

ABSTRACT

INSKEEP, TIFFANY KAY. Oral Vaccine Formulations Stimulate Mucosal and Systemic Antibody Responses against Staphylococcal Enterotoxin B using a Piglet Model. (Under the direction of Jack Odle).

Despite the potential for its use as an agent of bioterrorism, no approved vaccine against staphylococcal enterotoxin B (SEB) exists. Nontoxic, mutant forms of SEB have been developed, however it has been difficult to determine the efficacy of such subunit vaccine candidates due to the lack of superantigen activity of native SEB in rodents, and due to the limitations of primate models. Because pigs respond to SEB in a manner similar to that of humans, this study utilized this relevant animal model to investigate the safety and immunogenicity of a mutant form of SEB. We hypothesized that oral SEB vaccination with cholera toxin adjuvant would immunize against SEB in a piglet model. Two trials were performed at the NCSU Laboratory of Developmental Nutrition. Pigs were obtained at approximately 2 days of age and allotted to treatment according to a completely randomized design. Piglets were immunized orally with recombinant, mutant SEB (rmSEB) formulations (produced via transgenic soybeans) on day 0 and boosted 7, 14, and 24 days later. Pigs were bled post-immunizations to analyze serum antibodies.

Pigs experienced no change in overall growth performance during the vaccination regimen ($P < 0.05$). Following the first trial, pigs immunized with 1mg rmSEB had serum IgG antibodies that were not significantly different from control or 100ug CT + 1mg rmSEB, but tended to be different ($P = 0.052, 0.13$, respectively). Oral vaccination with 1mg doses of rmSEB resulted in serum IgG and fecal IgA responses ($P < 0.05$) by day 36 of Trial 2 that cross-reacted with native SEB. Surprisingly, the inclusion of the adjuvant, cholera toxin, in

vaccine formulations containing rmSEB did not result in increased antibody responses when compared to those using the immunogen alone. This rmSEB did not possess superantigen activity in pig lymphocytes. Furthermore, rmSEB was unable to compete with native SEB for binding in pig leukocytes. These in vitro studies suggest that rmSEB would be a safe, subunit vaccine. Taken together, these studies provide additional evidence for the potential use of nontoxic forms of SEB for use as vaccines.

Oral Vaccine Formulations Stimulate Mucosal and Systemic Antibody Responses against
Staphylococcal Enterotoxin B using a Piglet Model

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BIOGRAPHY

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CHAPTER I: LITERATURE REVIEW

Potential biological threat agent

Despite our best diplomatic efforts, the risk that biological agents will be used in warfare or terrorism remains quite high. Reasons for this include the relatively low degree of technological sophistication and expense required to produce a biological weapon compared to those of other weapons of mass destruction, such as chemical and nuclear arms.

Therefore, vaccines will continue to be one of our best defenses against such attacks. The development of new and improved vaccines, as well as treatments against the relatively small number of viable biological warfare agents will be needed in order to combat this potential terrorism (Cieslak et al., 2000). Biological warfare agents may be classified in several ways:

(1) operationally, as lethal or incapacitating agents, and as agents with or without potential for secondary transmission; (2) according to the intended target, as anti-personal, anti-animal, anti-plant, or anti-material; and (3) according to type, as replicating pathogens, toxins, or biomodulators (Cieslak et al., 2000). Staphylococcal enterotoxin B (SEB) is considered to be a viable incapacitating agent according to Cieslak et al. (2000). Although commonly known for its ability to cause food-borne disease, SEB capability to aerosolize categorizes it as a biological warfare agent. Research has shown SEB to have a very compact, stable protein structure, allowing it to survive the harsh environment of the gastrointestinal tract (Papageorgiou et al., 1998, Swaminathan et al., 1992). In addition, its stability to heat and denaturation allowed weaponization of this toxin for aerosol dispersal in the 1960s (Christopher et al., 1997). Following inhalation of aerosolized SEB, patients experience shortness of breath, chest pain, and some tachycardia (Rusnak et al., 2004). If exposure is

significant, pulmonary edema, high fever, and respiratory distress-like syndrome occur. With supportive medical intervention, death following inhalation is not common. However, symptoms and incapacitation can linger for up to two weeks following exposure. SEB has been characterized as “one of the two most important toxin threats on the battlefield or in bioterrorism” (Madsen 2001). SEB is especially dangerous as a biological agent because much lower quantities are needed to produce the desired effects than with synthetic chemicals. According to Madsen (2001) SEB is capable of incapacitating up to 80% of personnel in an area under attack. The effective dose, that which incapacitates fifty percent of the human population exposed, is 0.0004ug/kg by inhalation (Ulrich et al., 1997). No SEB vaccine is currently available for use in humans, although several approaches have been explored that will be discussed throughout this review.

Staphylococcal aureus and SEB

Since its discovery, *Staphylococcus aureus* has emerged as a potentially pathogenic Gram-positive bacterium that can cause a variety of diseases, ranging from minor infections of the skin to post-operative wound infections, bacteraemia, infections associated with foreign bodies and necrotizing pneumonia (Deurenberg et al., 2007). *S. aureus* commonly colonizes the skin and nasal passages of approximately 30% of the general population (Fraser and Proft 2008). Because *S. aureus* is gram-positive, half of the cell wall by weight is peptidoglycan. The peptidoglycan layer, specifically, protein A has endotoxin-like activity, stimulating the production of endogenous pyrogens, activation of complement, production of interleukin-1 (IL-1) from monocytes, and aggregations of neutrophils (Murray et al., 2005). The surface of most *S. aureus* strains are coated with protein A. This protein has a unique

affinity for binding to the Fc receptor of IgG. This effectively prevents antibody-mediated immune clearance of the organism (Murray et al., 2005).

The staphylococcal enterotoxins (SE) are a group of pyrogenic exoproteins produced by *S. aureus*. Exposure to staphylococcal enterotoxins initiates a range of clinical abnormalities from gastrointestinal upset to lethal toxic shock syndrome (TSS) (Van Gessel et al., 2004). SEs are stable to heating at 100°C for 30 minutes and are resistant to hydrolysis by gastric and jejunal enzymes (Murray et al., 2005). Currently there are at least twelve serotypes of staphylococcal enterotoxin described, which are named sequentially by letter (Jarraud et al., 2001). SEB is one of the most clinically significant and well-studied member of this family. SEB is known to induce typical food poisoning symptoms such as fever, vomiting and diarrhea. Food borne diseases have a major public health impact. It is estimated that in the US, each year food borne illnesses affect 6 to 80 million people, causing up to 9000 deaths, and cost about 5 billion dollars (Altekruse et al., 1997, Buzby and Roberts, 1997). Once introduced into host tissues, these proteins can elicit pathology in many different systems. Within four hours of ingestion of < 1 ug of SE, symptoms can be documented and include vomiting, diarrhea, nausea, and abdominal pain (Jett et al., 1990). Shupp et al. (2002) have shown that SEs are able to penetrate the gut lining and activate a local and systemic immune response. More specifically, SEB has been shown to poses the ability to bind and traverse protective intestinal epithelia (Hamad et al., 1997, McKay and Singh 1997) and transcytose to gain access to the circulation and systemic tissue. Characteristic histological changes in the stomach and jejunum include infiltration of neutrophils into the epithelium and underlying lamina propria, with loss of the brush border

in the jejunum. Stimulation of release of inflammatory mediators from mast cells is believed to be responsible for the emesis that is characteristic of staphylococcal food poisoning (Murray et al., 2005).

Immunology

The immune response is a complex series of steps that includes an inflammatory component and is aimed at identifying, attacking, and eliminating organisms and substances that invade our system or cause disease. The immune system is made up of a network of cells, tissues, and organs cooperating in the protection of the body. Immune cells are produced in the bone marrow and reside throughout the body in several locations, for example thymus, spleen, lymph nodes, and lymphoid tissues associated with various organs. When in the bloodstream, they are commonly termed white blood cells or leukocytes. These circulate through the body between organs and nodes through both the bloodstream and lymphatic vessels (Galli and Calder 2009).

The immune response is divided into innate and acquired immunity. Innate immunity is the first line of defense against an infectious agent; it is a type of generalized protection that is based on immune cells recognizing and responding to cells in a generic way. Phagocytic cells are a key component of the innate system, as well as the physical barriers and complement cascade (Janeway, Jr. and Medzhitov 2002). This type of immunity has no memory and is not influenced by previous exposures to infective agents (Janeway et al., 2008). According to Galli and Calder (2009) there are four steps in which the innate system responds:

- First, recruitment of immune cells to the sites of infection and inflammation through the production of chemical factors including cytokines, which are specialized chemical mediators.
- Second, activation of the complement cascade to identify bacteria, activate cells and promote clearance of dead cells and antibody complexes. The complement system is a biochemical cascade that complements the ability of antibodies to clear pathogens or mark them for degradation by other cells. The cascade is composed of plasma proteins synthesized by hepatocytes. These proteins trigger the recruitment of the inflammatory cells, tag the pathogens for destruction by opsonizing or coating the surface of pathogens, disrupt the plasma membranes of infected cells called cytolysis, causing pathogen death, and remove neutralized antigen-antibody complexes.
- Third, identification and removal of foreign substances present in tissues, organs, blood and lymph, by specialized white cell phagocytosis, most commonly carried out by macrophages.
- Fourth, activation of the adaptive immune system through antigen presentation. The cells forming the innate immune system are involved in inflammation.

Phagocytes (macrophages, neutrophils and dendritic cells) engulf pathogens and particles that are then incorporated into endosomes and merge with lysosomes releasing lytic enzymes and acids that kill and digest pathogens and particles (Janeway, Jr. and Medzhitov 2002). Activation of phagocytosis follows the response to cytokines produced by other cells and the expression of surface receptors specific for bacterial antigens. Macrophages in the tissues where the immune response takes place are derived from monocytes circulating in the

blood and are very efficient phagocytes producing reactive oxygen species (ROS), cytokines, and chemokines (Calder 2006). In addition to macrophages, Janeway (2008) also classifies neutrophils as a ROS. Dendritic cells are phagocytes in tissues in contact with the external environment (i.e., gastrointestinal tract) and are important in antigen presentation.

While protection by innate immunity is effective, some pathogens have developed the ability to escape detection or clearance by the innate system, leading to the activation of the adaptive system. Adaptive (or acquired) immunity develops throughout our lives, is highly specific and involves T and B lymphocytes. It allows for a stronger and more specific immune response and for development of immunological memory. This type of response is antigen-specific and requires the recognition of specific non-self antigens during antigen presentation, allowing responses that are tailored to specific antigens or pathogen-infected cells (Galli and Calder 2009). B cells are involved in the humoral immune response while T cells result in the cell-mediated response. Both types of lymphocytes carry surface receptors for a specific antigen and following activation, their function persists for several days after removal of the initiating antigen (Janeway et al., 2008). In contrast to the immunoglobulins, which interact with pathogens and their toxic products in the extracellular spaces of the body, T cells only recognize foreign antigens that are displayed on the surfaces of the body's own cells. These antigens can be of pathogens such as viruses or other intracellular bacteria, which replicate inside a cell, or from pathogens that have been consumed by endocytosis (Medzhitov and Janeway, Jr. 1997). T cells detect the presence of an intracellular pathogen because the infected cells display on their surface peptide fragments of the pathogen's proteins. These peptides are transported to the cell surface by the major histocompatibility

complex (MHC), class I and II, which are membrane bound proteins displayed on the surface of the antigen presenting cell (APC). T and B cells have receptors to recognize a specific antigen. A T cell receptor has only one antigen-binding site, whereas a B cell receptor has two. This type of recognition is the key to the high specificity of the immune response, where only a few T cells can recognize a specific antigen. T cell receptors (TCR) are never secreted, whereas B cells secrete antibodies (Janeway et al., 2008). Antibodies are soluble antigen-specific immunoglobulins (Medzhitov and Janeway, Jr. 1997) that prevent the attachment of microorganisms to host cells, thus neutralizing their pathogenic potential, and can also activate plasma complement proteins, which in turn promote phagocyte mediated destruction of a pathogen. As defined by Janeway et al. (2008) antibody molecules are Y-shaped molecules consisting of three equal-sized portions, connected by a flexible tether. The two arms of the Y end in regions that vary between different antibody molecules, the V regions, where antigen binds. The stem of the Y, or the constant, C, region is far less variable and it is the part that interacts with the effector cells. There are five different classes of immunoglobulins: IgM, IgD, IgG, IgA, and IgE, and can be distinguished by their C region. Each immunoglobulin is composed of two heavy chains and two light chains joined together by disulfide bonds and each heavy chain is linked to a light chain and the two heavy chains are linked together (Janeway et al., 2008). Activated B cells first produce IgM but undergo class switching to secrete antibodies of different classes. This does not affect antigen specificity, but alters the effector function that an antibody can engage by replacing one heavy chain C region with another. IgG is by far the most abundant immunoglobulin and is predominant in serum. IgA is found in mucosal areas such as the gastrointestinal,

respiratory, and urogenital tract (Burkey et al., 2009). The main effector function elicited by mucosal immunization is the stimulation of secretory antimicrobial or antitoxic IgA antibody responses and the associate mucosal immunologic memory (Eriksson and Holmgren 2002).

The V regions of any antibody molecule differ from those of every other. There are areas of higher variability in this region called hypervariable regions that exist in both the heavy and light chains. Both chains contribute to the antigen-binding site and therefore the immune system is able to generate antibodies of different specificities creating tremendous diversity (Janeway et al., 2008). Janeway et al. (2008) illustrates that the total number of antibody specificities available to an individual is known as the antibody repertoire. DNA sequences encoding each V region are generated by rearrangements of a relatively small group of inherited gene segments. The V region of an immunoglobulin heavy or light chain is encoded by more than one gene segment. These segments include the V, D (diversity), and J (joining) gene segments. There are multiples of each of these gene segments at each immunoglobulin locus that are rearranged to produce a multitude of diverse immunoglobulins. The process of VDJ recombination is found exclusively in lymphocytes in vertebrates, and allows the recombination of different gene segments into sequences encoding complete protein chains of immunoglobulins and T cell receptors (Janeway et al., 2008). Diversity is further enhanced by the process of somatic hypermutation in mature activated B cells. During B cell response, Janeway et al. (2008) defines somatic hypermutation as the result of higher affinity binding to the antigen in order to increase the antibody response. This increases the ability of an antibody to bind an invading pathogen and ultimately aid in the clearance of the pathogen from the body.

T cells respond to short amino acid sequences in proteins that cannot be identified by T cell receptors until the protein has been unfolded and processed into peptide fragments and bound to MHC molecules (Janeway et al., 2008). Galli and Calder (2009) describes that there are two classes of MHC molecules, I and II. MHC class I molecules consist of two polypeptide chains. One chain, the alpha chain, is encoded on the MHC and is noncovalently associated with a β_2 -microglobulin. MHC class II molecule consists of a noncovalent complex of two chains, alpha and beta. MHC class I molecules bind to the cell-surface protein CD8 that acts as a coreceptor while MHC class II molecules bind to CD4 on the T cell.

During the primary immune response, which occurs after the host encounters a pathogen for the first time, specific immunological memory is established. This ensures a rapid reinduction of antigen specific antibody and effector T cells on subsequent encounters with the same antigen. It is under this principle that vaccines work. Vaccines present a pathogenic organism to the body in controlled amounts to elicit an immune response and produce specific memory cells so when the pathogen is encountered again, it does not take as long to mount an immune response. More specifically, at the beginning of the secondary exposure, the source of antibodies is memory B cells generated from the previous exposure that are already switched from IgM to the more mature isotypes expressing IgG, IgA, or IgE, as well as have a higher level of MHC class II molecules than naïve B cells (Janeway et al., 2008).

Mucosal Immunology

The largest immune organ is situated in the gut where the mucosal immune system interacts with host and intestinal microbiota, playing an important role in protecting against pathogenic microorganisms (Moreto and Perez-Bosque 2009). The mucosal immune system maintains homeostasis by innate and adaptive immunity along the epithelial surface (Kiyono and Fukuyama 2004). One of the major functions of the immune system is to identify and eliminate pathogens; however, with respect to immune function within the gastrointestinal tract, it may be equally important to achieve a homeostatic balance between immune tolerance and immune responsiveness (Artis 2008). Because of the vast surface area of the gastrointestinal tract and the constant exposure to commensal and pathogenic microorganisms, mucosal immunity of the gastrointestinal tract has been the subject of great interest for the past several years. The mucosal surface of the gastrointestinal tract forms an intricate collaboration with the intestinal lumen. The diverse environment of antigenic dietary components, as well as the various populations of microbes within the tract, has facilitated the need for an evolving and sophisticated mucosal immune system. Burkey et al. (2009) claims that much of the burden on mucosal immunity is shouldered by the intestinal epithelial cells (IEC). The IEC monolayer provides anatomic and physiological barriers designed to maintain homeostasis within the gastrointestinal tract. The gastrointestinal tract must perform two distinct functions; the primary absorptive function, as well as discriminate and respond appropriately to enteropathogens in addition to food antigens or antigens from commensal organisms. Failure to do so can result in chronic inflammation, decreased digestive function, and a decreased growth rate. The importance of mucosal immunity is

clear when one considers that the gut contains greater than 10^{12} lymphocytes and has a greater concentration of antibodies than any other site in the body (Mayer 2000).

In addition to the IEC, the mucosal immune system also uses other gut-associated lymphoid tissue (GALT) to protect the organism and to mediate the immune response. GALT accounts for up to 80% of the mucosal immune system (Granger et al., 2006). A hallmark of mucosal immunity is the induction of an immune response in Peyer's patches and the subsequent production of secretory IgA by B cells in the lamina propria (Burkey et al., 2009). Subsequent to immune induction, the lamina propria has proven to function as the regulator of immune responses in the intestine (Makala et al., 2001). The gastrointestinal lamina propria is composed of smooth muscle cells, fibroblasts, blood vessels, and lymphatics that make up a highly vascular layer of loose connective tissue underlying and supporting the mucosal epithelium (Hunyady et al., 2000). The lamina propria also contains macrophages, dendritic cells, neutrophils, mast cells, and lymphocytes that participate in the effector function of the lamina propria. Pathogens can be taken up by M cells present in Peyer's patches, by dendritic cells of the lamina propria, or by transcytosis through the IEC. After induction in the Peyer's patch, mature T and B cells travel to mesenteric lymph nodes via lymphatic vessels before homing to the lamina propria (Hokari et al., 2001), where T cells can directly eliminate pathogens and where T and B cells can participate in the production of immunoglobulins and pro-inflammatory cytokines that amplify the immune response and induce cell recruitment (Moreto and Perez-Bosque 2009). After production of IgA from plasma cells, it is transcytosed across the IEC in vesicles that originate on the basolateral surface and fuse with the apical surface in contact with the intestinal lumen

(Janeway et al., 2008). In an inflammatory state, immune cells release reactive oxygen species to eliminate invaded pathogens; however, these agents also induced gut alterations and tissue destruction. Moreto and Perez-Bosque (2009) suggest that plasma protein supplementation can modulate the immune response at the luminal level and biologically active compounds present in plasma supplements can also interact directly with immune cells. They further claim plasma-induced changes in mucosal cytokine profile may prevent or reverse deleterious effects resulting from immune system activation when challenged with SEB in a rat model.

In humans, the majority of lamina propria T cells are CD4 T cells and expresses the T cell receptor. Lamina propria T cells differ from peripheral T cells in that they have a greater threshold of activation, produce increased concentrations of cytokines on stimulation, and have a phenotype associated with immunological memory (Wittig and Zeita 2003). Important differences in lamina propria lymphocytes exist between humans and swine that may relate to the function of these compartmentalized cells. Pig gut mucosa has a greater degree of organization compared with rodents and humans (Bailey et al., 2001). For example, Vegaz-Lopez et al. (1993), observed that plasma cells are preferentially localized to the intestinal crypts and T cells to the intestinal villi in the porcine intestine.

A key function of the intestinal epithelium is to serve as a selective barrier allowing the uptake of nutrients while excluding toxins and microorganisms. Mucosal permeability mainly depends on the capacity of tight junctions to efficiently seal the apical holes of epithelial cells (Moreto and Perez-Bosque 2009). Permeability of the intestinal epithelium is regulated by several stimuli. Increased permeability is associated with secretory diarrhea

(Toivola et al., 2004). Toxins from *Clostridium* and *Vibrio* change the localization of several tight junction proteins (Chen et al., 2002). Furthermore, enterotoxins can also have indirect effects by inducing the release of proinflammatory cytokines, such as $\text{INF}\gamma$ and $\text{TNF}\alpha$ (Moreto and Perez-Bosque 2009). SEB can also stimulate the secretion of $\text{INF}\gamma$ and $\text{TNF}\alpha$ from lymphocytes, which can disassemble tight junction protein complexes (Nusrat et al., 2000).

SEB is a mucosal pathogen with its primary route of entry through the oral route by way of contaminated food or through naso-pharyngeal route (Murray et al., 2005). Hence, the best defense against SEB would be a mucosal vaccine capable of inducing both systemic and mucosal immunity. Unfortunately, stimulation of mucosal immune responses by non-viable antigens is often inefficient and may in some instances result in immunological tolerance (Holmgren et al., 2003). Using an in vitro culture system to study the capacity of class II MHC human intestinal epithelial cells (Hamad et al., 1997) to transcytose several staphylococcal toxins, it was found that epithelial cells are capable of dose-dependent, facilitated transcytosis of SEB.

Superantigens

The bacterial superantigens are protein toxins that bind to MHC class II and the TCR in a nonspecific manner to stimulate a large number of T cells (Fraser and Proft 2008). The best characterized superantigens are those produced by the strains of *Staphylococcus aureus* and *Streptococcus pyogenes* and are the causative agents in toxic shock syndrome, an acute disease caused by sudden and massive release of T cell cytokines into the blood stream (Fraser and Proft 2008). Minute concentrations of superantigens can activate the immune

system receptors because they bind avidly to T cell antigen receptors and MHC class II molecules (Ulrich et al., 1997). The superantigens are noted by Fraser and Proft (2008) for their impressive ability to withstand denaturing conditions such as heat and acid and as a consequence are not completely destroyed by cooking of food, hence their potency in food poisoning. Instead of binding to the groove of the MHC class II, superantigens bind to the outer surface of the MHC and the variable domain- β ($V\beta$) region of the TCR cross-linking the two and therefore operate independently of the MHC-bound peptide (Fraser and Proft 2008). In conventional antigen specific binding, the antigen peptide is processed and presented to the TCR on the MHC class II molecule on the cell surface of the APCs. Bavari and Ulrich (1997) suggest that pyrogenic toxins may mimic CD4 binding and by doing so, stimulates large numbers of T cells in a manner independent of antigen recognition. This non-specific binding can activate up to 20% of the total T cell population (Fraser and Proft 2008) resulting in an over production of cytokines. The presence of even a small amount of superantigen in the blood stream rapidly elevates many cytokines to toxic levels, but IL-2, $INF\gamma$, and $TNF\alpha$ are believed to be the main determinants of toxicity (Jupin et al., 1988). The suddenness and magnitude of the cytokine release dictates the severity and outcome for the patient, and the most important survival factor is the level of pre-existing neutralizing antibodies that block the initial binding of the superantigen to the MHC class II (Fraser and Proft 2008).

Fraser and Proft (2008) affirm the notion that superantigens simultaneously bind to MHC class II and TCR is unrealistic. The superantigen must first bind to the MHC class II to concentrate onto the surface of the APC, making use of the adhesion and accessory

molecules that form the immunological synapse. Studies have shown that superantigen binding to APCs is extremely stable, particularly those that utilize zinc in their class II β -chain binding sites, with the superantigen remaining on the APC surface for up to 40 hours without any evidence for depletion (Li et al., 1998). Early after the APC binding, the surface concentration is sufficient to successively engage and cross-link multiple TCR molecules, resulting in strong TCR signaling, activation, and cytokine production. The affinity of superantigens towards MHC class II molecules is typically 10 to 100 fold higher than its affinities towards TCR (Fraser and Proft 2008).

Most staphylococcal superantigens have common structures for binding to a subunit of the human MHC class II molecule (Ulrich et al., 1998). Several studies have focused on the role of particular amino acid residues in SEB that are important in toxinogenic activity (Coffman et al., 2002). A hydrophobic binding loop, centered at a leucine residue (e.g. SEA L48, SEB L45, and TSST-1 L30), is conserved in all superantigens except streptococcal pyrogenic exotoxin C, and is essential for the recognition of the class II molecule. A second conserved structure is found in all the superantigens except TSST-1, and consists of a polar pocket that interacts with Lysine 39 of the class II molecule. These observations on superantigen-receptor complexes have resulted in the generation of mutant proteins that are immunogenic, but not toxic. Single mutations of key residues in the polar pocket or in the hydrophobic loop of SEB eliminated binding of the toxin to the MHC class II molecule, with minimal perturbation in SEB structure (Ulrich et al., 1998). This altered SEB molecule generated high levels of circulating antibody when injected into mice, and all immunized mice subsequently survived a challenge with wild-type SEB. A triple mutant carrying L45R,

Y89A, and Y94A was subsequently shown to induce immunity in nonhuman primates and to protect them against an aerosol challenge with wild-type SEB (Boles et al., 2003).

Vaccines

Research has shown that vaccines have saved millions of lives and represents the single greatest promise of biomedicine: disease prevention (Stern and Markel 2005).

Vaccine development, which began with Edward Jenner's observations in the late 18th century, is continually changing and exploring new methods for immunization. From the beginning vaccines held a promise to bring about an effective and safe method to prevent disease. Edward Jenner, a doctor in England, performed the first vaccination in 1796; taking pus from a cowpox lesion on a milkmaid's hand and inoculated a boy (Stern and Markel 2005). After additional experiments and case studies, Jenner proclaimed "that the cowpox protects the human constitution from the infection of smallpox (Stern and Markel 2005)."

This laid the ground work for modern vaccines. Following Jenner's work, a French chemist Louis Pasteur developed the rabies vaccine in 1885. These two works combined give the modern definition of vaccine as a suspension of live (usually attenuated) or inactivated microorganisms (bacteria or virus) or fractions thereof administered to induce immunity and prevent infectious disease (Advisory Committee on Immunization Practices and the American Academy of Family Physicians 2002, Stern and Markel 2005)."

There are basic requirements for an effective vaccine (Janeway et al., 2008) such as safe, protective, gives sustained protection, induces neutralizing antibodies and protective T cells, as well as practical considerations of low cost, stability, and few side effects. Vaccines must not cause illness or death, yet protect against illness resulting from exposure to live

pathogens that last for several years. In addition, vaccines must produce neutralizing antibodies in order to prevent future infection. Three classical types of vaccines are currently used including killed or inactivated vaccines, attenuated, and subunit vaccines. Killed or inactivated vaccines are safe because the organism is dead and hence cannot actively replicate in the host (Janeway et al., 2008). Frequent booster immunizations are often required because the organisms are rapidly cleared by phagocytic cells, shortening the exposure time to the host (Plotkin 2009). When producing an inactivated vaccine it is critical to preserve the structure of key antigens through the “inactivation” process. The dead organism must still be a faithful molecular image of the live pathogen. Attenuated vaccines utilize an organism that is rendered avirulent by introducing mutations in key virulence or regulatory genes (Plotkin 2009). These vaccines typically cause strong immune responses because the organism is alive and it grows in the host. This produces longer and higher levels of exposure to antigen when compared to inactivated forms; however, the potential for reversion to a pathogenic form must be considered (Janeway et al., 2008). Subunit vaccines make use of just a component rather than the whole organism. The antigen used must be sufficient to induce protective immunity, such as a key ligand for cell entry or toxin. These vaccines are considered safe because there is no living organism, but may require repeated booster immunizations. Classically, biochemical techniques are used to purify the subunit away from the organism. Today, expression of recombinant proteins can be used for protein antigens.

Current vaccines utilize the methods of attenuating or inactivating the organism’s virulence mechanisms in order to safely vaccinate an individual to stimulate an immune

response. Live attenuated vaccines have been among the most powerful for the purpose of disease control and even eradication, owing to the strong antibody and cellular response elicited by them (Plotkin 2009). However, these vaccines have also been associated with genetic instability and residual virulence (Ehrenfeld et al., 2009). A number of strategies are now being investigated for dealing with those issues including reassortment, reverse genetics, recombination, deletion mutants, transcutaneous vaccination, and adjuvants. Recombination allows the insertion of desirable genes of one microbe into the genome of another (Plotkin 2009).

Subunit vaccines in transgenic soybeans offer a unique platform with significant advantages. Transgenic plants expressing recombinant proteins from pathogenic microorganisms provide an inexpensive edible vaccine for induction of local immunity. Piller et al. (2005) was the first to demonstrate the expression and immunogenicity of a model subunit antigen in a soybean system. Soy milk formulations have been shown to have inherent buffering capacity (Lutchman et al., 2006, Park et al., 1991), which might aid in protein stability in the acidic environment of the gut. In addition to soybean, research has shown that corn has the ability develop recombinant proteins to induce an immune response (Streatfield et al., 2001). Transgenic recombinant protein antigens could have numerous beneficial affects including increased safety, stability, versatility and efficacy. A variety of immune responses have been recorded following oral delivery of plant-based vaccines including antigen specific serum IgG and IgA (Streatfield et al., 2001), as well as induction of intestinal mucosal IgG and IgA.

Oral Vaccines

Although most vaccines have traditionally been administered by intramuscular injection, mucosal administration of vaccines offers several important advantages. These include easier administration, reduced side effects, and the potential for frequent boosting (Singh and O'Hagan 1999). Hyland et al. (2004) affirm that protective immunity against enteric pathogens may be best induced by delivering vaccines directly to GALT via oral instead of parenteral administration.

There have been multiple attempts at edible vaccine development to protect against bacterial and viral pathogens responsible for diseases such as transmissible gastroenteritis (Streatfield et al., 2001) and foot and mouth disease (Dus Santos et al., 2002). Currently, there are only a handful of vaccines that are approved for human use that are administered mucosally: the oral polio vaccine, oral killed whole cell B subunit and live-attenuated cholera vaccines, an oral live-attenuated typhoid vaccine, and oral BCG vaccine (used in Brazil) and an oral adenovirus vaccine that is restricted to military personnel (Holmgren et al., 2003). Likewise, a few oral vaccines are currently on the market for swine, including ProSystem TGE/Rota, a combination of oral and IM vaccine for pregnant sows and gilts for transmissible gastroenteritis and rotavirus in neonatal piglets by Intervet, Schering-Plough. Boehringer Ingelheim Vetmedica, Inc has three oral vaccines including Enterisol SC-54 FF, an avirulent live vaccine to protect pigs against infection with *Salmonella choleraesuis*, Enterisol Ileitis FF, a live vaccine to protect against enteric diseases commonly caused by *Lawsonia intracellularis*, and Ingelvac ERY-ALC which immunizes pigs against erysipelas caused by the bacterium *Erysipelothrix rhusiopathiae*.

In addition, local immunization induces mucosal immunity at the sites where the majority of pathogens initially establish infection. Infections of this type include those of the gastrointestinal tract caused by *Helicobacter pylori*, *Vibrio cholerae*, enterotoxigenic *Escherichia coli*, *Salmonella*, and *Staphylococcal aureus* (Eriksson and Holmgren 2002). These infections still represent an enormous challenge for development of vaccines targeted to induce immunity that can prevent the infectious agent from attaching and colonizing at the mucosal epithelium, or from penetrating and replicating in the mucosa, and/or can block the binding and action of microbial toxins (Holmgren et al., 2003). In many cases, the main protective effector function elicited by immunizations is the stimulation of secretory IgA antibody response and associated mucosal memory.

Oral vaccines would be easier to administer than parental vaccines (Holmgren et al., 2003) and could be especially advantageous in isolated communities, where access to health care professionals is difficult (Singh and O'Hagan 1999). They would also carry less risk of transmitting the type of infections still associated with the use of injectable vaccines in several parts of the world, such as hepatitis B and HIV infections. Oral vaccines can also be expected to have much greater acceptability than injectable vaccines by causing no sore arms, etc (Holmgren et al., 2003).

A wide range of approaches are currently being evaluated for the mucosal delivery of vaccines (Pizza et al., 2001). The induction of an immune response at the mucosal surface is of major interest because of its ability to modulate colonization by commensals, as well as increase defenses against the penetration of pathogens through the epithelium. Efficacy has been demonstrated for the oral poliovirus vaccine (Bouvet et al., 2002), but only very few

other vaccines administered by the mucosal route are available commercially at present. Tremendous research efforts have now improved significantly the classical approach of these vaccines, and alternative methods of immunization, based on new concepts of mucosal immunity, are being developed.

In primates, oral mucosal immunization with microspheres induced protection against aerosol challenge with staphylococcal enterotoxin B (Tseng et al., 1995). In the experiment forty rhesus monkeys were immunized via three different routes: intramuscular (IM), intratracheal (IT), and oral (OR) seven weeks apart of two doses of microspheres containing 100ug of SEB toxoid. After priming and booster immunizations were performed, monkeys received a SEB aerosol challenge 3 to 4 weeks after the booster. One week before challenge monkeys were bled to collect serum to determine antibody response prior to challenge and were likewise bled after challenge. Antibodies from plasma were quantified by enzyme-linked immunosorbent assay (ELISA). Monkeys primed IM followed by an IT booster all survived the SEB challenge. Tseng et al. (1995) also demonstrated relatively high survival rates in monkeys primed via the IT or OR route and boosted IT with microspheres. Before challenge, monkeys boosted via the IT route showed the highest levels of circulating IgG and IgA antibodies. However, statistically, these high levels of IgG and IgA antibodies were not significantly different from those of monkeys with an IM booster. After challenge, there were dramatic reductions in the levels of all antibody isotypes. Prominent reductions in circulating IgA and IgG antibodies were seen in survivors. When prechallenged levels of circulating antibodies in survivors and non-survivors were compared, the survivors had significantly higher average levels of IgG and IgA antibodies than the non-survivors.

Noticeably, monkeys orally immunized had lower antibody titers than monkeys with IT or IM booster. Altogether these results suggest that antibodies in the circulation play an important role in protective immunity. However, these attempts at toxoid-based vaccines have been abandoned because these toxoids were not reproducibly protective (Tseng et al., 1995).

On the contrary, a previous study with mice orally immunized against SEB produced just as high antibodies when compared to other routes of administration (Stiles et al., 2001). This study explored the possibility of nasally and orally immunizing mice with a recombinantly attenuated SEB vaccine (SEBv). SEB-specific antibodies in the saliva and sera were detected by ELISA, and the mice were finally challenged intraperitoneally or mucosally (via aerosol) with a lethal dose of wild-type SEB. Earlier studies clearly demonstrated that antibodies elicited by parentally administered vaccines for SEB, or premixing of SEB-specific antisera with toxin before injection into naïve mice, protect animals against SEB-induced lethal shock (Ulrich et al., 1998). Stiles et al. (2001) determined if three nasal or oral doses of SEBv (20 or 50ug each), without cholera toxin adjuvant, could effectively elicit SEB-specific antibodies. None of the animals developed SEB-specific antibodies in their serum or saliva and were not protected against a lethal intraperitoneal challenge. In contrast, there was SEB-specific IgA, IgG, and IgM in sera after three immunizations (20ug of SEBv each) given intraperitoneally with aluminum, or nasally and orally with cholera toxin (CT). Additionally, only the nasally and orally vaccinated mice developed SEB-specific IgA and IgG in their saliva, unlike mice injected intraperitoneally with 20ug of SEBv plus aluminum. After challenge, oral vaccinations were statistically

effective toward a mucosal SEB challenge. Therefore, Stiles et al. (2001) demonstrated that oral administration of SEB could induce an anti-SEB mucosal and systemic antibody response when immunized with an adjuvant of aluminum or cholera toxin. In an unpublished experiment by Bost and Piller, oral immunizations with soy milk formulations made from recombinant mutated SEB induced high levels of serum and mucosal antibodies in mice. In addition, CT was utilized as an adjuvant and was able to induce high levels of specific anti-SEB antibodies.

Adjuvants

Purified antigens are not usually strongly immunogenic on their own, and most vaccines require the addition of adjuvants, which are substances that enhance the immunogenicity of antigens (Janeway et al., 2008). Adjuvants can be used to improve the immune response to vaccine antigens in many different ways. They can increase the immunogenicity of weak antigens, enhance the speed and duration of the immune response, stimulate cell-mediated immunity and promote the induction of mucosal immunity (Singh and O'Hagan 1999). For years, mineral salts and oil emulsions were the only acceptable adjuvants for vaccines. Freund's classical adjuvant contains mineral oil and bacterial cell walls. Emulsions hold the antigen and slowly allow release so the body has prolonged exposure to the antigen being presented (Janeway et al., 2008). There are two types of Freund's adjuvant, complete and incomplete. Complete contains oil-water emulsion with dead mycobacteria while the incomplete adjuvant does not contain mycobacteria (Janeway et al., 2008). However, there are many poor side effects such as a large inflammatory response and animals tend to get sores at site of injection (Broderson 1989). Boderson (1989)

performed a retrospective evaluation of lesions after the use of Freund's adjuvant formulations in rabbits and monkeys. Inflammation was consistently present at the sites of inoculation and complications frequently included focal necrosis and ulceration of the skin. Mineral salts, for example, aluminum hydroxide, forms an aluminum precipitate with the protein slowing processing and presentation (Janeway et al., 2008). Immunization often activates several complex cascades of immune effectors, only some of which are relevant to the induction of a particular antigen specific response. Determining which adjuvant best fits each vaccine antigen is often complicated. A desirable feature of an adjuvant is that it should specifically enhance the immune response to the vaccine antigen with which it is co-administered. An adjuvant with broad nonspecific effects has more potential for the induction of adverse immunological events. Many alternative adjuvants have been evaluated for mucosal delivery of vaccines, including the bacterial toxins from *Virbrio cholerae*, cholera toxin (CT), and *Escherichia coli* heat-labile toxin (LT) which are the most potent mucosal adjuvants available because of their intense immunogenicity (Singh and O'Hagan 1999). CT and lipopolysaccharide (LPS) have been used to enhance anti-PRRSV immune response because of these bacterial components' ability to activate antigen presenting cell's (APC) antigen presentation and T cell stimulation (Charerntantanakul 2008). Hyland et al. (2004) state that few proteins are immunogenic when administered orally. One exception is CT which is one of the most widely used mucosal adjuvants in animals. Cholera toxin is a potential oral immunogen in a number of species including pigs (Foss and Murtaugh 1999). Previous studies claim that CT is a potent oral antigen and adjuvant in swine, inducing secretory IgA responses in the gut and oral cavity, systemic IgG, and cell mediated immune

responses (Foss and Murtaugh 1999, 2000). Hyland et al. (2004) demonstrated the feasibility of using orally administered CT for the induction of immunity to reproductive pathogens. Research by Verdonck et al. (2004) showed that oral immunization of pigs with CT resulted in the significant induction of CT-specific serum antibodies to F4(K88) *E. coli*. Foss and Murtaugh (1999) observed CT-B specific IgA and IgG antibodies in jejunal mucus and saliva of pigs that were orally immunized with CT. On the contrary, a study by Challa et al. (2007) revealed that higher IgG antibodies were produced against foot and mouth in treatment groups that did not include CT versus treatments with CT as an adjuvant.

Research has shown CT to be a potential adjuvant for mucosal immunogenicity. The cholera toxin is a complex A-B toxin that is structurally and functionally similar to the heat-labile enterotoxin of *E. coli*. A ring of five identical B subunits of CT binds to the ganglioside (G_{m1}) receptor on the surface of the intestinal epithelial cells. The active portion of the A subunit enters the cell and interacts with G proteins that control adenylyl cyclase so that it is activated leading to the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) along the cell membrane. The cAMP causes hypersecretion of sodium, chloride, potassium, bicarbonate, and water out of the cell into the intestinal lumen (Murray et al., 2005). CT has been found to affect several steps in the induction of the mucosal immune response. These effects include: increased permeability of the intestinal epithelium leading to enhanced uptake of co-administered antigen; enhanced antigen presentation by a variety of cells; promotion of isotype differentiation in B cells leading to increased IgA formation; and complex stimulatory as well as inhibitory effects on T cell proliferation and cytokine production (Holmgren et al., 2003). Holmgren et al. (2003)

explains that CT has also been shown to increase antigen presentation by DC, macrophages, and B cells. Therefore, the next cohort of vaccines should include the addition of carrier or adjuvant molecules such as CT secreted from *Vibrio cholerae*, and heat-labile enterotoxin (LT) secreted from *Escherichia coli*. Both of these toxins contain ADP-ribosylating enzymes, and their non-toxic forms can be used as mucosal adjuvants because of their ability to bind to receptors on the epithelial cell surface and reach the underlying mucosal tissue (Lycke et al., 2006).

Piglet Model

Bi et al. (2009) explored the weanling piglet model of SEB intoxication and reported that piglets exposed to intravenous SEB exhibited typical and severe pathological signs including prostration, emesis, diarrhea, and pyrexia as well as hypotension and death. This model is superior to rodent and nonhuman primate models in terms of mirroring the human clinical course and physical signs of SEB intoxication. Van Gessel et al. (2004) also demonstrated that the piglet model of SEB overcomes the shortcomings of the current rodent and nonhuman primate model. This model more realistically parallels SEB intoxication in people than described in mouse models, and piglets are easier to obtain, maintain and handle than the nonhuman primate model (Van Gessel et al., 2004). Van Gessel et al. (2004) showed that the piglet exhibits a biphasic clinical response to SEB intoxication that is virtually identical to people but not mice. Although SEB intoxication has been achieved in mice, none of these models exhibit the typical initial gastrointestinal signs seen in humans. Furthermore, the small size of mice makes many clinical measurements such as repeat routine hematology, serum, blood pressure, and body temperature difficult (Van Gessel et al.,

2004). While nonhuman primates, especially rhesus macaques, exhibit similar signs of humans, they have their own unique set of limitations. Most notable limitations include high cost, limited supply, and bio-safety concerns. In addition, the aggressive nature makes heavy sedation or anesthesia necessary for routine procedures. In contrast, piglets are more easily obtained and relatively inexpensive. The nature of pigs allows routine procedures to be performed with out sedation and with minimal stress to the animal and handler (Van Gessel et al., 2004).

Current research focus: SEB vaccine in a piglet model

In an effort to develop a needle-less vaccine formulation, a triple SEB mutant examined in a piglet model to test oral immunization strategies. Unlike mouse models, native SEB functions as a superantigen in pigs, demonstrating the relevance of using this animal species for evaluation and development of vaccination strategies for use in humans. For development of such vaccines, the FDA requires animal studies in at least two animal models. Therefore, we used the piglet model to address the possibility that an oral vaccine formulation consisting of triple mutant of SEB (rmSEB) and oral adjuvant, cholera toxin, would combine to stimulate an antibody response against SEB.

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Chapter II
Oral Vaccine Formulations Stimulate Mucosal and Systemic Antibody Responses against
Staphylococcal Enterotoxin B using a Piglet Model

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INTRODUCTION

Staphylococcus aureus produces several exotoxins that are important determinants of pathogenicity (Dinges et al. 2000). The staphylococcal enterotoxins are among these exotoxins, and are produced by *S. aureus* strains growing in contaminated food, with staphylococcal enterotoxin B (SEB) being the most potent of the exotoxins. SEB mediates its toxicity by linking MHC class II molecules with T cell receptors outside the antigen binding site (Papageorgiou et al., 1998). Several families of T lymphocytes expressing certain V beta T cell receptors can be stimulated by this toxin, which can include up to 20% of the total T cell population. The term “superantigen” has been given to SEB and similar toxins which have this ability to bridge MHC class II molecules and T cell receptors, stimulating a large percentage of T lymphocytes in this unconventional manner (Herman et al., 1991). One result of this toxin-induced T lymphocyte activation is the overproduction of certain cytokines, which contribute to the clinical symptoms of SEB-induced toxicity and shock (Drake et al., 1992). The most common natural exposure to SEB is through ingestion of contaminated foods. Symptoms following ingestion include anorexia, nausea, vomiting, and diarrhea, which may present with hypotension, tachycardia, and hyperperistalsis (Marrack and Kappler 1990).

SEB has several characteristics which make it a candidate for possible use as an agent of biowarfare or bioterrorism. SEB has a very compact, stable protein structure, allowing it to survive the harsh environment of the gastrointestinal tract (Papageorgiou et al., 1998 and Swaminathan et al., 1992). In addition, its stability to heat and denaturation have allowed the weaponization of this toxin for aerosol dispersal in the 1960s (Christopher et al., 1997).

Following inhalation of aerosolized SEB, patients experience shortness of breath, chest pain, and some tachycardia (Rusnak et al., 2004). If exposure is significant, pulmonary edema, high fever, and a respiratory distress-like syndrome occurs. With supportive medical intervention, death following inhalation is not common. However symptoms and incapacitation can linger for up to two weeks following exposure. SEB has been characterized as “one of the two most important toxin threats on the battlefield or in bioterrorism” (Madsen 2001).

At present there are no approved vaccines for SEB. Early attempts at toxoid-based formalin-inactivated vaccines have been abandoned since these toxoids were not reproducibly protective (Tseng et al., 1995). This has led to more recent investigations using engineered, non-toxic mutant forms of SEB. These mutant forms of SEB have been designed and tested, based on the considerable data defining the structure-function relationships for this toxin (Kotb 1998, Papageorgiou and Acharya 2000 and Papageorgiou and Acharya 1997). Specifically, several studies have focused on the role of particular amino acid residues in SEB that are important in toxinogenic activity (Baker et al., 2002, Ulrich et al., 1998). Most staphylococcal superantigens have common structures for binding to a subunit of the human MHC class II molecule (Ulrich et al., 1998). A hydrophobic binding loop, centered at a leucine residue (e.g. SEA L48, SEB L45, and TSST-1 L30), is conserved in all superantigens except streptococcal pyrogenic exotoxin C, and is essential for the recognition of the class II molecule. A second conserved structure is found in all the superantigens except TSST-1, and consists of a polar pocket that interacts with Lysine 39 of the class II molecule (e.g. SEA Y92, Y108, D70; and SEB Y89, Y115, E67). These

observations on superantigen-receptor complexes have resulted in the generation of mutant proteins that are immunogenic, but not toxic. Single mutations of key residues in the polar pocket (e.g. Y89A) or in the hydrophobic binding loop (e.g. L45R) of SEB eliminated binding of the toxin to the MHC class II molecule, with minimal perturbation in SEB structure (Ulrich et al., 1998). This altered SEB molecule generated high levels of circulating antibody when injected into mice, and all immunized mice subsequently survived a challenge with wild-type SEB. A triple mutant carrying L45R, Y89A, and Y94A was subsequently shown to induce immunity in nonhuman primates, and to protect them against an aerosol challenge with wild-type SEB (Boles et al., 2003).

In an effort to develop a needle-less vaccine formulation, this triple SEB mutant (L45R, Y89A, Y94A) was used in a piglet model to test oral immunization strategies. Unlike mouse models (Boles et al., 2003, Stiles et al., 1993, Stiles et al., 2001 and Ulrich et al., 1998), native SEB functions as a superantigen in pigs (Bi et al., 2009, Hammamieh et al., 2004, Mateu de Antonio et al., 1998 and Van Gessel et al., 2004), demonstrating the relevance of using this animal species for evaluating vaccination strategies for use in humans. This study used the piglet model to address the possibility that an oral vaccine formulation consisting of the recombinant triple mutant of SEB (rmSEB) and the oral adjuvant, cholera toxin, would combine to stimulate a mucosal and systemic antibody response against SEB.

MATERIALS and METHODS

Protocol

All animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of North Carolina State University. Two trials were conducted to

determine oral vaccine formulations against SEB. Seven-day-old mixed gender commercial crossbred piglets (N=40 per trial) were obtained from Spring Meadow Farms (Springfield, NC) and individually housed in 0.6 x 1.5 m pens. Piglets were maintained under controlled lighting (15-hour light and 9-hour dark cycle) and ambient temperature (ranged from 22-34°C). Piglets were assigned to one of six (Trial 1) or one of five (Trial 2) treatment groups according to a completely randomized design. For each trial, bodyweights were recorded on days 0, 1, 2, 3, 7, 8, 9, 10, 14, 15, 16, 17, 24, and 36. Piglets were fed a milk replacer liquid diet free of antibiotics and acidifiers (Milk Specialties Company, Carpentersville, IL), which exceeded NRC requirements (APPENDIX, Table 1), each morning, afternoon and evening for the first twenty-four days. Feed amounts and feed weigh back amounts were recorded at each feeding. After the final vaccination piglets were switched to a dry pellet feed (Ralco Nutrition, Marshall, MN) and had access to feed and water ad libitum.

Expression and purification of recombinant mutant SEB

Recombinant mutant SEB (L45R, Y89A, Y94A) (rmSEB) was expressed in *E. coli* and purified as a C-terminal 6x-histidine-tagged (6X-His) fusion protein. The plasmid harboring the fusion protein was created by PCR amplification of *seb* with the primers SEB-PCR-F2 (5'-CC TCT AG ATG GAG TCA CAG CCA GAC CCC AAG C-3') and SEB-PCR-R (5'-AAC TCG AGT CAG TGG TGG TGA TGG TGG TGG CCA CCC TTC TTC TTA GTA GTA AGG TAC ACC TCG-3') and *PfuUltra* high-fidelity DNA polymerase (Stratagene) using pRG5 plasmid DNA as a template (Figure 1). The amplified PCR product was digested with *XbaI* and *XhoI*, and subcloned into the vector backbone of pET303/CT-His (Invitrogen, Carlsbad, CA). The plasmid pET303/R-SEB harboring rmSEB-6xHis was used

to transform *E. coli* (BL21 Star strain) for the heterologous protein expression. rmSEB was purified from the cleared bacterial cell lysate using His-Select™ nickel affinity gel (Sigma-Aldrich, Saint Louis, MO) according to the manufacturer's protocol. rmSEB was further purified by polyacrylamide gel electrophoresis (PAGE) for its use as an immunogen. Protein quantification was performed using a modified Lowry protein assay (Pierce, Rockford, IL) and using a capture ELISA.

For the capture ELISA, plates were coated overnight with a monoclonal anti-SEB antibody (clone S222, Abcam, Cambridge, MA). After blocking plates with PBS-1% BSA, dilutions of rmSEB or known amounts of native SEB (Sigma-Aldrich) were added to the wells. Bound material was detected using a horseradish peroxidase-conjugated polyclonal anti-SEB antibody (Abcam). Following washing, plates were incubated with TMB substrate (BioFX, Owings Mills, MD) at room temperature. The enzymatic reactions were stopped by addition of 1M sulfuric acid and absorbances were read at 405 nm. The quantity of rmSEB present in each dilution was determined by extrapolation from standard curves.

Immunization of piglets

Groups of eight day old (Trial 1) and seven day old (Trial 2) piglets were orally immunized on day 0, and then boosted 7, 14, and 24 days with formulations of rmSEB with or without 100 ug of the oral adjuvant, cholera toxin (CT). The immunogen, with or without adjuvant, was formulated in soy milk to facilitate oral delivery to the piglets. Control piglets for both trials received soy milk only as a vehicle control. For Trial 1, piglets were immunized with 50µg CT, 100µg CT, 50µg CT + 1mg rmSEB, 100µg CT + 1mg rmSEB, or

1mg rmSEB alone. For Trial 2, piglets were immunized with 1mg rmSEB, 10mg rmSEB, 1mg rmSEB + 100 μ g CT, or 10mg rmSEB + 100 μ g CT.

Sample collections and Laboratory Analyses

Piglets were bled on days 0, 7, 14, 24, and 36 post-immunization to analyze serum antibodies. On day 36, piglets were euthanized and fecal material collected for ELISA analyses. In addition, spleen tissues were collected to analyze superantigen activity in pigs.

ELISA to detect anti-SEB antibodies

To determine the anti-SEB titers in immunized animal serum and fecal samples, microtiter plates were coated with 200 ng/well of native SEB (Sigma-Aldrich) in 100 μ L of carbonate buffer overnight at 4°C. Wells were then blocked with 1% BSA in PBS. After washing, serial dilutions of sera or fecal extracts were incubated in wells for 2 hours at room temperature. After washing off unbound material, a horseradish peroxidase-conjugated goat anti-swine IgG (Southern Biotech, Birmingham, AL) was added for two hours. Following washing, plates were incubated with TMB substrate (BioFX) at room temperature. The enzymatic reactions were stopped by addition of 1M sulfuric acid and absorbances were read at 405 nm. Endpoint titers were defined as the last serum dilution with an absorbance double that of animals which received vehicle only.

Fecal extracts were diluted in PBS (7 μ L/mg fecal pellet) supplemented with protease inhibitors (1 mM PMSF, 1 mM IAA, 1 mM Pepstatin A and 5 mM EDTA). Following homogenization, particulate matter was removed by passage over nylon wool columns, followed by centrifugation (13,000 x g). The soluble fraction was lyophilized, and reconstituted for determination of total IgA using a capture ELISA. A monoclonal anti-

swine IgA antibody (US Biological, Swampscott, MA) was coated onto microtiter plates, and dilutions of fecal samples added. Total bound IgA was detected using a horseradish peroxidase-conjugated polyclonal anti-swine IgA antibody (US Biological). Total IgA levels present in each fecal sample were then determined by comparison to a standard curve. For the determination of anti-SEB fecal IgA levels, ELISA were performed as described above except that 1 ug of total IgA from each fecal sample was incubated on SEB-coated plates, and a horseradish peroxidase-conjugated polyclonal anti-swine IgA antibody (US Biological) was used to determine reactivity with native SEB.

Induction of interferon gamma production by native SEB and rmSEB

To assess the ability of native SEB and rmSEB to function as a superantigen, mononuclear leukocytes were isolated from normal pig spleen tissue. Following aseptic removal of spleens, single cell suspensions were made by pressing tissue through 30 gauge wire mesh screens, followed by passage over nylon wool to remove cellular debris. Cells were then pelleted, and mononuclear leukocytes isolated by centrifugation on Histopaque 1077 density medium (Sigma-Aldrich). After washing, cells were counted and plated at 500,000 per well (Corning, Corning, NY), and incubated in RPMI-1640 (Mediatech, Manassas, VA) supplemented with 10% fetal calf serum (Atlanta Biologicals, Atlanta, GA).

Varying concentrations of native SEB (10 – 0.01 ug/ml, Sigma-Aldrich), rmSEB (100 – 0.1 ug/ml) were added to wells in triplicate as indicated. For Trial 1, cells were cultured with media or 100ug of rmSEB. Culture supernates were harvested 40 or 60 hours later (Trial 1 and Trial 2, respectively), and porcine interferon gamma production quantified using

an ELISA (R&D Systems, Minneapolis, MN) and the instructions supplied by the manufacturer.

Statistical Analysis

For analysis of growth performance, data were analyzed using the MIXED procedure of Statistical Analysis System (SAS 9.1, Cary, NC) using a T-test for comparison of means according to a completely randomized design with pig as the experimental unit. Significance was declared when $P < 0.05$. Initial bodyweight was used as a covariate. Data are presented as least-square means with pooled standard error of the mean.

For analysis of immune responses, one-way ANOVA was performed followed by the post hoc Tukey-Kramer test using Graph Pad Prism 4.0 software (Innotech; Schonaich, Germany). Statistical significance was declared when $P < 0.05$ and data are presented as mean \pm standard error.

RESULTS

Trial 1

Animal Performance

Because initial piglet body weights were not similar among treatments ($P < 0.05$) they were used to covariately adjust the animal performance data. Immunization with the oral vaccine did not affect overall growth performance for Trial 1. Average daily feed intake (ADFI, Table 1) was calculated on dry matter (DM) basis. Following the first immunization, ADFI for piglets immunized with 50ug CT was significantly less ($P < 0.05$) than piglets immunized with 100ug CT and 1 mg SEB (202 g/day, 228 g/day, and 224 g/day, respectively). However, these differences were not sustained throughout the entire length of

the trial. After the fourth vaccination, control pigs were consuming slightly more (327 g/day) than other treatments.

Average daily gain (ADG, Table 1) was affected ($P < 0.05$) by treatment after the first and fourth vaccination with control piglets gaining the fastest at 319 and 625 g/day, respectively. After the first immunization, control piglets gained significantly more ($P < 0.05$), 319 g/day, compared to piglets immunized with 50ug CT and piglets vaccinated with 100ug CT + 1mg SEB (247 g/day and 248 g/day, respectively). Gains among treatments after the second and third vaccinations were not significantly different. During the fourth vaccination period, control pigs gained the most, 625 g/day, and were significantly higher ($P < 0.05$) than pigs immunized with 100ug CT + 1mg SEB, 437 g/day. However, overall ADG did not differ among treatments ($P > 0.05$).

Feed efficiency (G: F, Table 1) following the first immunization was greatest in the control piglets with a value of 1.37 and was significantly higher ($P < 0.05$) than pigs immunized with 50ug CT and 100ug CT + 1mg SEB which each had a G:F value of 1.07. During the third week of the trial, feed efficiency was at its lowest, 0.89, with pigs immunized with 1mg SEB. Over the entire 36 day trial, G: F was not significantly affected by immunization regimens ($P > 0.05$).

Antibody Response

Immunization with the oral vaccine had a significant effect ($P < 0.05$) on anti-CT IgG antibodies produced in the sera (Figure 2). Blood sampled at the completion of the trial showed animals immunized with 100ug CT + 1mg SEB had antibody titers of 1.407 at a dilution of 1:3,000 which was significantly higher ($P < 0.001$) compared to control pigs and

those immunized with 1mg of SEB, 0.064 and 0.066, respectively. However, oral vaccination with rmSEB did not have significant effect ($P > 0.05$) on anti-SEB IgG antibody titers in the sera at the end of 36 days on trial (Figure 3). Pigs immunized with 1mg SEB without an adjuvant had the highest titers of anti-SEB IgG antibodies with a value of 0.6995 at a dilution of 1:100. These pigs tended to be significantly higher ($P = 0.052, 0.13$) than control pigs and pigs immunized with the highest does of CT with SEB with values of 0.2766 and 0.3997, respectively.

Superantigen Activity

Figure 4 shows the interferon gamma ($IFN\gamma$) response of pig splenic leukocytes following in vitro re-stimulation. Little difference is observed in control pigs versus treatment groups immunized with 1mg SEB. Limited in vivo immune responses resulted in limited in vitro antigen stimulation.

Trial 2

Animal Performance

Because initial piglet body weights were not similar among treatments ($P < 0.05$) they were used to covariately adjust the animal performance data. Oral immunization against SEB had an affect ($P < 0.05$) on overall ADFI (Table 2) with pigs receiving 1mg SEB + 100ug CT consuming less than pigs immunized with 10mg SEB + 100ug CT (314 g/day and 324 g/day, respectively). Similar differences were observed following the first vaccination. Yet, these treatments did not differ from one another after the second, third, and fourth vaccinations. In addition, neither of these treatments was significantly different than the controls.

ADG (Table 2) was affected by treatment ($P < 0.05$) after the second immunization for pigs vaccinated with 1mg SEB + 100ug CT. These pigs gained on average only 340 g/day which was significantly less than pigs immunized with 1mg SEB, 10mg SEB, and 10mg SEB + 100ug CT (370 g/day, 373 g/day, and 383 g/day, respectively). Piglets consumed roughly the same amount of feed during the first, third, and fourth week of the trial. Interestingly, pigs vaccinated with the highest dose of SEB + 100ug of CT had the highest gains following the fourth and final immunization, 516 g/day, including the control pigs which gained only 447 g/day. Furthermore, gains were not significantly different ($P > 0.05$) when data was averaged across the overall 36 day trial.

Accordingly, feed efficiency (G: F, Table 2) was affected by treatment ($P < 0.05$) during the second week of the trial with pigs immunized with 1mg SEB + 100ug CT having the lowest efficiency (1.13). These pigs' efficiency was significant less compared to pigs immunized with 1mg SEB, 10mg SEB, and 10mg SEB + 100ug CT (1.23, 1.24, and 1.27, respectively). Throughout the entire trial, efficiency was continually greatest for pigs vaccinated with the highest dose of SEB + 100ug CT, except following the third vaccination where 1mg SEB + 100ug CT immunized pigs had the highest efficiency (1.19 and 1.20, respectively). Overall there was not a significant affect ($P > 0.05$) of treatment on feed efficiency.

Body temperature (Figure 5) was affected by treatment ($P < 0.05$) after the second and fourth vaccinations. Piglets' surface body temperatures were roughly the same following the first and third immunizations. After the second vaccination, pigs immunized with 1mg SEB and 1mg SEB + 100ug CT temperatures were significantly less than pigs immunized

with the highest dose of SEB + CT (35.8 °C, 35.9 °C, and 36.8 °C, respectively). Control pigs' body temperatures were significantly lower following the fourth vaccination compared to the treatment of pigs immunized with 1mg SEB + 100ug CT (34 °C and 34.6 °C).

Antibody Response

Oral administration of rmSEB resulted in significant antibody responses. Figure 6 (Panel A) shows that on day 36 following the initial exposure to this antigen, significant levels ($P < 0.05$) of IgG antibody against native SEB were present in sera of pigs immunized with SEB without CT when compared to piglets that had received only the soy formulation. Consistent with the results obtained from serum antibody, Panel B of Figure 2 shows significant ($P < 0.05$) IgA titers were observed in fecal extracts of groups immunized with 1mg of SEB, with a value of approximately 0.20 titers, when compared to control piglets having a lower titer near 0.05.

Figure 7 shows specific sera IgG response to CT and SEB, Panels A and B, respectively. Pigs that received adjuvant formulated with immunogen produced the highest titers (near 70,000) of serum IgG antibodies against CT ($P < 0.05$) compared to control pigs and pigs immunized with only 1mg SEB (Panel A). Figure 7, Panel B shows that pigs immunized with 1mg SEB with and without adjuvant, CT, had high anti-SEB IgG titers, a value of approximately 1800. These titers were significantly higher ($P < 0.05$) than control pigs.

Superantigen Activity

Figure 8 shows that pig leukocytes from the spleen are quite responsive to native SEB with as little as 1ug/ml of the toxin stimulating a robust IFN γ response. Leukocytes that

were stimulated with higher concentrations, 3.3 ug/ml and 10 ug/ml, of the native toxin produced a response that surpassed 40 ng/ml of IFN γ . In contrast, rmSEB concentrations as high as 100ug/ml induced no detectable secretion of this cytokine. In addition, no significant reduction in cytokine secretion was observed when rmSEB was co-cultured with native SEB, even when a 100 fold excess of rmSEB (100 ug/ml) was present in the native SEB-stimulated cultures (Figure 9).

DISCUSSION

SEB has been characterized as “one of the two most important toxin threats on the battlefield or bioterrorism” (Chapter I). In fact, it is one of the few agents which has actually been weaponized (Christopher et al., 1997), and its effects on human subjects documented (Ulrich et al., 1997). While debilitating, exposure to the toxin is usually not fatal. However the nature of the symptoms and the ability to incapacitate victims for days to weeks following exposure, likely heightens the dangerousness of this toxin for weaponization.

No approved vaccine currently exists for SEB, and the lack of such a vaccine likely stems from at least two facts. First, widespread immunization of the populous against this toxin has not been justified, especially in westernized societies where the incidence of SEB-induced hospitalizations and deaths is circumscribed (Mead et al., 1999). Second, the threat of large-scale bioterrorism has only recently received renewed attention (Salerno et al., 2007), and efforts to prevent and protect from such attacks will require time and effort to achieve. Therefore, the availability of an efficacious vaccine for use by the military or other at-risk populations would represent a significant deterrent for those considering the use of SEB to induce injury.

Growth Performance

Immunization with an oral vaccine had transient effects on piglet growth performance with no net effect observed overall. As expected, there were no overall significant differences in ADFI (not including Trial 2), ADG, or feed efficiency. Slight differences in these data after the first vaccination could be attributed to acclimation of a new environment. Differences observed in ADG after the fourth vaccination were possibly due to switching from liquid feed to dry pelleted feed immediately following this immunization. Interestingly, the highest dose of SEB + CT often had the highest gains, feed efficiencies, and feed intake. Because weight loss over time can be a sensitive measure of adverse treatments, these results indicate the safety of using rmSEB, with or without the adjuvant, CT, as an oral vaccine.

Body temperature can also be an indication of health following an immune response. Piglet body temperatures were significantly different following two of the four immunizations. This could be due to the “heat lamp effect.” Piglets had access to a heat lamp during the first three immunizations. Temperatures were recorded on the surface of the pig with little account of the environment the pig had just been in (i.e. whether or not it was under a heat lamp). Therefore, differences could easily be derived from the environment of the pig and not the oral vaccine.

While many subunit vaccines candidates cannot survive the harsh environment of the gastrointestinal tract to stimulate immune responses, SEB has a very compact, stable protein structure (Papageorgiou et al., 1998, Swaminathan et al., 1992). Previous studies have demonstrated that SEB mutant, L45R, Y89A, and Y94A, was antigenically related to native SEB, and that immunization of mice and rhesus macaques resulted in the production of

antibodies (Boles et al., 2003, Tseng et al., 1995). In the present study, it was demonstrated that rmSEB can function as an immunogen and produce antibodies in swine that cross-react with native SEB. In addition, results surprisingly indicate that an oral vaccine formulation containing rmSEB could induce a systemic IgG (Figures 3, 6A, and 7B) and a mucosal IgA (Figure 6B) antibody response following an immunization regimen.

rmSEB lacks superantigen activity for pig lymphocytes

Fortunately, much is known regarding the structure-function relationships of this protein mediating its pathology (Li et al., 1998). The ability of SEB to bridge MHC class II molecules with the β chain of T cell receptors results in immune activation, cytokine secretion, and toxin-induced illness. By altering key amino acid residues which allow SEB to bind these molecules, no bridging can occur, and the toxic activity can be eliminated. Safety of human vaccine formulations is a paramount concern (WHO Technical Report Series, 2005), and nonclinical evaluations of vaccines using relevant animal models is required prior to their use. For mutant forms of SEB, the piglet provides an excellent model to assess the safety of non-toxic mutants such as rmSEB (Bi et al., 2009, Van Gessel et al., 2004). Using cultured splenic leukocytes, this study questioned whether rmSEB showed any detectable superantigen activity. Results from Trial 2 indicated that pig leukocytes are quite responsive to native SEB, but induce no detectable response against rmSEB. In addition, lack of significant reduction in IFN γ secretion (Figure 9) when rmSEB and native SEB are co-cultured, strongly suggests that rmSEB could not function as a competitive inhibitor of native SEB binding to class II MHC molecules. These in vitro studies suggested that rmSEB would be non-toxic to piglets when used in subunit vaccine formulations.

Oral vaccines superior to parenteral vaccines

Oral immunization strategies have some significant advantages when compared to conventional vaccinations; however there are some hurdles which remain when trying to develop efficacious mucosal vaccines. Most pathogens and toxins enter the host by mucosal surfaces (Chapter I), and it is logical to suggest that existing immunity at such surfaces would prevent or limit entry. Unfortunately, parenteral immunization regimens do not routinely stimulate high levels of mucosal IgA antibodies or cellular immunity at mucosal surfaces. Efficacious oral vaccinations can not only stimulate mucosal immunity, but often result in the induction of peripheral IgG and cellular responses as well. In this manner, mucosal immunizations can have the advantage on providing local, as well as systemic, protection against a particular pathogen or toxin. Injection-based vaccines must contend with the problems and limitations associated with the use of needles for delivery (Ryan et al., 2001). The concept of needle-less, oral administration is an attractive one that would reduce the need for medically trained personnel, eliminate disease transmission by contaminated needles, and provide the safest method of delivery. Despite these advantages, the development of efficacious oral immunization strategies has been slowed by the recognition that subunit protein antigens can be degraded in the gut, that these proteins are poorly immunogenic, and that new mucosal adjuvants must be developed to augment the response.

One class of oral adjuvants which may have the most promise for use in efficacious oral vaccine formulations are the α - β bacterial toxins, including CT (Chapter I). However, the ability of CT to function as an oral adjuvant in swine appears to depend on the antigen which is being co-administered. In a previous study using swine, co-administration of CT

was shown to improve the response against a fimbriae protein of *E. coli* following an oral immunization (Verdonck et al., 2005). In the present study, however, no adjuvant effect was observed when CT was added to oral vaccine formulations containing rmSEB (Figures 3, 7B). Despite the presence of substantial anti-CT antibody levels in piglets co-administered this bacterial protein (Figures 2, 7A), this exposure was not sufficient to augment the rmSEB response.

The inconsistency of adjuvant activity for co-administrated cholera toxin illustrates some hurdles which still need to be overcome when constructing efficacious oral vaccine formulations (Neutra et al., 2006, Ryan et al., 2001). In addition to discovering new oral adjuvants for human use, it will also be important to consider additives which might facilitate the delivery of immunogens safely through the gastrointestinal tract to mucosal antigen presentation cells. Many subunit protein vaccines can be destroyed or altered following oral administration, often making them poor immunogens by themselves. The results from this study were somewhat surprising because they demonstrate that rmSEB remained sufficiently intact once administered that it could stimulate a systemic (Figure 6A) and mucosal (Figure 6B) antibody response without aid of an adjuvant (Figure 7B). To facilitate oral delivery of rmSEB to the piglets, this immunogen was formulated with soy milk. Whether such a formulation contributed to the stability of rmSEB as it traversed the gastrointestinal tract will need further study. Soy milk formulations have been shown to have inherent buffering capacity (Lutchman et al., 2006, Park et al., 1991), which might aid in protein stability in the acidic environment of the gut. These studies clearly demonstrate that soy formulations containing rmSEB can induce an immune response against SEB when given orally.

Piglet Model

Previous work has suggested that swine would be an excellent model for a SEB challenge. During the course of immunization, little to no significant alteration in food intake or weight gain was noted in groups of control versus immunized piglets, demonstrating the safety of using oral rmSEB in vivo. Coupled with no superantigen activity and a robust systemic and mucosal antibody response being documented in the present study, a case has been made for the use of a piglet model. The piglet model is superior to mouse models which require LPS to potentiate SEB toxicity (Boles et al., 2003), or has limitations in mirroring the biphasic clinical response and overall pathology observed in humans (Bi et al., 2009, Van Gessel et al., 2004). The advantages of using the pig when compared to the rhesus macaque model (Tseng et al., 1995) include high expenses, complexity during experimental manipulations, and some biosafety concerns. Therefore, the piglet model is ideal for use in studies to determine whether oral administration of a formulation containing rmSEB could stimulate an immune response following an immunization regimen. Future studies should be aimed at using this model to demonstrate the efficacy of vaccination formulations and regimens by challenging immunized pigs with native toxin therapy.

IMPLICATIONS

Oral vaccines can provide an adequate mucosal and systemic antibody response against SEB without decreasing animal performance. Needless vaccines could have numerous implications for human use as well as agricultural use. Potential impacts of oral vaccines in the swine industry would minimize labor, provide immunization to a large number of animals, and would mean fewer injections resulting in less stress on the pig and ultimately better overall well being for the animals.

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Table 1. Trial 1 Growth Performance.

	Day	Control	50ug CT	100ug CT	50ug CT + 1mg SEB	100ug CT + 1mg SEB	1mg SEB	Pooled SEM
ADFI, g/day	0-7	212 ^{a,b}	202 ^a	228 ^b	219 ^{a,b}	218 ^{a,b}	224 ^b	11.00
	7-14	308	308	309	305	303	308	4.84
	14-24	409	406	410	408	403	408	3.86
	24-36	319	273	243	277	275	307	68.88
	0-36	327	321	328	325	327	329	4.84
ADG, g/day	0-7	319 ^a	247 ^b	251 ^{a,b}	265 ^{a,b}	248 ^b	287 ^{a,b}	34.73
	7-14	328	328	300	336	325	341	39.27
	14-24	484	445	429	453	436	447	30.99
	24-36	625 ^a	525 ^{a,b}	516 ^{a,b}	532 ^{a,b}	437 ^b	597 ^{a,b}	88.77
	0-36	417	393	395	401	378	380	26.72
Gain Feed ratio	0-7	1.37 ^a	1.07 ^b	1.08 ^{a,b}	1.14 ^{a,b}	1.07 ^b	1.24 ^{a,b}	0.15
	7-14	1.03	1.03	0.94	1.05	1.02	1.07	0.12
	14-24	1.27	1.17	1.12	1.19	1.14	1.17	0.08
	24-36	1.09	1.00	1.02	1.12	0.96	0.89	0.18
	0-36	1.11	1.04	1.05	1.07	1.00	1.01	0.07

^{a,b} Means within a row lacking a common superscript differ (P<0.05)

¹ Initial bodyweights used as a covariate. Initial body weights in grams: Control = 2757, 50ug CT = 2751, 100ug CT= 2587, 50ug CT + 1mg SEB = 3054, 100ug CT + 1mg SEB = 2399, 1mg SEB = 2824.

Table 2. Trial 2 Growth Performance.

	Day	Control	1mg SEB	10mg SEB	1mg SEB + 100ug CT	10mg SEB + 100ug CT	Pooled SEM
Feed Intake, g/day	0-7	227 ^{a,b}	225 ^{a,b}	221 ^{a,b}	215 ^a	233 ^b	8.31
	7-14	296	295	297	290	298	4.02
	14-24	396	396	396	394	395	1.03
	24-36	301	318	306	297	328	23.03
	0-36	320 ^{a,b}	321 ^{a,b}	320 ^{a,b}	314 ^a	324 ^b	3.94
Bodyweight gain, g/day	0-7	279	254	268	269	299	27.55
	7-14	363 ^{a,b}	370 ^b	373 ^b	340 ^a	383 ^b	14.21
	14-24	454	451	454	456	454	13.04
	24-36	447	493	474	463	516	53.51
	0-36	368	373	372	361	389	18.17
Gain Feed ratio	0-7	1.20	1.10	1.16	1.16	1.29	0.12
	7-14	1.21 ^{a,b}	1.23 ^b	1.24 ^b	1.13 ^a	1.27 ^b	0.05
	14-24	1.19	1.18	1.19	1.20	1.19	0.03
	24-36	1.14	1.23	1.20	1.22	1.32	0.14
	0-36	0.94	0.95	0.95	0.92	0.99	0.05

^{a,b} Means within a row lacking a common superscript differ (P<0.05)

¹ Initial bodyweights used as a covariate. Initial body weights in grams: Control = 2773, 1mg SEB = 2731, 10mg SEB = 2745, 1mg SEB + 100ug CT = 2552, 10mg SEB + 100ug CT = 2340.

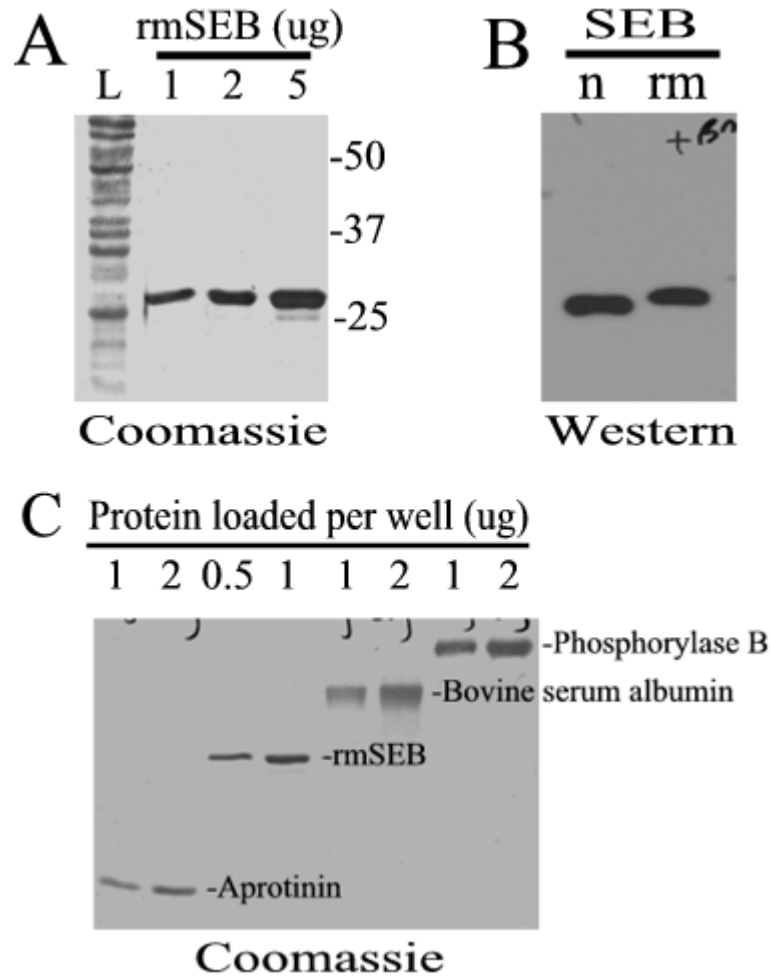
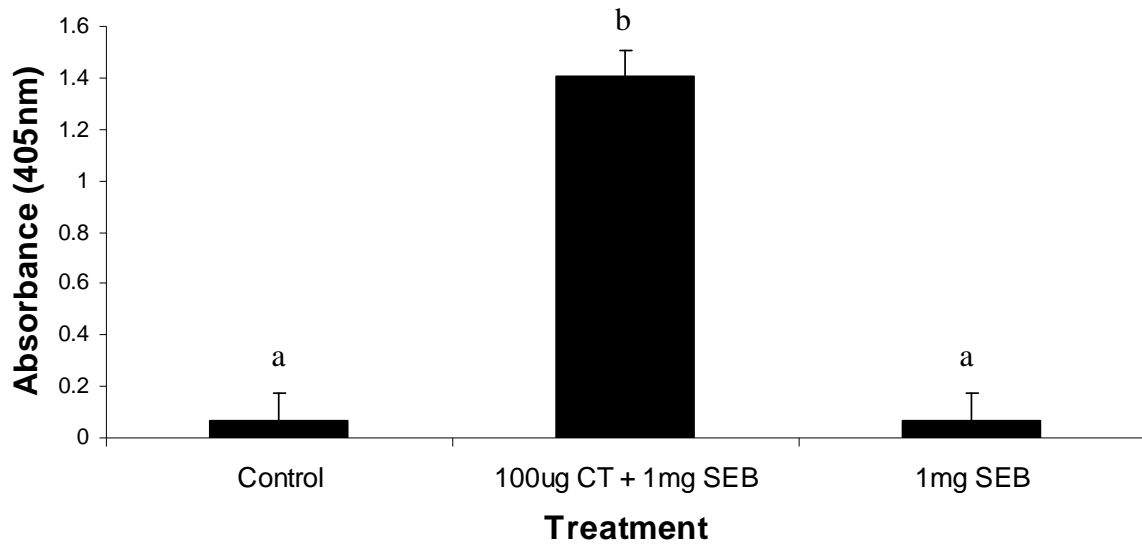


Figure 1. Characterization and quantification of SEB. Recombinant, mutant SEB (rmSEB), was expressed in *E. coli*, and purified as a C-terminal 6x-histidine-tagged fusion protein. Coomassie-stained SDS-PAGE of purified rmSEB showed a single band migrating at approximately 28 kDa, when compared to the migration protein standards shown to the left of the gel (Panel A). Western blot analyses demonstrated that rmSEB (rm) could be recognized by the same antibodies used to detect native SEB (n, Panel B). Known quantities of standard proteins were co-electrophoresed on coomassie-stained SDS-PAGE to estimate quantities of rmSEB (Panel C).

Anti-CT IgG Pig Sera



^{a,b} Bars lacking common superscript differ ($P < 0.05$)

Figure 2. Effect of oral immunization on sera antibody response to anti-CT IgG for Trial 1. Results are presented as least square mean absorbance values (\pm SEM) using a sera dilution of 1:3,000.

Anti-SEB IgG Pig Sera

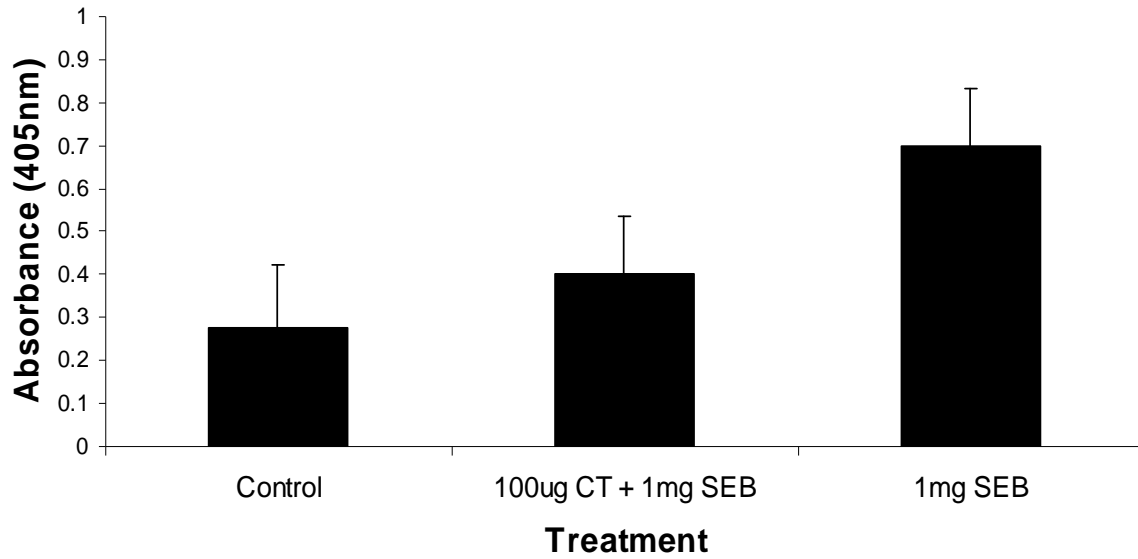


Figure 3. Effect of oral immunization of sera antibody response to anti-SEB IgG for Trial 1. Treatments did not differ significantly ($P > 0.05$), however, pigs ($n = 7$) immunized with 1mg SEB tended to be different ($P = 0.052, 0.13$). Results are presented as least square mean absorbance values (\pm SEM) using a sera dilution of 1:100.

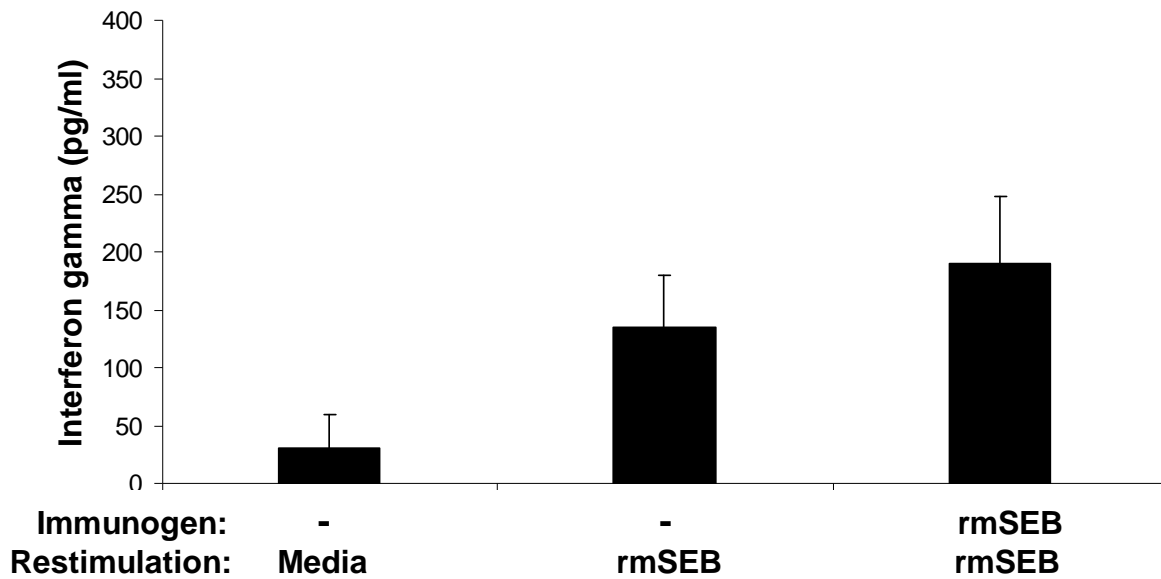
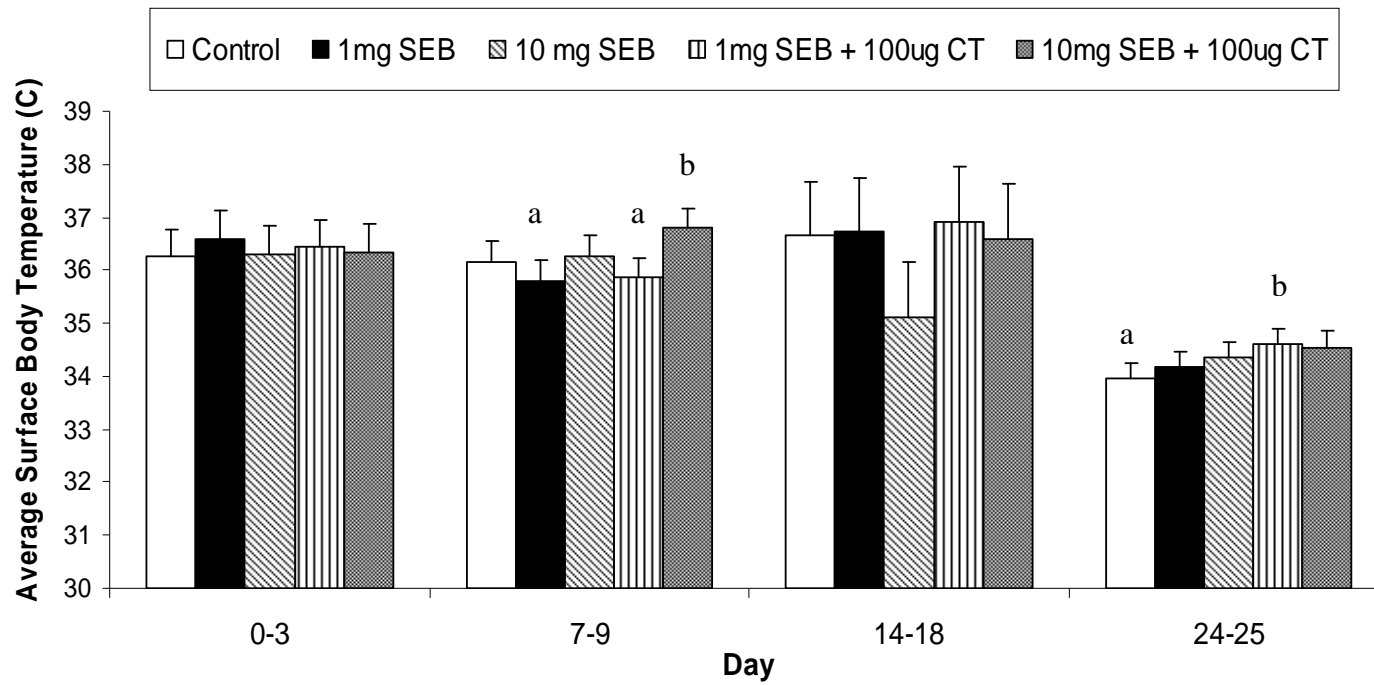


Figure 4. Effect of oral immunization on superantigen activity in pig leukocytes. Total pig leukocytes were isolated from individual spleens using lymphoprep density gradients. Triplicate wells from each animal containing 500,000 cells per well were cultured in the presence of media (RPMI – 10% FCS) or with 100ug of rmSEB. After 60 hours in culture, supernates were removed and the level of porcine interferon gamma was quantified by ELISA. Results are presented as mean values of 4 different pigs per treatment group with standard errors indicated.



^{a,b} Bars lacking common superscripts differ ($P < 0.05$)

Figure 5. Effect of oral immunization on surface body temperatures. Results are presented as least square means (\pm SEM).

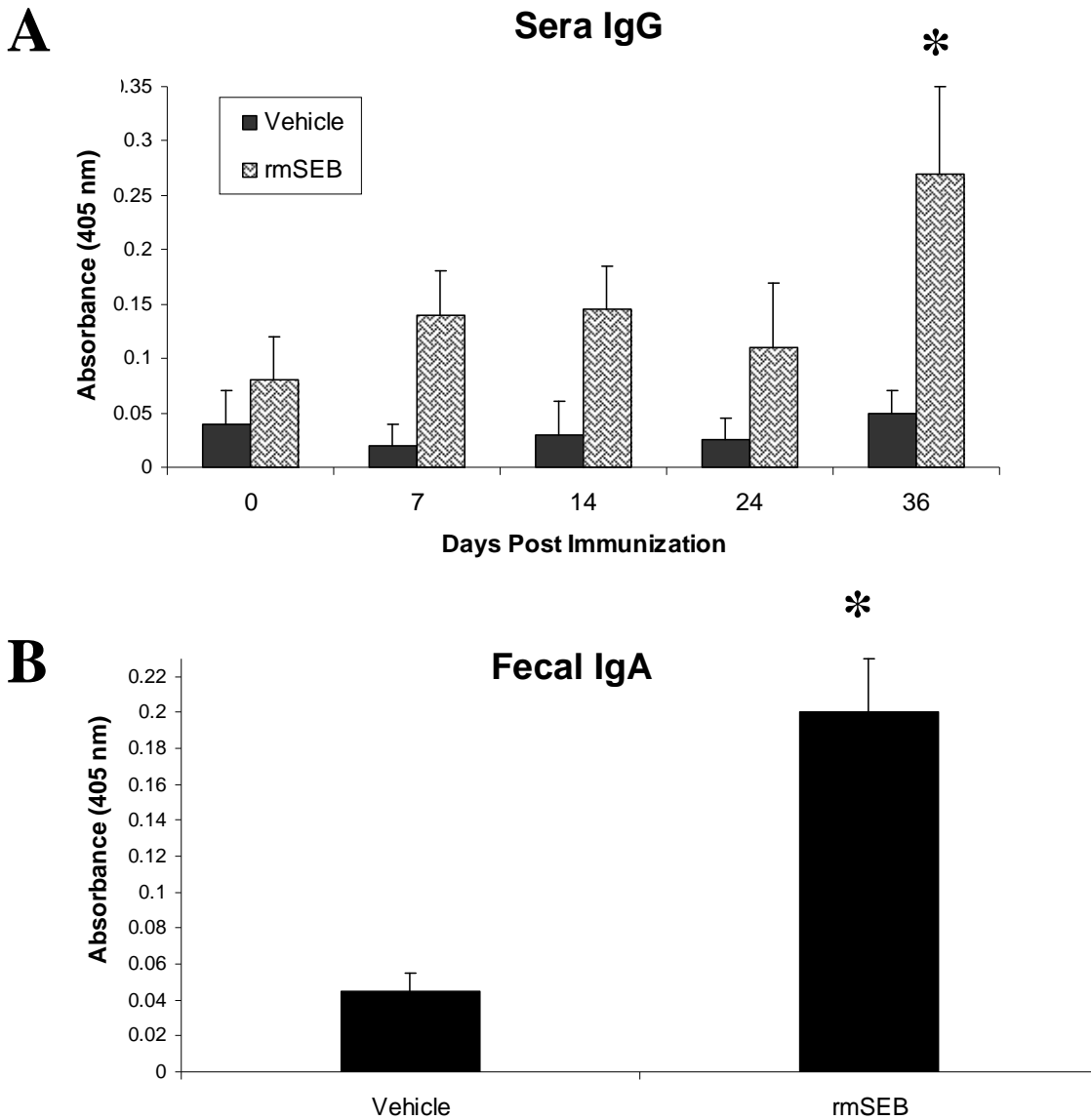


Figure 6. Oral administration of rmSEB results in significant antibody responses. Groups of seven day old piglets ($n = 8$) were orally immunized (day 0), and then boosted 7, 14, and 24 days later with formulations of 1mg of rmSEB formulated in soy milk (rmSEB) or with soy milk alone (vehicle). Each animal was bled at days 0, 7, 14, 24, and 36 post-immunization to obtain sera. Fecal samples were also collected at euthanasia (day 36) for ELISA to determine IgG (Panel A) or fecal IgA (Panel B) anti-SEB reactivity respectively. Results are presented as mean absorbance values (\pm SEM) using a sera dilution of 1:200 or a total fecal IgA level of 1 μ g/ml. * indicates a statistically significant difference ($P < 0.05$) when compared to all other determinations.

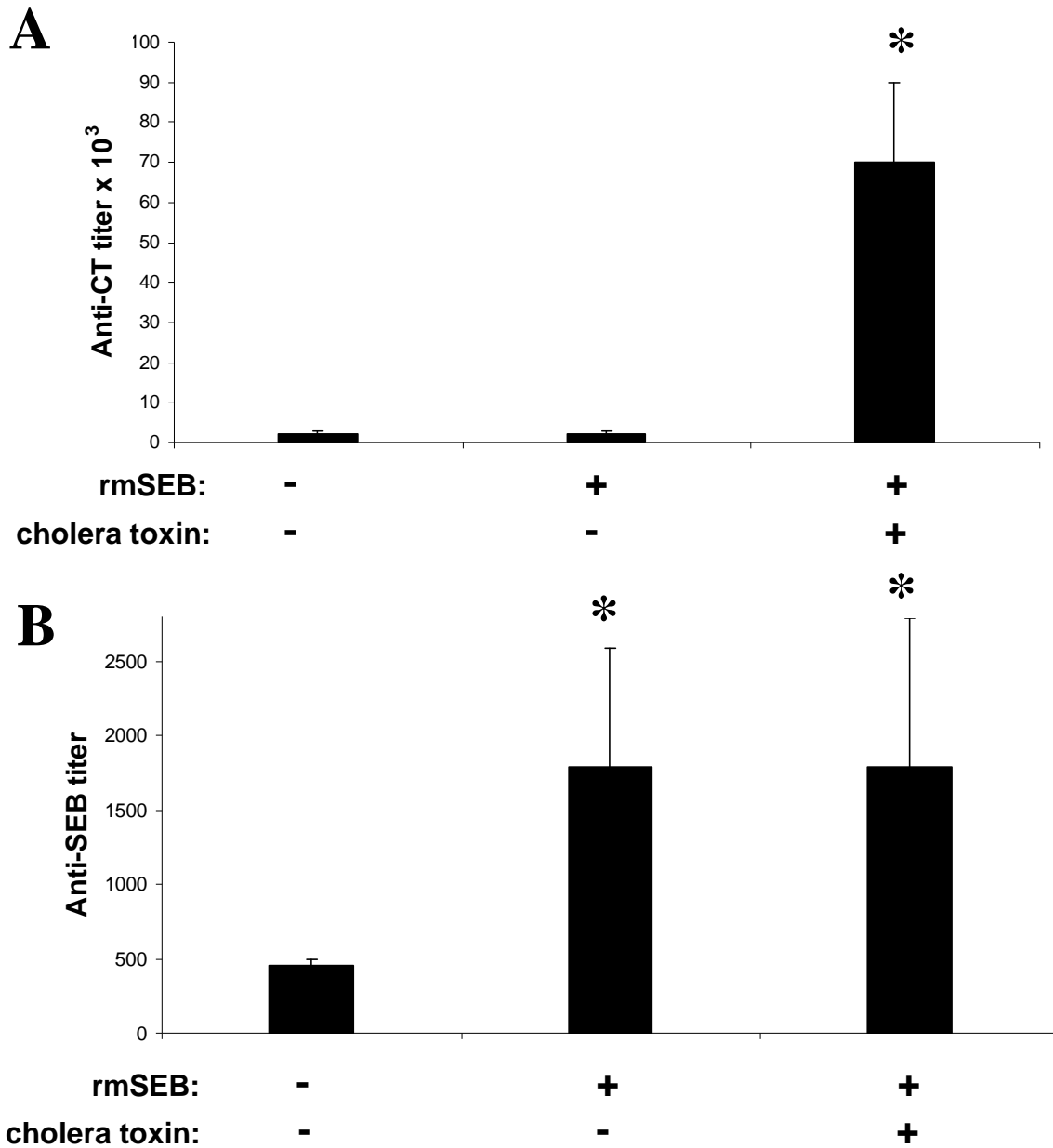


Figure 7. Oral administration of rmSEB plus the adjuvant, cholera toxin, does not augment the antibody response. Piglets were immunized with formulations of 1mg of rmSEB formulated in soy milk (rmSEB) with (+) or without (-) 100ug of cholera toxin (CT). On day 36 post immunization, piglets were euthanized and sera collected for ELISA to determine serum IgG anti-CT (Panel A) or serum IgG anti-SEB (Panel B) titers, respectively. Higher immunization dosages (10mg SEB and 10mg SEB + 100ug CT) produced no further increases in antibody titer (data not shown). Endpoint titers were defined as the last serum dilution with an absorbance double that of animals which received vehicle only. Results are presented as mean titers (\pm SEM). * indicates a statistically significant difference ($P < 0.05$) when compared to animals receiving vehicle only.

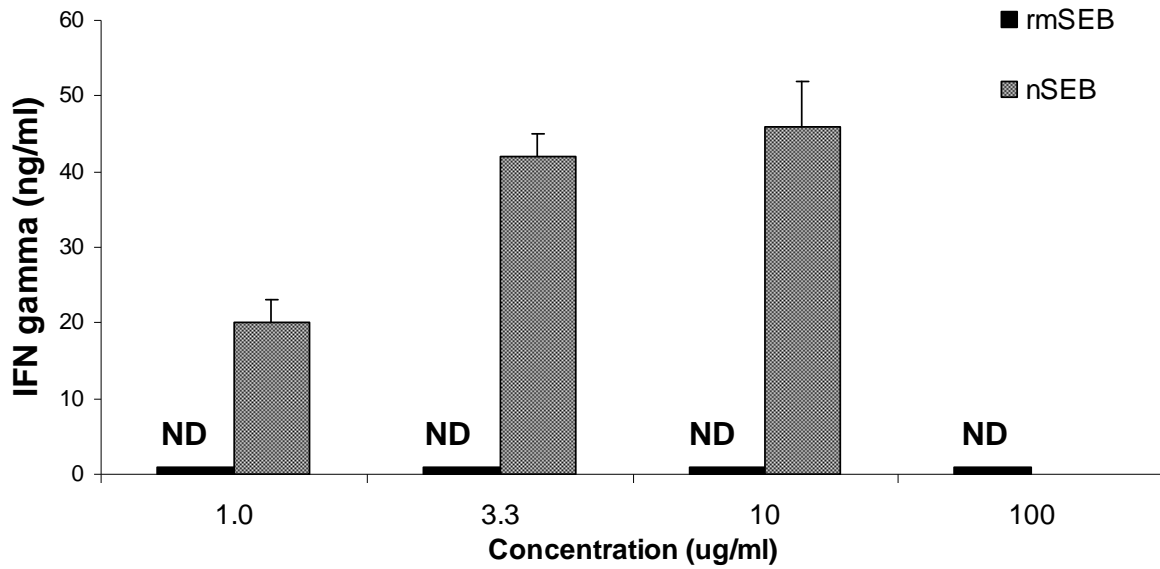


Figure 8. rmSEB lacks superantigen activity for pig lymphocytes. Pig splenic leukocytes were isolated, and cultured in the presence of the indicated concentrations of native SEB (nSEB) or rmSEB. After 40 hours of culture, supernates were taken and porcine interferon gamma (IFN gamma) secretion determined using an ELISA. Results are presented as mean values (\pm SEM) for triplicate determinations. Levels of interferon gamma that were below 50 pg/ml detection limit for this ELISA were designated as non-detectable (ND).

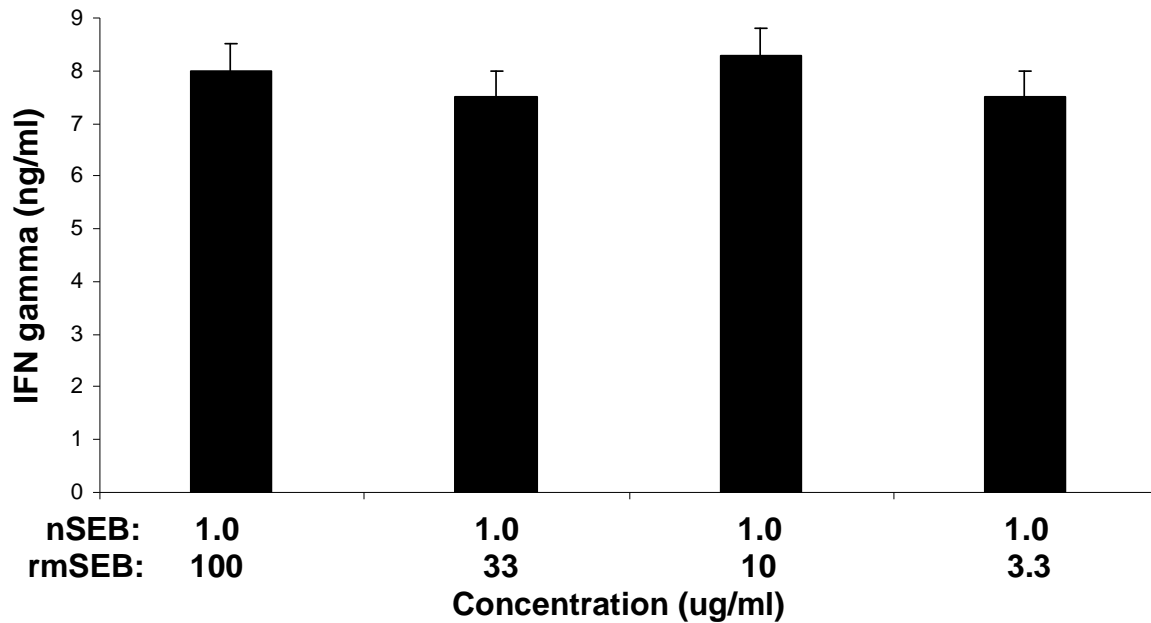


Figure 9. rmSEB is not a competitive inhibitor of native SEB for pig leukocytes. Pig splenic leukocytes were isolated, and co-cultured in the presence of 1.0 ug/ml of native SEB (nSEB) with the indicated concentrations of rmSEB. After 40 hours of culture, supernates were taken and porcine IFN γ secretion determined using ELISA. Results are presented as mean values (\pm SEM) for triplicate determinations.

APPENDIX

Table 1. Baby Pig Milk Replacer Diet provided by Milk Specialties Company (Carpentersville, IL)

Formula Code	Description	Species Code	Date Stored	Ver
GW0908-A	Jack Odle- Special High-End Pig MR	5	9/12/2008	3
	Amount Code Name		Per.	
	450.0000 103 NA CASEINATE 89%		11.2500	
	572.8008 144 DELAC 21.5% 8/28/08		14.3200	
	726.4457 203 12/28		18.1612	
	584.8608 215 13/60 MR		14.6215	
	75.0000 504 DICAL PHOS (ANHYD)		1.8750	
	13.0000 514 CALCIUM CHLORIDE 27% Ca		0.3250	
	1.5000 615 VITAMIN E 100,000 PREMIX		0.0375	
	20.0000 629 PRESTARTER MINERAL		0.5000	
	3.0000 669 VEAL ST VIT FX		0.0750	
	1.0000 808 LIQ CALF-ADE C *		0.0250	
	18.0000 819 POTASSIUM SORBA*		0.4500	
	19.7314 827 DL-METHIONINE		0.4933	
	1,614.8610 PB14 AGGLOM. AM PB14		37.8665	

	4,000.0000			
	Nutrient Name	Actual	Units	
1	Weight	1.0000	Lbs	
2	Dry Matter	96.7235	%	
3	Crude Protein	27.6946	%	
4	Crude Fat	18.0000	%	
5	Crude Fiber		%	
6	Ash	8.9865	%	
7	Gross Energy	4,717.6120	KCAL/Kg	
9	Lactose	36.3774	%	
12	Delactosed Whey	20.0000	%	
14	Citric Acid Inclusion		%	
21	Calcium	1.2111	%	
22	Phosphorus	1.1451	%	
23	Ca/P	1.0576	%	
25	Copper	11.8848	Mg/Kg	
26	Zinc	122.8090	Mg/Kg	
27	Selenium	0.3678	Mg/Kg	
28	Sodium	1.0416	%	
32	Cobalt	1.0518	Mg/Kg	
33	Iodine	6.1312	Mg/Kg	
34	Iron	106.5601	Mg/Kg	
35	Magnesium	0.1052	%	
36	Manganese	29.0908	Mg/Kg	
37	Potassium	2.0114	%	
38	Sulfur	0.3773	%	
45	Vitamin A	11.3278	KIU/LB	
46	Vitamin D3	2.2500	KIU/LB	
47	Vitamin E	56.2571	IU/lb	
48	Vitamin K	3.8363	Mg/Kg	
58	Thiamine	4.9677	Mg/Kg	
59	Riboflavin	21.4916	Mg/Kg	
60	Pyridoxine	5.1110	Mg/Kg	
61	Vitamin B12	72.3791	Mcg/Kg	
62	Pantothenic Acid	58.5601	Mg/Kg	
63	Niacin	28.8659	Mg/Kg	
64	Folic Acid	2.6900	Mg/Kg	
65	Vitamin C	90.1848	Mg/Kg	
66	Biotin	0.3742	Mg/Kg	
67	Choline	2,006.0570	Mg/Kg	
75	Alanine	1.2001	%	
76	Arginine	0.8478	%	
78	Aspartate	2.7729	%	
79	Cystine	0.3903	%	
80	Glutamate	5.6858	%	
82	Glycine	0.5557	%	
83	Histidine	0.6662	%	
85	Isoleucine	1.6486	%	
86	Leucine	2.8714	%	
87	Lysine	2.4500	%	
88	Methionine	1.0797	%	
89	Phenylalanine	1.1576	%	
90	Proline	2.3144	%	
91	Serine	1.3535	%	
92	Threonine	1.6468	%	
93	Tryptophan	0.4596	%	
94	Tyrosine	1.0326	%	
95	Valine	1.7719	%	
96	Met + Cys	1.4700	%	
156	C13:0 Tridecanoic	0.0604	%	
157	C14:0 Myristic	0.3929	%	
158	C14:1 Myristoleic	0.0002	%	
161	C16:0 Palmitic	4.5384	%	
162	C16:1 Palmitoleic	0.5663	%	
165	C18:0 Stearic	2.2850	%	
166	C18:1 Oleic	7.9342	%	
167	C18:2 Linoleic	1.7642	%	
168	C18:3 Linolenic	0.0267	%	
169	C20:0 Arachidic	0.0338	%	