression level of OmpA:TOP.

Staining *S. typhimurium* was problematic due to full length LPS of *S. typhimurium*. The majority of clones were not stained (Table 3-5). Only clones 4, 5, 6, 9 and 10 were stained. Even for these clones, the percentage of cells fluorescently labeled was low compared to *E. coli* strain. Less than half of cells were labeled, except for clone 5 (52.7 percent).

4. Hybrid Constructs for Immunization

One of my goals in this study was to investigate how the exposure of a foreign epitope on the bacterial surface would influence the immunogenicity of the epitope. How would the immune system react to the epitope which was exposed on the bacterial surface? Would the immune system be able to differentiate the level of the epitope exposure and respond in a different manner? We were especially interested in humoral response. Our strategy was to immunize mice with selected hybrid constructs and analyze mice sera for IgG responses. The selection of hybrid constructs for immunization was based on the mean fluorescence intensities of different constructs. We selected clones with higher and lower mean fluorescence intensities to investigate if they would elicit different humoral responses in mice.

For LamB:V3 constructs, we selected a clone expressing LamB with no V3 fusion, a clone expressing a LamB:V3 hybrid encoded by the starting vector pX1V3, and three other LamB:V3 chimeric proteins (pX1V3-8, pX1V3-22, pX1V3-23) generated by PCR mutagenesis. In cells carrying pX1V3-8 plasmid, the V3 epitope was poorly recognized on the cell surface, but the chimeric protein was expressed in a normal level. On the other hand, the passenger epitope was well recognized on the surface of cells carrying pX1V3-22 and pX1V3-23. *E. coli* strain pop6510 or attenuated *S. typhimurium* strain SL3261 carrying these five plasmids were administered intraperitoneally to two groups of mice (ten mice in

each group). When *E. coli* was used for immunization, a heat-killed formulation was used and each mouse was given 10^7 cells. 5 x 10^6 cells of live SL3251 were given to each mouse, and each antigen was administered into twenty mice.

For OmpA:TOP constructs, we selected the vaccines based on mean fluorescence intensity data obtained for SL3261 strain. Clones 4, 5, 6 and 9 were chosen because of the accessibility of the mouse monoclonal anti-gp120 to their cell surface exposed TOP epitope. Clones 7, 8, and 12 were selected for the opposite reason. SL3261 that did not express OmpA:TOP was also chosen as a negative control. All constructs were expressed in attenuated SL3261 strain for mice immunization.

5. Western Blot Analysis of Mouse Sera

A. Anti LamB:V3 Sera

Mouse sera were collected from immunized mice and sera from 10 mice that received the same antigen were pooled and tested for the reactivity toward outer membrane preparation from *E. coli* cells expressing wild type LamB or LamB:V3 chimeric protein as encoded by the vector plasmid pX1V3. A protein profile of the outer membrane preparations is shown in Figure 3.9. Major proteins in the outer membrane(OM) preparation besides LamB or LamB:V3 were porins, and iron-regulated proteins. When analyzed by Western blot, both anti-*E.*

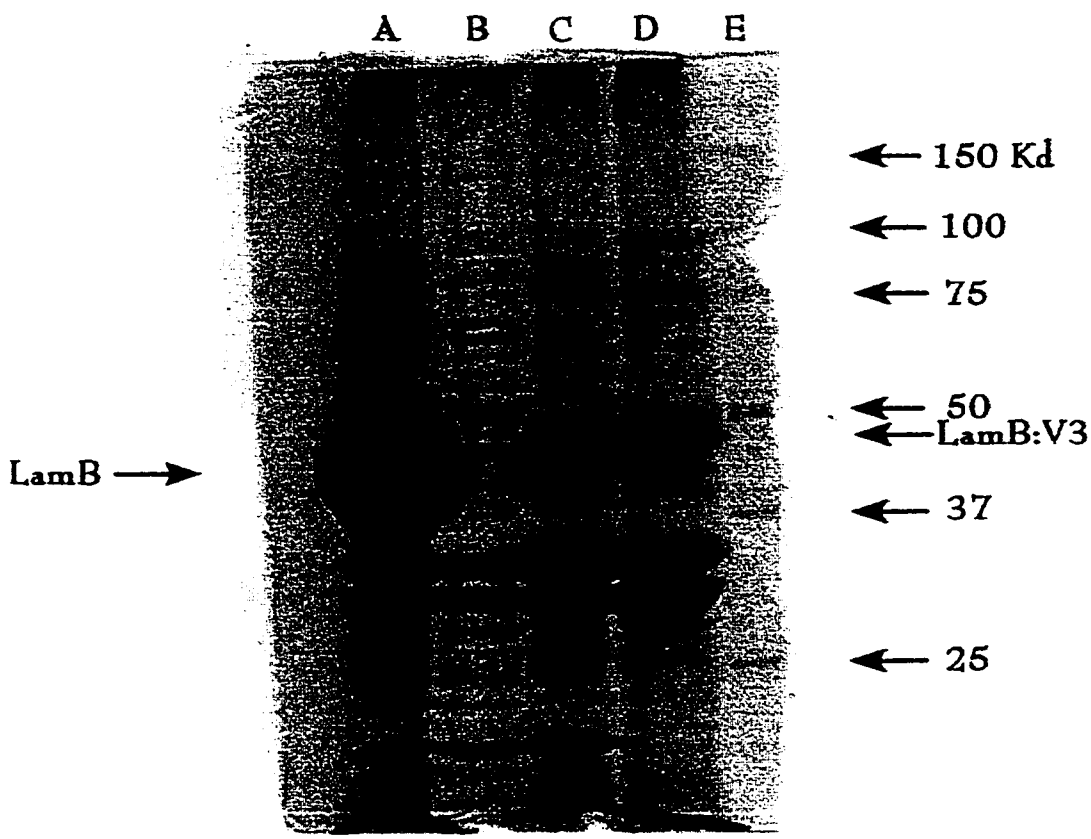

Figure 3.9 Protein profile of OM preparation. Outer membrane was prepared from *E. coli* and an aliquot was loaded on SDS-PAGE gel. The gel was stained with Coomassie Blue. A - OM was prepared from cells expressing wild type LamB; B - OM from pop6510; C and D - OM from cells expressing LamB:V3 hybrid; E - molecular weight marker.

coli and anti-*Salmonella* sera show strong reactivity to these major proteins in the OM preparation (Figure 3.10A, Figure 3.10B) which was consistent with well established facts that OmpA and the iron-regulated proteins are major antigens of the Gram-negative bacteria (Fernandez-Beros et al., 1989; Ortiz et al., 1989) (Brown and Hormaeche, 1989). All sera (anti-*E. coli* and anti-*Salmonella*) showed similar reactivity against wild type LamB (Figure 3.10A). Mouse sera against *E. coli* carrying pX1V3-23 (Figure 3.10A, lane 6) and mouse sera against *Salmonella* carrying pX1V3-23 (Figure 3.10A, lane 5) reacted with wild type LamB in a similar manner. When mouse sera was tested for the reactivity against LamB:V3 hybrid, the difference was clear. The sera against *Salmonella* carrying pX1V3-22 and pX1V3-23 (Figure 3.10B, lane 4, and 5) showed strong reactivity with LamB:V3, whereas sera against *Salmonella* carrying pDV3 encoding a mutant LamB without V3 fusion did not react with LamB:V3 (Figure 3.10B, lane 2). The reactivity of the anti-*Salmonella* sera with LamB:V3 decreased in the order of pX1V3-23, pX1V3-22, pX1V3, pX1V3-8 and pDV3. This was the exact order of the mean fluorescence intensities of *E. coli* cells carrying these plasmids. Thus, the immunogens (pX1V3-23, pX1V3-22) that projected V3 epitope in such a way that the epitope was most accessible to mouse anti-gp120 elicited the strongest humoral response against the epitope. The immunogen (pX1V3-8) with low mean fluorescence intensity only elicited a meager humoral response toward the V3 epitope. Therefore, the reactivity of mouse sera with LamB:V3

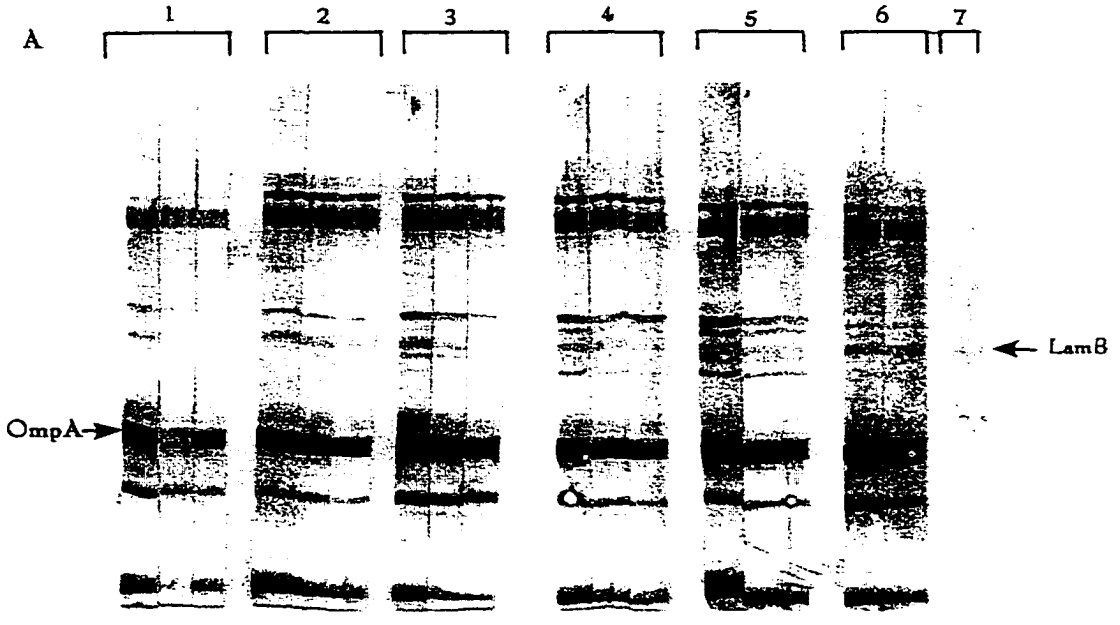

Figure 3.10A Recognition of LamB by mouse sera raised against LamB:V3
S. typhimurium strain SL3261 was transformed with plasmids of interest and individual clones were isolated and inoculated into groups of mice. Antisera were collected, and individual sera within the groups were pooled, diluted, and assayed against OM extracts from pop6510/pAC1. The blot shows titration of 6 pools of sera from groups of mice immunized with SL3261 carrying pX1V3, pDV3, pX1V-8, pX1V-22, pX1V-23, and pop6510/pX1V3-23 (panels 1-6, respectively). The three panels in panels 1-5 represent (left to right) 1/200-, 1/1000-, 1/2000-fold dilutions of the pooled sera. In panel 6, the dilutions are 1/1000 and 1/2000. Lane 7 was exposed to rabbit anti-LamB.

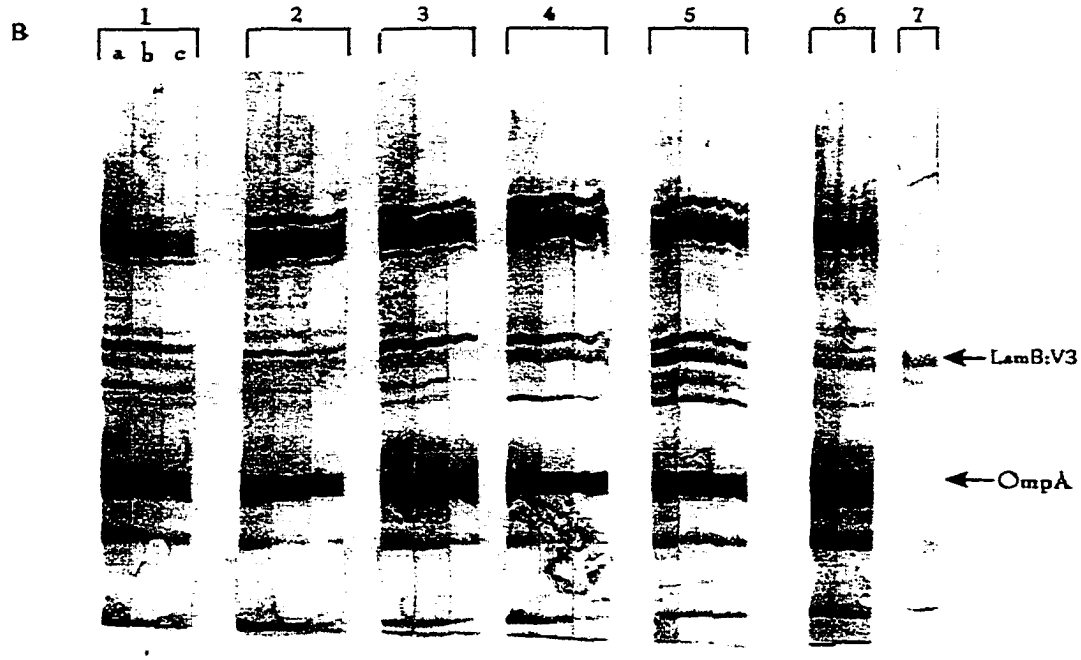

Figure 3.10B Recognition of TOP epitope by mouse sera raised against LamB:V3 *S. typhimurium* strain SL3261 was transformed with plasmids of interest and individual clones were isolated and inoculated into groups of mice. Antisera were collected, and individual sera within the groups were pooled, diluted, and assayed against OM extracts from pop6510/pX1V3. The blot shows titration of 6 pools of sera from groups of mice immunized with SL3261 carrying pX1V3, pDV3, pX1V-8, pX1V-22, pX1V-23, and pop6510/pX1V3-23 (panels 1-6, respectively). The three panels in panels 1-5 represent (left to right) 1/200-, 1/1000-, 1/2000-fold dilutions of the pooled sera. In panel 6, the dilutions are 1/1000 and 1/2000. Lane 7 was exposed to sheep anti-gp120.

did correlate with the exposure of the immunogens. The immune system did react in a different manner toward the epitope that was exposed to the cell surface at different degrees, as indicated by the different mean fluorescence intensities of cells expressing the epitope. All sera showed a weak reactivity towards the carrier protein LamB to a similar extent, which obviously resulted from a low concentration of antibodies against LamB in the mouse sera. The data suggested that the humoral immune response towards the epitope can be selectively enhanced, by the optimization of the exposure of vaccine epitopes on the cell surface. The sera raised against *E. coli* that was harboring pX1V3-23 also showed the reactivity toward LamB:V3 (Figure 3.10B, lane 6), although its reactivity was a little weaker than that of the sera raised against *Salmonella* harboring the same plasmid.

B. Anti-OmpA:TOP Sera

Sera from mice immunized with *Salmonella* expressing OmpA:TOP chimeric proteins were evaluated by Western blot. When tested against purified OmpA or the outer membrane preparation, all sera showed a strong reactivity towards OmpA (Figures 3.11A, 3.11B, 3.11C and 3.11D), and there was no perceivable difference among the sera regarding their reactivity towards OmpA. Mouse sera displayed a very weak reactivity towards wild type LamB (Figure 3.11B). Although LamB was not the carrier protein, SL3261 strain expresses wild

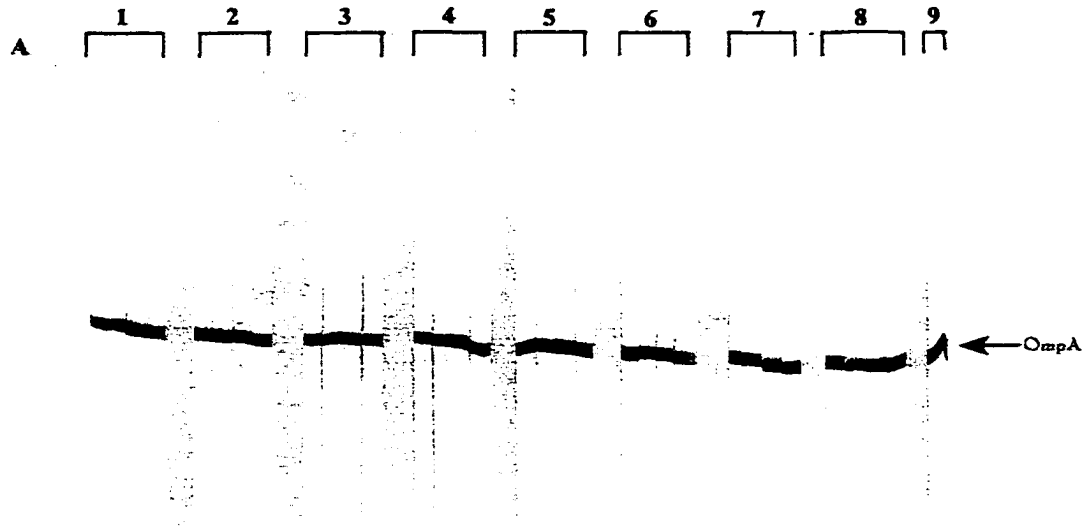

Figure 3.11A Recognition of OmpA by mouse anti-OmpA:TOP sera. Figure shows the interaction between purified OmpA and mice sera obtained from mice immunized with SL3261 expressing OmpA:TOP antigen. Panels 1-8 represent eight different antisera raised against SL3261/pL3TOP4, pL3TOP5, pL3TOP6, pL3TOP7, pL3TOP8, pL3TOP9, pL3TOP12, and SL3261 without plasmid, respectively. Panel 9 was exposed to mouse anti-OmpA. Each panel in panels 1-8 has 4 columns, representing four different sera dilultions. From left to right, the dilution is 1/2000, 1/4000, 1/8000 and 1/16000 folds, respectively.

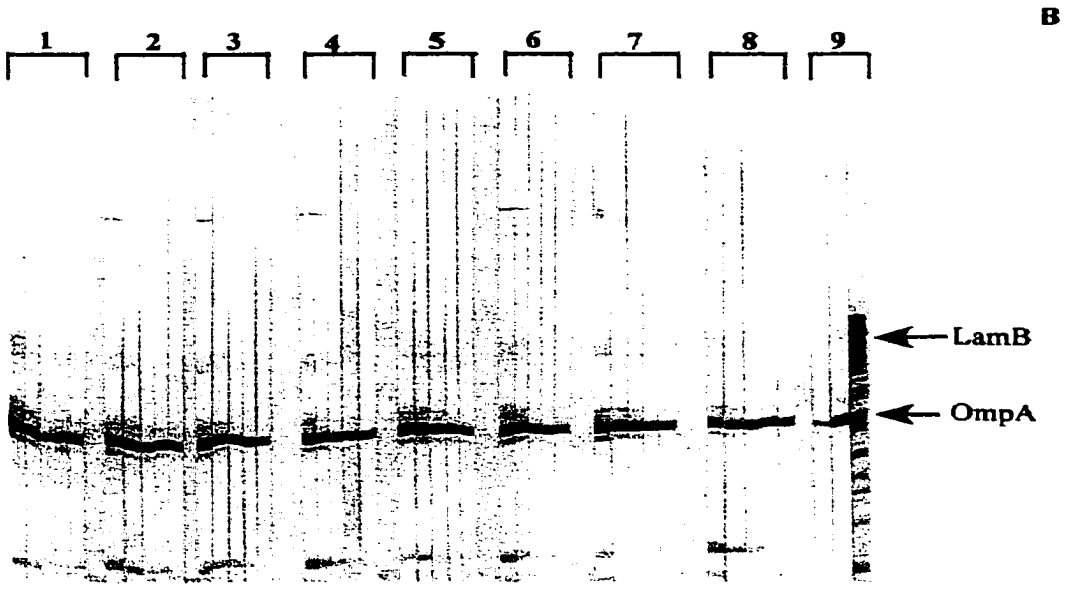

Figure 3.11B Recognition of LamB by mouse anti-OmpA:TOP sera. The Figure shows the interaction between OM prepared from *E. coli* pop6510 carrying pAC1 and mice sera obtained from mice immunized with SL3261 expressing OmpA:TOP antigen. Panels 1-8 represent eight different antisera raised against SL3261/pL3TOP4, pL3TOP5, pL3TOP6, pL3TOP9, pL3TOP7, pL3TOP8, pL3TOP12, and SL3261 without plasmid, respectively. Panel 9 was exposed to mouse anti-LamB. Each panels in panels 1-8 has 5 columns, representing five different sera dilultions. From left to right, the dilution is 1/2000, 1/4000, 1/8000, 1/16000 and 1/32000 folds, respectively.

type LamB from the chromosome. The sera from mice immunized with *Salmonella* horboring the OmpA-TOP hybrids only weakly recognized wild type LamB.

The sera were next analyzed for reactivity towards LamB:V3 (Figures 3.11C, 3.11D). One might expect that it would be better to test the sera against OmpA:TOP. However, because of the highly immunogenic nature of OmpA and the high Ig concentration specific for OmpA in the mouse sera, the reactivity between OmpA and the sera would totally mask the reactivity of the sera with TOP epitope. On the other hand, because of the very low Ig concentration specific for LamB, the reactivity of the sera towards LamB:V3 indicates the specific reaction between the sera and the V3 loop. All mouse sera specifically recognized the V3 loop (Figure 3.11C, lanes 1, 2, 3, 4, 5, 6 and 7), except anti-SL3261 (Figure 3.11C, lane 8), which was obtained from the mice immunized with SL3261 that did not express OmpA:TOP hybrid. From Figure 3.11C, it was impossible to determine which clone had a higher reactivity towards TOP epitope. To pursue any reactivity difference that might exist among the sera towards the TOP epitope, the sera were diluted further and the result is shown in Figure 3.11D. Panels 1 (clone 4), 2 (clone 5), 3(clone 6), 4(clone7), and 5 (clone 9) did show a better reactivity towards the TOP epitope than lanes 6 and 7 (clone 8, and clone 12). Based on the cell staining results (Table 5), clones 4, 5, 6, and 9 had epitope better exposed than clones 7, 8 and 12. Data from clones 4, 5, 6, 9, 8 and

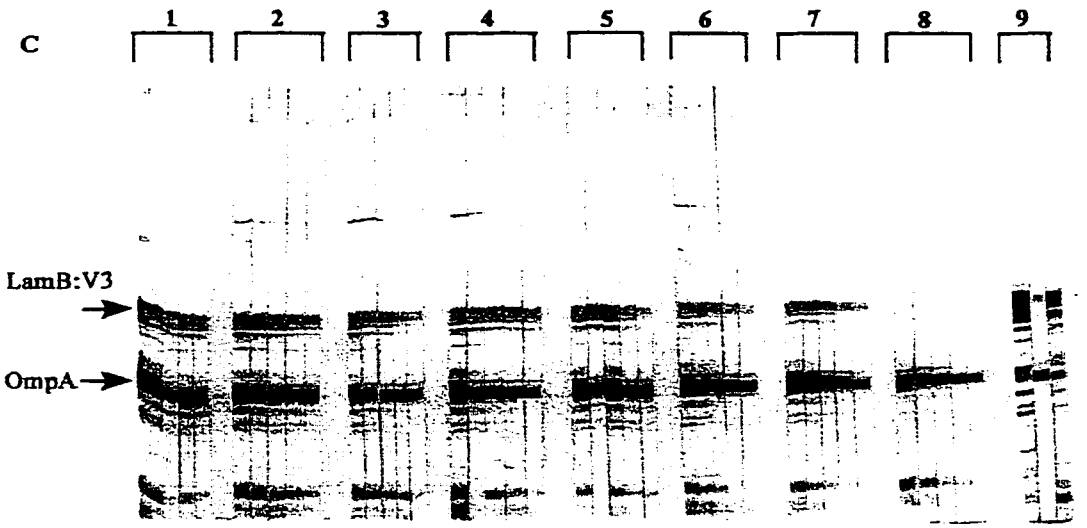

Figure 3.11C Recognition of the V3 loop by mouse anti-OmpA:V3 sera. The Figure shows the interaction between OM prepared from *E. coli* pop6510 carrying pX1V3 and mice sera raised against SL3261 expressing OmpA:TOP antigen. Panels 1-8 represent eight different antisera raised against SL3261/pL3TOP4, pL3TOP5, pL3TOP6, pL3TOP9, pL3TOP7, pL3TOP8, pL3TOP12, and SL3261 without plasmid, respectively. Each panel in panels 1-8 has 5 columns, representing five different sera dilultions. From left to right, the dilution is 1/2000, 1/4000, 1/8000, 1/16000 and 1/32000 folds, respectively. Panel 9 has three columns. From left to right, each was exposed to a mouse anti-gp120, a mouse anti-OmpA, and a rabbit anti-LamB, respectively.

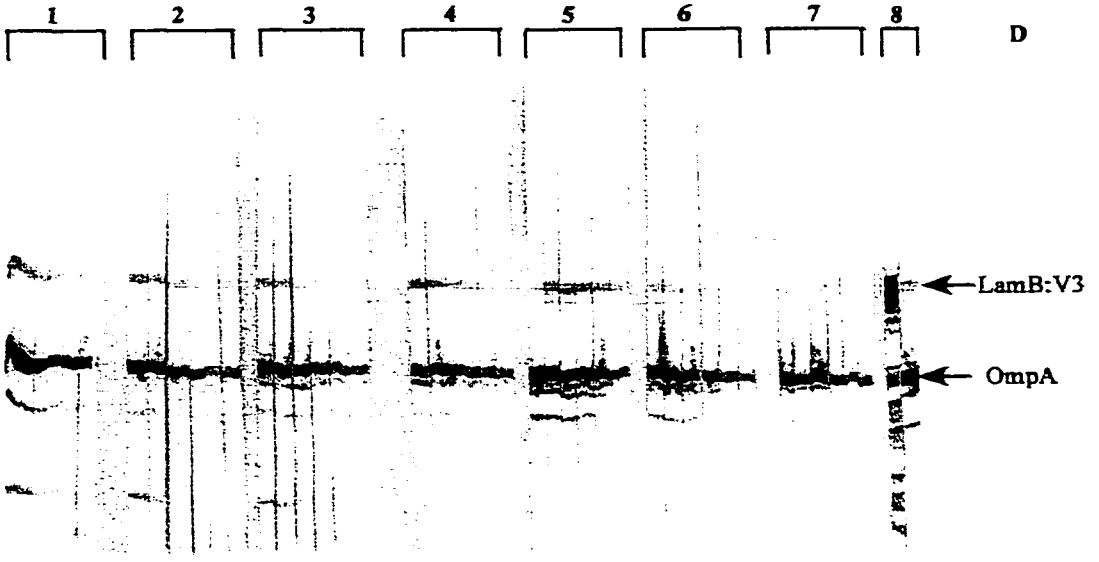

Figure 3.11D Titration of mouse anti-OmpA:TOP sera. The figure shows the interaction between OM prepared from *E. coli* pop6510 carrying pX1V3 and mouse sera raised against SL3261 expressing OmpA:TOP antigen. Panels 1-7 represent seven different antisera raised against SL3261/pL3TOP4, pL3TOP5, pL3TOP6, pL3TOP9, pL3TOP7, pL3TOP8, and pL3TOP12 respectively. Each panel in panels 1-8 has six columns, representing six different sera dilultions. From left to right, the dilution is 1/8000, 1/16000, 1/32000, 1/64000 and 1/128000 and 1/256000 folds, respectively. Panel 9 has two columns. From left to right, each was exposed to a mouse anti-gp120 and a mouse anti-OmpA, respectively.

12 suggested that cells carrying more optimized vaccine epitopes elicited a stronger humoral response against the epitopes. The only exception was clone 7. The mean fluorescence data for clone 7 in JM101 was 14.1, and the epitope was not recognized in *Salmonella*. As mentioned earlier, staining *Salmonella* is difficult because of its full length LPS. The mean fluorescence data for the *Salmonella* strain might not reflect the exposure of the epitope at all. In the fluorescence data for *E. coli*, all clones showed very similar fluorescence intensity, from 13.3 (clone 4) to 15.7 (clone 12). The exception was clone 9, which had an intensity of 20.2. Because of small variation in the fluorescence, I concluded that the clones did not show a large variation with regard to optimization of the epitope. All clones (except clone 9) projected the epitope on the cell surface in a similar manner, and the little variation of the reactivity of the sera towards the TOP epitope confirmed that the epitope was presented on the cell surface approximately in a similar fashion.

6. ELISA Analysis of Mouse Sera

Sera obtained from mice immunized with *E. coli* and *Salmonella* expressing LamB:V3 chimeric proteins were also analyzed by ELISA. In this analysis, the purified envelope protein-gp120 from HIV-1 was coated on ELISA plates. The gp120 coated plates were incubated with the sera obtained from the mice immunized with *E. coli* or *Salmonella* that expressed LamB:V3 chimeric

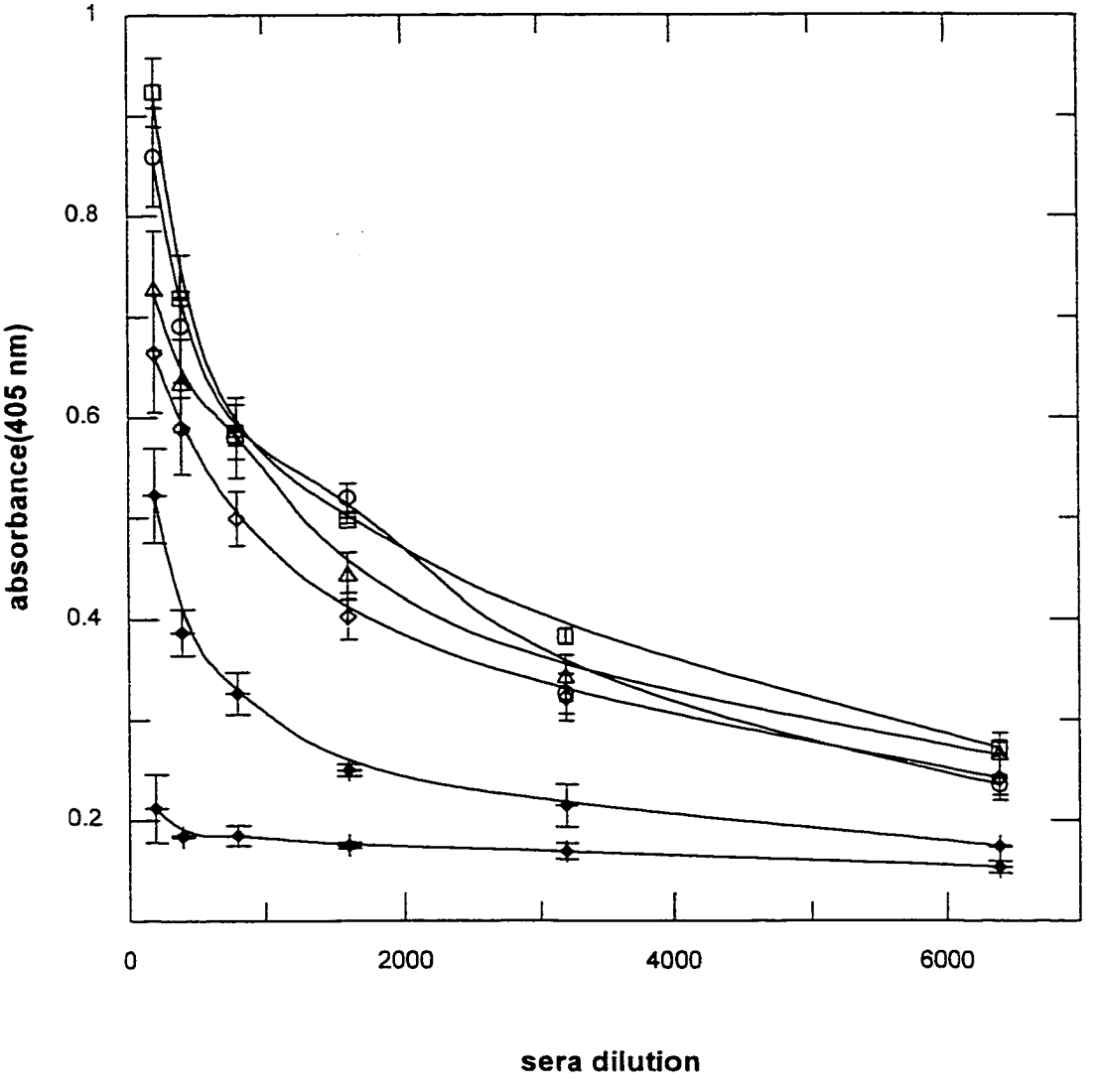

Figure 3.12 Humoral response to *E.coli*/LamB:V3 antigen. ELISA plates were coated with the envelope protein gp120 from HIV-1. The sera from a group of mice immunized with *E.coli* carrying LamB:V3 antigen was pooled. The ELISA plates were incubated with the pooled sera and developed with goat anti-mouse IgG (phosphatase conjugate). The average from three experiments is shown. (○) pX1V3; (●) pDV3; (□) pX1V3-23; (△) pX1V3-22; (◇) pX1V3-8; (✦) unimmunized mouse sera..

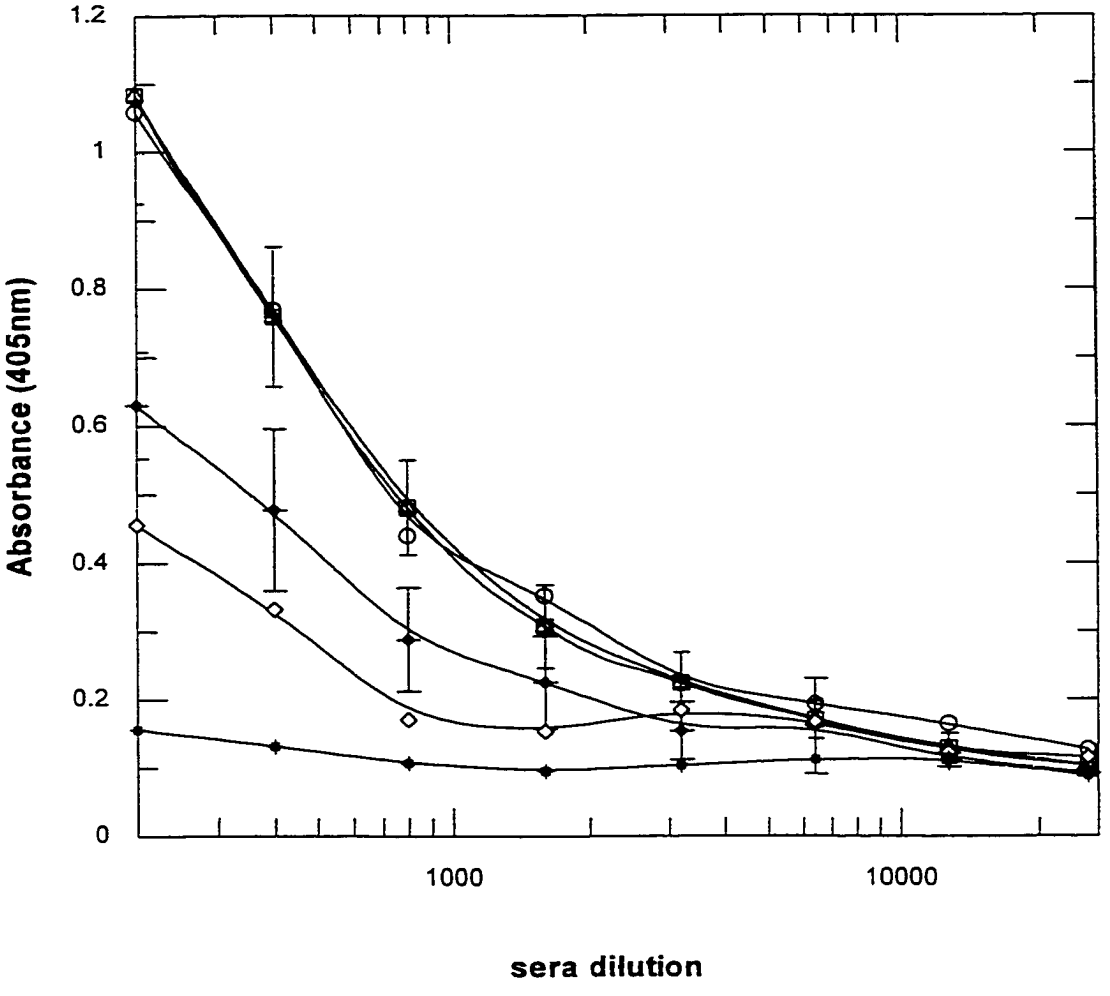

Figure 3.13 Humoral response to *Salmonella*/LamB:V3 antigen. ELISA plates were coated with the envelope protein gp120 from HIV-1 and incubated with the sera obtained from mice immunized with *SL3261* carrying LamB:V3 antigen. The serum from individual mouse was analyzed and the average from ten sera is plotted on the graph. (O) pX1V3; (✦) pX1V3-8; (□) pX1V3-23; (△) pX1V3-22; (◇) pDV3; (✦) unimmunized mouse sera..

proteins on their surface. All LamB:V3 constructs expressed in *E. coli* showed activity towards gp120, compared to the sera collected from unimmunized mice (Figure 3.12). The anti-pDV3 sera exhibited the lowest titer, whereas the most optimized LamB:V3 vaccine construct, pX1V3-N23 showed the highest titer. The serum from each individual mouse immunized with attenuated *Salmonella typhimurium* strain SL3261 was analyzed by ELISA, and the average of the ten sera against each different LamB:V3 constructs is plotted in Figure 3.13. All constructs that were expressed in SL3261 elicited a slight stronger humoral immune response than those that were expressed in *E. coli* strain pop6510, except for pDV3 constructs. The highest anti-gp120 antibody titer that was raised against pX1V3-23 and pX1V3-22 was 2500, whereas the lowest anti-gp120 antibody titer that was induced against pX1V3-8 was 900. The least recognized construct by anti-gp120 antibody, pX1V3-8, induced a weaker humoral response. Two constructs, pX1V3-N22, and pX1V3-23 oriented the vaccine epitope-V3 in such a manner that the epitope was most accessible to the cell surface, and elicited a stronger humoral response in both *E. coli* and *Salmonella*, suggests that optimized vaccine epitopes elicited a stronger humoral immune responses. For unknown reasons, the sera raised against construct pDV3, that encoded a mutant of LamB without the L9 loop, also slightly reacted with gp120. A conceivable explanation is that mouse sera contained antibody that cross reacted against

gp120. However, this non-specific reaction between the sera and gp120 did not obscure the specific reaction between the sera and the V3 loop.

CHAPTER IV

DISCUSSION

Viruses and pathogenic bacteria frequently infect hosts through mucosal membranes. Effective new vaccines should elicit both systemic and mucosal immune responses. Vaccines can only achieve this goal by making contact with mucosal surfaces. Attenuated live *Salmonella* vaccines can be delivered to hosts by oral route to induce humoral, cellular and mucosal IgA immune responses. For this reason, attenuated live *Salmonella* has become the most popular carrier for heterologous antigens. Antigens from viruses, bacteria, parasites and fungi expressed in attenuated *Salmonella* elicit humoral, cellular and mucosal immune responses to a variety of heterologous antigens. These foreign antigens were fused to various *Salmonella* proteins such as flagella, outer membrane porins, and periplasmic proteins) and expressed as chimeric proteins. Alternatively, they were

expressed in *Salmonella* as plasmid-encoded whole antigens. Not only can heterologous proteins be expressed in *Salmonella*, but foreign epitopes can also be delivered to the immune system using *Salmonella* as a live vaccine. This strategy of subunit vaccine has great potential in multivalent vaccine design. It is difficult to co-express different antigens from different microorganisms in either *Salmonella* or *E. coli*. However, by adopting the subunit vaccine strategy, many foreign epitopes from different microorganisms can be fused to carrier proteins and expressed as chimeric antigens in *Salmonella*.

The exact factors that contribute to the efficacy of subunit vaccines based on attenuated *Salmonella* are not well defined. Besides the immunogenicity of the chosen epitope, the methods that present the epitope to the immune system may play a vital role in defining immunogenicity of vaccines. Generally speaking, foreign epitopes can be presented to the immune system by *Salmonella* as a secreted antigen, a chimeric protein expressed on the bacterial surface, or as a chimeric protein that resides in the periplasm. It was reported that a secreted protein antigen provided better protective efficacy than the same antigen expressed intracellularly in *Salmonella* (Hess et al., 1996). The result supported the hypothesis that antigens that are extracellularly accessible are better recognized by the immune system than antigens that reside inside bacteria. In our study, we developed a method to present a heterologous epitope to the immune system as a chimeric protein expressed on the bacterial surface. We chose two

outer membrane proteins of *E. coli*, LamB and OmpA, as the carriers for the vaccine epitope V3 loop (residues 293 to 334) of gp120 of HIV-1 and TOP epitope (a part of V3 loop, residues 309 to 320). The use of LamB as an expression vector for heterologous epitopes has been extensively studied [for a review, see (Charbit et al., 1997)]. The structure of LamB and other outer membrane porins (Weiss et al., 1991; Cowan et al., 1992; Weiss and Schulz, 1992; Kreusch et al., 1994) have been solved. Using the structural data of these proteins, heterologous epitopes can be inserted into the cell surface loops of LamB or OmpA LamB or OmpA exposing the epitope to the extracellular environment. A common structural feature of these proteins is that they all contain multiple external loops with different degrees of magnitude and exposure to the extracellular environment. Loop 9 (L9) of LamB exhibits hypervariability at the top. Therefore, it was expected that this loop would be able to accommodate significant alterations in its primary structure. Experiments with LamB mutants have suggested that this loop is important to maltoporin structure and physiology (Klebba et al., 1994).

Based on these results, the C-terminal loop of maltoporin appeared to be an ideal site for epitope insertions. The chimeric proteins constructed by insertion of epitopes into the loop 4 (L4) of maltoporin resulted in poorly expressed unstable chimeras (Charbit et al., 1993). This low level of expression of L4-based chimeric proteins was probably caused by structural defects introduced by

the insertion of polypeptides. In our study, we replaced the L9 (residues 379 to 401) of LamB with the V3 epitope of HIV gp120. The advantage of our approach is obvious: LamB:V3 hybrids were stably expressed in both *E. coli* and *Salmonella*: no degradation was observed.

How can we optimize the exposure of the inserted epitope on the bacterial surface, making it more accessible to the extracellular environment and will be well recognized by the immune system? The orientation and the exposure of the inserted epitope depend on the amino acid sequences surrounding the epitope, because the bends and turns created by the neighboring amino acids will orient and project the epitope differently. In order to optimize the exposure of the inserted epitope, we inserted three amino acids immediately upstream and downstream to the epitope, at its junction with the carrier protein by PCR random mutagenesis. With this method, we obtained a LamB:V3 or OmpA:TOP chimeric protein library in which all members were different from each other only at the flanking regions between the carrier protein and the epitope. Therefore, we expected the orientation and exposure of the epitope in each chimeric protein to be different.

For LamB:V3 chimeric protein library, we analyzed 21 clones and the majority of the hybrids was expressed stably and at wild type levels. Degradation was observed for clones 7 and 13. On the other hand, for OmpA:TOP chimeric protein library, the expression of hybrids proved to be problematic. Degradation

was observed in some clones, and was often proportional to their OmpA:TOP expression level. Another problem we faced regarding OmpA:TOP constructs, was that the generation of the chimeric library proved more difficult than expected. The majority of bacteria transformed by mutagenized plasmids did not express full length OmpA:TOP protein. Rather, they expressed a truncated protein because of stop codons incorporated in either upstream or downstream of the epitope-specifying DNA sequence. This contrasts with the results obtained for LamB, in which only 2 of 21 clones sequenced, contained stop codons in their junctions. Apparently, bacteria expressing truncated OmpA or OmpA:TOP protein had a growth advantage over those expressing normal length OmpA:TOP protein and dominated the culture. We originally tried to express OmpA:TOP hybrid in an *ompA*$^-$ strain *of E. coli*, but without success. The transformation efficiency was extremely low and the expression level of OmpA:TOP was barely detectable by Western blot. This led us to transform an *ompA*$^+$ strain with the plasmids encoding OmpA:TOP hybrids. Although the transformation of *ompA*$^+$ strain posed no problem, bacteria carrying the plasmid grew slow on LB-ampicillin plates and cell lysis was observed when bacteria were re-inoculated in LB-ampicillin liquid media. However, when the bacteria were subcultured into liquid LB media with ampicillin and grown overnight, no cell lysis was observed. The mechanism behind this phenomena remains unclear. A conceivable explanation for the problems encountered with the OmpA:TOP expression is that

the over-expression of OmpA:TOP is toxic to the cells. It was less likely that insertion of TOP epitope into the L3 loop distorted the structure of the porin, since TOP epitope was recognized by an anti-gp120 monoclonal antibody on the bacterial surface, and could be labeled by fluorescent probes.

To optimize the exposure of the epitopes on the bacterial surface, we created a chimeric protein library for LamB:V3 fusion and a library for OmpA:TOP fusion. We expected that the V3 loop or TOP epitope was oriented on the bacterial surface differently and exposed to the extracellular environment in to different degrees. We confirmed this idea by measuring the mean fluorescence intensity of bacteria expressing the chimeras labeled by the fluorescence probe. Because mice anti-gp120 was available and recognized both V3 and TOP epitope, bacteria expressing LamB:V3 or OmpA:TOP chimeric proteins were incubated with anti-gp120, and followed by incubation with FITC conjugated goat anti-mouse IgG.

Theoretically, if V3 loop or TOP epitope is accessible to the extracellular environment, antibodies can bind to them and the cells will be labeled by the fluorescence probe. The fluorescence intensity of the cells will be directly proportional to the amount of antibody molecules that bind to the epitope, which depends on the expression level of the chimeric protein and the degree of epitope exposure to the bacterial surface. If the expression level of the chimeric protein is equivalent among the different chimeric proteins, the fluorescence intensity of the

cells will be determined by the degree of the exposure of the epitope to the extracellular environment. Therefore, by measuring the mean fluorescence intensity of the cells, the degree of the exposure of the epitope to the bacterial surface will be evaluated.

We measured the mean fluorescence intensity of *E. coli* cells expressing LamB:V3 chimeric proteins, cells that did not express LamB:V3 hybrid, and a LamB mutant (pDV3). The results showed the mean fluorescence intensity for LamB:V3 chimeric proteins varied from 2.1 to 19.80, whereas the intensity for pDV3 was 0.11. Due to variations of cell growth and labeling conditions, fluorescence intensities varied in repeated experiments, but were always consistent when comparing with pDV3 and with each other. The difference of the mean fluorescence intensity among cells expressing different LamB:V3 constructs was significant and reflected the differentiation of the accessibility of V3 loop to extracellular environment caused by the difference of amino acid sequences at their junction regions among different constructs.

The mean fluorescence intensity for *E. coli* expressing OmpA:TOP hybrids was less heterogeneous. Three clones were not labeled because the expression of OmpA:TOP chimeric proteins was undetectable by Western blot. The levels for the remaining18 clones varied from 13.3 to 23.2. The clone with the highest mean fluorescence intensity also had the highest OmpA:TOP expression level and observable degradation of the chimeric protein. Staining

Salmonella expressing OmpA:TOP chimeric proteins was hindered by full length LPS of *Salmonella*. The majority of clones were not stained at all. Although a few clones were stained, the percentage of the stained cells in the population was low, at just about 50%. The mean fluorescence intensity varied from 5.47 to 8.47 for clones with a stained cell population of around 50%.

The next objective in our study was to investigate the correlation between the exposure of the epitopes on bacterial surface and the immunogenicity of the epitopes. How would the immune response be influenced by optimization of the exposure of the epitopes on the bacterial surface? Could the immune system differentiate the level of the epitope exposure on the cell surface and react differently towards antigens with the different degrees of epitope exposure? To address these questions, we chose constructs with high fluorescence intensity and constructs with low fluorescence intensity as vaccines for mice immunization. We analyzed the sera from immunized mice by Western blot and ELISA.

When analyzed by Western blot, the optimized vaccines, *Salmonella* carrying pX1V3-23 and pX1V3-22 elicited specific antibody response against V3 loop, whereas *Salmonella* carrying pX1V3-8 with the least accessible V3 loop on their surface, induced a very weak humoral response against gp120. ELISA data showed the same pattern. Vaccines with epitopes maximally exposed to the bacterial surface elicited anti-V3 antibody with a higher titer; vaccines with epitopes less exposed to the bacterial surface induced anti-V3 antibody with a

lower titer. The data suggested that the degree of the exposure of the epitope to bacterial surface correlates with immunogenicity of the epitope. The most exposed epitope is more immunogenic than a less exposed epitope.

The data from the sera obtained from mice, immunized with *Salmonella* expressing various OmpA:TOP constructs, was less conclusive. All constructs elicited anti-TOP antibody with a similar titer. It seemed that the data undermined conclusions we made, based on our experiments with LamB:V3 constructs. Upon further examination of all the data, we concluded that this is not the case. The mean fluorescence intensity for the OmpA:TOP constructs that were used in mice immunization varied from 13.3 to 20.2 in *E. coli*. This indicates that the epitope for all constructs was exposed to the cell surface, and well recognized by mice anti-gp120 antibody. The differentiation of the epitope exposure among these clones was not significant to elicit a different magnitude of immune response towards different constructs. On the other hand, they all raised antibody that specifically recognized TOP epitope. It would be better to analyze more clones by fluorescence measurement. This would give a better chance to find the clones with large differentiation in regard to the exposure of the epitope on bacterial surface. The fluorescent cell sorting technique would be an ideal method for selecting clones with the greatest epitope exposure. With fluorescence cell sorting, a large population of bacteria expressing chimeric proteins could be analyzed. The selection of clones with the most optimized epitope exposure

would not be limited to a few clones that were picked randomly at the beginning. It is quite likely that clones with maximized epitope exposure were not present in our analysis. Nevertheless, the vaccine vector we developed, the method we utilized to optimize the exposure of heterologous epitope, and the finding that immunogenicity of an epitope correlates to the exposure of the epitope on the bacterial surface, will play a significant role in new vaccine design and development.

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ABSTRACT

A peptide library was screened for the reactivity with a monoclonal antibody raised against the LPS of *Salmonella typhimurium*, and three peptides that reacted with O5-specific antibody were identified. These peptides were conjugated to bovine serum albumin (BSA) and ovalbumin (OVA) with divinysulfone (DVS), 1-ethyl-3-dimethylaminopropyl carbodiimide hydrochloride (EDAC) or glutaraldehyde, respectively. Mice were immunized intraperitoneally with the BSA-peptide conjugates and sera was collected. The reactivity of the sera towards OVA-peptide conjugates and *S. typhimurium* bacteria strain SL 3261 (O5+) and SL7517 (O5-) was analyzed by ELISA and cytofluorimetry. Early (10 days) sera showed cross reactivity between the different peptide immunogens, and for O5+, but not O5- LPS. The anti-peptide antibodies displayed the cross reactivity towards peptides.

CHAPTER V

PEPTIDE VACCINES

INTRODUCTION

Lipopolysaccharide (LPS) of Gram-negative bacteria is the outermost physical barrier of the bacteria. It protects cells from some deleterious agents such as antibiotics, host-defense proteins such as lysozyme, hydrophobic dyes, and detergents such as bile salts. LPS consists of three components: Lipid A, core oligosaccharide, and O-antigen (Rietschel, et al., 1992). In pathogenic bacteria, LPS plays an important role in the interaction between bacteria and their host and has great influence on the immune system. The lipid A moiety is the major contributor to the endotoxic effects of LPS, whereas O-antigen is the most immunogenic portion of the molecule (Rietschel, et al., 1992). The O antigen of

Salmonella consists of a polymer of a tetrasaccharide composed of galactose, rhamnose, and mannose in the main chain and abequose (3.6-dideoxy-glactose) attached to the mannose residue (Hellerqvist, et al, 1968). Serologically, *Salmonella typhimurium* O antigen is classified into three groups: O4, O5, and O12. The O12 antigen is complex but involves the trisaccharide of the main chain (Jorbeck, et al., 1979; Sevenson, et al., 1978). The abequose residue confers the O4 serotype (Jorbeck, et al., 1979), and if the abequose residue is acetylated on the 2 hydroxyl group, this confers the O5 serotype (Hellerqvist, et al, 1968).

LPS is a determinant of virulence for Gram-negative bacteria. Rough *Salmonella* mutants are less virulent because of the synthesis of incomplete LPS molecules, but may still retain their ability to survive intracellularly. Antibodies against LPS play a very important role against infection caused by Gram-negative bacteria. The O-antigen of LPS is capable of eliciting a humoral response by cross-linking antigen receptors on B cells. It has been evaluated as a potential vaccine against infection caused by Gram-negative bacteria. However, because O antigen is a T cell-independent antigen (Mitchell and Andrew, 1995; Andersson and Blomgren 1971), the humoral response is not boosted by repeated immunization. We studied the efficacy of peptides that mimic the O5-antigen of *Salmonella typhimurium*, for their ability to elicit an anti-LPS immune response. Using three peptides that were isolated from a phage display library with a monoclonal antibody against O5-antigen, we generated mouse antisera and tested

the reactivity of these antibodies against *S. typhimurium* O5 antigen. This research focused on the cross-reactivity of the antisera between the peptides and LPS.

MATERIAL AND METHODS

1. Bacterial Strains

Salmonella typhimurium strain SL3261 (*aroA his*, Hoiseth, et al., 1981), SL7517 were obtained from Stocker, B.A.D and used in cytofluorimetry analysis of mice sera.

2. Polypeptides

The amino acid sequences for three peptides used in this study and the nomenclature of peptide-protein conjugates are given in Table 5.1.

Table 5.1. Peptide sequence and peptide-protein conjugate nomenclature

Name	Coupling reagent	Peptide Sequence
BSA-D-Pep1	DVS	C-A-A-A-E-Y-W-K-F-K-E-R-COOH
BSA-D-Pep2	DVS	Ac-C-(G-L-W-P-N-L-C)-A-A-A-COOH
BSA-D-Pep3	DVS	C-G-L-W-P-N-L-C-A-A-A-COOH
BSA-G-Pep1	Glutaraldehyde	C-A-A-A-E-Y-W-K-F-K-E-R-COOH
BSA-G-Pep2	Glutaraldehyde	(Ac-C-G-L-W-P-N-L-C)-A-A-A-COOH
BSA-G-Pep3	Glutaraldehyde	C-G-L-W-P-N-LC-A-A-A-COOH
BSA-E-Pep1	EDAC	C-A-A-A-E-Y-W-K-F-K-E-R-COOH
BSA-E-Pep2	EDAC	(Ac-C-G-LW-P-N-L-C)-A-A-A-COOH
BSA-E-Pep3	EDAC	C-G-L-W-P-N-L-C-A-A-A-COOH
PL-G-pep2	Glutaraldehyde	(Ac-C-G-LW-P-N-L-C)-A-A-A-COOH

3. Coupling Reagents

Divinysulfone (DVS)

DVS is a homobifunctional reagents. Cross-links occurs through amino group and -SH group. Coupling peptides to DVS is a two-step process. In the first step, carrier protein (p) is activated with DVS; in the second step, peptide is coupled to DVS activated protein (A). Activation of a carrier protein by DVS is illustrated by the following:

$$p\text{-}CH2CH2CH2CH_2NH_2 + CH_2{=}CH\text{-}SO_2\text{-}CH{=}CH_2$$

$$\downarrow$$

$$p\text{-}CH_2CH_2CH_2CH_2NH\text{-}CH_2CH_2\text{-}SO_2\text{-}CH{=}CH_2 \quad (A)$$

where p stands for protein. The coupling reaction can occurs in a number of different ways, depending on the pH of the buffer (Houen and Jensen, 1995):

1. at pH 10

$$A + HO\text{-}R \rightarrow p\text{-}CH_2(CH_2)_3NH\text{-}CH_2\ CH_2\text{-}SO_2CH_2CH_2\text{-}O\text{-}R$$

2. at pH 8

$$A + HS\text{-}R \rightarrow p\text{-}CH_2(CH_2)_3NH\text{-}CH_2\ CH_2\text{-}SO_2CH_2CH_2\text{-}S\text{-}R$$

3. at pH 9

$$A + H_2N\text{-}R \rightarrow p\text{-}\ p\text{-}CH_2(CH_2)_3NH\text{-}CH_2\ CH_2\text{-}SO_2CH_2CH_2\text{-}NH\text{-}R$$

Glutaraldehyde

Glutaraldehyde also serves as a homobifunctional linker (Dent and Aslam, 1998) and the reaction mechanism is illustrated by Figures 5.1 and 5.2.

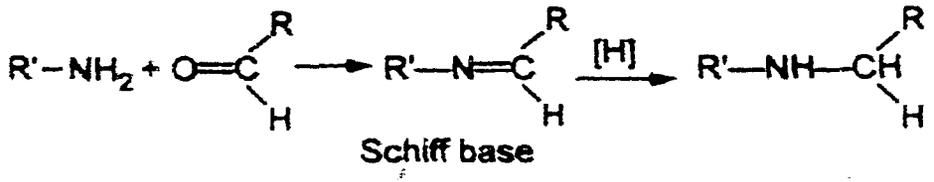

Figure 5.1. Reaction of amines with aldehydes.

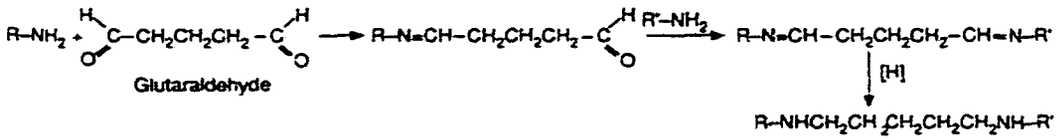

Figure 5.2. Use of glutaraldehyde as a homobifunctional reagent.

1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC)

EDAC (Figure 5.3) is a carbodiimide (Figure 5.4). The reactions of carbodiimide with nucleophiles are illustrated (Dent and Aslam, 1998) by Figure 5.5.

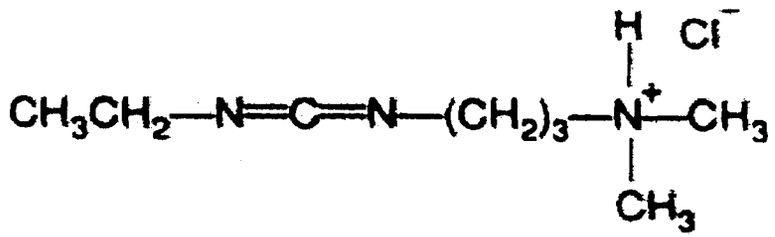

Figure 5.3. Structure of EDAC hydrochloride.

Figure 5.4. Structure of Carbodiimides.

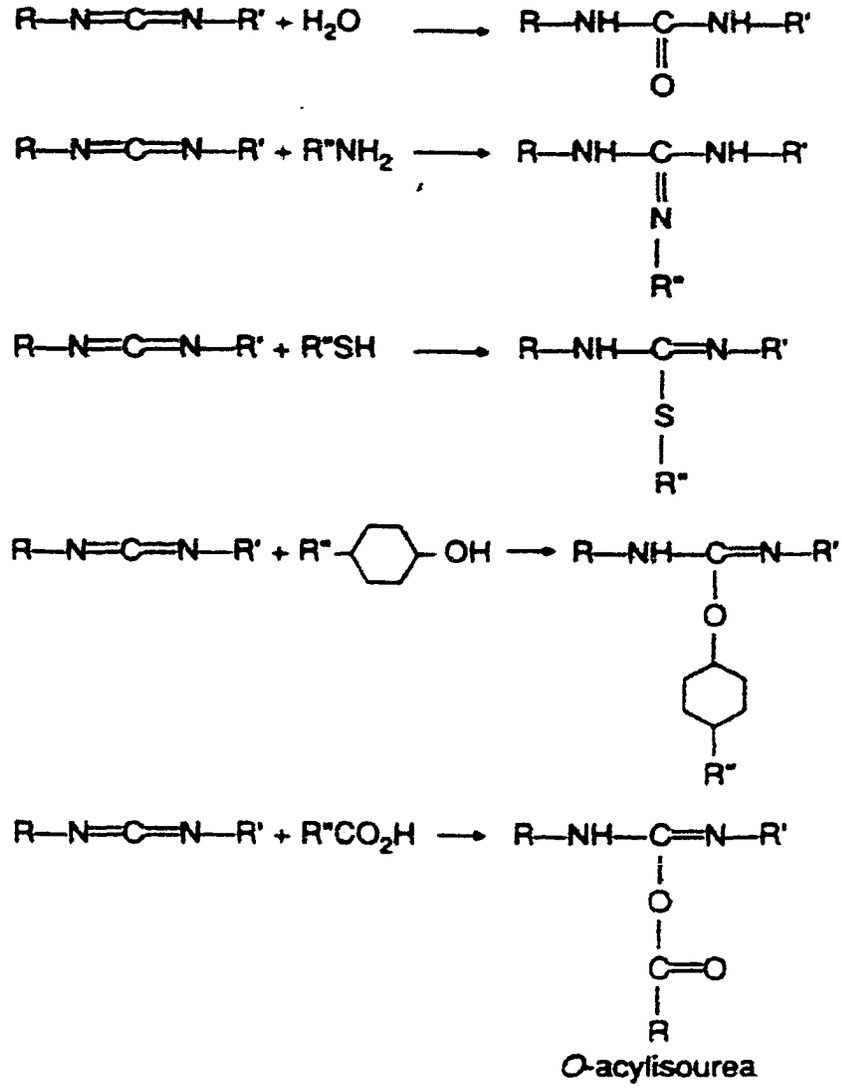

Figure 5.5. Reactions of carbodiimides with water, amines, thiols and carboxylic acids.

4. Conjugation of Peptides

A. DVS Preactivation of BSA and OVA

100 mg of bovine sera albumin (BSA) or ovalbumin (OVA) were dissolved in 10 ml 0.1 M Na_2CO_3 (pH 10.0) and 1ml of divinysulfone (DVS) was added to the solution. The mixture was stirred gently and kept at room temperature overnight. The reaction mixture was loaded on a G-50 column equilibrated with PBS (pH 7.4). The fractions were collected and the absorbance at 280 nm of each fraction was measured. Two or three fractions with the highest absorbances were consolidated and used for the coupling of peptides.

B. Iodination of Peptide

In order to monitor the efficiency of the peptide-protein, the peptide was iodinated before coupling. The iodination takes place at tyrosine residues and the mechanism of iodination (Dent and Aslam, 1998) is illustrated in Figure 5-6. Peptide 1 was dissolved in PBS buffer, to a final concentration of 1mg/mL. Forty µl of 0.3 M Na_2HPO_4, 10 µl of ^{125}I (1 mCi), and 100 µl of chloramine T (2 mg/mL) were added to 200 µl of the peptide solution The reaction was kept at room temperature for 1 minute and quenched by adding 200 µl of sodium metabisulfite ($Na_2S_2O_5$, 4.8 mg/ml in H_2O), and 200 µl of saturated tyrosine solution (in PBS). Finally, the reaction mixture was loaded on a 10 ml G-50 column equilibrated in 50 mM Tris-HCl (pH7.6), 0.1% BSA. Fractions with 40

drops each were collected. 5 μl of each fraction was removed for to γ counting (Figure 7). Two fractions with the highest γ counting were pooled together and used in coupling reactions.

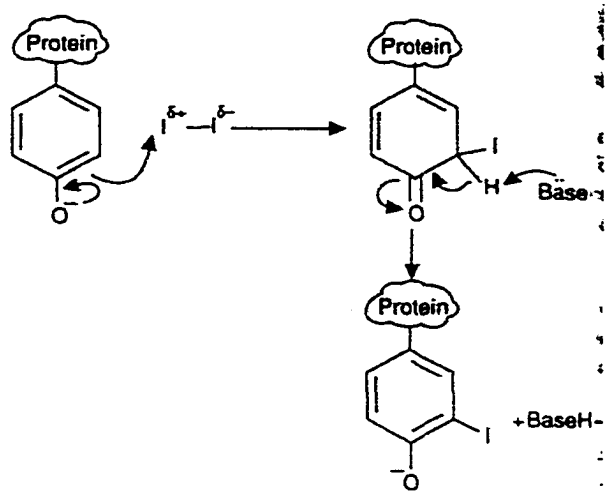

Figure 5.6. mechanism of iodination of protein tyrosine based on molecular iodine.

C. Conjugation of Peptides to BSA with DVS

100 μg of peptide were dissolved in 100 μl of 0.05M NaH_2PO_4. 50 μl of iodinated peptide(10^6 cpm) and 1.5 mL(1.5 mg/mL) DVS-preactivated BSA were added to the solution and the mixture was kept at room temperature overnight. BSA-DVS-peptide conjugate was purified on a sephadex G-50 column (Figure 5.8).

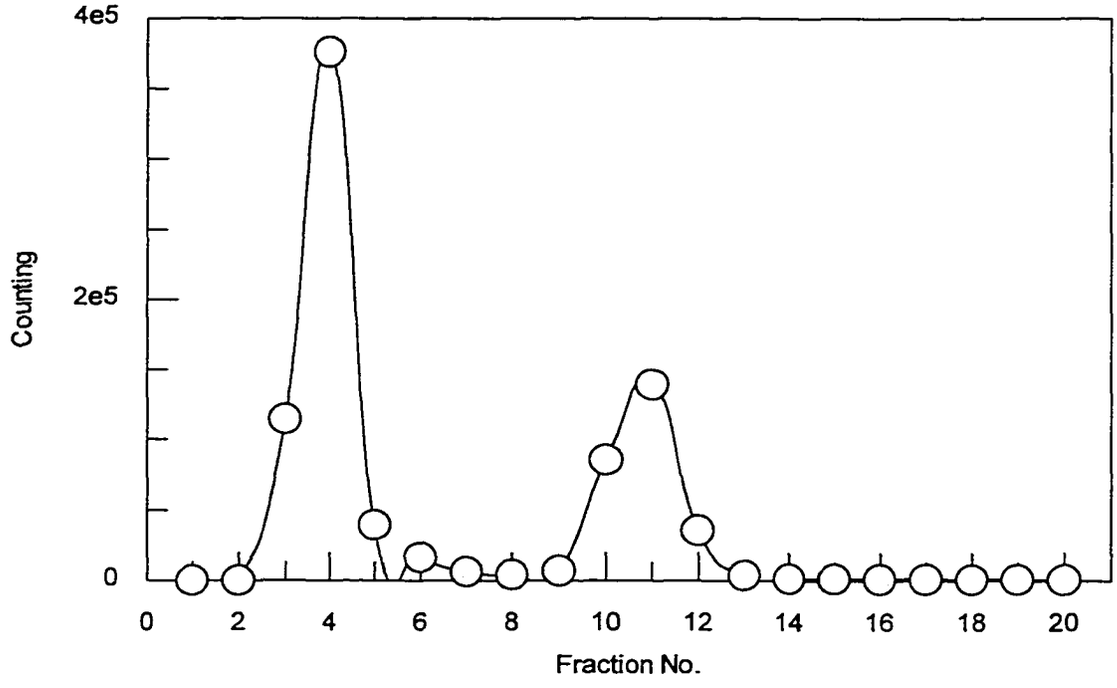

Figure 5.7. Iodination of peptide. peptide 1 was iodinated by chloramine T method and purified on a G-50 column. 5 μl of each fraction were taken to do γ counting.

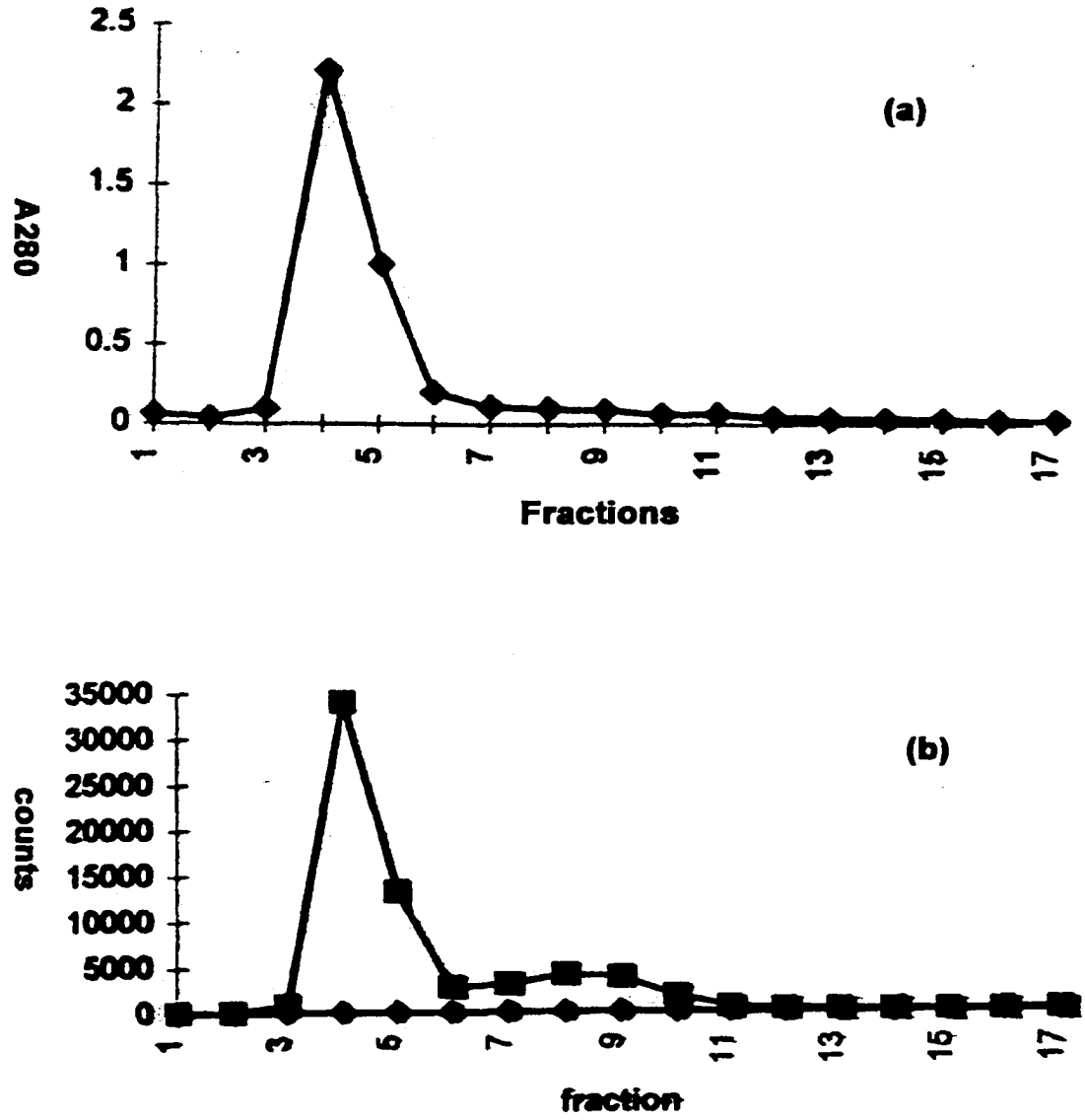

Figure 5-8. Coupling peptide to BSA-DVS 100 μg of peptide in 100 μl solution in 0.05M NaH_2PO_4, 50 μl of iodinated peptide (10^6 cpm) were mixed with 1.5 ml(1.5 mg/ml) of DVS-activated BSA and reacted overnight at room temperature. The BSA-DVS-peptide conjugate was purified on a sephadex G-50 column. **A.** absorbance (280 nm) of the of G-50 column fractions. **B.** γ counting (cpm) of 10 μl of G-50 column fractions.

D. Conjugation of Peptides to BSA with Glutaraldehyde

One mg of peptide and 1 mg of BSA were dissolved in 1 mL PBS. An equal volume of 2% of glutaraldehyde was added into the solution drop by drop with constant stirring. After 1 hour, the reaction was stopped by addition of sodium borohydride ($NaBH_4$) to a final concentration of 10 mg/mL. The mixture was kept at 4^0C for 1 hour and dialyzed against PBS (three changes) and purified on a G-50 column

E. Conjugation of Peptides to BSA with EDAC

Peptide and BSA were dissolved in 0.1M MES(2-[N-morpholino]ethanesulfonic acid, pH 6.0) to a final concentration of 4 and 10 mg/mL, respectively. To the mixture of 500 µl of peptide solution and 200 µl of BSA solution, 10 mg EDAC were added. The reaction mixture was stirred at room temperature for two hours. Peptide-BSA conjugate was purified on a sephadex G-50 column.

F. Conjugation of Peptides to Polylysine with Glutaraldehyde

22 µl glutaraldehyde (10% in PBS) were added to a mixture of 200 µl peptide solution (1 mg/mL in PBS) and 2 mL polylysine (PK) solution (1 mg/mL). The reaction was kept at 4^0C overnight and dialyzed against PBS (three changes).

5. Immunization of Mice

A total of six antigens were selected to immunize mice: BSA-D-pep1, BSA-D-pep2, BSA-D-pep3, BSA-G-pep1, BSA-G-pep2, BSA-G-pep3. For each antigen, ten mice were immunized intraperitoneally four times over a four week period. Each mouse received 60 μg of peptide conjugation per injection. Freund's complete adjuvant was used for the 1st injection, and for the subsequent immunizations, Freund's incomplete adjuvant was used. Immunized mice were bled at day 10 after the 1st injection, and one week after the last injection. Mice that were immunized with BSA-D-Pep1 conjugate were boosted with free peptide Pep2 twice, late in the immunization regime. One week after each injection, sera was collected .

6. Analysis of Mouse Sera

A. ELISA

Twenty microgram of antigens (peptide-Ovalbumin conjugates) were diluted to 20 mL with 0.01M NH_4Ac, 0.01M Na_2CO_3, pH 8.3. Each well of microtiter plates (Nunc, Roskilde, Denmark) were coated with 50 μl of the antigen solution. When using *Salmonella typhimurium* as an antigen, microtiter plates were coated with 50 μl suspension of bacteria (10^8 cells/mL) in 0.01M

NH_4Ac, 0.01M Na_2CO_3, pH 8.3. The plates were kept overnight at 37°C. The plates were blocked with 200 µl of TBS-0.1% OVA. After 1 hr, the plates were emptied and refilled with 50 µl of TBS-0.1%OVA. 5 µl of the sera dilution were added to each well and incubated at room temperature for 3 hours. Plates were washed three times with PBS-Tween-20 (0.05%). One hundred microliters of Goat α-Mouse Ig-alkaline phosphatase conjugate (1 to 1000 dilution with TBS-0.1% OVA) were added to each well. The plates were kept at room temperature for 1 hour, and washed three times with PBS-Tween-20 (0.05%). 50 µl of p-nitrophenyl phosphate (Sigma 104) 1mg/mL in substrate buffer (0.1M Tris pH 9.5, 0.1M NaCl, 5mM $MgCl_2$) were added to each well. The reaction was allowed to proceed for one to two hours and stopped by addition of 50 µl of 1M NaOH. Absorption at 405 nm was measured on the Titertek Multiskan MCC mircrotiter plate reader.

B. Cytofluorimetry Assay

Bacteria strains of SL7517 05- and SL3261 05+ were grown in LB medium overnight at 37°C. 10^8 cells were harvested in 1.5 mL eppendorff tubes by centrifuging for 1 minute at 14,000 rpm. The supernatant was discarded and cells were resuspended in 50 µl mice sera [1:500 dilution in TBS-1% gelatin (TBSG)] and incubated for 45 minutes at room temperature. After adding 1 mL of TBS, cells were centrifuged and resuspended in 50 mL fluorescein (FITC)

conjugated goat anti-mouse IgG (1:50 dilution, in TBSG). After 40 minutes of incubation, 1 mL of TBS were added to the cells. The cells were pelleted and resuspended in 0.5 mL TBS, 0.5 mL TBSG. The mean fluorescence intensity of FITC-labeled cells was measured with a Coulter EPICS ELITE cytofluorimeter.

RESULT

1. Analysis of Mouse Sera by ELISA

Mice were immunized with peptide conjugated to BSA and analyzed against OVA or polylysin conjugates to avoid the reaction between the sera and BSA which masks the specific reaction against the peptide. The reactivity of the sera raised against BSA-EDAC-peptide2 and BSA-DVS-peptide1 conjugates with various antigens is given in Figures 5.9, 5.10, and 5.11. Sera anti-BSA-EDAC-peptide2 reacted strongly with polylysine-glutaraldehyde-pep2 conjugate (Figure 5.9), which means that the sera specifically recognized peptide2. Sera anti-BSA-DVS-peptide1 recognized OVA-Glutaraldehyde-pep1 (Figure 5.10). The most interesting result was obtained from mice anti-BSA-DVS-pep3. This sera not only recognized peptide 3, but it also reacted strongly with OVA-DVS-peptide1, almost with the same intensity as peptide 3. Although we also observed the cross- reactivity displayed by the sera raised against peptides 1 and 2, the sera raised against BSA-DVS-pep3 demonstrated phenomenal cross-reactivity. We also noticed that the sera raised against peptide 2 recognized peptide 3 very well, and vice versa. This was not at all surprising, because peptides 1 and 3 only differ at the N-termini. The N-termini of peptide 3 was acetylated. This data indicated

that specific antibodies against peptide1, peptide 2,and peptide 3 were elicited in mice. However, all three sera did not show any specific activity towards *Salmonella* SL3261 05+ or SL7517 O5-.

To further investigate the cross reactivity of sera, we analyzed the reactivity of various mice sera toward two peptide antigens: OVA-DVS-peptide3, and OVA-Glutaraldehyde-peptide1. The result for the reactivity of OVA-DVS-peptide3 is given in Figure 5.12. This antigen displayed the highest reactivity towards the sera raised against BSA-DVS-peptide 1, but showed no reactivity towards the sera raised against BSA-Glutaraldehyde-peptide1 conjugate. The reactivity of the antigen with the sera raised against BSA-glutaraldehyde-3 was lower than with the sera raised against BSA-DVS-peptide 1. Figure 5.13 shows that the highest reactivity of OVA-glutaraldehyde-peptide1 with various sera did not come from the sera raised against the same peptide. Rather, it came from a different peptide, BSA-glutaraldehyde-peptide3. This data clearly demonstrated that a serum raised against a peptide could recognize a peptide that had different amino acids due to the similarities of structure of two peptides.

2. Analysis of Mouse Sera by Cytofluorimetry

SL3261 O5+ and SL7517 O5- bacteria were incubated with sera raised against three peptides and stained with a fluorescent probe. The result is given in

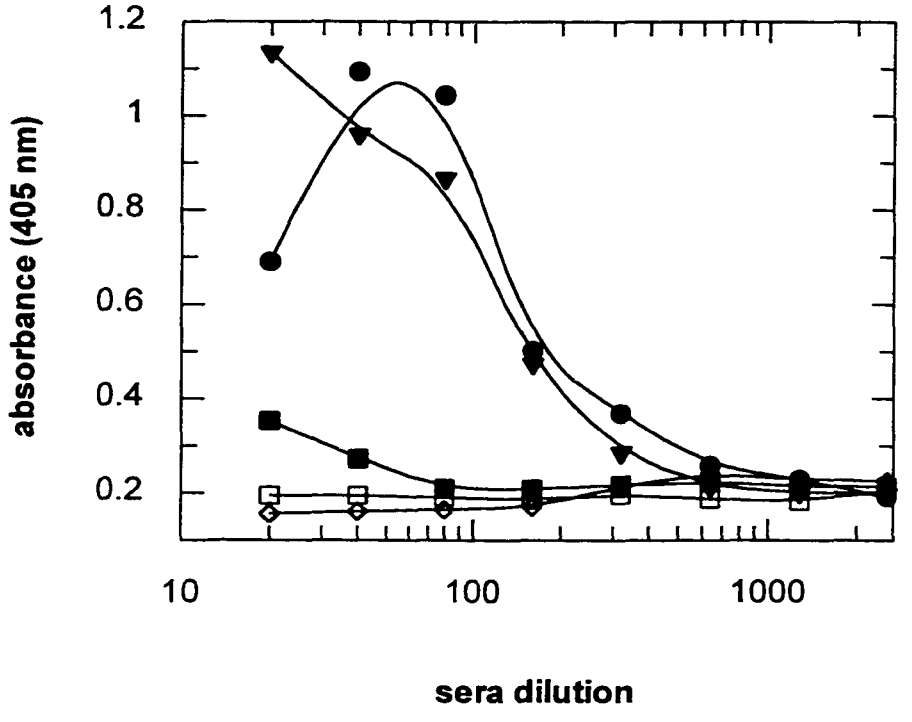

Figure 5.9. Analysis mouse anti BSA-E-pep2 sera from mice immunized with BSA-E-pep2 antigen was analyzed for the reactivity with various peptide conjugates by ELISA. (●) polylysine-G-pep2, (▼) OVA-D-pep3; (■) OVA-D-pep1; (□) SL3261 O5+; (◇) SL7517 05-

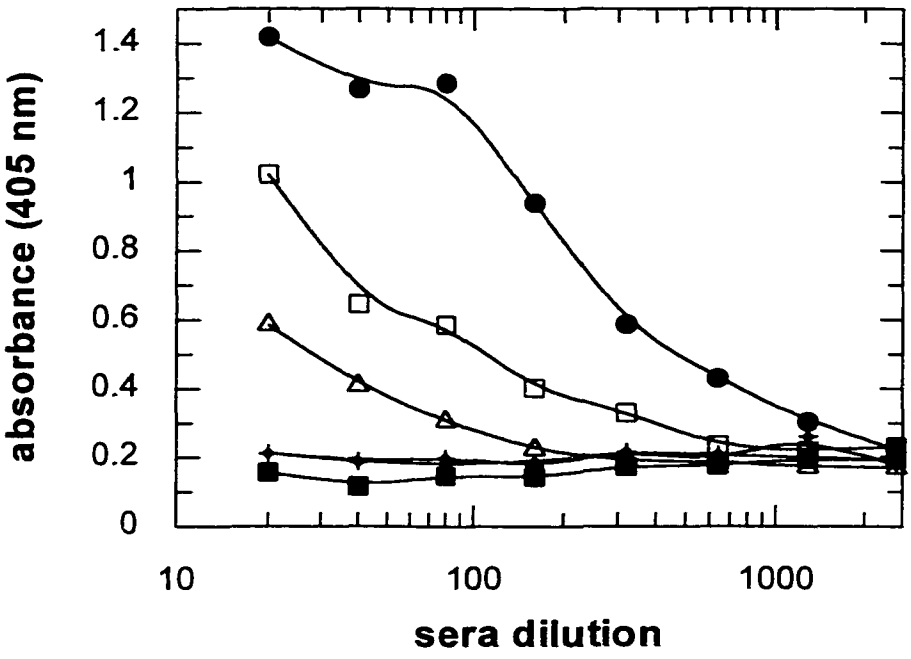

Figure 5.10. Analysis of mouse anti-BSA-D-pep1. sera from mice immunized with BSA-DVS-pep1 antigen was analyzed for the reactivity with various peptide conjugates by ELISA. (●) OVA-G-pep1; (□)OVA-D-pep1; ()(△) OVA-D-pep3; (○) polylysine-E-pep2; (✦)SL3261 O5+; (■) SL7517 05-

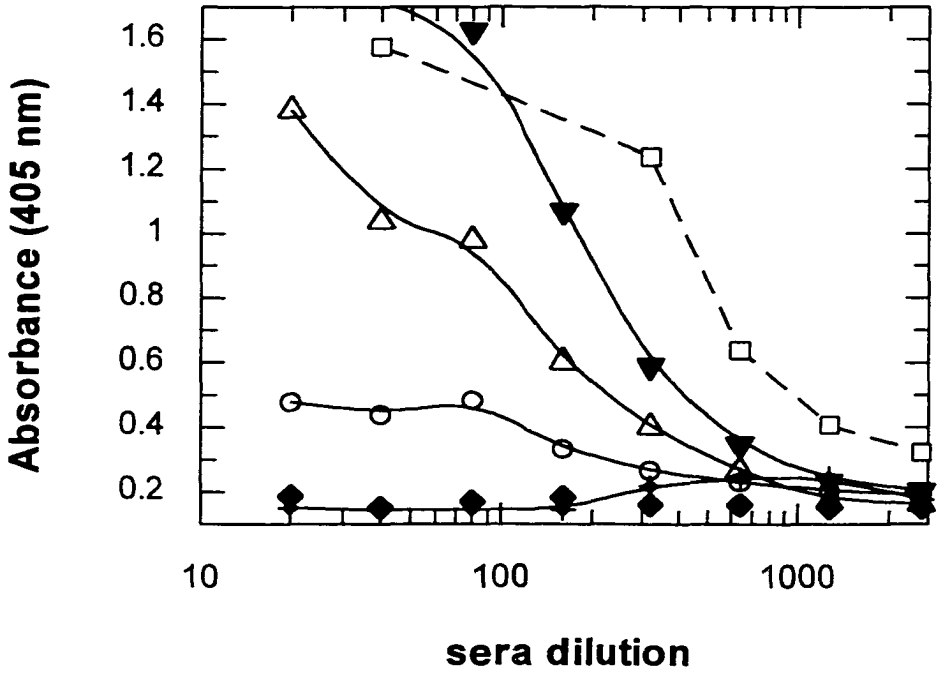

Figure 5.11. Analysis of mouse anti-BSA-D-pep3. sera from mice immunized with **BSA-DVS-pep3** antigen was analyzed for the reactivity with various peptide conjugates by ELISA. (O)polylysine-E-pep3; (□)OVA-D-pep3; ()(△) OVA-G-pep1; (▼) OVA-D-pep1; (✦)SL3261 O5+; (◆) SL7517 05-.

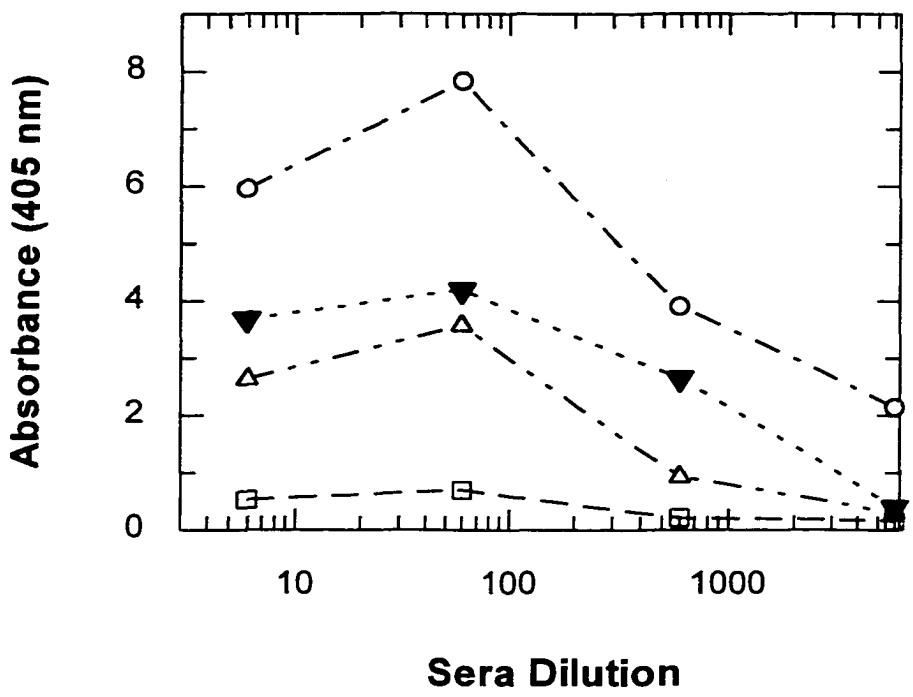

Figure 5.12. The reactivity of mouse sera toward OVA-D-pep3 conjugate. ELISA plates was coated with OVA-DVS-peptide3 conjugate and tested for the reactivity towards mice sera raised against various peptide conjugates. (▼) BSA-G-pep3; (□) BSA-G-pep1; (△)BSA-D-pep3; (○)BSA-D-pep1.

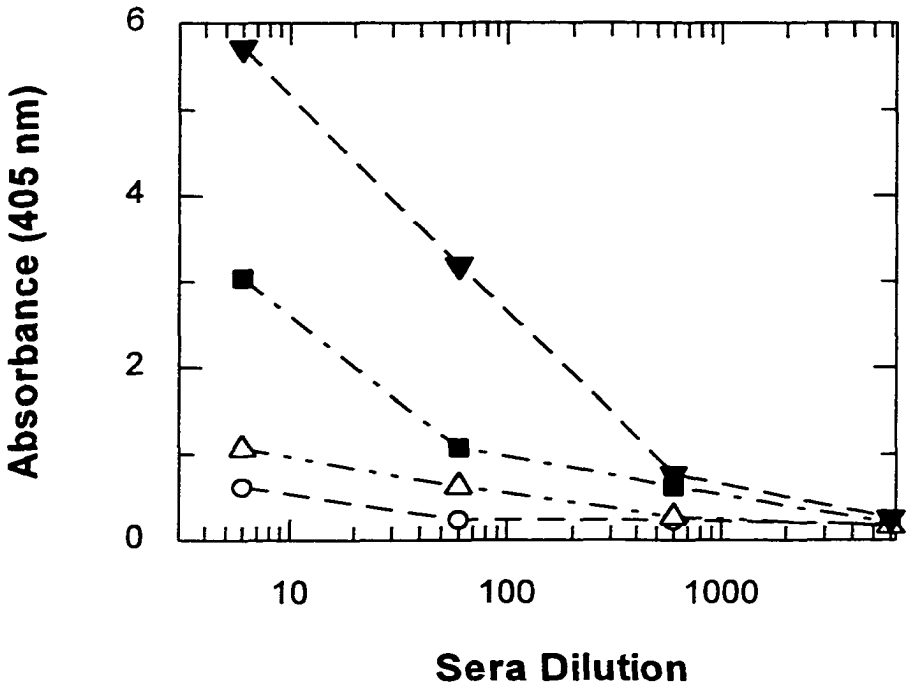

Figure 5.13. The reactivity of mouse sera toward OVA-G-pep1 conjugate. ELISA plates was coated with OVA-glutaraldehyde-peptide1 conjugate and tested for the reactivity towards mice sera raised against various peptide conjugates. (▼) BSA-G-pep3; (■) BSA-G-pep1; (Δ) BSA-D-pep3; (O)BSA-D-pep1;

Table 5.2. Sera raised against BSA-glutaraldehyde-peptide 2 and BSA-glutaraldehyde-peptide1 bound to SL3261 O5+ strain, but not SL7517 O5- strain. However, the percentage of cells labeled was low, only 21 and 10.2%, respectively.

Table 5.2. The reactivity of mice sera with LPS O5+ and LPS O5- antigens

sera	strain	percent	fluorescence intensity	percent	fluorescence intensity
BSA-D-pep2	O5+	96.1	0.2		
BSA-G-pep2	O5+	76.1	0.2	21.2	39.2
BSA-D-pep1	O5+	98.1	0.2		
BSA-G-pep1	O5+	87.8	0.2	10.2	46.4
BSA-E-pep1	O5+	95.5	0.2		
presera	O5+	97.6	0.2		
BSA-D-pep2	O5-	97.1	0.3		
BSA-G-pep2	O5-	96.2	0.3		
BSA-D-pep1	O5-	98.3	0.2		
BSA-G-pep1	O5-	97.2	0.3		
BSA-E-pep1	O5-	98.2	0.2		
presera	O5-	99.9	0.2		

DISCUSSION

The synthetic peptides used in the present work were defined as cross-reactive with *Salmonella typhimurium* LPS by screening a phage-display library with a monoclonal antibody specific for the O5 determinant. Two different peptides were synthesized and conjugated to BSA by different coupling reagents. To avoid the detection of the carrier protein in our ELISA assays, we coupled each of the peptides to two different carriers, bovine serum albumin (BSA) and ovalbumin (OVA). Polylysine was also used as a carrier for peptide 2, which has the same amino acid sequence as peptide 3, except that its N-termini was acetalyted. BSA-peptide conjugates were used for mice immunization and OVA-peptide conjugates were used for analysis of mice sera. Our results show high cross-reactivity between peptides 1 and 3, which indicates they share some common antigenic components. Since both peptides were primarily isolated through the use of the same monoclonal antibody, our results are not surprising: they confirm the potential of the technique of phage display for the selection of antigens sharing common epitopes.

However, some of our findings are not what we expected: antigen OVA-DVS-peptide3 displayed a stronger reactivity towards the sera raised against BSA-DVS-peptide1 than towards any sera raised against peptide 3; antigen OVA-glutaraldehyde-peptide1 showed stronger reactivity towards the sera raised against BSA-glutaradehyde-peptide3 than towards sera raised against peptide 1. We noticed that for both situations, the antigen and the conjugates used to immunize mice shared the same coupling reagents. However, we are sure that this "super" cross reactivity was not caused by antibodies specific for coupling reagents that might be in sera. If this was true, antigen OVA-DVS-peptide3 would had displayed a stronger reactivity towards BSA-DVS-peptide3, not towards BSA-DVS-peptide1 for obvious reasons. This "super" cross-reactivity might be caused by how it was linked to a carrier and the carrier that was linked to. The conformation of a peptide on a carrier protein should influence the immunogenicity of the peptide and its ability to react towards a sera raised against a peptide with different amino acid composition, but sharing similar conformation.

Two sera displayed specific binding to SL3261 O5+ strain as shown in cytofluorimeter analysis of mouse sera. However, the significance of the data is undermined by the fact that only a small population of the bacteria was labeled. We experienced difficulties in labeling epitopes on the bacterial surface of *Salmonella typhimurium* strain SL3261 because of full length LPS. LPS may

shield antigenic epitopes from being recognized by antibodies. Moreover, cells expressing full LPS are difficult to pellet under the conditions of fluorescence labeling.

Antibodies raised against Peptide 1 and Peptide 3 were unable to react specifically with *Salmonella typhimurium* O5+ strain in whole-cell ELISA. This was not what we expected. Peptides used in this study share structural similarities with each other, indicated by the fact that they reacted with the monoclonal antibody raised against *Salmonella typhimurium* O5-antigen. We expected that sera raised against these peptide would specifically recognize *Salmonella typhimurium* O5-antigen. The molecular basis for the interaction between an antigen and an antibody molecule lies on the complement of their structure, just as a protein-protein and enzyme-substrate interaction. The antibody repertoire of the immune system pre-exists before antigen exposure. Theoretically, when the immune system encounters an antigen, those B cells whose antigen receptors have the highest affinity for the specific antigen are activated. Cross-reactivity of antibodies arises because of an enormous antigen repertoire and the structural similarities among different antigens, which is the basis for the reactivity between these two peptides and antibodies raised against O5-antigen. We expected there would be structural similarities between antibodies raised against the peptides and O5-antigen. As a result, we expected that antibodies raised against the peptide would have reacted with O5-antigen. We hope that our findings regarding

"super" cross-reactivity will encourage people who are doing similar research and our data from whole-cell ELISA will not discourage people from trying harder.

The current vaccine against *Streptococcus pneumoniae* consists of purified pneumococcal capsular polysaccharide. The major shortcoming of polysaccharide vaccines is that they do not confer protection for children under age 2 (Mitchell and Andrew, 1995; Andersson and Blomgren 1971). This is caused by the fact that polysaccharide is T-independent antigen. Vaccines consisting of peptides have the potential to overcome the shortcomings of polyssacharides vaccine. Cross-reactivity between peptides and the antibody raised against O5-antigen, and cross reactivity between sera raised against a specific peptide and a homologous peptide gives us some hope that peptide vaccines may do the job.

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APPENDIX

I participated in several other research projects in Dr. Klebba's laboratory in past several years. My contribution to those projects led to my co-authorship on the publica-tions listed below:

Scott, D. C., Cao, Z., **Qi, Z.**, Bauler, M., Igo, J. D., Newton, S. M., and Klebba, P. E. (2001). Exchangeability of N-Termini in the Ligand-Gated Porins of Escherichia Coli. *J.Biol.Chem.* 276(16):13025-13033.

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Sprencel, C., Cao, Z., **Qi, Z.**, Scott, D.C., Montague, M. A., Ivanoff, N., Xu, J., Raymond K. M., Newton S. M., and Klebba, P. E. (2000). Binding of ferric enterobactin by the *Escherichia coli* periplasmic protein FepB. *J Bacteriol.* 182(19):5359-5364.

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