THE EVALUATION OF TYPE III SECRETION APPARATUS COMPONENTS AS SUBUNIT VACCINE CANDIDATES AGAINST *SHIGELLA* AND *SALMONELLA* INFECTION IN MICE

By

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CHAPTER I

Introduction

Diarrheal diseases impact millions of people worldwide every year. Global mortality from diarrheal diseases is estimated between 4 and 6 million people per year according to the World Health Organization (WHO, 2003). Shigellosis and salmonellosis are two of the most important diarrheal diseases (Table 1.1). The gram-negative bacillus *Shigella flexneri* is a causative agent of shigellosis, a severe form of bacillary dysentery. According to the Centers for Disease Control and Prevention, there are approximately 14,000 confirmed cases and perhaps 450,000 total cases of shigellosis occurring in the United States annually (CDC, 2008). This disease is endemic in the developing world with an estimated 1 million deaths per year, the majority of these being children under the age of five. *Salmonella enterica* serovar Typhimurium, one of the most common of the approximately 2500 serotypes of the genus *Salmonella*, is a gram-negative bacillus that causes nontyphoidal *Salmonella* infection, which is a severe gastroenteritis in humans. The Centers for Disease Control and Prevention report an estimated 1.4 million cases of nontyphoidal salmonellosis annually in the United States, with 40,000 confirmed cases reported. Worldwide estimates of nontyphoidal *Salmonella* infections range from

Table 1.1- Common Foodborne Bacterial Pathogens Causing Diarrheal Disease In the United States	
Species of bacteria	**Incidence per 100,000**
Salmonella	7,444
Campylobater	5,825
Shigella	3,029
Shiga toxin producing *Escherichia coli* (0157)	513
Shiga toxin producing *Escherichia coli* (Non-0157)	205
Yersenia	164
Listeria	135
Vibrio	131

Table 1.1-Many bacterial pathogens cause diarrheal disease. While not all inclusive, this table lists some of the most common bacteria, in order of prevalence, that cause diarrhea in the United States per 100,000 according to the CDC (2009).

200 million to 1.3 billion annually. The death toll worldwide is approximated at 3 million each year (Coburn, Grassl, and Finlay, 2007).

Salmonella and *Shigella* infections represent major global public health burdens and substantial expenses to societies around the world. As strains are emerging with more resistance to antibiotics, vaccine development research for these pathogens has become crucial. Both *Salmonella* and *Shigella* use a type III secretion system (TTSS) to invade host cells. The TTSS is made up of a basal body spanning the bacterial inner and outer membranes and an extracellular needle-like structure used to recognize and inject effector proteins into the host cell. The exposed portions of the TTSS could potentially be targeted for vaccine development.

Historical Evaluation of *Shigella*

The first account of the characterization and isolation of the causative agent of bacillary dysentery was made in Japan by Kiyoshi Shiga in 1897 while studying *skeiri* (dysentery) outbreaks. This work was done at the Institute of Infectious Disease on 36 dysentery patients (Shiga, 1897). From the stool of these patients, he isolated a gram-negative bacillus that fermented dextrose, did not ferment mannitol, and had a negative indole reaction (Niyogi, 2005). Shiga continued characterization of the bacillus and originally called it *Bacillus dysenteriae* (Shiga, 1906). This discovery led researchers Flexner, Boyd and Sonne, in the next 40 years, to define and place three additional species of this organism into the Enterobacteriaceae whose names were attributed to their researchers: *S. flexneri, S. boydii, and S. sonnei* into the genus *Shigella* (Hale, 1991). The genus was first recognized in *Bergey's Manual of Determinative Bacteriology* in the 1930

edition and was officially accepted into the Congress of the International Association of Microbiologists in 1952 (*Shigella Commission*).

With the establishment of the genus, defining and characterizing the mechanism of virulence became the focus of *Shigella* research in the 1950s. The natural host range of shigellosis is limited to humans and other higher primates. In the latter part of the 1950s and into the 1960s the first animal models began to emerge. The corneal epithelia of guinea pigs were first successfully infected with *Shigella* spp. (Sereny test)(Sereny, 1957). Soon following, it was established that *Shigella* spp. could be grown in cultured mammalian cells (Gerber and Watkins, 1961). High doses of *Shigella* have more recently been found to induce pneumonia and diarrhea in rats if presented nasally or rectally, respectively (Mallet *et al.*, 1993) (Kamgang *et al.*, 2005).

It was assumed until 1964 that the pathogenicity of *Shigella* was a result of the release of toxins while the bacteria were adhering to the surface of the epithelial cells of the intestine (Watkins, 1960). LaBrec *et al*. fed guinea pigs virulent strains of *S. flexneri*, and found they caused ulceration and penetration of the epithelial cells of the colon and lamina propria. This showed that in fact, it was through invasion of the colonic epithelium that *Shigella* accomplished pathogenicity. (LaBrec *et al*., 1964).

It was not until 1981 that the genetic basis for *Shigella* pathogenesis was discovered. Sansonetti and his colleagues detected the presence of a large plasmid in *S. flexneri* and *S. sonnei.* The loss of this plasmid resulted in a loss of invasion in cultured mammalian cells (Sansonetti, 1981). All *Shigella* species have a large 200-kb virulence plasmid that contains the necessary genes that are required for invasion (Buchrieser *et al*.,

2000). Current research on *Shigella* is mostly focused on classifying and understanding the genes and proteins encoded on this plasmid.

Historical Evaluation of *Salmonella*

Salmonella organisms are gram-negative, rod-shaped bacteria that live in the intestines of many animal types and have evolved with their hosts to cause gastroenteritis and/or enteric fever (Ohl and Miller 2001). Accounts of *Salmonella* infection can be dated back to ancient times and are well described throughout history (Cunha, 2004). William Jenner clarified differences between typhus and typhoid based on epidemiology and symptomology in 1850 (Ellermeier and Slauch, 2006, 2006). Discovery of the Typhi serovar occurred in 1880, when Karl Eberth observed rod shaped organisms in the lymph and spleens of typhoid patients (Ellermeier and Slauch, 2006, 2006). In 1895 Theobald Smith, working under veterinarian Daniel E. Salmon at the USDA, isolated what became known as *Salmonella* serovar Choleraesuis while looking for the causative agent of hog cholera (Ellermeier and Slauch, 2006, 2006). Now known as *S. enterica*, the organism is one of two species, the other being *S. bongori*, recognized as members of Enterobacteriaceae (Ellermeier and Slauch, 2006, 2006).

The evolution of the genus has been delineated by a large number of investigators (Selander *et al*., 1996)(Baumler *et al*., 1998)(Edwards *et al*., 2002)(Porowollik *et al*., 2002)(Chan *et al*., 2003) and can be summarized as follows: *Salmonella* diverged from *E. coli* around the time mammals first appeared, 120-160 million years ago. Roughly 25-40 million years ago, *Salmonella* obtained the *Salmonella* pathogenicity island 1 (SPI1) which encodes the type III secretion system involved the invasion of the intestinal

epithelium. At this time *Salmonella* was an intracellular pathogen affiliated with cold-blooded vertebrates. *Salmonella* subsequently acquired the Salmonella pathogenicity island 2 (SPI2), which has a type III secretion system that permits the organisms to colonize deeper tissues by surviving in macrophages. The presence of the SPI2 is a defining characteristic of *Salmonella* spp. The seven subspecies of S. enterica (I, II, IIIa, IIIb, IV, VI, and VII) continued to evolve to fit their respective host niches. The ancestor of subspecies I, II, IIIb and VI developed the ability to phase shift between multiple structural subunits of flagella, the organism's principal organ of motility. This provided *S. enterica* with the ability to evade the host immune system further. The subspecies I ancestor then acquired the ability to colonize warm-blooded animals, a major advance with marked repercussions for human health. The subspecies I strains have subsequently evolved to colonize a variety of hosts. Serovars such as Typhi are host specific, infecting only humans, however, serovar Typhimrium is less specific, and is able to colonize and cause disease in a variety of mammalian species. *Salmonella* serovar Typhimurium usually causes a self-limiting gastroenteritis in humans, but in mice it causes a systemic disease similar to typhoid fever. This species of *Salmonella* has therefore offered a valuable animal model for the study of these invasive pathogens and allowed genetic, biological, and biochemical analysis of its infection process (Brummell and Finlay, 2000).

Epidemiology of *Shigella* and *Salmonella*

Shigellosis is exclusive to humans and other higher primates and infection is limited to the intestinal mucosa. Common symptoms can include watery diarrhea with mucus, fever, malaise, abdominal cramping, and ulceration of the mucosa resulting in

bloody stools (Niyogi, 2005). Symptoms exhibited are dependent on the species and quantity of the organisms ingested and additional complications can ensue including: septicemia, bacteremia, dehydration, hypoglycemia, uremic and hemolytic syndrome, and toxic megacolon (Phalipon and Sansonetti, 2007). Shigellosis is widespread throughout the world, infecting approximately 165 million individuals, 163 million of which inhabit developing countries. Over 1 million of these instances result in death, and children under the age of five account for over 600,000 deaths. This mortality rate due to dehydration and is exacerbated by malnutrition (World Health Organization). *S. flexneri* is most prevalent in developing nations, while *S. sonnei* is more common in industrialized countries (Niyogi, 2005). In the United States the majority of outbreaks of *Shigella* affect children in day care facilities, migrant workers, travelers, custodial workers, homosexual men, and persons living in community homes and prisons (Niyogi, 2005).

Salmonella species can infect an extensive range of animals, and those infections can cause distinctive diseases in different hosts, producing gastroenteritis, typhoid fever and bacteremia. Certain infections can be host specific, although *Salmonella* serovar Typhimuruim has the ability to infect a wide range of animal hosts (Brummell and Finlay, 2000). Symptoms of gastroenteritis caused by *Salmonella serovar* Typhimurium include diarrhea, which can be watery or bloody, abdominal cramps, fever, nausea and vomiting. *Salmonella* can occasionally establish a localized infection or enter the blood, causing bacteremia. The bacteria often remain in the intestine and are excreted for several weeks (CDC). 1.4 million cases of nontyphoidal salmonellosis are annually reported in the United States. Worldwide estimates of nontyphoid *Salmonella* infection range from 200 million to 1.3 billion. The death toll worldwide is estimated to be 3 million each year

(Coburn, Grassl, and Finlay, 2007). *Salmonella* can infect anyone, at any age, but elderly individuals, infants and those with a compromised immune system are the most at risk for a *Salmonella* infection (Wallis and Galyov, 2000).

Both *Shigella* and *Salmonella* infections are acquired via the fecal-oral route and are primarily linked to poor hygienic practices and contaminated food or water. Overcrowding further contributes to outbreaks around the world. The incidence of both infections is greater in the developing world for the above reasons and this compounds already existing problems in these underdeveloped nations (DuPont, 1989).

***Shigella flexneri and Salmonella serovar Typhimurium*-The Pathogens**

Species of *Shigella* and *Salmonella* belong to the family Enterobacteriaceae. (Kreig and Holt, 1984) (Haimovich and Venkatesan, 2006). Each organism is a gram-negative, faculatively anaerobic, oxidase negative, gastrointestinal pathogen with the ability to reduce nitrate and ferment glucose (Kreig and Holt, 1984). *Shigella* spp. can be differentiated serologically based on species-specific O-antigen components that each contain (Kreig and Holt, 1984). There are four species: A, B, C, and D belonging to *S. dysenteriae, S. flexneri, S. boydii* and *S. sonnei*, respectively (Niyogi, 2005). *Shigella* spp. are extremely infectious requiring as few as 10-100 organisms to elicit shigellosis (DuPoint *et al.*, 1989).

The more serologically complicated *Salmonella* spp. are classified based on the Kauffman-White Classification Scheme. *Salmonella* is divided into 2 species, *S. bongori* and *S. enterica*, which are further divided into 2501 serovars based on surface antigens. O-antigen type is first determined followed by H-antigen expression classification. The

H-antigen expressed is dependent upon the flagellar protein type made and this can change through a process called phase variation. The phase, 1 or 2, denotes the organism's motile or non-motile phenotype, respectively. *S. borgori* is further divided into 21 serovars. *S. enterica* is futher divided into 6 subspecies: I, *S. enterica* subsp. *enterica*,(1,478 serovars); II, *S. enterica* subsp. *salamae*, (498 serovars); IIIa, *S. enterica* subsp. *arizonae*, (94 serovars); IIIb, *S. enterica* subsp. *diarizonae*, (327 serovars);IV, *S. enterica* subsp. *houtenae*,(71 serovars); and VI, *S. enterica* subsp. *indica*. (12 serovars) (Centers for Disease Control and Prevention, 2006).

Shigellosis and salmonellosis are self-limiting infections with symptoms lasting 5-7 days or longer if untreated (Hueck, 1998) (Bullock et al., 2006). If treated with antibiotics, the duration of symptoms is lessened and the period that the organisms are shed following recovery is shortened, however, as long as the individual is shedding the organisms, the diseases can easily be spread (Sur, 2004) (Brands, 2006). Individuals with symptoms of diarrhea for salmonellosis and shigellosis are most responsible for transmission of the disease. Poor sanitation and hygiene practices and contaminated food or water sources are common causes of outbreaks of these diseases (Niyogi, 2005) (Brands, 2006). Oral antibiotics are the most preferred means of treatment for *Shigella* and *Salmonella* infections. Unfortunately, due to the ability of bacteria to adapt and overcome host defenses, there has been an emergence of strains that have acquired antibiotic-resistance, which is limiting the number of drugs available for treating each infection (Hueck, 1998).

Genetics of Virulence

All species of *Shigella* harbor a large virulence plasmid. Genes encoded on this plasmid and those of the pathogenicity islands located on its chromosome, provide the bacteria with antibiotic resistance, iron acquisition and proteases (Schroeder and Hilbi, 2008). *Shigella* virulence and its ability to invade human intestinal cells are made possible by genes on the 200-kb virulence plasmid, which contains approximately 100 genes (Maurelli *et al.*, 1985) (Yoshikawa *et al.*, 1988). Genes included on this plasmid are required for bacterial uptake and propagation within the colonic epithelium (Wantanabe, 1990). The type III secretion system facilitates bacterial uptake and is encoded by the 31-kb entry region of the virulence plasmid (Schroeder and Hilbi, 2008). This entry region also contains the Mxi-Spa and Ipa-Ipg operons, which encode the type III secretion apparatus, and its secreted effectors, and the translocators IpaA, IpaB, IpaC, IpaD along with the molecular chaperone IpgC (Espina, *et al*., 2006) (Figure 1.1). If this virulence plasmid is not present, *Shigella* are not infectious (Yoshikawa, et al., 1988). Noninvasive *E. coli* strains ahve been shown to become invasive upon receiving the *Shigella* virulence plasmid (Sansonetti 1982).

There are four categories of genes in the entry region of the virulence plasmid, which are distinguished based on their functions (Schroeder and Hilbi, 2008). Ipa (invasion plasmid antigen) genes function as effector and translocator proteins secreted by the TTSA make up the first group and these are involved in the cytoskeletal

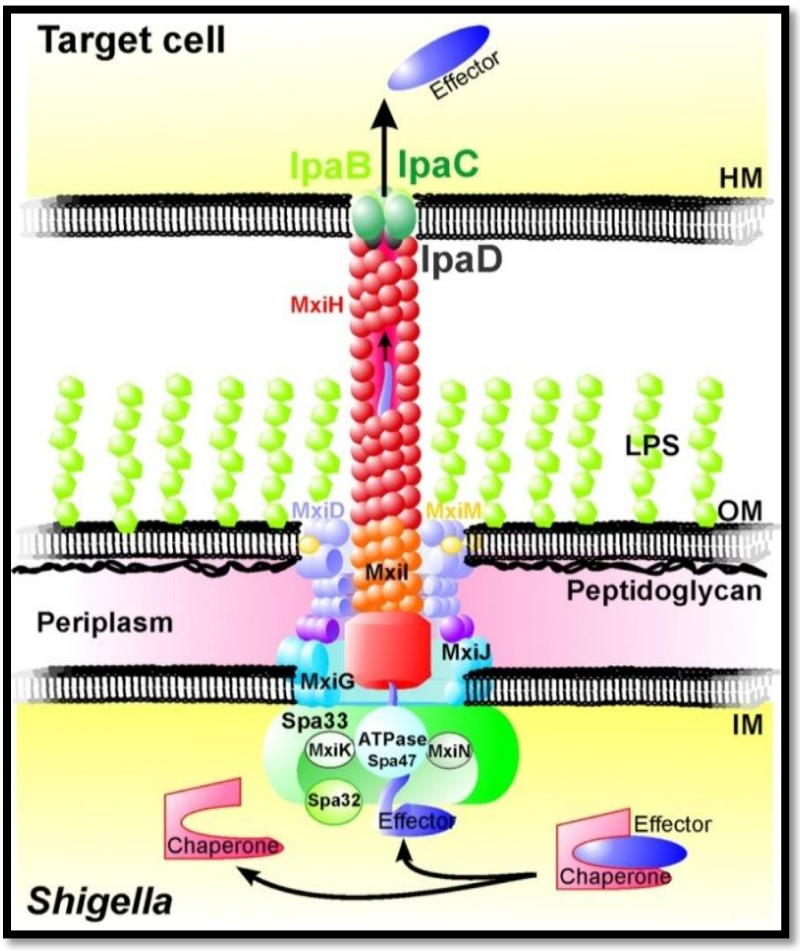

Figure 1.1-The type III Secretion System of *Shigella flexneri*

The TTSS is made up of a basal body; spanning the inner and outer bacterial membrane, a needle complex; facilitating injection of effector proteins, and a pore complex which allows host cell recognition and translocation of effectors into host cells (Schroeder and Hilbi, 2008).

rearrangements, and the cell membrane ruffling which result in bacterial uptake. The structural proteins Mxi (membrane expression of Ipa proteins) and Spa (surface presentation of Ipa antigens) make up the second group (Espina *et al.,* 2006) (Schroeder and Hlbi, 2008). Mxi and Spa structural protein polymers comprise the basal body and needle of the TTSA (Espina, *et al*., 2006). Transcriptional activators control expression of the early and late entry region genes, and these make up the third group. The molecular chaperones then fall into the fourth group, and they function to protect effector and translocatorproteins while they are stored in the cytoplasm prior to secretion (Schroeder and Hilbi, 2008).

The invasion of human cells and subsequent intracellular replication by *Salmonella* is multifaceted. There are approximately 200 genes including those on the five chromosomal pathogenicity islands (SPI-1 to SPI-5) on the *Salmonella serovar* Typhimurium chromosome that are essential for virulence. SPI-1 and SPI-2 encode two of the type III secretion systems that facilitate invasion by *Salmonella* species. SPI-1 is necessary for intestinal epithelium invasion and SPI-2 is required for survival within macrophages (Haimovich and Venkatesan 2006). SPI-1 encodes over 30 proteins, including those that comprise the TTSS-1 and its secreted effectors. *Salmonella* invasion proteins are encoded by the Sip/Sic operon on SPI-1 (Galan, 1996) (Hueck, 1998). Secreted proteins SipA, SipB, SipC and SipD and the molecular chaperone, SicA are homologues to *Shigella* Ipa proteins and IpgC., Each of the four required effectors and the chaperone function in host cell invasion in the same way their *Shigella* homologues (Espina, et al., 2006). *Salmonella* serovar Typhimurium mutants deficient in SPI-1 are

incapable of invading M cells and other intestinal epithelial cells, stressing the necessity of SPI-1 genes in intestinal colonization (Galan, 1996) (Hueck, 1998).

Following invasion of host cells, SPI-2 genes are expressed and are necessary for intracellular survival of *Salmonella* in both epithelial cells and macrophages (Haimovich and Venkatesan 2006). SsrA-SsrB is encoded by SPI-2 and regulates the TTSS-2 which is required for intracellular survival and subsequent replication within the host. Mutants lacking SPI-2 genes resulted in a reduction of virulence and the inability to colonize the spleens of infected animals (Anthea, Detweiler and Falkow, 2000). It is believed that the systemic infection in mice caused by *Salmonella* infection is reliant upon its ability to colonize and replicate within macrophages, and this is one of the traits setting apart *Salmonella* infection from gut-confined shigellosis (Haimovich and Venkatesan 2006).

Cellular Invasion by *Shigella flexneri* and *Salmonella serovar* Typhimurium

After ingestion, *Shigella flexneri* is protected from the stomach due to its high acid tolerance. It then facilitates its way to the large intestine using as set of enterotoxins called sen and set (Kotloff, *et al.,* 2000). In the large intestine, *S. flexneri* invades the colonic epithelium (Sur, 2004). . Invasion requires that the organism reach the basolateral face of the epithelial cells. Once in the large intestine, *S. flexneri* is taken up in vacuoles by microfold cells (M cells) via macropinocytosis in the colon (Owen, 1986) (Wassef, 1989). The organisms then escape the vacuole into the cytosol, and travel to underlying macrophages that are associated with M cell-associated lymphoid follicles (Sansonetti, *et al*., 1996). *Shigella* is transcytosed into an intraepithelial pocket by M cells, (Schroeder and Hilbi, 2008) where it is then taken up by a resident macrophage, which is triggered to

undergo apoptosis. This allows escape of the pathogens to the basal side of the epithelium. Death of the macrophage is initiated via a caspase 1-dependent pathway, and this leads to the release of the proinflammatory cytokines, Interleukin-1β (IL-1β) which triggers inflammation in the lower intestine, and Interleukin-18 (IL-18) which activates NK cells. Production of IL-8 follows this inflammation trigger, which recruits polymorphonuclear cells. PMNs initiate tissue damage and allow more bacteria to bypass the M cells and reach the submucosa via destabilization of the epithelial lining (Schroeder and Hilbi, 2008) (Sansonetti, 2000). The tight junctions of epithelial cells are also altered and loosened, exacerbating the initial invasion of *S. flexneri*. All of this leads to the bloody diarrhea that the organism is known for (Schroeder and Hilbi, 2008). After crossing the epithelial barrier and inducing ingestion by the epithelial cells of the colon, *Shigella* uses actin-based motility to move through the cytoplasm laterally from cell to cell (Mounier et al., 1992). This further enhances inflammation and ulceration of the mucosa in the colon. This is attributed to shigellosis, but it is actually a result of the host's own immune response to the infection (Wassef et al., 1989) (Islam et al., 1997). The goal of *Shigella flexneri* is to replicate within its host cell's cytoplasm, and invade neighboring cells by spreading laterally through the colonic epithelium. This intracellular, replicative niche of *S. flexneri* is also what protects the organism from the extracellular immune system components (Schroeder and Hilbi, 2008).

Salmonella serovar Typhimurium is not so resistant to the acid from the stomach and thus a much higher inoculum is required for invasion unless there is a deficiency of stomach acid, which can be caused by various factors including improper nutrient absorption, stress or alcohol consumption (Jones and Falkow, 1996) (Hersh, 1999).

Salmonella serovar Typhimurium achieves invasion of the colonic epithelia by the same mechanism as *S. flexneri,* but it does not necessarily stop there. The goal of *Salmonella serovar* Typhimurium is to penetrate the mucosal barrier and interact with the cells of the immune system (Bowe, Lipps, Tsolis, Groisman, Heffron, and Kusters, 1998). *Salmonella* serovar Typhimurium has adapted mechanisms to survive and replicate within the vacuoles of these cells. In particular, infection of macrophages allows *Salmonella* serovar Typhimurium to disseminate beyond the mucosal surface to initiate systemic infection (Brumel and Finlay, 2000). Following invasion of host cells, *Salmonella* serovar Typhimurium becomes localized in the *Salmonella*-containing vacuole (SCV) within which the organism replicates and is protected from stomach acid and the immune system. It eventually lyses the host cell and escapes to the extracellular environment to infect its next host (Fields, Swanson, Haidaris and Heffron, 1986). This ability to survive and replicate within phagocytic cells, such as the macrophage, is another example of what differentiates *Salmonella* infections in humans from gut-contained shigellosis (Haimovich and Venkatesan, 2006).

The Type Three Secretion System

Bacteria carry secretion systems as part of their natural functions. Many gram-negative bacterial pathogens use needle-like, highly homologous secretion systems to manipulate host cell signals and eventually transport effector molecules or virulence factors into targeted eukaryotic host cells (translocation) or possibly into the extracellular environment (secretion). There are currently six recognized classed of bacterial secretion systems (numbered I to VI). *Shigella* and *Salmonella* spp. possess type III secretion systems and their invasion of human cells is dependent on the translocation of effector

proteins that manipulate a targeted host's cytoskeleton, thereby inducing membrane ruffling and subsequent bacterial uptake. *Salmonella* serovar Typhimurium also harbors a second type III secretion system which facilitates its survival and replication within host cell phagosomes (Monack, Raupach, Hromockyj and Falkow, 1996). Additionally, *S. flexneri* and *Salmonella* serovar Typhimurium are capable of inducing apoptosis of macrophages, which is facilitated by their type III secretion systems and which allows their escape after crossing the intestinal epithelium (Ruckdeschel, et al., 1997).

The type III secretion system is made up of a basal body that spans the inner and outer membranes of the bacterium and an extracellular needle complex that bacteria use as a molecular syringe to deliver effectors directly into the target cell's cytoplasm and promote bacterial uptake or host cell killing (Hueck, 1998)(Cornelis, 2006) (See Fig. 1.1 and 1.2) The needle is a polymer that is approximately 50 nm long, 7 nm wide, and contains an inner channel with a diameter of about 2.5 nm (Epler *et al.*, 2009). Type III secretion is contact dependent and is an ATPase-driven system. The ATPase elaborates protein unfolding, chaperone release, and transmembrane transport of the substrate proteins (Blocker *et al.*, 2001) (Cornelis, 2006).

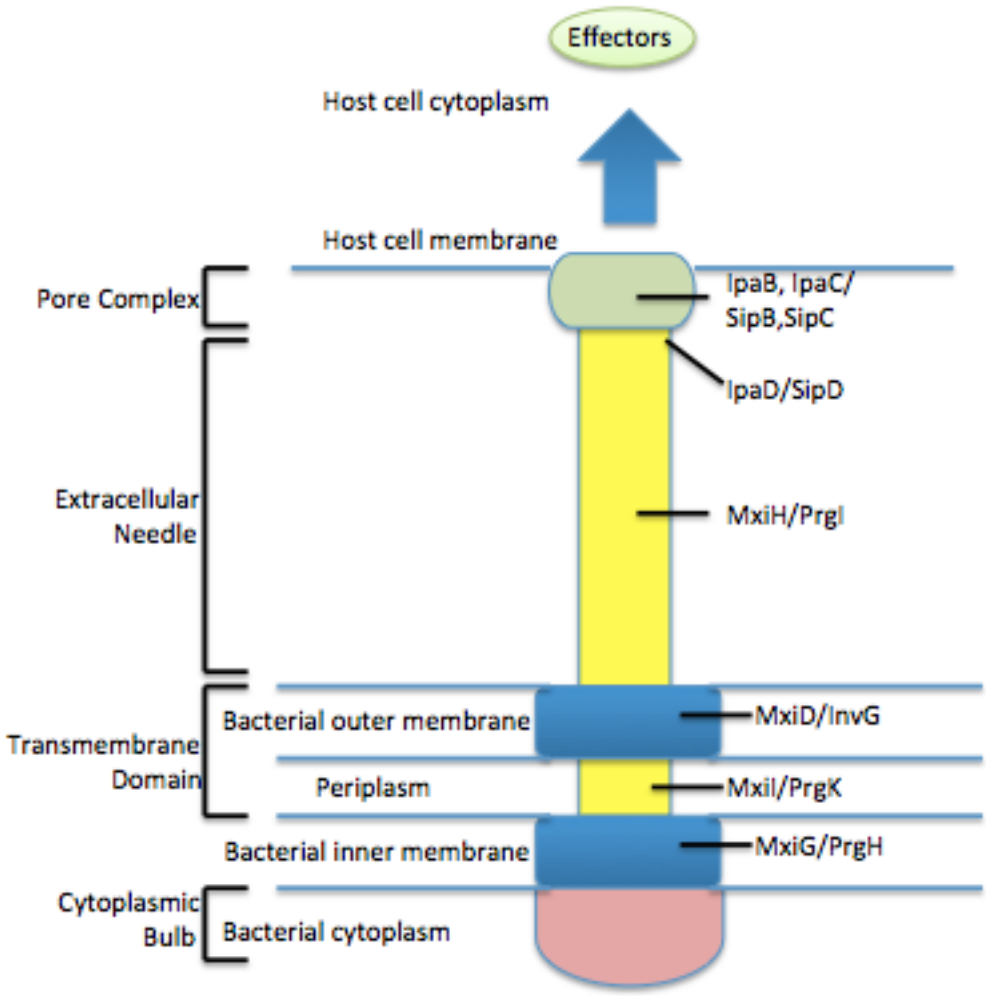

Figure 1.2. Components of the TTSS

An overview of the TTSS including: the basal body, spanning the inner and outer membrane; extracellular needle; and tip complex for *Salmonella* (Sip, Prg and Inv proteins) and *Shigella* (Ipa and Mxi proteins).

The Ipa and Sip Proteins

Shigella and *Salmonella* use closely related type of type III secretion systems to deliver effector proteins into host cells. The homologous proteins that make up the needle complex for *Shigella* and *Salmonella* are MxiH and PrgI, respectively. Due to their fundamental role in surface exposure and virulence, they are attractive vaccine component candidates (Darboe, *et al.*, 2006). Effector proteins that are secreted and translocated during interaction of *Shigella* with the host cell are IpaD, IpaB, IpaC, IpaA and the molecular chaperone IpgC. Based on nucleotide sequencing *Salmonella* have genes that encode for homologues of these five *Shigella* proteins used in their own TTSS: SipD, SipB, SipC, SipA and SicA, respectively, which assemble and function in the TTSS in the same way and are required for invasion (Kaniga, Trollinger and Galan, 1995). *Salmonella* further diverges from *Shigella* in that it uses a second TTSS to survive and replicate within the macrophage to induce systemic infection (Monack, Raupach, Hromockyj and Falkow, 1996).

To promote bacterial uptake, *Shigella* exploits secreted effector proteins that facilitate entry into the target cell and seize control of host cytoskeletal proteins. IpaD sits at the tip of the MxiH needle apparatus, where it senses environmental signals, initiates secretion induction, (Espina et al., 2006) and is an important secretion substrate in *Shigella* type III secretion. As IpaD binds and thereby senses intestinal bile salts, IpaB is secreted and recruited to the tip of the TTSA needle (Stensrud *et al.*, 2008). It is at this point that the TTSS of *Shigella* is equipped for full induction of type III secretion. Following host cell contact, additional secreted effectors include the final translocator protein, IpaC which is recruited to the needle tip and is secreted into the host cell

membrane (Epler *et al.*, 2009) (van der Goot *et al.*, 2004). Studies in our lab have shown that the presense of bile salts, found in high concentrations in the small intestine, induces the secretion of IpaB and leads to the co-localization of IpaB and IpaD at the distal tip of the TTSA needle (Olive et al., 2007). This is said to be a priming step that prepares the needle for interaction with the host cell via cholesterol binding by IpaB (Olive, 2007) (Hayward *et al.*, 2005).

The molecular chaperone IpgC (invasion plasmid gene) binds to IpaB prior to IpaB secretion by *S. flexneri* (Birket *et al.*, 2007), thus preventing aggregation of IpaB within the bacterial cytoplasm, and its binding to IpaC, the final translocator protein at the TTSA needle tip (Menard *et al.*, 1994) (Page *et al.*, 1999) (Lunelli *et al.*, 2009). Association between IpaB and IpgC has been shown to maintain the pre-secretion state of IpaB for its consequent passage through the needle of the TTSS (Menard *et al.*, 1994) (Page *et al.*, 1999). IpgC additionally acts as a chaperone for IpaC, the final translocator/effector protein. IpaC joins with IpaB to form a translocon pore component (Menard *et al.*, 1994) (Page *et al.*, 1999) and it is involved with host cell signaling (Tran Van Nhieu *et al.*, 1999).

The preliminary proteins secreted by the *Salmonella* Pathogenicity Island-1 (SPI-1) TTSS are called Sips (*Salmonella* invasion proteins), and are homologous to *Shigella* proteins and function in invasion of the host cell and secrete effectors in the same manner (refer to Figure 1.2). Invasion by *Salmonella* requires SipD, SipB, SipC, and the molecular chaperone SicA, just as homologues, IpaD, IpaB, IpaC,and IpgC, respectively, are essential for *Shigella* invasion (Kaniga, Trollinger and Galan, 1995)(Kaniga, Tucker, Trollinger and Galan, 1995). SipB and SipC are secreted through the *Salmonella* TTSA

and form a translocon pore in the host membrane (Cossart and Sansonetti, 2004) (Galan, 2001). As with the *Shigella* homologues, mutations in *Salmonella* proteins SipD, SipB, SipC, and SicA individually, prevent the SipB/SipC translocon from inserting into the host cell membrane, which is why each protein is required for translocon formation (Cossart and Sansonetti, 2004).

IpaB and SipB are important to study because they have several functions that are crucial to *Shigella* and *Salmonella* pathogenesis. Both serve as secreted effector proteins, both regulate type III secretion, and both function as structural proteins in the formation of the translocon pores that form in the host cell membrane (Schroeder and Hilbi, 2008). After initially crossing M cells, *S. flexneri* and *Salmonella* serovar Typhimurium secrete IpaB and SipB, respectively, to induce apoptosis via a caspase1-mediated pathway and allow escape from the macrophage (Hilbi *et al*., 1998) (Hersh *et al*., 1999). This apoptotic (pyroptotic) pathway results in the massive release of IL-1β, which provides the signal for induction of the inflammatory processes that lead to the inflammation and tissue damage that give rise to symptoms (Sansonetti, 2000). IpaB and SipB also serve to control effector regulation, as do IpaD and SipD (Menard *et al.*, 1994) (Galan, 2001). IpaB and SipB are also each a component of the two-part translocon pore that is inserted into the host cell membrane after intial contact. The other translocon components for each are IpaC and SipC (Schroeder and Hilbi, 2008). IpaD and SipD allow this pore to remain in contact with the TTSA needle tip (Schroeder and Hilbi, 2008) (Kaniga, Trollinger and Galan, 1995)(Kaniga, Tucker, Trollinger and Galan, 1995), thus forming a conduit through the membrane to the host cell's cytoplasm, channeling direct injection and secretion of later effectors from the bacterium to the host cell's cytoplasm (Menard *et*

al., 1994) (Zychlinsky *et al.*, 1994) (Blocker *et al.*, 1999)(Kaniga, Trollinger and Galan, 1995)(Kaniga, Tucker, Trollinger and Galan, 1995).

Research Focus

Shigellosis and salmonellosis are two of the most common diarrheal diseases in the world, causing over 4 million deaths per year with the majority of these being in children. The development of vaccines for these diseases is dependent on overcoming several significant barriers. There is currently no vaccine for *Salmonella* serovar Typhimurium or *S. flexneri* available for use in humans. This problem is confounded further by the need for a vaccine that is safe and effective in children. Development of vaccines for salmonellosis and shigellosis is also difficult due to the massive number of serovars of the pathogen. Vaccine development is hampered due to the lack of an appropriate animal model. The current mouse model relies upon a pulmonary induced *Shigella* infection. This is much different than the gastroenteritis that occurs in humans. The mouse, employed as a model for human typhoid fever, develops a systemic disease when infected with *Salmonella* serovar Typhimurium, with no diarrhea (Santos *et al*., 2003). Constructing a vaccine that is applicable in the developing world has also been a challenge because of differences in the gut flora and nutrition status as compared to the developed world. There are also concerns with the use of a live-attenuated vaccine. It is not advantageous due to the serotype specificity of these vaccines and the need for a vaccine that can be safely administered to children.

Formulating a vaccine that is protective against *Shigella* and *Salmonella* infections in mice is the initial step needed for development of a practical and successful vaccine in humans. This research is focused on the assessment of the translocators of the

type three-secretion system as protective antigens against *Shigella* and *Salmonella* infection in respective mouse models. My hypothesis is that the exposed parts of the *Shigella* and *Salmonella* TSSAs can be targeted for development of an effective subunit vaccine. In *Shigella*, this means targeting MxiH (needle), IpaD (tip protein), and IpaB (tip-associated translocator protein) and in *Salmonella* homologues, PrgI (needle), SipD (tip protein), and SipB (tip-associated translocator protein).

CHAPTER II

Materials and Methods

Reagents and Buffers

Coomassie Blue Protein Gel Stain
250 mL methanol
1.25 g Coomassie brilliant blue
75 mL acetic acid
175 mL diH_2O

8X His-Tag Binding Buffer
2.72 g imidazole
237 g NaCl
19.36 g Tris
Adjust to 1.00 L with diH_20

4X His-Tag Charge Buffer
52.56 g $NiSO_4$
Adjust to 500 mL with diH_2O

4X His-Tag Elution Buffer
136 g imidazole
58.44 g NaCl
4.84 g Tris
Adjust to 500 mL with diH_2O
pH to 7.9

4X His-Tag Strip Buffer
74.4 g EDTA
58.44 g NaCl
4.84 g Tris
Adjust volume to 500 mL with diH_2O
pH to 7.9

8X His-Tag Wash Buffer

5.44 g imidazole
117 g NaCl
9.68 g Tris
Adjust volume to 500 mL with diH_20
pH to 7.9

Luria-Bertani Broth (LB)
25.0 g LB broth
1.00 L diH_2O

Luria-Bertani Agar (LB)
40.0 g LB agar
1.00 L diH_20

10X Phosphate Buffered Saline (PBS)
85.0 g NaCl
10.7 g sodium phosphate, dibasic
3.90 g sodium phosphate, monobasic
1.00 L diH_2O

12% SDS-PAGE Separating Gel
3.00 mL diH_20
2.50 mL 1.5 M Tris-HCL, pH 8.8
100 µL 10% SDS
4.00 mL 30% Bis:Acrylamide

15% SDS-PAGE Separating Gel
2.50 mL diH_20
2.50 mL 1.5 M Tris-HCL, pH 8.8
100 µL 10% SDS
5.00 mL 30% Bis:Acrylamide

5% SDS-PAGE Stacking Gel (2)
2.85 mL diH_2O
1.25 mL 0.5 M Tris-HCl, pH 6.8
50.0 µL 10% SDS
1.00 mL 30% Bis:Acrylamide

SDS-PAGE Running Buffer
2.42 g Tris
14.41 g glycine
10.0 mL SDS
1.00 L diH_2O

1X Sodium Phosphate Dialysis Buffer (NaP buffer)
20 mL .5 M NaP
8.77g NaCl
5 mL OPOE
Adjust to 1 L with 1X PBS

10 mM Histidine buffer
2.1 g Histidine
8.77 g NaCl
in 1L H20

1x PBS + 0.5% Tween
1L 10x PBS
5 mL Tween 20
added to 9L di H20

10%NFDM
20 g Best Choice Non-fat dry milk
200mL PBS or PBS + 0.5% Tween
Mix on stir plate for 40 minutes

TMB Peroxidase Substrate
TMB peroxidase substrate- 0.4 g/L 3,3',5,5'- tetramethylbenzidine.
Peroxidase Substrate Solution B - 0.02% H2O2 in Citric Acid buffer.

Peroxidase-Labeled Antibody To Mouse IgG
Affinity purified antibody isolated from a pool of serum from goats immunized with purified mouse IgG was labeled with peroxidase by the periodate method by KPL.

Phosphoric Acid Stop Solution
55.2 mL H3PO4
944.8 mL diH20

Construction/Production of Recombinant Proteins

mxiH, ipaB, ipaD, prgI, sipB and *sipD* were subcloned from the pUC-based plasmid pHS2 into pET15b. The resulting constructs were then transformed into Nova Blue *E. coli*. Transformants were screened with T7 promoter and terminator primers. Plasmids containing inserts were then purified using a QIAGEN QIAquick™ plasmid purification kit. *ipaB/plasmid* and *ipgC/pACYC* were then co-transformed, and

sipB/plasmid and sicA/pACYC were co-transformed, both into Tuner (DE3) *E. coli*. Transformations were plated on LB plates containing either ampicillin for bacteria containing genes on pET15b or ampicillin and chloramphenicol for those containing genes on both pET15b and pACYC plasmids. Several colonies were selected and frozen as permanent stocks. A loop of the permanent stock was resuspended in 50 mL of LB and grown overnight at 37 °C with shaking. The 50 mL culture was then divided evenly between four liters of LB, each containing either 100 μg/mL ampicillin or 100 μg/mL ampicillin and 25 μg/mL chloramphenicol. Cultures were grown to mid-log phase (OD_{600} = 0.6) and the protein expression was induced using 0.5 M isopropyl-thio-2-D-galactopyranoside (IPTG). Induced cultures were grown for an additional three hours. The bacteria were then collected by centrifugation and resuspended in 40 mL/L of culture, with 1X His-tag binding buffer. The bacterial suspension was frozen at –80°C.

Ni+ Affinity Protein Purification of His-Tag Containing Proteins

Frozen bacterial suspensions were thawed, sonicated and clarified by centrifugation for 15 minutes at 10,000 x g. Three mL of iminodiacetic acid Sepharose resin were washed with five column volumes (CV) of distilled water. The resin was charged with three CV of 1X His-tag charge buffer and then five CV of 1X His-tag binding buffer. Clarified supernatants of IpaD and SipD were applied to the column, which was washed with an additional five CV of binding buffer. Non-specifically bound proteins were removed with five CV of 1X His-tag wash buffer and bound proteins were then eluted in three CV of 1X His-tag elution buffer. Fractions were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to

check for presence of protein. The resin was stripped with three CV of 1X His-tag strip buffer and stored at 4°C.

Ni+ Affinity Protein Purification of His-Tag Containing Proteins with OPOE

Frozen bacterial suspensions were thawed, sonicated and clarified by centrifugation for 15 minutes at 10,000 x g. Three mL of iminodiacetic acid Sepharose resin were washed with five CV of distilled water. The resin was charged with three CV of 1X His-tag charge buffer and then five CV of 1X His-tag binding buffer. Clarified supernatants of IpaB and SipB were applied to the column; three mL of binding buffer + 1% n-octyl-poly-oxyethlene (OPOE) was added. The column was allowed to rock overnight in a cold room at 4°C. Non-specifically bound proteins were removed with five CV of 1X His-tag wash buffer with 1% OPOE and bound proteins were then eluted in three CV of 1X His-tag elution buffer with 1% OPOE. Fractions were collected and analyzed by SDS-PAGE gel to check for presence of protein. The resin was stripped with three CV of 1X His-tag strip buffer and stored at 4°C for future use.

Ni+Affinity Protein Purification of His-Tag Containing Proteins with 6M Urea

Frozen bacterial suspensions were thawed, sonicated, homogenized and clarified by centrifugation for 15 minutes at 10,000 x g. Three mL of iminodiacetic acid sepharose resin were washed with five CV of distilled water. The resin was charged with three CV of 1X His-tag charge buffer and then five CV of 1X His-tag binding buffer. Clarified supernatants of MxiH and PrgI were applied to the column; three mL of binding buffer + 6M Urea was added. Non-specifically bound proteins were removed with five CV of 1X His-tag wash buffer with 6M urea and bound proteins were then eluted in three CV of 1X His-tag elution buffer with 6M urea. Fractions were collected and analyzed by

SDS-PAGE gel to check for presence of protein. The resin was stripped with three CV of 1X His-tag strip buffer and stored at 4°C for future use.

Dialysis of Proteins Following Purification

Proteins were placed in a Thermo Scientific Slide-A-Lyzer dialysis cassette. All dialysis steps were done at 4°C. Cassettes were dialyzed against 500 mL of 10mM Histidine or 1x PBS dialysis buffer for IpaD and SipD and 500 mL of 10mM Histidine buffer + 1% OPOE or 1x PBS +1% OPOE for IpaB and SipB, overnight at 4°C, stirring constantly. MxiH and PrgI were placed in dialysis tubing and dialyzed overnight at 4°C, stirring constantly in 1L of 1x PBS + 4M urea and continued to dialyze stepwise, decreasing the concentration of urea by ½ each day. This was done until buffer contained only 1x PBS. Purified proteins were removed from dialysis were placed into a 1.5 mL Eppendorf tube and stored at 4°C.

The concentration of each protein was then determined by the Beer-Lambert Law: ($A=\varepsilon bc$); where A is the absorbance at 280nm, ε is the extinction coefficient of the protein, b is the path length and C is concentration. The extinction coefficient measures the amount of light a protein absorbs at a given wavelength. From the amino acid composition of the protein and the known molar extinction coefficients of Tyrosine (Tyr), Tryptophan (Trp), and Cysteine extinction coefficients of native proteins can be calculated. The formula for this calculation is:

Extinction coefficient (Protein) = (Number of Tyr)*(Extinction coefficient of Tyr) + (Number of Trp)*(Extinction coefficient of Trp) + (Number of Cysteines)*(Extinction coefficient of Cysteine)

$$\varepsilon \text{ (prot)} = (\#Tyr)(5{,}500) + (\#Trp)(1{,}490) + (\#Cysteine)(125)$$

ProtParam is a tool in ExPASy Proteomics Server used by our lab to calculate the extinction coefficient. Using the native protein sequence provided, the program calculates the extinction coefficient of proteins based on their amino acid composition. Using the equation below and by solving for c, the concentration for each protein was calculated by dividing the absorbance by the extinction coefficient (Table 2.1)(Gasteiger, *et al.*, 2005). When multiplied by the molecular weight of the protein, the product is the protein concentration in mg/mL.

C (concentration) = (absorbance) / (extinction coefficient)

Calculation of Protein Concentrations		
Protein	Molecular weight (Da)	Extinction coefficient (M^{-1} cm^{-1})
MxiH	10,330	9,970
IpaD	36,634	36,900
IpaB	64,349	11,460
PrgI	8,856.9	12,950
SipD	37,112	38,390
SipB	62,451	16,960

Table 2.1 The proteins used in Experiment 2, their molecular weights, and extinction coefficients. Absorbance at $A_{280,}$ is divided by the extinction coefficient and concentration is calculated based on Beer-Lambert Law ($A=\varepsilon bc$). Multiplying the concentration (C) by the molecular weight of the protein yields the protein's concentration in mg/mL.

Animal Protocols

Prior to this current study, Dr. Wendy Picking and Dr. Julian Kissman conducted Experiment 1. These preliminary trials were used to determine the 50% mouse lethal dose (LD50) and evaluate *Salmonella* proteins PrgI and SipD along with *Shigella* proteins MxiH and IpaD, as potential protective antigens. This research was incorporated in the development of Experiments 2 and 3. Six to eight-week old female Balb/c mice (*Mus musculus*) were housed in groups of five in microisolator cages in the OSU animal facility. Animals were fed and watered *ad libidum* and they were ear-tagged for identification and weighed initially.

There were 300 mice involved with Experiment 2. Adjuvants used in Experiment 2 are listed in Table 2.2. Flow charts illustrating Experiment 2 are depicted in Figures 2.1-2.4. Each chart follows a basic procedural outline and includes: number of mice per group, group classification, adjuvants used, bleeding schedule, immunization days, challenge, and monitoring of animals post challenge. Groups were established for intramuscular and intranasal vaccines. One hundred and forty mice were grouped for intramuscular vaccines and 160 mice were grouped to receive intranasal vaccines. Each animal group was comprised of ten mice (n=10). Classification of these groups was based on the vaccine components for the group. Proteins used for *Salmonella* vaccines were PrgI, SipD and SipB. *Shigella* vaccines incorporated the proteins MxiH, IpaD and IpaB. Intramuscular immunizations were formulated with Freund's adjuvant, Freund's Incomplete Adjuvant, and Monophosphoryl lipid A (MPL). Intranasal immunizations were formulated with cholera toxin adjuvant, MPL and MPL with chitosan. Intramuscular immunizations were administered on days 0, 14 and 28. Intranasal

immunizations were given on days 0, 14, 28 and 42. Mice were bled via facial vein two days prior to all immunizations and challenge. Mice receiving *Shigella* vaccines were challenged intranasally with *Shigella* strain 2457T. Mice that were given *Salmonella* vaccines were challenged ororgastrically with *Salmonella* strain SL1344. All animals were challenged 28 days after receiving the final immunization. Mice were monitored for up to two weeks post challenge. During this period, mice were scored based on health and weights were recorded daily. Mice losing 25% of initial body weight were promptly euthanized by cervical dislocation. All survivors were euthanized on day15 day post challenge per Institutional Animal Care and Use Committee regulations.

Adjuvants used for Experiment 2	
Alhydrogel-Aluminum hydroxide (AH) -10 mg/mL	[IM]
Cholera toxin (CT) -1mg/mL	[IN]
Freund's (CFA)-1mg/mL [IM]	
Incomplete Freund's (FA) -1mg/mL [IM]	
Monophosphoryl lipid A- (MPL) -1mg/mL	[IM and IN]
Chitosan -2mg/mL	[IN]

Table 2.2. Adjuvants used in Experiment 2 for intramuscular (IM) and intranasal (IN) vaccinations. AH, CFA , IFA and MPL were used in intramuscular immunizations with and with out antigens. CT, MPL and Chitosan formulated with and without antigens were components of intranasal immunizations. All adjuvants for Experiment 2 were provided by the University of Kansas.

Flow Charts for Experiment 2

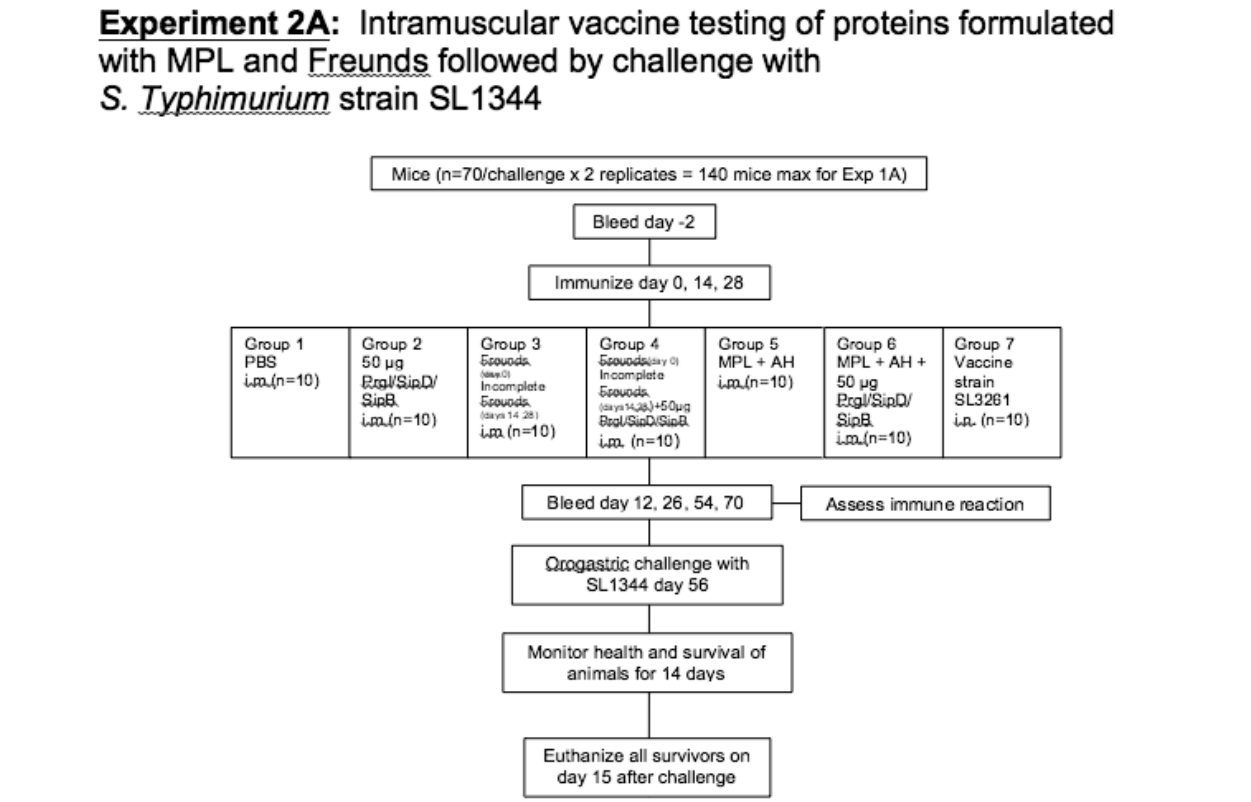

Figure 2.1. 70 mice were given intramuscular immunizations on days 0,14 and 28. Mice were bled 2 days prior to immunizations and orogastric challenge with *S. typhimurium* strain SL1344. Groups of 10 mice were given vaccines formulated with and without *Salmonella* proteins: PrgI, SipD and SipB; using Freund's and MPL+AH as adjuvants (refer to Table 2.2). Animals were monitored for up to 14 days post challenge.

Flow Charts for Experiment 2, continued

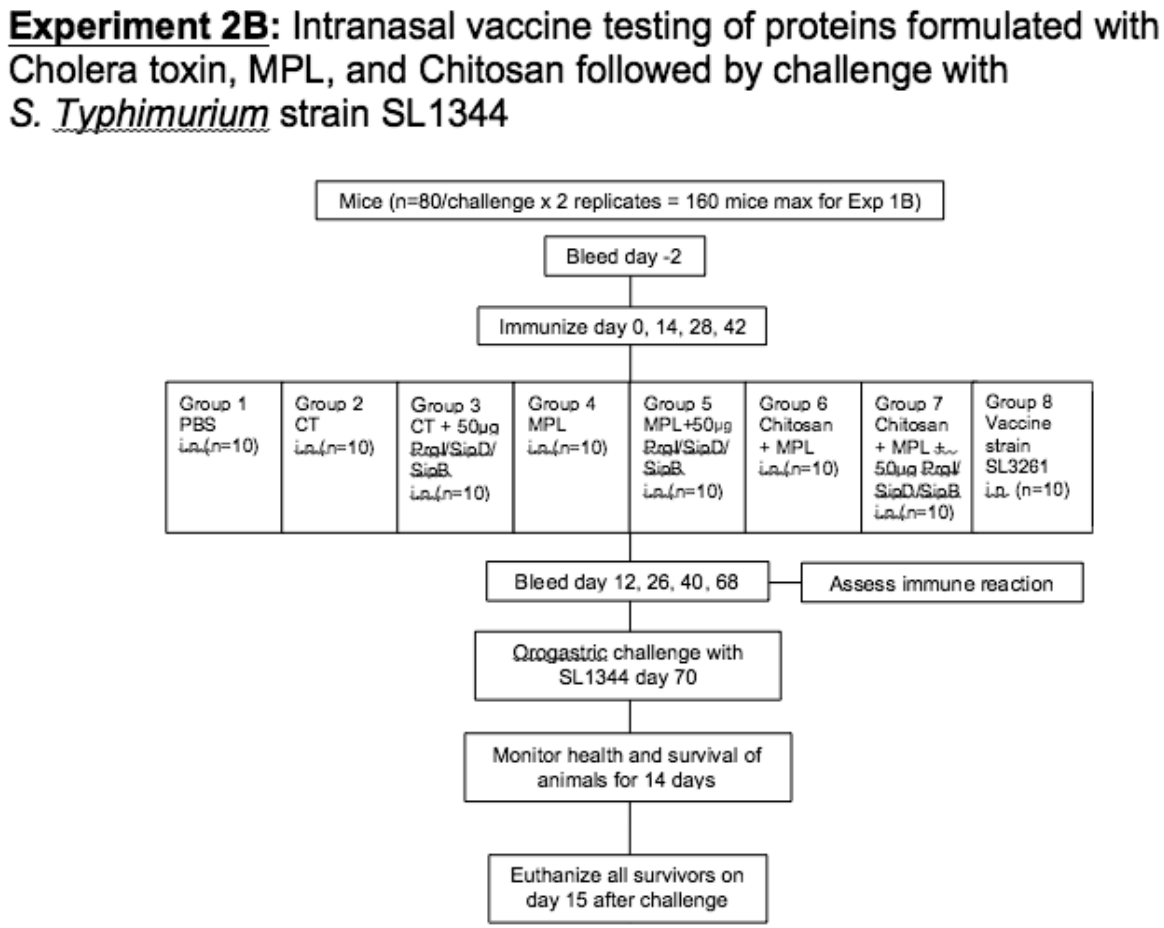

Figure 2.2. 80 mice were given intranasal immunizations on days 0,14 and 28. Mice were bled 2 days prior to immunizations and orogastric challenge with *S. typhimurium* strain SL1344. Groups of 10 mice were given vaccines formulated with and without *Salmonella* proteins: PrgI, SipD and SipB; using Cholera toxin and MPL and Chitosan + MPL as adjuvants (refer to Table 2.2). Animals were monitored for up to 14 days post challenge.

Flow Charts for Experiment 2, continued

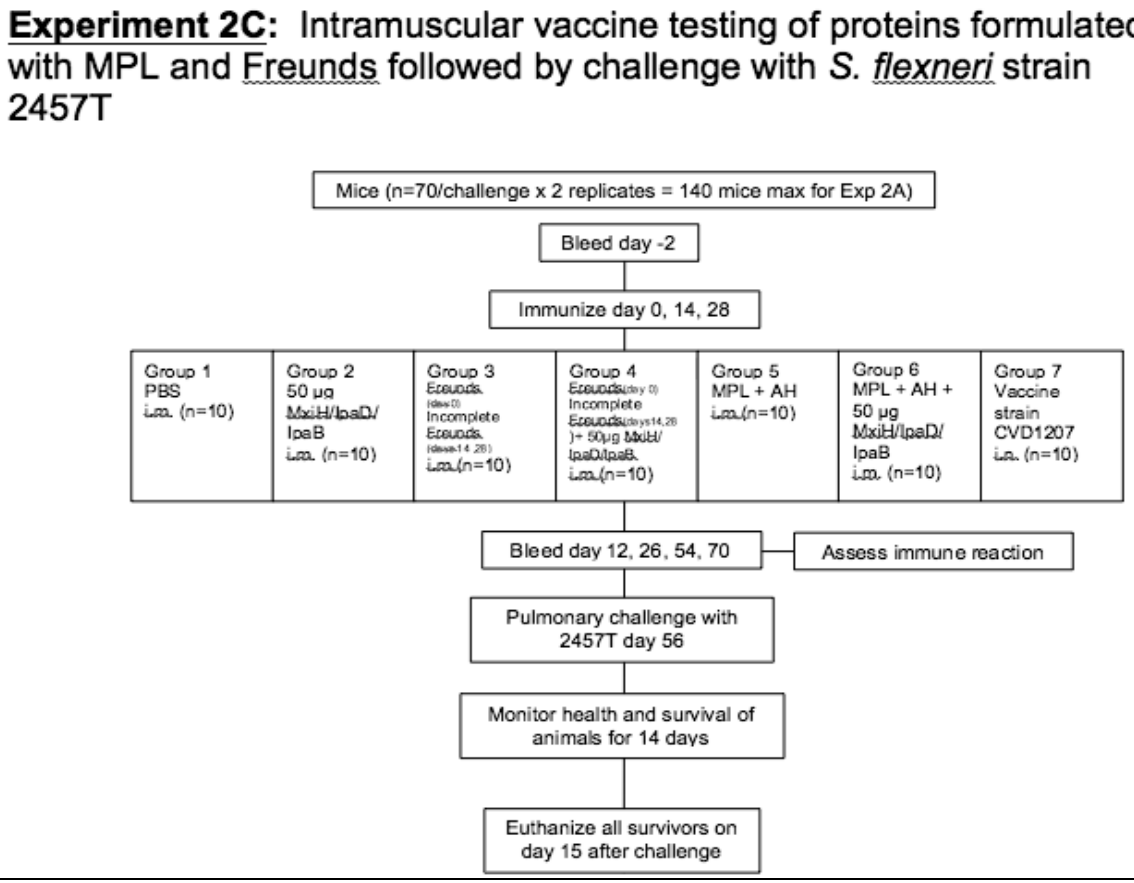

Figure 2.3. 70 mice were given intramuscular immunizations on days 0,14 and 28. Mice were bled 2 days prior to immunizations and intranasal challenge with *S. flexneri* strain 2457T. Groups of 10 mice were given vaccines formulated with and without *Shigella* proteins: MxiH, IpaD and IpaB; using Freund's and MPL+AH as adjuvants (refer to Table 2.2). Animals were monitored for up to 14 days post challenge.

Flow Charts for Experiment 2, continued

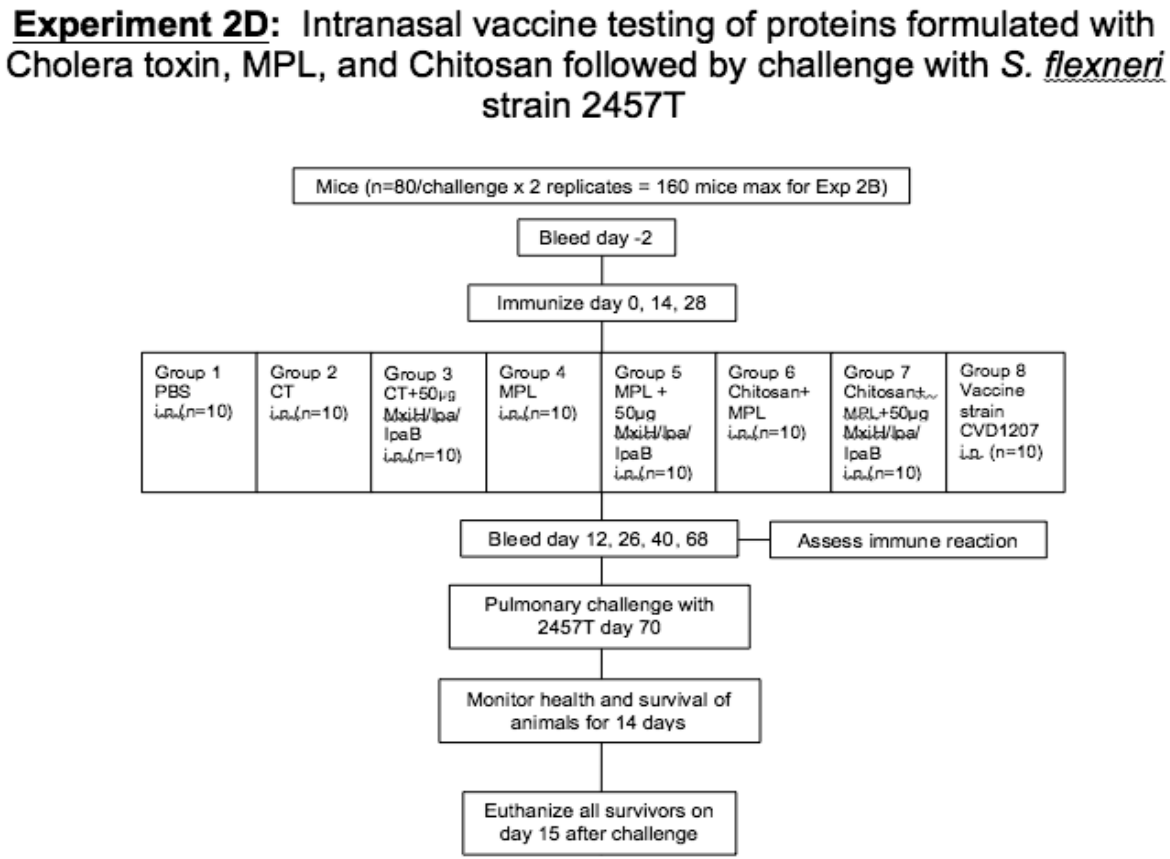

Figure 2.4. 80 mice were given intramuscular immunizations on days 0,14 and 28. Mice were bled 2 days prior to immunizations and intranasal challenge with *S. flexneri* strain 2457T. Groups of 10 mice were given vaccines formulated with and without *Shigella* proteins: MixH, IpaD and IpaB; using Cholera toxin, MPL and Chitosan + MPL as adjuvants (refer to Table 2.2). Animals were monitored for up to 14 days post challenge.

Vaccine Formulation for Experiment 2

Vaccines for Experiment 2 were mass formulated by group in 1.5mL, sterile Eppendorf tubes. Tables 2.3-2.6 provide the quantities of adjuvants (listed in Table 2.2) used in microliters for each immunization. Amounts of each protein used at calculated concentrations (A=εbc) are given in microliters in these tables as well. For intramuscular vaccines alhydrogel (AH) was initially bound to the antigens by mixing with gentle agitation for 30 minutes, which ensured maximal antigen binding to Monophosphoryl lipid A (MPL). Vaccines formulated with these adjuvants contained 30μg of both AH and MPL. Freund's complete (FCA) and Freund's incomplete adjuvants (FA) were used for intramuscular immunizations and were mixed at a 1:1 ratio with antigens by pipetting with subsequent vortexing. For intranasal immunizations, 1mg of cholera toxin (CT) adjuvant was reconstituted with 1 mL deionized water. Reconstituted CT was then mixed with antigens by simple pipetting. Vaccines formulated with CT contained 2.5μg for each immunization. The adjuvant chitosan was used with MPL in intranasal immunizations. Each vaccine was formulated with 10ug of each chitosan and MPL. Chitosan was first vortexed into 1x histidine buffer to dissolve, prior to mixing with antigens by pipetting. MPL was then added and mixed by pipetting with subsequent vortexing.

All *Salmonella* intramuscular immunizations containing antigens were formulated with 25μg PrgI, 25μg SipD, and 10μg SipB. The first three *Salmonella* intranasal immunizations were formulated with a total of 10μg PrgI, 10μg SipD and 2.5μg SipB. The fourth intranasal booster contained 10μg SipD and 2.5μg SipB only.

All *Shigella* intramuscular immunizations containing antigens were formulated with 25μg MxiH, 25μg IpaD, and 10μg IpaB. *Shigella* intranasal immunizations 1-3

contained antigen amounts of 10μg MxiH, 10μg IpaD and 2.5μg IpaB. The fourth booster contained 10μg IpaD and 2.5μg IpaB only. The quantity of each protein (in μL) at calculated concentrations (mg/mL) that were used in each immunization is given in Tables 2.3-2.6. PBS and histidine buffers were added as appropriately needed to bring each intramuscular vaccine to a final volume of 100μL. Each intranasal vaccine had a final volume of 30μL per dose. Vaccine group tubes were kept at 4°C until they were administered.

Table 2.3. Expt 2A Intramuscular Immunization 1-3-*Salmonella*		Protein Concentration			
Group/Adjuvant	Adjuvant Quantity	PrgI 1.12 mg/mL	SipB 0.435 round 1/ 0.862 round 2/3 mg/mL	SipD 2.32 mg/mL	Total Immunization Volume
1- PBS	-	-	-	-	100µL
2-Protein	-	22.3 µL	23/11.6µL	10.78 µL	100µL
3-Freund's (1)/ Incomplete Freund's (2,3)	44µL	-	-	-	100µL
4-Freund's (1)/ Incomplete Freund's (2,3) + protein	44µL	22.3 µL	23/11.6µL	10.78 µL	100µL
5-MPL+AH	30µL + 3µL	-	-	-	100µL
6-MPL+AH + protein	30µL + 3µL	22.3 µL	23/11.6µL	10.78 µL	100µL
7-Vaccine strain -SL-3261	-	-	-	-	100µL-Gavage

Table 2.3. Quantities and concentrations of *Salmonella* proteins and quantities of adjuvants used in formulations of intramuscular vaccines. There were three rounds of immunizations which required that we purify additional SipB for rounds two and three. The total volume of each vaccine was 100µL. Vaccines for each group were mass formulated in 1.5mL Eppendorf tubes. PBS and histidine buffers were added to complete formulation quantities to 100µL per dosage, when necessary.

Table 2.4. Expt 2B Intranasal Immunization 1-3 - *Salmonella*		Protein Concentration			**Immunization 4- Boost** SipB and SipD only		
Group/ Adjuvant	Adjuvant quantity	PrgI 1.12 mg/mL	SipB 0.435 round 1/0.862 round 2-3 mg/mL	SipD 2.32 mg/ mL	SipB 9.35 mg/ mL	SipD 3.97 mg/ mL	Total Immunization Volume
8- PBS	-	-	-	-	-	-	30µL
9-Cholera Toxin	2.5 µL	-	-	-	-	-	30µL
10-Cholera Toxin + Protein	2.5 µL	8.93 µL	5.75/2.9µL	4.31 µL	0.3µL	2.5µL	30µL
11-MPL	10µL	-	-	-	-	-	30µL
12-MPL+ protein	10µL	8.93 µL	5.75/2.9µL	4.31 µL	0.3µL	2.5µL	30µL
13-Chitosan + MPL	5µL + 10µL	-	-	-	-	-	30µL
14-Chitosan + MPL + protein	5µL + 10µL	8.93 µL	5.75/2.9µL	4.31 µL	0.3µL	2.5µL	30µL
15-protein	-	8.93 µL	5.75/2.9µL	4.31 µL	0.3µL	2.5µL	30µL

Table 2.4. Quantities and concentrations of *Salmonella* proteins and quantities of adjuvants used in formulations of intranasal vaccines. There were three rounds of immunizations plus a fourth booster with only SipB and SipD. It was required to purify additional SipB for rounds two and three, and additional SipB and SipD for the fourth boost. The total volume of each vaccine was 30µL. Vaccines for each group were mass formulated in 1.5mL Eppendorf tubes. PBS and histidine buffers were added to complete formulation quantities to 30µL per dose, when necessary.

Table 2.5. Expt 2C Intramuscular Immunization 1-3 - *Shigella*		Protein Concentration			
Group/Adjuvant	Adjuvant quantity	MxiH 3.76 mg/mL	IpaB 0.65 mg/mL	IpaD 3.15 mg/mL	Total Immunization Volume
1- PBS	-	-	-	-	100µL
2-Protein	-	6.65µL	15.4µL	7.94µL	100µL
3-Freund's (1)/ Incomplete Freund's (2,3)	30µL	-	-	-	100µL
4- Freund's (1)/ Incomplete Freund's (2,3) + protein	30µL	6.65µL	15.4µL	7.94µL	100µL
5-MPL+AH	30µL + 3µL	-	-	-	100µL
6-MPL+AH + protein	30µL + 3µL	6.65µL	15.4µL	7.94µL	100µL
7-Vaccine strain-1207	-	-	-	-	30µL

Table 2.5. Quantities and concentrations of *Shigella* proteins and quantities of adjuvants used in formulations of intramuscular vaccines are listed above. There were three rounds of immunizations. The total volume of each vaccine was 100µL. Vaccines for each group were mass formulated in 1.5mL Eppendorf tubes. PBS and histidine buffers were added to complete formulation quantities to 100µL per dose when necessary.

Table 2.6. Expt 2D Intranasal Immunization 1-3- *Shigella*		Protein Concentration			**Immunization 4- Boost** IpaB and IpaD only		
Group/ Adjuvant	Adjuvant quantity	MxiH 3.76 mg/mL	IpaB 0.65 mg/mL	IpaD 3.15 mg/ mL	IpaB 4.28 mg/mL	IpaD 7.25 mg/mL	Total Immunization Volume
8- PBS	-	-	-	-	-	-	30µL
9-Cholera Toxin	2.5µL	-	-	-	-	-	30µL
10-Cholera Toxin + Protein	2.5µL	6.65µL	3.85µL	3.17 µL	0.6 µL	1.38µL	30µL
11-MPL	10µL	-	-	-	-	-	30µL
12-MPL+ protein	10µL	6.65µL	3.85µL	3.17 µL	0.6 µL	1.38µL	30µL
13-Chitosan + MPL	5µL + 10µL	-	-	-	-	-	30µL
14-Chitosan + MPL + protein	5µL + 10µL	6.65µL	3.85µL	3.17 µL	0.6 µL	1.38µL	30µL
15-protein	-	6.65µL	3.85µL	3.17 µL	0.6 µL	1.38µL	30µL

Table 2.6. Quantities and concentrations of *Shigella* proteins and quantities of adjuvants used in formulations of intranasal vaccines. There were three rounds of immunizations plus a fourth booster with only IpaB and IpaD. It was required to purify additional IpaB and IpaD for the fourth boost. The total volume of each vaccine was 30µL. Vaccines for each group were mass formulated in 1.5 mL Eppendorf tubes. PBS and histidine buffers were added to complete formulation quantities to 30µL per dose when necessary.

Experiment 3

Dr. Wendy Picking completed all vaccine formulations and immunizations for Experiment 3. The experimental design followed the basic procedures of Experiment 2. There are however several significant differences involved in the antigen/adjuvant combinations used. Needle proteins for *Salmonella* and *Shigella* (PrgI and MxiH respectively) are not used in Experiment 3. The reason for this is that these proteins yielded no protective value in Experiment 2. In addition to SipB and SipD, *Salmonella* protein variants of SipB were used with SipD for some of the immunizations in Experiment 3.These variants included SipB/SicA which stabilizes SipB, and a newly identified stable SipB fragment (amino acid residues 77-237). Modifications also included the use of fewer adjuvants in Experiment 3. Freund's adjuvants, both complete and incomplete, as well as chitosan were not used in Experiment 3. These adjuvants did not seem to contribute to protection when immunized with antigens in Experiment 2. Adjuvants used in Experiment 3 are listed in Table 2.7. The less toxic, double mutant heat-labile toxin (dmLT) was used in place of cholera toxin. Monophosphoryl lipid A (MPL) was used once again along with alhydrogel (AH). Decisions on changes made in Experiment 3 were loosely based on the data from Experiment 2. The overall goal of both experiments was to close in on an efficient antigen/adjuvant combination using the proper route of administration. Flow charts 2.5-2.7 depict the general process for Experiment 3, each chart follows the same basic procedure as Experiment 2 including: number of mice per group, group classification, adjuvants used, bleeding schedule, immunization days, challenge and monitoring of animals post challenge.

Table 2.7- Ex 3- Immunizations 1-4			Adjuvants
Protein	Amount	Buffer	
IpaB	200µg	His	Alhydrogel-Aluminum hydroxide (AH) - 10 mg/mL
IpaD	350µg	His	
IpaD	600µg	PBS	
SipB	200µg	His	Monophosphoryl lipid A (MPL)-1mg/mL
SipB	300µg	PBS	
SipB/SicA	200µg	His	
SipB/SicA	150µg	PBS	Double mutant heat-labile toxin (dmLT)
SipD	350µg	His	
SipD	550µg	PBS	

Table 2.7. Adjuvants and protein amounts for Experiment 3. Antigens were formulated with listed adjuvants for intranasal and intramuscular immunizations for *Shigella* and *Salmonella* test groups by Dr. Wendy Picking. MPL and AH were used with and without antigens for intramuscular *Shigella* vaccines, rounds 1-4. Intranasal immunizations with and without antigens for *Salmonella* vaccines 1-4 were also formulated with MPL and AH. dmLT was used in *Salmonella* intranasal immunizations for vaccine rounds 1-4.

Flow Charts for Experiment 3

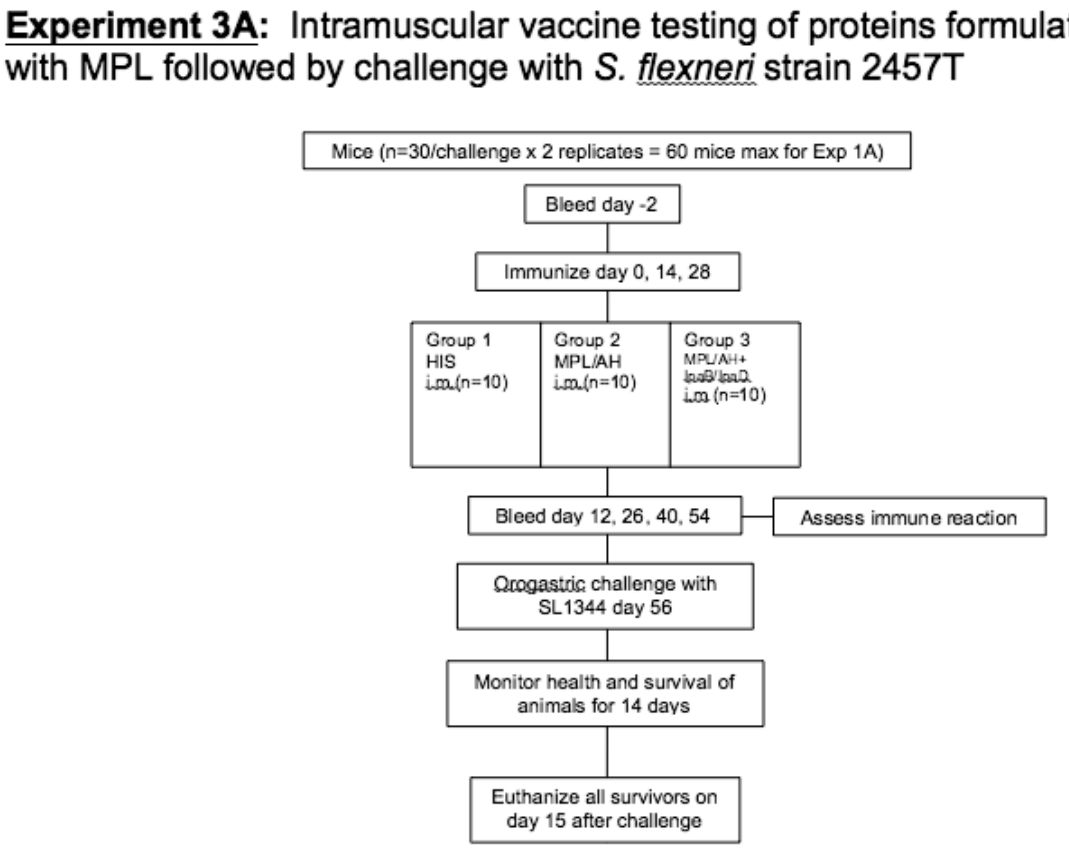

Figure 2.5. 30 mice were given intramuscular immunizations on days 0,14 and 28. Mice were bled 2 days prior to immunizations and intranasal challenge with *S. flexneri* strain 2457T. Groups of 10 mice were given vaccines formulated with and without *Shigella* proteins: IpaD and IpaB; using MPL + AH a as adjuvants (refer to Table 2.6). Animals were monitored for up to 14 days post challenge.

Flow Charts for Experiment 3, continued

Experiment 3B: Intramuscular vaccine testing of proteins formulated with MPL and Alhydrogel followed by challenge with *S. Typhimurium* strain SL1344

/

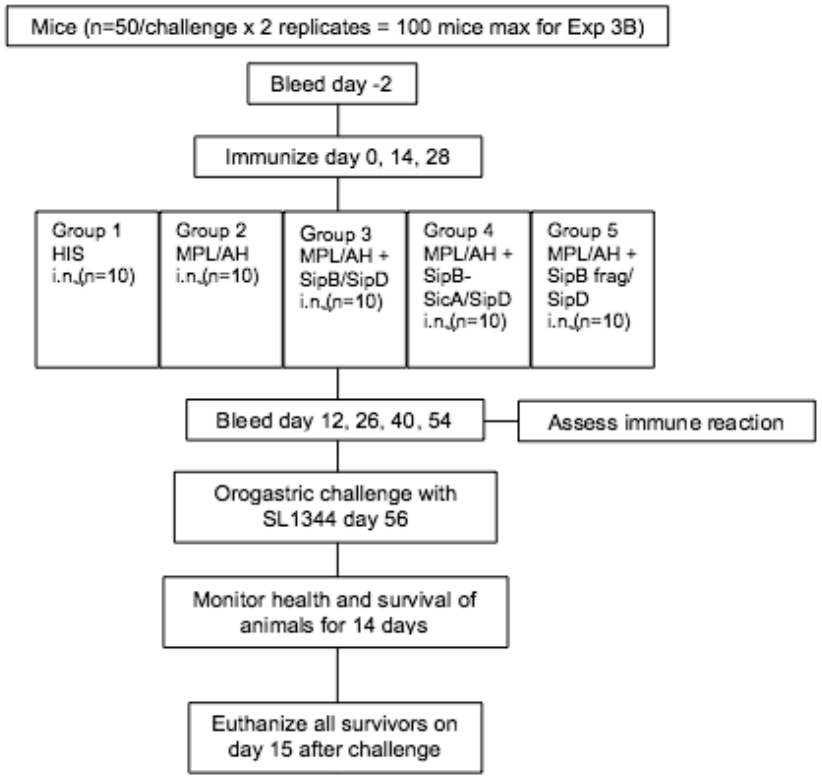

Figure 2.6. 50 mice were given intranasal immunizations on days 0,14 and 28. Mice were bled 2 days prior to immunizations and orogastric challenge with *S. typhimurium* strain SL1344. Groups of 10 mice were given vaccines formulated with and without *Salmonella* proteins: SipB and SipD, SipB/SicA with SipD and SipB fragment with SipD; using MPL with alhydrogel as adjuvants (refer to Table 2.6). Animals were monitored for up to 14 days post challenge.

Flow Charts for Experiment 3, continued

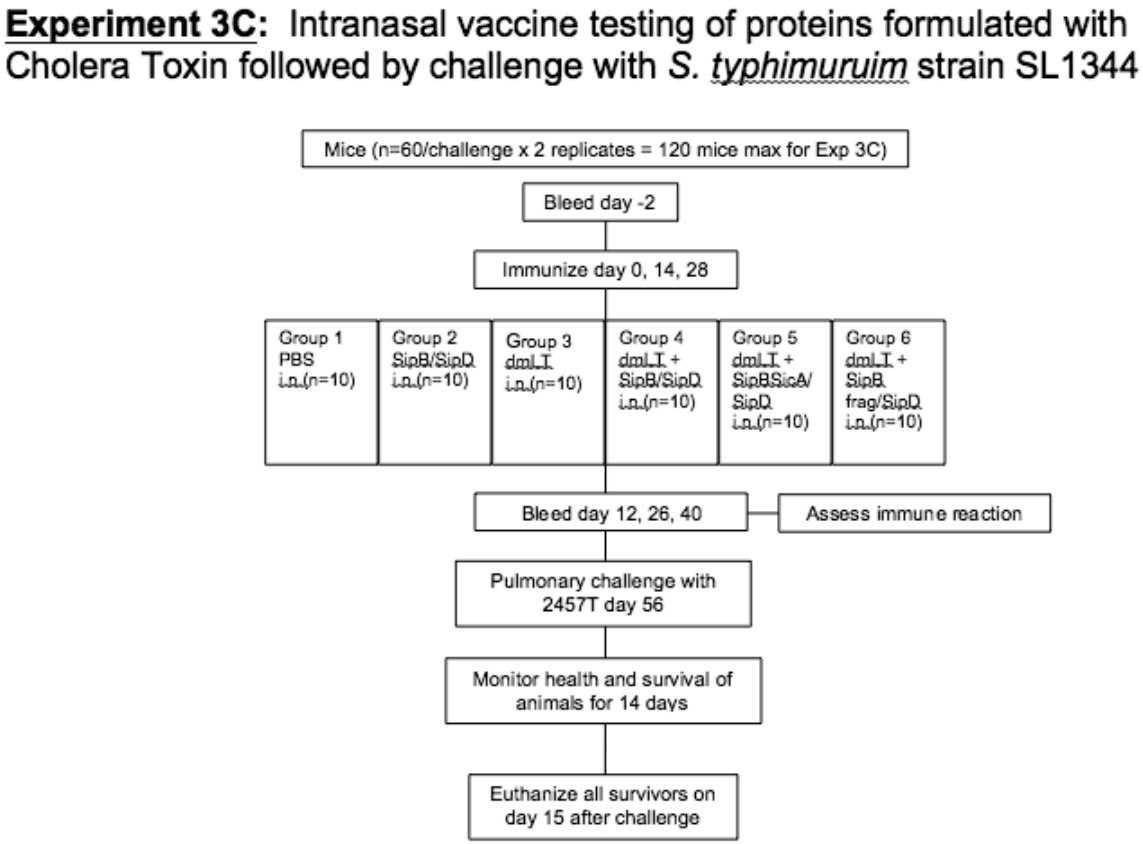

Figure 2.7. 60 mice were given intranasal immunizations on days 0,14 and 28. Mice were bled 2 days prior to immunizations and orogastric challenge with *S. typhimurium* strain SL1344. Groups of 10 mice were given vaccines formulated with and without *Salmonella* proteins: SipB and SipD, SipB/SicA with SipD and SipB fragment with SipD; using dmLT as the adjuvant (refer to Table 2.6). Animals were monitored for up to 14 days post challenge.

Immunization

For Experiment 2, the animals receiving the intramuscular vaccine were given three immnizations every two weeks beginning with day 0. Twenty-eight days after the third immunization, animals were challenged with either *Salmonella* serovar Typhimurium (SL1344) or *S. flexneri* (2745T). Animals receiving the intranasal vaccine were given a fourth boost containing tip protein antigens and then subsequently challenged 28 days later with SL1344 and 2457T.

All groups receiving intramuscular vaccines were immunized with a 100μL injection into the biceps femoris using a 1 mL syringe with a 27-gauge needle. Intranasal immunizations and the *Shigella* vaccine strain (1207) were administered as 30μL doses. Immunizations were applied to nares of mice via micropipette tip. To immunize with the *Salmonella* (SL3261) vaccine strain, 100μL of the bacterial strain was given orogastrically by passing 20-guage bulb-tipped gavage needle through the mouth into the stomach.

Vaccine Strain

Salmonella serovar Typhimurium strain SL3261 (attenuated vaccine strain) was grown to mid-log phase overnight at 37°C with shaking at 100rpm in 100mL of LB. Bacteria were collected by centrifugation at 3200rpm. Bacteria were then resuspended in 1mL of 1X PBS. Absorbance of the culture was taken at a dilution of 1:100 (10μL bacteria in 990μL of PBS) and further diluted with PBS to reach desired A600 of 0.15. 100μL of *Salmonella* serovar Typhimurium vaccine strain SL3261 was administered orogastrically by gavage for each vaccine round.

The *S. flexneri* vaccine strain 1207 was grown to mid-log phase overnight. Guanine was added to media at 37°C with shaking at 200rpm in 100mL of TSB. Bacteria were collected by centrifugation at 3200rpm. Bacteria were then resuspended in 1mL of 1X PBS. Absorbance of bacteria was taken at dilution of 1:100 and diluted further with PBS to reach desired A600 of 6.6. 30μL of *S. flexneri* vaccine strain 1207 was administered intranasally for each vaccine round.

Blood Sampling

Blood was collected via submandibular vein lancet technique. Mice were manually restrained as 0.2mL of blood obtained and drip collected into 1.5mL serum separation tubes. The samples were centrifuged at 12,000 rpm and sera were collected after separation from clotted red blood cells. One hundred μL of serum was collected from each animal and the samples were pooled, labeled, and stored in the freezer at -20°C for each experimental group. The remaining 100μL was labeled and stored for each individual specimen. Animals were bled two days prior to each immunization to allow recovery from the stress.

Challenge

Log-fold dilution experiments were performed to determine bacterial strain concentration for challenge dose. One hundred-fold dilutions of bacterial strains SL1344 and 2457T plated on LB agar and left overnight to incubate at 37°C. Colony forming units were quantified to determine the bacteria/mL to allow for administration of a uniform dose per animal. Animals were challenged 28 days after last immunization.

Salmonella serovar Typhimurium strain SL1344 was grown to mid-log phase overnight at 37°C with shaking at 100rpm in 200mL of LB. Bacteria were collected by

centrifugation at 3200rpm. Bacteria were then resuspended in 10mL of 1X PBS. Absorbance at 600nm was taken of bacteria at a dilution of 1:100 (10μL bacteria in 990μL of PBS) and further diluted with PBS to reach desired A600 of 0.15. One hundred μL of *Salmonella serovar* Typhimurium vaccine strain SL1344 was administered orogastrically by gavage for challenge. Animal appearance and weights were monitored and recorded for up to two weeks post challenge.

S. flexneri strain 2457T was grown to mid-log phase overnight at 37°C with shaking at 200rpm in 200mL of TSB. Bacteria were collected by centrifugation at 3200rpm. Bacteria were resuspended in 10mL of 1X PBS. Absorbance of the culture was taken at dilution of 1:100 (10μL bacteria in 990μL of PBS) and diluted further with PBS to reach desired A600 of 6.6. Thirty μL of *S. flexneri* vaccine strain 2457T was administered intranasally for challenge using the accepted mouse lung infection model.

Monitoring

Animals were examined for appearance twice-daily post challenge. Weights were recorded once daily at which time the animals were scored based on degree of sickness. Moribund animals were euthanized promptly by cervical dislocation as per Institutional Animal Care and Use Committee regulations. All surviving animals were euthanized after the 15^{th} day post challenge.

Immunology

Upon completion of vaccine trials, serum collected from animals at each preimmunization time-point was analyzed for immune response by enzyme-linked immunosorbent assay (ELISA). All ELISA's were performed following the Standard Operating Procedures (SOPs) provided by the Center for Vaccine Development at the

University of Maryland School of Medicine. In conjunction with the SOPs, each assay was documented, step-by-step using the Qualitative ELISA Worksheet: AI-01. This template provides the basic ELISA protocol but can be individualized for each assay. The form also provides a place for documentation of: 1) coating of plates with antigens, 2) blocking of plates with 10% non-fat dry milk, 3) incubation of plates with specimens, 4) conjugate incubation of plates with secondary antibody, 5) binding of substrate, 6) addition of stop solution to end the reaction. ELISA's for Experiments 2 and 3 were completed using these guidelines.

On day one antigens were diluted into 10mL of PBS to achieve 0.1μg/100μL. Ninety-six well Immulon* Immunoassy U-bottom plates were coated with antigen. Each well was coated with 100μL of antigen and incubated for 3 hours at 37°C. Following incubation, plates were then washed 6 times with 1x PBS + 0.5% Tween. The plates were aspirated between each wash. Plates were coated with 10% non-fat dry milk (NFDM) blocking buffer in the amount of 250μL/well. Plates were incubated at 4°C overnight. On day two plates were washed 6 times with 1x PBS + 0.5% Tween. The plates were aspirated between each wash. Serum samples from previously bled mice were used as primary antibody. The lowest dilution for mouse serum accepted is 1:50 (Standard Operating Procedure provided by the Center for Vaccine Development). Five μL of mouse serum was diluted into 250uL 10% NFDM + 1x PBS + 0.5% Tween. Each well was coated with 100μL of diluted serum. Samples were laid down in pairs following pre-determined template (Figure 2.8). Row H contained 10% NFDM + 1x PBS + 0.5% Tween to serve as blank for each plate. Columns 11and 12 were reserved for the standard control. This control is titered down the columns by beginning with 200μL of diluted

control in row A of columns 11 and 12. Rows B-G in columns 11 and 12 contained 100μL of 10% NFDM + 1x PBS + 0.5% Tween. One hundred μL was taken from row A and diluted by mixing into row B. This 100μL dilution was followed through row G in columns 11 and 12.Plates were incubated for 1 hour at 37°C, and washed 6 times with 1x PBS + 0.5% Tween. One hundred μL of peroxidase-labeled, goat anti-mouse IgG was laid down on plates as secondary antibody. Each well received 100μL of secondary antibody at a concentration of 1mg/mL diluted 1:1000 in 10% NFDM. Plates were incubated for 1 hour at 37°C, and washed 6 times with 1x PBS + 0.5% Tween. Equal volumes of TMB Peroxidase Substrate and Peroxidase Substrate Solution B were mixed to form the substrate. One hundred μL of substrate was placed in each well and incubated on shaker at room temperature for 15 minutes, covered. One hundred μL 1M phosphoric acid stop solution was added to plates with bound substrate. Plates were read immediately on multi-scan plate reader taking the optical density at 450nm. Values for each well were pasted into Qualitative ELISA regression template, provided by the Center for Vaccine Development, which assigned titers for each sample based on the optical density at 450nm.

ELISA Plate Template

Table 5-Plate Template:

	1	2	3	4	5	6	7	8	9	10	control
A	Sample 1		Sample 8		Sample 15		Sample 22		Sample 29		Titered standard control
B	Sample 2		Sample 9		Sample 16		Sample 23		Sample 30		
C	Sample 3		Sample 10		Sample 17		Sample 24		Sample 31		
D	Sample 4		Sample 11		Sample 18		Sample 25		Sample 32		
E	Sample 5		Sample 12		Sample 19		Sample 26		Sample 33		
F	Sample 6		Sample 13		Sample 20		Sample 27		Sample 34		
G	Sample 7		Sample 14		Sample 21		Sample 28		Sample 35		
H				B	L	A	N	K	S		

Figure 2.8. ELISA plate template. Template is used for each ELISA run and made before beginning assay. Row H is always reserved for the plate blank. Rows A-G in columns 11-12 are always reserved for the standard control for the plate.

CHAPTER III

The Overall Results

Animals immunized with the PrgI, SipD and SipD (from *Salmonella*) or MxiH, IpaD and IpaB (from *Shigella*) showed increasing antibody titers with each additional immunization. Overall results of Experiments 2 and 3 are best presented in tables illustrating serum antibody titers resulting from ELISA assays for each bleed followed by plotting the accompanying survival rates for vaccination regimen. These tables are divided into groups of mice immunized with *Salmonella* proteins (intramuscular and intranasal immunizations) and mice immunized with *Shigella* proteins (intramuscular and intranasal immunizations).

This chapter also reports the titer values determined using ELISA's for each group and each bleed. Titer values reflect the detectable level of antibody present in the serum. Titers are determined based on optical density of samples at 450nm according to the protocols in the previous chapter. Results must fall between the standard control curves to be assigned a titer value. Titers are used here as an assessment of the immune response to a given antigen. It is assumed here that higher levels of antibodies will yield protection, but that cannot be verified without also determining the survival rates in challenge experiments. Survival curves are listed for each route of administration for both *Salmonella* and *Shigella.* Each curve plots survival percentage for a given group of

animals versus time post challenge with the final data collected by Dr. Wendy Picking.

***Salmonella* Results for Experiment 2**

Results of intramuscular *Salmonella* vaccines are presented in Figure 3.1. Results show no protection using Freund's adjuvant with Salmonella proteins PrgI, SipD, and SipB (I/D/B). There was 90% survival within the group immunized with Monophosphoryl lipid A (MPL) + Ahlydrogel (AH) + I/D/B. Mice immunized with MPL + AH alone had 56% surivial within the group. Freund's adjuvant was not anticipated to offer protection when immunized with I/D/B and results in Figure 3.1 confirm this. Protection was anticipated with the use of MPL + AH +I/D/B and the 90% survival rate looks promising. The 56% survival of mice immunized with MPL + AH alone brings protection of the group immunized with MPL + AH +I/D/B into question, however, since there should be no reason for these mice to survive the challenge at this high rate.

The titers shown in Table 3.1 reflect antibodies to SipB and SipB generated post-ELISA at each bleed interval. Each bleed was completed two days prior to immunizations. All animals whose immunizations included I/D/B showed increasing antibody production by by bleed four as expected. Mice immunized with I/D/B alone had the lowest titers of the animals receiving antigens and these values correlated with a low survival rate. Titers for immunizations with Freund's + I/D/B had the highest titers but the lowest of all the survival rates. Titers for the group immunized with MPL + AH +I/D/B displayed a modest correlation with survival.

It is generally expected that the titers of mice immunized with I/D/B should increase with each bleed. Although mice in each group given I/D/B show such increases,

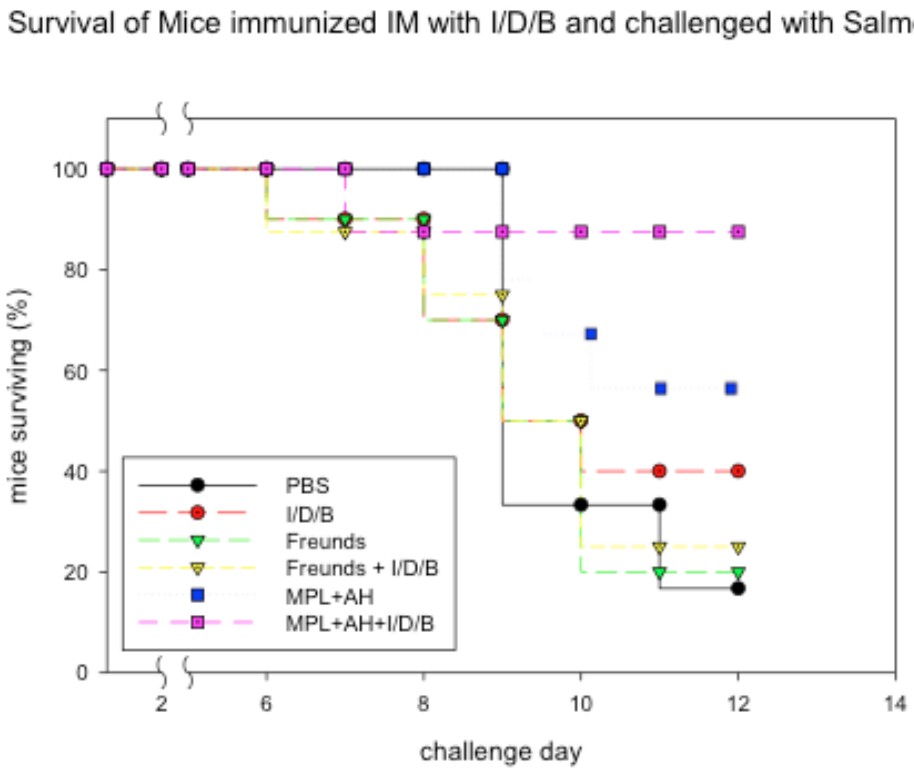

Figure 3.1. Survival of mice immunized intramuscularly with *Salmonella* proteins: PrgI, SipB and SipD, Freund's and Incomplete Freund's with and without antigens, and MPL with and without antigens

Antibody Titers for *Salmonella* Immunizations

Salmonella Sip B IgG							Salmonella Sip D IgG					
Group	Bleed 1	Bleed 2	Bleed 3	Bleed 4	Bleed 5		Group	Bleed 1	Bleed 2	Bleed 3	Bleed 4	Bleed 5
1		25.00	2,146.41	2,804.16		PBS i.m.	1		12.50	341.45	349.00	
2		148.69	12,116.25	24,242.28		B/D/I i.m.	2		152.49	3,820.60	9,070.99	
3		12.50	12.50	12.50		CFA/IFA i.m.	3		12.50	25.00	12.50	
4		197.27	23,587.58	96,596.87		B/D/I CFA/IFA i.m.	4		136.84	8,047.86	19,977.75	
5		12.50	12.50	25.00		MPL-AH i.m.	5		12.50	12.50	25.00	
6		187.11	8,901.50	45,011.09		B/D/I MPL-AH i.m.	6		244.37	9,864.26	22,676.57	
7		12.50	12.50	62.07		VS	7		12.50	12.50	25.00	
8		12.50	12.50	12.50	12.50	PBS i.n.	8		12.50	12.50	12.50	12.50
9		12.50	12.50	12.50	12.50	CT i.n.	9		12.50	12.50	12.50	12.50
10		12.50	12.50	2,193.57	44,790.62	B/D/I CT i.n.	10		12.50	12.50	1,044.85	7,593.09
11		25.00	25.00	12.50	12.50	MPL i.n.	11		12.50	12.50	12.50	12.50
12		95.10	564.51	10,801.01	20,143.84	B/D/I MPL i.n.	12		12.50	25.00	25.00	140.08
13		12.50	12.50	12.50	12.50	Chitosan/MPL i.n.	13		12.50	12.50	12.50	12.50
14		12.50	12.50	123.51	2,253.53	B/D/I Chit/MPL i.n.	14		12.50	12.50	12.50	66.93
15		12.50	12.50	234.66	2,892.16	B/D/I i.n.	15		12.50	12.50	235.98	2,890.00

Table 3.1. Assigned titers reflecting the quantity of antibodies to SipB and SipD based on ELISA. TMB (3,3',5,5'-tetramethylbenzidine) soluble substrate was used for enzyme catalysis of peroxidase-labeled goat anti-mouse IgG. This reaction yields a blue color when peroxidase is detected. The color then changes to yellow with the addition of phosphoric acid with maximum absorbance at 450 nm. Quantified optical densities were used to calculate titers.

they did not show strong rates of survival during the challenge. The MPL + AH titers were low, as expected; however a large number (56%) of these animals survived. This was not expected. No titers were expected for the control group given PBS, but for an unknown reason, had titers beginning at bleed three.

Results of intranasal *Salmonella* vaccines are presented in Figure 3.2. Mice immunized with cholera toxin (CT) had 50% survival. Mice given CT +I/D/B had a 60% survival, which is only 10% more survival than CT alone. Mice immunized with MPL had a survival of 60% and mice given MPL + I/D/B had a survival of 80%. Immunizations with MPL + Chitosan resulted in a 40% survival within the group and this was greater than the survival seen for mice given MPL + Chitosan + I/D/B (10% survival. Titers reflect poor antibody response for this group.

It was anticipated that groups given immunizations formulated with I/D/B would have substantially higher survival than those immunized without I/D/B, however, this was not the case. While groups given immunizations containing I/D/B had higher survival (excluding the MPL + Chitosan + I/D/B group) than that of their corresponding control groups, none were substantially higher and offer no definitive results with regard to vaccine efficacy.

Titers for mice given intranasal immunizations are shown in Table 3.1. Antibodies to SipB and SipD generated post ELISA are evident at each bleed interval. All animals whose immunizations included I/D/B showed increasing titer values by bleed four as expected, however, these increases were not as substantial as the increases seen for the intramuscular immunizations. Survival of mice within groups did not appear to correlate closely with antibody titers (Table 3.1) and the survival rates were not

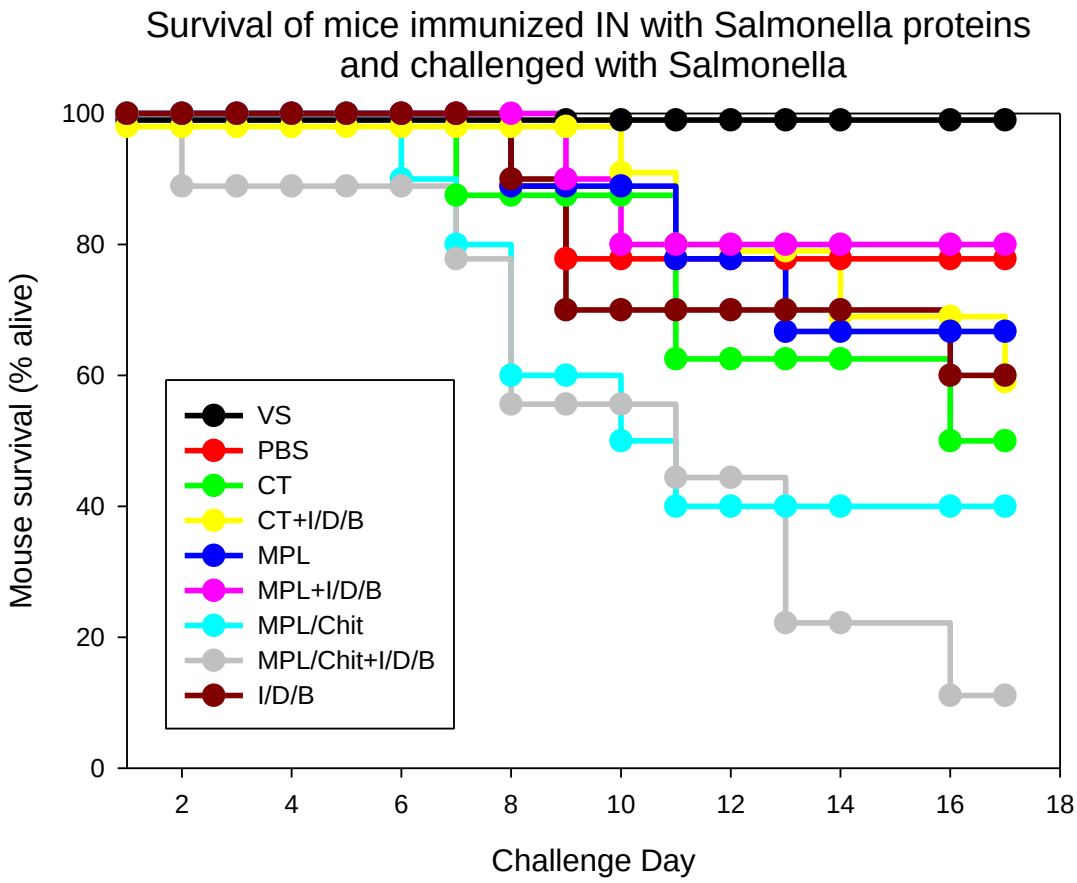

Figure 3.2. Survival of mice immunized intranasally with *Salmonella* proteins: PrgI, SipB and SipD, Cholera toxin with and without antigens, MPL with and without antigens, and Chitosan + MPL with and without antigens.

substantial when compared to adjuvants alone. These results are not consistent due to discrepancies in the survival versus the titer values. For example, based on lower titers than the group given MPL+ Chitosan+ I/D/B, animals given MPL+ Chitosan alone should not have survived, yet they did and in fact had higher survival than the group given MPL+ Chitosan+ I/D/B. All other groups given immunizations with I/D/B had higher titers and survival than that of their controls, but none were great enough to yield substantial results.

Shigella Results for Experiment 2

Mice immunized intramuscularly with *Shigella* proteins did not survive the challenge. There are no results of survival for this section due to the death of all groups of mice. This outcome was not expected and was unfortunate. It was assumed based on high titer values, that mice given immunizations with H/D/B would survive the challenge. The titers for mice immunized intramuscularly with *Shigella* proteins are listed in Table 3.2. Titers reflect antibodies to IpaB and IpaD generated post-ELISA at each bleed interval. Each bleed was completed two days prior to the subsequent immunization. All animals whose immunizations included MxiH, IpaD, and IpaB (H/D/B) showed increasing titers by bleed four as expected, however, while titers for all animals immunized with H/D/B were high, there is no sign of vaccine efficacy if there is no survival.

The intranasal immunizations with the *Shigella* proteins and cholera toxin (CT) provided the most substantial results for Experiment 2. The immunizations with H/D/B + (CT) were successful in providing complete (100%) protection against challenge (Fig

3.3). The H/D/B + CT group antibody titers were 5,041,306.68 against IpaB and 2,667.69 against IpaD after the fifth bleed (Table 3.2), which was anticipated and certainly correlates with survival in this section of the experiment. CT alone had only 30% survival within the group, which is higher than expected but substantially lower than CT with the proteins. Immunizations formulated with MPL, and MPL + Chitosan with H/D/B did not fare well in the challenge. These results from Experiment 2 seem to indicate that the intranasal immunizations with *Shigella* proteins and CT provide considerable protection against challenge with *S. flexneri*.

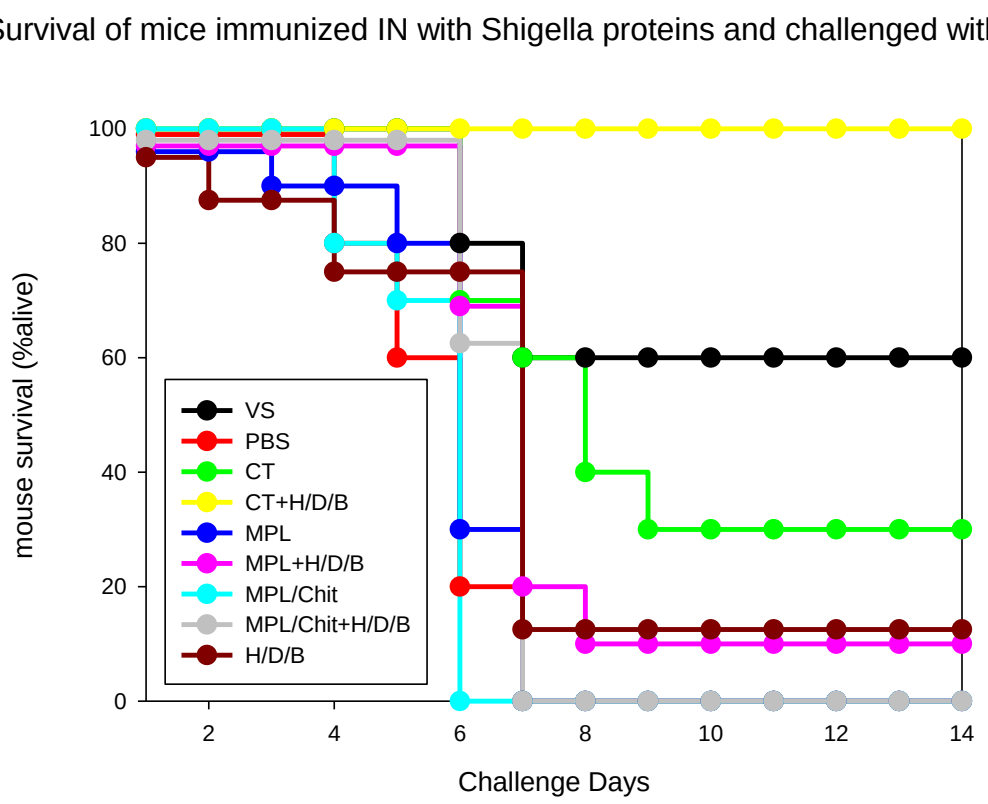

Figure 3.3. Survival of mice after intranasal immunizations with *Shigella* proteins MxiH, IpaB, and IpaD. The adjuvants used for immunizations included: cholera toxin with and without proteins; MPL with and without proteins; and Chitosan + MPL with and without proteins. The graph illustrates survival rate versus time. The survival rate of the mice begins on day one post challenge.

Antibody titers for *Shilgella* immunizations

Shigella Ipa B IgG							Shigella Ipa D IgG					
Group	Bleed 1	Bleed 2	Bleed 3	Bleed 4	Bleed 5		Group	Bleed 1	Bleed 2	Bleed 3	Bleed 4	Bleed 5
16	25.00	12.50	12.50	12.50		PBS i.m.	16	12.50	12.50	12.50	12.50	
17	12.50	21,369.91	371,253.81	433,573.58		B/D/H i.m.	17	12.50	12.50	6,633.23	9,555.01	
18	12.50	12.50	-	25.00		CFA/IFA i.m.	18	12.50	12.50	12.50	12.50	
19	12.50	66,938.05	1,086,751.75	1,697,396.73		B/D/H CFA/IFA i.m.	19	12.50	25.00	15,855.40	25,074.45	
20	12.50	25.00	57.53	88.73		MPL-AH i.m.	20	12.50	12.50	12.50	25.00	
21	12.50	25.00	866,744.61	1,724,074.87		B/D/H MPL-AH i.m.	21	12.50	25.00	19,441.70	178,910.39	
22	12.50	25.00	25.00	259.13		VS	22	12.50	12.50	12.50	12.50	
23	12.50	12.50	12.50	12.50	12.50	PBS i.n.	23	12.50	12.50	12.50	12.50	12.50
24	12.50	12.50	12.50	12.50	12.50	CT i.n.	24	12.50	12.50	12.50	12.50	12.50
25	12.50	12.50	403.01	54,388.16	5,041,306.68	B/D/H CT i.n.	25	12.50	12.50	12.50	57.86	2,667.69
26	12.50	12.50	12.50	12.50	12.50	MPL i.n.	26	12.50	12.50	12.50	12.50	12.50
27	12.50	236.78	1,118.27	102,004.82	377,835.77	B/D/H MPL i.n.	27	12.50	12.50	12.50	82.81	4,521.32
28	12.50	12.50	12.50	12.50	12.50	Chitosan/MPL i.n.	28	12.50	12.50	12.50	12.50	12.50
29	12.50	25.00	178.57	1,922.17	2,579.89	B/D/H Chit/MPL i.n.	29	12.50	12.50	12.50	12.50	131.24
30	12.50	12.50	813.82	25,419.02	25,419.02	B/D/H i.n.	30	12.50	12.50	12.50	12.50	243.65

Table 3.2 lists assigned titers reflecting the quantity of antibodies to IpaB and IpaD based on ELISA assay and optical density at 450nm.

Shigella **Results for Experiment 3**

Shigella immunizations for Experiment 3 were only done intramuscularly (see Figure 3.4). Animals given IM immunizations formulated with Monophosphoryl lipid A (MPL) + AH + IpaD and IpaB (D/B) had 60% survival within the group. Only 10% of mice immunized with MPL + AH alone survived the challenge. These results are based upon and reinforce anticipated potential using MPL as an adjuvant for IM vaccination. Titers for mice immunized intramuscularly with *Shigella* proteins are listed in Table 3.3. These titers show strong antibody responses to IpaB and IpaD at each bleed interval. Each bleed was completed two days prior to the subsequent immunization. All animals whose immunizations included IpaD and IpaB (D/B) showed increasing titer values by bleed four as expected. High antibody titers provide a foundation for the observed protection.

Salmonella **Results for Experiment 3**

In Figure 3.5 intramuscular *Salmonella* immunizations were formulated with MPL + AH alone and with SipB and SipD (B/D), with SipB/SicA and SipD (B-A/D), and with a SipB fragment and SipD (B-frag/D). Unfortunately, none of the mice survived the challenge. High titer values for the groups immunized with SipB and its variants with SipD did not correlate with a high rate of survival (Table 3.4). These results were not expected since such high titers were generated.

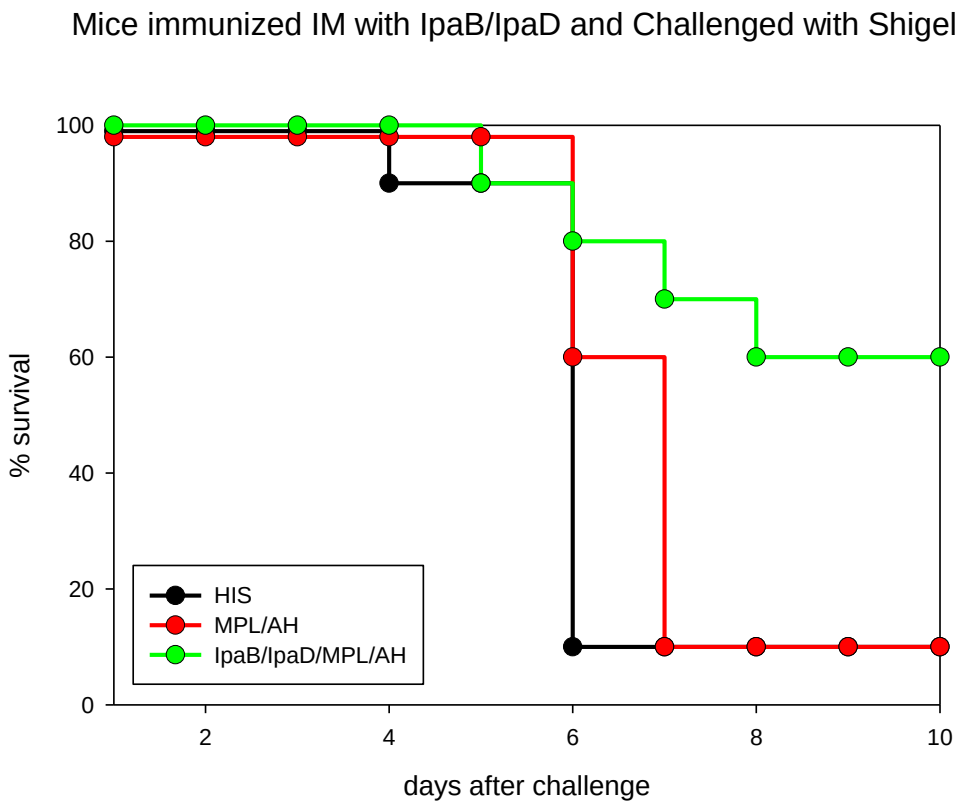

Figure 3.4. Survival of mice after intramuscular immunizations with *Shigella* proteins: IpaB and IpaD with and without MPL.

Antibody Titers for *Shilgella* Immunizations

Shigella Ipa B IgG						Shigella Ipa D IgG				
Group	Bleed 1	Bleed 2	Bleed 3	Bleed 4	IM	Group	Bleed 1	Bleed 2	Bleed 3	Bleed 4
11	12.50	25.00	12.50	12.50	His	11	12.50	25.00	12.50	12.50
12	12.50	12.50	25.00	25.00	MPL/AH	12	12.50	12.50	25.00	12.50
13	25.00	19,736.07	297,357.28	1,562,970.93	MPL/AH+B/D	13	12.50	2,320.16	50,584.95	256,493.54

Table 3.3. *Shigella* antibody titers listed above reflect the quantity of antibodies to IpaB and IpaD based on ELISA assay and optical density at 450nm.

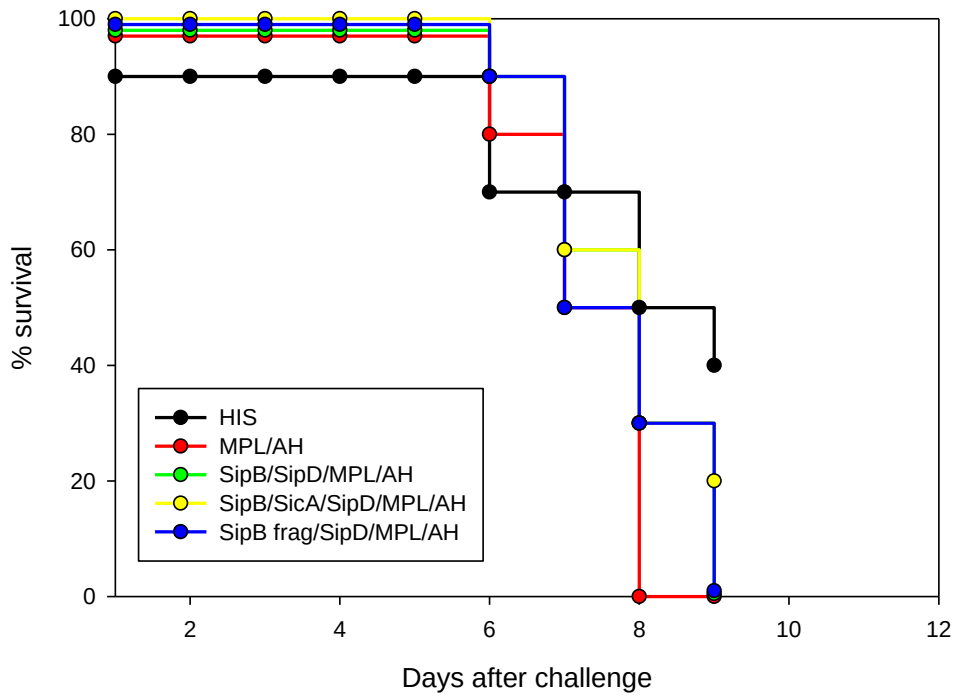

Figure 3.5. Survival of mice immunized intramuscularly with *Salmonella* proteins SipB and SipD with and without MPL, SipB-SicA complex with MPL and SipD, SipB-fragment and SipD with MPL.

Intranasal immunizations with *Salmonella* proteins in Figure 3.6 show mice immunized with the SipB-SicA complex and SipD + dmLT had a survival rate of 50%. No other groups had higher than 30% survival, however the group given dmLT alone had only 20% survival within the group. Titers for the group immunized with the SipB-SicA complex along with SipD + dmLT were very high against SipB at 1,280,663.67 (Table 3.4). They were the highest titers out of all the mice in this experiment. It was expected that with such high titers this group would have the highest rate of survival. These results show promise for immunizations formulated with the SipB-SicA complex.

Substantially high antibody titer levels for both immunization regimens in Experiment 3 show that immunity is developed against IpaD, IpaB, SipD and SipB. In addition to high titer levels, survival rates of animals immunized with with IpaD/IpaB + MPL+ AH and the SipB-SicA complex and SipD + dmLT had higher survival rates compared to any of the other groups tested. Although the *Salmonella* intramuscular immunizations yielded high titer values, there was no survival in these experiments.

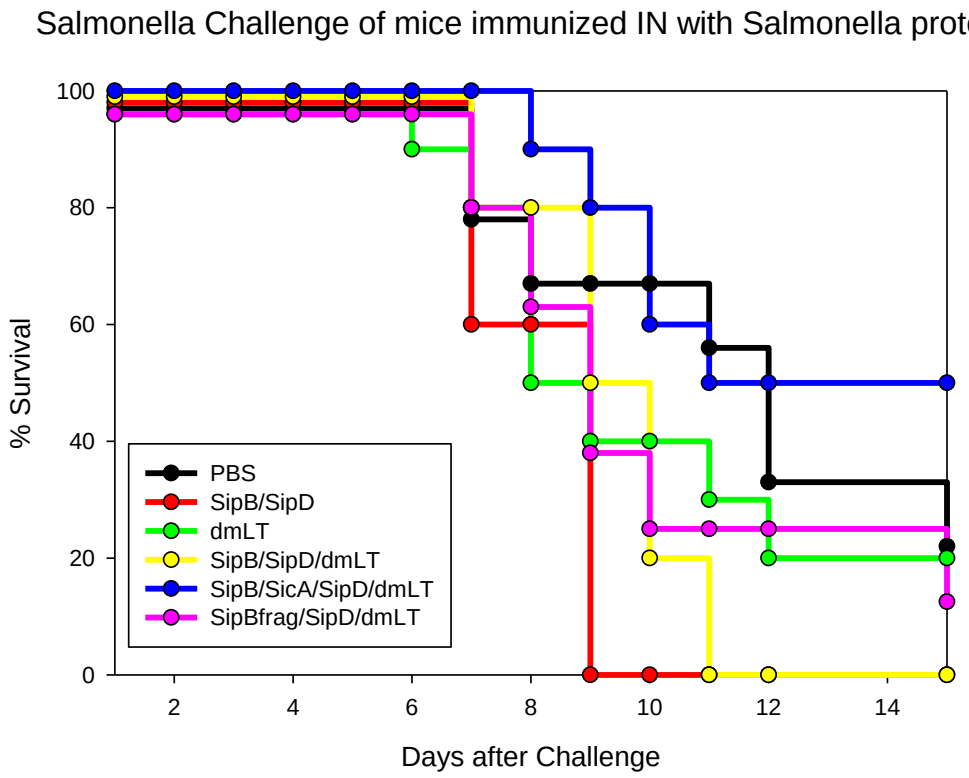

Figure 3.6. Survival of mice immunized intranasally with Salmonella proteins SipB and SipD with and without dmLT, SipB-SicA complex with dmLT and SipD, SipB-fragment and SipD with dmLT.

Antibody titers for *Salmonella* immunizations

Salmonella Sip B IgG						Salmonella Sip D IgG				
Group	Bleed 1	Bleed 2	Bleed 3	Bleed 4	IM	Group	Bleed 1	Bleed 2	Bleed 3	Bleed 4
1	12.50	12.50	12.50	12.50	His	1	12.50	12.50	12.50	12.50
2	12.50	82.21	67.25	65.03	MPL + AH	2	12.50	12.50	67.34	121.17
3	12.50	27,406.71	172,175.08	2,526,396.78	MPL/AH + B/D	3	12.50	20,775.16	391,821.29	5,428,563.40
4	12.50	49,194.56	1,205,474.90	4,207,556.21	MPL/AH + B-SicA/D	4	12.50	12.50	41,336.19	351,604.72
5	25.00	33,341.90	748,659.21	926,38.49	MPL/AH + B-frag/D	5	12.50	12.50	32,425.02	155,028.72
					IN					
6	12.50	12.50	25.00	66.43	PBS	6	12.50	12.50	12.50	12.50
7	12.50	12.50	233.78	23,584.86	B/D	7	12.50	12.50	25.00	152.80
8	12.50	12.50	12.50	12.50	dmLT	8	12.50	12.50	12.50	12.50
9	12.50	QNS	1,012.43	787,538.77	dmLT+B/D	9	25.00	QNS	152.80	8,670.81
10	12.50	12.50	5,400.84	1,280,663.67	dmLT+B-sicA/D	10	12.50	12.50	25.00	8,303.09
19	12.50	12.50	1,702.69	928,604.36	dmLT+B-frag/D	19	12.50	12.50	3,202.75	99,089.83

Table 3.4 lists assigned titers reflecting the quantity of antibodies to SipB and SipD based on ELISA assay and optical density at 450nm.

CHAPTER IV

Discussion and Future Directions

The goal of Experiment 2 was to determine the effectiveness of different adjuvants in combination with type III secretion needle, translocator and effector proteins as subunit vaccines to protect against *Salmonella* serovar Typhimurium and *Shigella flexneri* infections. Both intranasal and intramuscular immunizations were tested to determine the influence that the route of administration has on protective efficacy. PrgI, SipB, and SipD were used as antigens for protection against *Salmonella* serovar Typhimurium and MxiH, IpaB, and IpaD were the antigens used to protect against *S. flexneri* infection. These proteins comprise the essential extracellular components of the type three-secretion apparatuses used by these pathogens. These virulence factors are required for invasion of host cells by these facultative intracellular pathogens. The adjuvants chosen for this experiment stimulate the innate and adaptive immune responses in different ways. Freund's adjuvant is heat killed, dried mycobacterium components dispersed in a water in oil emulsion and it can potentially be toxic or cause tissue damage; however, it is known to be an effective adjuvant in animals immunized by the intramuscular route. It was only used for a primary immunization because it is known to be very effective in stimulating the initial immune responses in animals when co-administered with antigens. Incomplete Freund's adjuvant lacks the mycobacterial

components but continues to boost the immune system after the initial immunization for these animals. Monophosphoryl lipid A (MPL), is derived from the lipid A portion of *Salmonella* LPS and is known to enhance specific immunity and stimulate the synthesis of cytokines after recognition by toll-like receptor 4 (TLR-4). Alhydrogel (aluminum hydroxide) is currently approved for use in human vaccines because it is very stable, it readily adsorbs proteins and it rarely yields adverse effects in animals or humans. Cholera toxin (CT) promotes long-term mucosal immunity when used in intranasal vaccine formulations; however, it is not approved for use in humans due to toxicity issues. Nevertheless, *E. coli* heat-labile enterotoxin (LT) is almost identical to CT and a mutated derivative of LT (called dmLT). It is greatly reduced in toxicity and it may be promising for future use in humans for eliciting mucosal immune responses against co-administered antigens. Chitosan was also used as an adjuvant because it has been shown to stimulate cytokines that enhance the immune response while providing a nontoxic and natural mucosal delivery vehicle for protein antigens. Live-attenuated *Salmonella* and *Shigella* vaccine strains were used in these experiments as positive controls. Survival of these mice demonstrates antibody production to the lipopolysaccharide components of these strains.

A major outcome of Experiment 2 was that it has provided a foundation upon which additional experiments could be developed for identifying a fully efficacious adjuvant/immunization combination. Only the mice immunized intranasally with *Shigella* proteins MxiH, IpaB, and IpaD formulated with CT provided complete protection against lethal challenge with *S. flexneri* 2457T. This level of protection was even better that that seen for a known protective live, attenuated *Shigella* vaccine strain. CT is known to be a

strong systemic and mucosal adjuvant (Vajdy and Lycke, 1992). Another encouraging result within Experiment 2 involved the intramuscular immunizations in which MPL and AH were combined with *Salmonella* proteins PrgI, SipD and SipB. These immunizations appeared to provide partial protection against *Salmonella* challenge. Unfortunately this adjuvant combination alone also provided partial protection. Although the overall result was inconclusive, it does provide promise that MPL and AH can be used as intramuscular adjuvants for anti-salmonellosis vaccines and suggests that PrgI, SipD, and/or SipB are effective and protective antigens.

The data from Experiment 2 that were not successful still provided valuable information with respect to future direction of research. The low survival rate of the group immunized intramuscularly with *Salmonella* proteins PrgI, SipD and SipB with the Freund's was somewhat surprising since the MPS plus AH adjuvnats appeared to work with these antigens. The intranasal vaccines formulated with MPL with PrgI, SipD and SipB for *Salmonella* could not be evaluated unfortunately due to the high survival rates of the negative controls (adjuvants only). In contrast, the intranasal administration of MPL with MxiH, IpaD and IpaB clearly offered no substantial protection against *Shigella* challenge, perhaps implying that MPL may be better served when used as an adjuvant for intramuscular vaccines. Chitosan was added to intranasal immunizations formulated with MPL and *Salmonella* and *Shigella* proteins with the expectation that it would offer enhanced delivery and presentation of these proteins. The low survival rate of these groups showed that it did not enhance protection at all. In the end, Experiment 2 only offered two possible vaccine insights. First, the proteins PrgI, SipD and SipB with MPL and AH may provide protection against *Salmonella* infection when administered

intramuscularly. Second, MxiH, IpaD and IpaB may provide protection when administered intranasally with CT as the adjuvant.

The approach of Experiment 3 focused more on the TTSS needle tip proteins as vaccine components rather than the needle proteins. For protection against *Shigella* infection, this meant focusing on IpaD and IpaB. In this experiment, we also focused on vaccinating intramuscularly for protection against shigellosis. *Shigella* proteins IpaB and IpaD were used as antigens for intramuscular immunizations. Mice immunized intramuscularly with MPL with AH and *Shigella* proteins IpaB and IpaD had 60% survival with high antibody titers against IpaB and IpaD. Survival of mice given MPL with AH alone was only 10%. These results appear to validate the use of MPL with AH as successful intramuscular adjuvants, and show that protection against shigellosis be possible with an intramuscular vaccine.

The goal for the *Salmonella* immunizations in Experiment 3 was to provide a more stable form of the antigen that was being delivered. This involved using the SipB/SicA complex along with SipD, and a SipB fragment that is known to be stable alone with SipD as the antigens for both intramuscular and intranasal immunizations. Double mutant heat-labile toxin (dmLT) was used as an intranasal adjuvant in these experiments instead of CT. dmLT is considered safe and effective and the mice immunized intranasally with the *Salmonella* SipB-SicA complex and SipD with dmLT had the greatest survival at 50%. This shows some promise for intranasal immunizations using the more stable SipB-SicA complex and SipD with dmLT, however, several of the negative control animals showed high rates of survival too (30% for PBS alone).

Unfortunately, the Experiment 3 intramuscular immunizations for protection against *Salmonella* challenge were not successful. It was expected that high antibody levels and protection would be achieved by using the adjuvants MPL and AH with the *Salmonella* proteins, however there was a high rate of death among the groups. Possible reasons for this may again be due to too high a challenge dose. Alternatively, lack of survival could be due to problems with the mouse model, since *Salmonella* causes a typhoid type of disease in mice instead of the gastroenteritis that occurs in humans.

The dire need for a vaccine for humans against *S. flexneri* and *Salmonella* serovar Typhimurium infections drives research in this field. These experiments will continue since there is still a need for determining the optimal antigen/adjuvant combinations for formulating vaccines against these important enteric pathogens. The more stable SipB/SicA complex will be used in future experiments since it shows some early promise in providing protection against *Salmonella* challenge in mice. It may be necessary, however, to consider using an alternate (gastroenteritis) model for this work. Further work with MPL as an adjuvant is also a direction worth pursuing. Taking these initial steps in formulating an effective way to combat against these bacterial diseases will prove valuable for global public health. Though this portion of research is concluded, there is much more to be done in hopes of an eventual vaccine that will save countless lives.

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VITA

Rian Mae Perryman

Candidate for the Degree of

Master of Science

Thesis: THE EVALUATION OF TYPE III SECRETION APPARATUS COMPONENTS AS SUBUNIT VACCINE CANDIDATES AGAINST *SHIGELLA* AND *SALMONELLA* INFECTION IN MICE

Major Field: Microbiology

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Completed the requirements for the Bachelor of Science in Biology at Northeastern State University, Tahlequah, OK/USA in 2009.

Experience: Working with Dr. Wendy Picking on vaccine trials and evaluation of the TTSA in *Shigella* and *Salmonella.*

Name: Rian Perryman Date of Degree: July, 2011

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: The Evaluation of the Type III Secretion Apparatus Components as Subunit Vaccine Candidates Against *Shigella* and *Salmonella* Infection in Mice

Pages in Study: 91 Candidate for the Degree of Master of Science

Major Field: Microbiology

Scope and Method of Study:

This study focuses on the assessment of the translocators of the type III secretion system (TTSS) as protective antigens against *Shigella* and *Salmonella* infections in mice. Mice were given intramuscular and intranasal subunit vaccines formulated using extracellular components of the TTSA and various adjuvants. In Experiment 2 purified MxiH (needle), IpaD (tip protein) and IpaB (tip-associated translocator protein) were targeted in *Shigella* immunizations and in immunizations using purified *Salmonella* homologues, PrgI (needle), SipD (tip protein), and SipB (tip-associated translocator protein). Intramuscular immunizations were formulated with Freund's adjuvant, Freund's Incomplete Adjuvant, and Monophosphoryl lipid A (MPL). Intranasal immunizations were formulated with cholera toxin (CT), MPL and MPL with chitosan. Experiment 3 focused on using the TTSS needle tip proteins as vaccine components. IpaB and IpaD were used with MPL and AH as antigens for *Shigella* intramuscular immunizations. SipB, the SipB/SicA complex, and a SipB fragment with SipD were used with MPL and AH in *Salmonella* intramuscular vaccines and with double mutant heat-labile toxin (dmLT) for intranasal vaccines. Mice receiving *Shigella* vaccines were challenged intranasally with *Shigella* strain 2457T. Mice given *Salmonella* vaccines were challenged ororgastrically with *Salmonella* strain SL1344. Mice were monitored for up to two weeks post challenge.

Findings and Conclusions:

Experiment 2 offers two possible vaccine insights. First, the proteins PrgI, SipD and SipB with MPL and AH may provide protection against *Salmonella* infection when administered intramuscularly. Second, MxiH, IpaD and IpaB may provide protection when administered intranasally with CT as the adjuvant. Findings for Experiment 3 offer further understanding by using tip proteins as vaccine components. IpaD and IpaB in combination with MPL and AH appear to validate that protection against shigellosis may be possible with an intramuscular vaccine. Furthermore, the *Salmonella* SipB-SicA complex and SipD administered intranasally with dmLT may provide protection against *Salmonella* infection.

ADVISER'S APPROVAL: Dr. Wendy Picking