

Development of Vaccines Against Uropathogenic *Escherichia coli* Toxins to
Reduce Morbidity Associated with Urinary Tract Infections

by

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DEDICATION

Dedicated to Carrie.

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ABSTRACT

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Reduce Morbidity Associated with Urinary Tract Infections

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Urinary tract infections (UTIs) are estimated to affect eighty percent of women in the United States annually with a resulting cost of billions of dollars spent on physician visits, treatment, and lost work hours. Uropathogenic *E. coli* (UPEC) are the leading cause of UTIs. Previous research in our laboratory demonstrated that female C3H/HeOuJ mice that are transurethrally infected with UPEC strain CP9 into the urinary bladder develop a suppurative cystitis with severe submucosal edema within 24 hours of bacterial inoculation. Researchers in our laboratory also reported that CP9 strains with inactivated Cytotoxic Necrotizing Factor 1 (CNF1) and/or the Hemolysin (HlyA) genes evoke less severe pathology in the urinary bladder than does the wild type strain. CNF1 and HlyA are toxins that contribute to UPEC pathogenesis. CNF1 enzymatically causes

constitutive activation of small Rho family GTPases that leads to cytoskeletal changes in host cells. HlyA forms membrane pores and lyses mammalian cells. CNF1 and HlyA contribute to the robust inflammatory response in the bladders of mice challenged for 24hrs with the UPEC strain CP9. We hypothesized that antibodies against CNF1 and/or HlyA will reduce the pathology caused by CP9 infection of the bladder. Therefore, we actively immunized mice with a HlyA toxoid in which two essential lysine residues were changed to glutamic acid. Female C3H/HeOuJ mice were vaccinated subcutaneously twice with HlyA toxoid in adjuvant and boosted once or twice more without adjuvant. We collected serum prior to each boost, and we observed increases in anti-HlyA titers over time by ELISA and elevations in neutralizing antibody levels as assessed by erythrocyte lysis neutralization after the second boost. Two weeks after the final boost, we challenged the mice intraurethrally with CP9 and sacrificed them 24 hr later. We observed a reduction in urine colonization in HlyA-vaccinated mice compared to sham-vaccinated mice but the reduction was not statistically significant. There was no difference in kidney colonization between the two groups. HlyA-vaccinated mice exhibited significantly reduced cystitis when compared to sham-immunized mice. We also actively immunized mice with a CNF1 toxoid in which an essential cysteine residue within the catalytic site was changed to serine. Mice were immunized subcutaneously once with CNF1 toxoid in adjuvant and boosted three times with CNF1 toxoid combined with sublethal doses of CNF1 without adjuvant. We collected serum prior to each boost and observed an increase in anti-CNF1 titers over time by ELISA and an enhancement of neutralizing antibody titers as assessed by inhibition of multinucleation of HEp-2 cells, a CNF1-mediated phenotype. Two weeks after the final CNF1 toxoid/ CNF1 boost, we

challenged the mice. We observed no differences in urine bacterial counts or in kidney counts between the CNF1-vaccinated mice and sham-vaccinated mice. There was also no reduction in cystitis in CNF1-vaccinated mice. To determine whether antibodies against CNF1 could reduce cystitis, we passively administered a CNF-neutralizing monoclonal antibody intraperitoneally to mice 24hr and 4hr prior to intraurethral challenge with CP9. After 24hr, we collected urine, sacrificed the mice and harvested the bladder and kidneys. Enumeration of bacteria in the urine and kidneys showed that the anti-CNF1 antibody did not affect colonization. However, the anti-CNF1 antibody caused a significant reduction in cystitis when compared to bladders of mice given an isotype control antibody. Taken together, our data show that antibodies against CNF1 or HlyA reduce the bladder pathology caused by UPEC.

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CHAPTER 1: Introduction

IMPACT OF URINARY TRACT INFECTIONS

Millions of women and men are afflicted with urinary tract infections (UTIs) annually. In fact, half of all women will experience a UTI in their lifetime (63; 73). Bacterial UTIs are the most common infection in humans (74). UTIs cause pain and discomfort, lost work time and productivity, and can lead to serious sequelae such as kidney damage, bacteremia and sepsis (63; 73; 152). Clinicians usually treat UTIs with antibiotics. As a consequence, antibiotic resistance of UTI strains is a growing problem. Indeed, more than 10% of UTI strains are multiply antibiotic resistant (64; 191). There are few alternatives to antibiotics. In spite of years of active study and dozens of candidates, UTI research has not yielded an approved vaccine candidate (27). There continues to be a need to develop UTI treatments and preventions and to better understand the urinary tract and its pathogens.

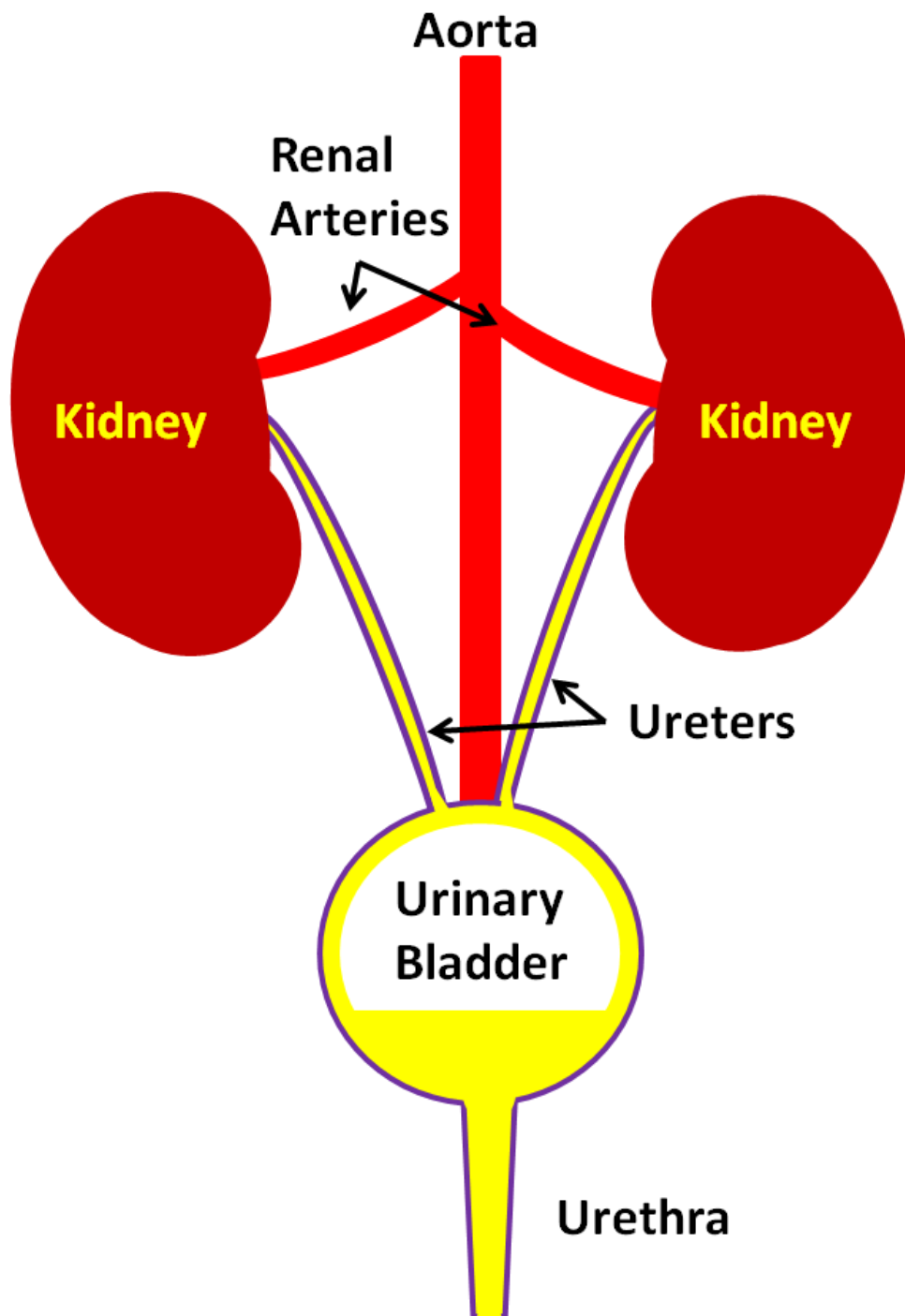
EPIDEMIOLOGY OF URINARY TRACT INFECTIONS

Anatomy the urinary tract

The urinary tract is composed of the kidneys, ureters, urinary bladder, and urethra. The kidneys create an ultrafiltrate of plasma that is then processed into urine in the tubules of the nephron, the functional unit of the kidney. Urine flows from the nephron into collecting ducts that empty into the renal pelvis. The renal pelvis is continuous with the ureter, a tubular duct that conducts urine into the urinary bladder. Urine is stored in the urinary bladder and ultimately voided through another tubular duct, the urethra (Figure 1) (11). The ureter, urinary bladder, and urethra are composed of layers of smooth muscle with a mucosal lining. The mucosal lining is composed of stratified

Figure 1. Diagram of the Mammalian Urinary Tract

Two kidneys create urine from a filtrate of blood supplied through the renal arteries. The ureters are tubes lined by smooth muscle (purple) that conducts urine from the kidney to the urinary bladder for temporary storage. The urinary bladder is a highly distensible sac of layered smooth muscle with a specialized mucosal lining that is impervious to urine. The urethra is the muscular tube that serves as the passage for urine to exit the body.



urothelial cells beginning at the renal pelvis and ending at the distal urethra. The mucosa of the distal urethra transitions to stratified squamous at the mucocutaneous junction. The urethral opening is in close proximity to the openings of the intestinal tract and the genital tract, especially in women. Bacterial pathogens from these systems and the integument can access the urinary tract through the urethra. These organisms can ascend the urethra to the urinary bladder, colonize, and cause inflammation called cystitis. They can further ascend the ureters and into the kidneys and infect the renal pelvis (pyelonephritis). Eventually the bacteria (typically) can access the bloodstream to cause bacteremia with or without septic manifestations (septicemia). Clinically, UTIs can be defined as either uncomplicated or complicated.

Uncomplicated versus complicated UTIs

Uncomplicated UTIs occur in patients who have no underlying disease that affects the urinary tract and is usually limited to the lower urinary tract (urethra and urinary bladder). Clinical signs of uncomplicated UTIs include painful urination (dysuria), increased frequency and perceived urgency to urinate, and the presence of pus (pyuria), blood (hematuria), and bacteria (bacteriuria) in the urine (84). Complicated UTIs affect patients who have a disorder of the urinary tract. These disorders can be of the urinary tract itself, such as anatomic anomalies, urinary obstructions, or the presence of a urinary catheter. Clinically, patients can experience signs that refer to the bladder, as detailed above under uncomplicated UTI, or the kidney such as flank pain, back pain, and tenderness at the costovertebral angle. Patients with complicated UTIs can also have signs of systemic illness such as fever, chills, and malaise (84). In addition, asymptomatic UTIs can occur in which bacteriuria exists without clinical signs (148).

Uncomplicated UTIs are often self-limiting, but treatment with antimicrobial therapy reduces the time course as well as the risk of recurrence and pyelonephritis (63).

Complicated UTIs usually require antimicrobial therapy. Asymptomatic UTIs do not require antibiotics with the exception of pregnant women as they more often progress to symptomatic UTIs than do other asymptomatically infected individuals (153).

The most common clinical presentation of UTIs are uncomplicated UTIs that affect women (63). Up to 50% of women will suffer from at least one UTI in their lifetime. Risk factors for this group include age (20-30), behavior (sexual intercourse), genetic factors (ABO blood-group antigens, family history), antibiotic usage, and the use of spermicides (58; 63). In 2000, an estimated seven million women were treated for UTIs at a cost of almost three billion dollars (63; 73). Women usually recover from uncomplicated UTIs, but 25% progress to pyelonephritis and/or experience recurrence (64; 65).

Complicated UTIs affect many subpopulations. Use of a urinary catheter increases the risk of a UTI. Catheter-associated UTIs are the most common nosocomial infection in hospitals and nursing homes (213). In addition to hospitalized patients, individuals with spinal cord injuries are at a higher risk of UTIs. Spinal cord injury can cause a neurogenic bladder, a syndrome that is due to a loss of innervation of that organ. Bladder emptying is under control of both sympathetic and parasympathetic spinal nerves that arise from the lumbar and sacral spinal cord. Without innervation, there is a loss of control of bladder emptying, reduced sensation of a full bladder, and incomplete emptying. Spinal cord patients manage a neurogenic bladder by long-term or frequent intermittent catheterization; this requirement for catheterization further increases the risk

of UTIs (106). Individuals afflicted with multiple sclerosis can also develop a neurogenic bladder, and they too experience a higher incidence of UTIs (131). Diabetes predisposes women to UTIs. The pathogenesis of this link is not yet clear, but glycosuria, diabetic neuropathy, and impaired immune function may play a role (155; 167). Diabetic UTIs can have unusual and more severe clinical forms such as emphysematous cystitis and pyelonephritis (167).

The incidence of UTIs also changes with age groups. A small percentage of prepubescent children are affected by UTIs (233; 237). Prepubertal girls are at increased risk of recurrent UTIs and, unlike adult women, renal scarring (129; 158). The elderly also share a higher incidence of UTIs, especially those in institutional settings (149-151). There are a number of age-related exposures/changes that increase the risk of UTIs; these factors include greater chance of hospitalization, chronic neurologic disease leading to a neurogenic bladder, prostatic disease in men, and menopause leading to decreased estrogen (153). Sex hormones influence bladder emptying in women (11). Pregnancy increases the risk of UTIs, likely due to hormonal changes and increased pressure at the pelvic brim that may hinder bladder emptying (39).

Another population with increased incidence of UTIs includes individuals with anatomic anomalies within the urinary tract. Some anomalies are congenital (ectopic ureters, congenital valves, bladder diverticula) and some can be acquired (cystoceles) (151). Urinary tract obstructions also disrupt the process of bladder emptying and predispose individuals to UTIs. Obstructive conditions include bladder stones, enlarged prostate, neoplasms, pregnancy, and urethral strictures (secondary to catheterization) (216). People with immunodeficiency, including acquired immune deficiency syndrome,

have an increased incidence of UTIs (63; 194). Individuals with the risk factors listed in the above paragraphs are the most represented in the scientific literature in relation to their propensity to develop UTIs.

ETIOLOGY OF URINARY TRACT INFECTIONS

Overview of etiologies

UTIs can be caused by a variety of organisms including bacteria, viruses, fungi, and parasitic worms (74; 93). Adenovirus infection is associated with hemorrhagic cystitis (163). The yeast *Candida albicans* is the most common fungal infection of the urinary tract (205). Both viral and fungal causes of UTIs are rare in healthy people and are usually associated with complicating risk factors. Parasites, including the trematode *Schistosoma haematobium*, and the sexually-transmitted protozoa *Trichomonas vaginalis* also infect the urinary tract (16; 93). However, the most common etiological agents of UTIs are bacteria, and among these, intestinal bacteria are linked most frequently with UTIs. In uncomplicated UTIs in otherwise healthy individuals, the most frequently isolated bacteria are *Escherichia coli* (*E. coli*), *Staphylococcus saprophyticus*, *Klebsiella* spp., and *Enterococcus faecalis* (64; 179). *E. coli* has been responsible for 70-80% in this category of UTIs for more than 30 years with *S. saprophyticus* a distant second at 5-10%; other bacterial causes of UTI are each represented in single percentage points (64; 102; 179). Complicated UTIs with more diverse predisposing host factors also have a more varied set of bacterial etiologies. In addition to the organisms listed as causes of uncomplicated UTIs, *Enterobacter cloacae*, *Serratia marcescens*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and others are often causes of complicated UTIs (37; 64; 179).

However, even in most categories of complicated UTI, *E. coli* is still the most common cause of illness (74; 154).

Uropathogenic *Escherichia coli*

E. coli are Gram-negative bacilli in the Enterobacteriaceae family. These organisms typically inhabit the intestinal tracts of humans and animals without causing harm and in some cases may even provide a benefit to the host (15; 185). These commensal *E. coli* strains rarely cause disease because they do not possess virulence factors. Intestinal pathogenic *E. coli* cause outbreaks of gastroenteritis and are classified into six pathotypes: Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Diffusely adherent *E. coli* (DAEC), and Enteroaggregative *E. coli* (EAEC) (96; 185). EHEC, well known by the public as a cause of foodborne illness, is responsible for hemorrhagic colitis with the hemolytic uremic syndrome as a possible sequela. Intestinal pathogenic strains rarely cause disease outside of the gut (96). Yet another diverse set of *E. coli* pathotypes have evolved the capacity to cause disease outside the intestinal tract, the extraintestinal pathogenic *E. coli* (ExPEC).

The ExPEC can colonize the intestine as commensal strains, but once outside the gut they can cause a variety of diseases depending on their location (96; 197). These conditions include bacteremia, sepsis, neonatal meningitis and sepsis, wound infections, hospital-acquired infections and pneumonia, peritonitis, prostatitis, and UTIs (96; 182). UTIs are not only the most common of these infections but also the most common bacterial infection of women (74). The causal *E. coli* strains are called Uropathogenic *E.*

coli (UPEC). Researchers have studied the many aspects of UPEC pathogenesis through a variety of models.

MODELS OF URINARY TRACT INFECTIONS

Investigators have applied both in vitro systems and animal models to study the pathogenesis as well as the effectiveness of antimicrobial therapies and vaccinations against UTIs caused by UPEC. For example, a number of different cell culture systems have been used to study attachment, toxicity, and molecular mechanisms of UPEC. The types of cells employed for such investigations include: urothelial cells (PD07i and 5637 cells), renal cells (Vero, A498, ACHN), ureter epithelial cells (SV-HUC-1), Chinese hamster ovary cells (CHO), intestinal epithelial cells (T84 and Caco-2), mouse adrenal cells (Y-1), and other epithelial cells (HEp-2 and HeLa) (17; 26; 30; 48; 68; 83; 89; 116; 133). In one study, Smith et al. used an organoid lined by human urothelial cells as a UPEC model (203).

In animals, ascending infections can be mimicked by direct inoculation of a bacterial culture into the urinary bladder (intravesicular) through the urethra with a urinary catheter. Scientists have developed primate models for a variety of UTIs studies. Indeed, both cynomolgus macaques and African green monkeys have been used to investigate the role of bacterial adherence to epithelial cells in cystitis and pyelonephritis and to evaluate immune responses related to adhesins (87; 100; 175-177). African green monkeys are also the source for Vero cells. Moreover, cynomolgus and rhesus macaques have been used as challenge models for whole cell mucosal vaccines against UPEC (86; 219; 220), while male macaques have been used to study ascending prostatitis caused by UPEC (45; 145). Clinical trials have also been conducted in volunteers to assess their

responses to agents that can treat or prevent UTIs. For example, several groups of investigators have conducted trials on whole cell UPEC vaccines delivered mucosally and parenterally to participants in those studies (13; 27; 77; 217; 218).

Although not as directly relevant to human UTIs as primate models, a number of small animal models have been adapted for the investigation of UPEC pathogenesis.

Although rabbits have been used as a model for urinary catheter-associated UTIs (160), overall, mice and rats are the most common types of small animal models. In fact, rats have been used to: model cystitis and pyelonephritis, evaluate therapeutics against UTIs, study UTIs associated with neurogenic bladder (spinal cord injury), examine the effect of UPEC infections on the motility of ureters and the urinary bladder, and monitor changes in clinical chemistries associated with UTIs (36; 62; 120; 200; 232). Male rats have also been used as models for ascending bacterial prostatitis caused by UPEC strains (147; 172). Nevertheless, over the last several decades, mouse models have dominated UTI research. Scientists have used mouse models to parse out the roles of the many aspects of UPEC biology, individual virulence factors, and host-parasite interactions. Mice have also served as models for cystitis, pyelonephritis, sepsis subsequent to UPEC infection, and as UTI vaccine challenge models (27; 88; 95; 198; 202; 212; 235).

UROPATHOGENIC *E. COLI* AND THE CYCLE OF INFECTION

The source of UPEC is the intestine, an origin of infection that has led some investigators to consider UTIs as potential foodborne illnesses (85; 157). UPEC can colonize the periurethral integument and distal urethra, and then migrate into the urinary bladder. Through the application of mouse models of UTIs, Anderson et al. discovered that, once in the urinary bladder, UPEC use a cyclic infection process to persist in that

environment (Figure 2) (6). In the initial step of this cycle, UPEC attach to the superficial epithelial cells of the bladder mucosa. UPEC are then internalized into a phagocytic vacuole within these uroepithelial cells. UPEC can then escape from the vacuole into the cytoplasm and proliferate to form biofilm-like colonies called intracellular bacterial communities (IBC). The IBCs go through a maturation process in which UPEC first replicate into few loosely arranged cells with rod-like morphology. This type of replication occurs during the first few hours after formation of IBCs. Within about 8 hours after IBC formation, the colonies become tightly packed “pods” filled with bacterial cells that have a coccoid shape. By 12 hours after IBC formation, the UPEC cells return to the rod-shaped bacilli morphology, become motile, and leave the IBC. This exit is termed “fluxing”, and these now extracellular bacteria move on to invade other bladder epithelial cells and repeat the cycle. A subset of UPEC can also take on a septated filamentous morphology from which rod-shaped daughter cells can be released. UPEC in IBCs can also become quiescent and may serve as a reservoir for future infection (5; 88; 99; 143). This cycle has been studied in mice, but IBCs have also been seen in human bladders (88; 178; 180). While UPEC are going through this infection cycle, the host attempts to mount a protective response.

HOST DEFENSES IN THE URINARY TRACT

UPEC must overcome several layers of host defenses (Table 1). From their origin in the intestine, UPEC must colonize and ascend the urethra through a region that contains a unique commensal microflora (35; 127). In women, vaginal microflora, predominantly *Lactobacillus spp*, can inhibit uropathogens. Indeed, researchers have found that lactobacilli can produce hydrogen peroxide and lower pH by lactic acid

Figure 2. UPEC Cycle of Infection

Mouse urinary bladders 24 hours following infection with UPEC. Panel A. UPEC enters the urinary bladder and attaches to and invades the superficial urothelial cells (umbrella cells). Panel B. UPEC proliferates forming intracellular bacterial communities (IBC); note the neutrophil nearby (arrow). Panel C. Fluxing, UPEC leaving the IBC into the lumen of the bladder to repeat the cycle. Panel D. Multiple morphologies of UPEC. Densely packed bacilli are present in the IBC, but filamentous UPEC are present in the surrounding lumen of the bladder. Panel E. UPEC may enter a quiescent state and persist in the bladder. Hematoxylin and eosin, Panel B (top) and D. Giemsa, Panel A, B (bottom), C, and E. Magnification 400X.

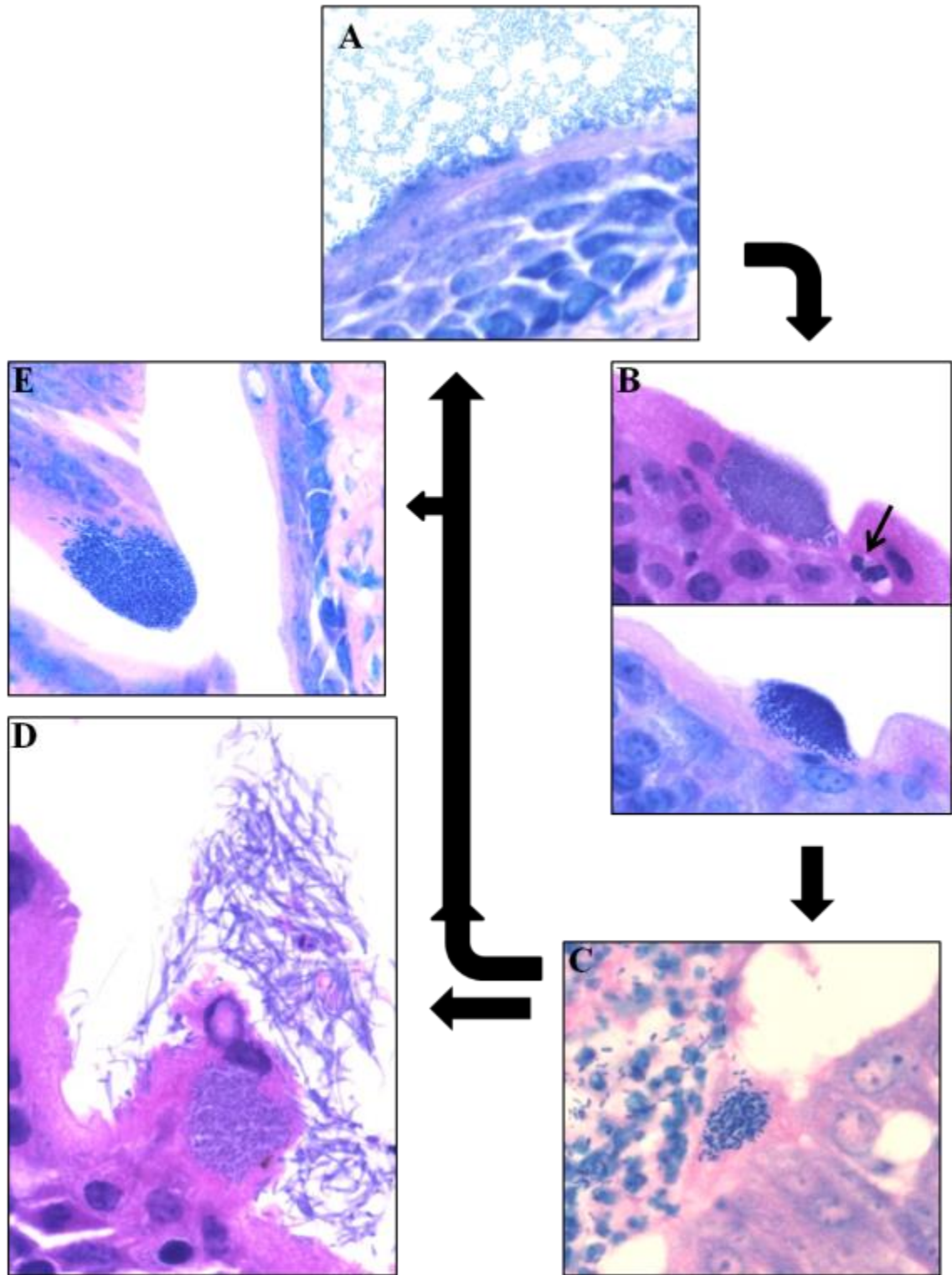


Table 1. Host Defenses Against Uropathogenic *Escherichia coli*

Host defense mechanism	Function	Example/effector	References
Commensal flora of distal urethra and vagina	Create unfavorable conditions for UPEC by H ₂ O ₂ and lactic acid production	<i>Lactobacillus</i>	(98; 126)
Mechanical defenses	Expulsion of UPEC	Urothelial cell apoptosis and exfoliation	(133; 140; 143)
	Anti-adherence factors	Urothelial glycosaminoglycans, Uromodulin	(92; 121; 164-166; 186)
	Flushing action	Urine flow	(144)
Antimicrobial peptides (AMP)	UPEC lysis	Defensins, ribonuclease-7, cathelicidin, lipocalin	(3; 206; 240; 241)
	Iron sequestration	Lactoferrin	(2)
Innate immune response	Initiation of acute inflammation	Cytokine production (IL-1, IL-6, IL-8, IL-17, TNF- α)	(48; 81; 113; 199; 207)
	UPEC phagocytosis	Neutrophils, macrophages, dendritic cells	(49; 50; 79)
	UPEC antigen presentation	Macrophages, dendritic cells	(49; 79)
	UPEC lysis by mediators	Neutrophils, natural killer cells	(78)
Adaptive immune response	Antibody production IgG, IgA, IgM	B Lymphocytes	(86; 87; 103)
	Helper response	T Lymphocytes	(134)

production to create a hostile environment for UPEC (9; 98; 126; 238). Moreover, one strain of *Lactobacillus*, *Lactobacillus rhamnosus* GR-1, can enhance the NF- κ B response through the toll-like receptor 4 (TLR-4) of cultured bladder epithelial when triggered by UPEC. This augmented NF- κ B response results in higher levels of TNF- α than when cells are exposed to UPEC alone (104; 105). This enhancement of the innate immune response could potentially result in a more rapid clearance of UPEC. The flushing action of urine represents another, albeit mechanical, defense against UPEC, as UPEC must overcome this flow to reach the bladder and kidneys (144). Once UPEC reach the bladder, urothelial cells provide yet another level of defense against these organisms. Urothelial cells are coated in a mucous layer that is rich in GAGs (glycosaminoglycans), substances that inhibit the adhesion of UPEC (121; 164-166). Additionally, the urinary tract produces several anti-microbial and anti-infective peptides. Uromodulin, otherwise known as Tamm-Horsfall protein, is produced in the kidneys and is the most abundant protein in human urine. One function of uromodulin is to prevent attachment of UPEC to urothelial cells by binding to adhesive pili (92; 186). An assortment of anti-microbial peptides are produced by urothelial cells and inflammatory cells in the urinary tract that include defensins, cathelicidin, lipocalin, ribonuclease 7, and lactoferrin (3; 206; 240; 241). When urothelial cells become infected, they can undergo apoptosis and detach in an attempt to remove the bacteria (133; 140; 143). If UPEC succeed in infecting the urinary tract, the host's innate immune response will be activated to produce proinflammatory cytokines and an influx of inflammatory cells (189; 190). Such cytokines include IL-1, IL-6, IL-8, IL-17, and TNF- α that together attract and activate neutrophils, natural killer cells, and lymphocytes (78; 81; 113; 134; 199; 207; 222).

Activation of innate immune responses against UPEC results in inflammation.

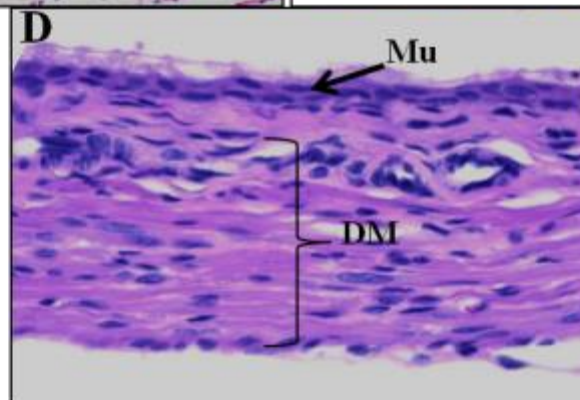
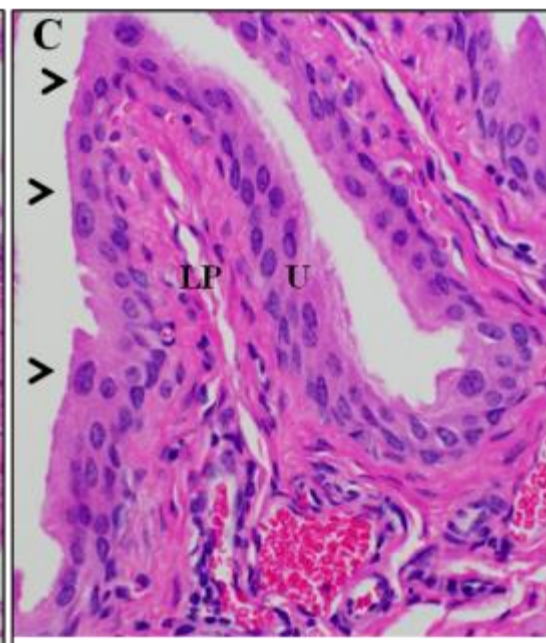
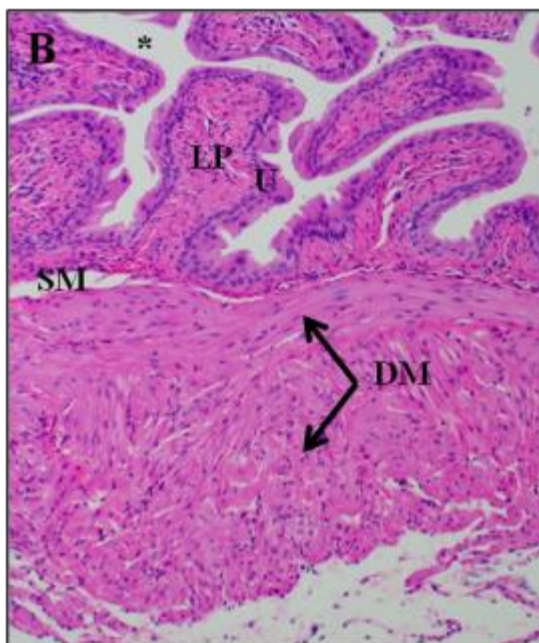
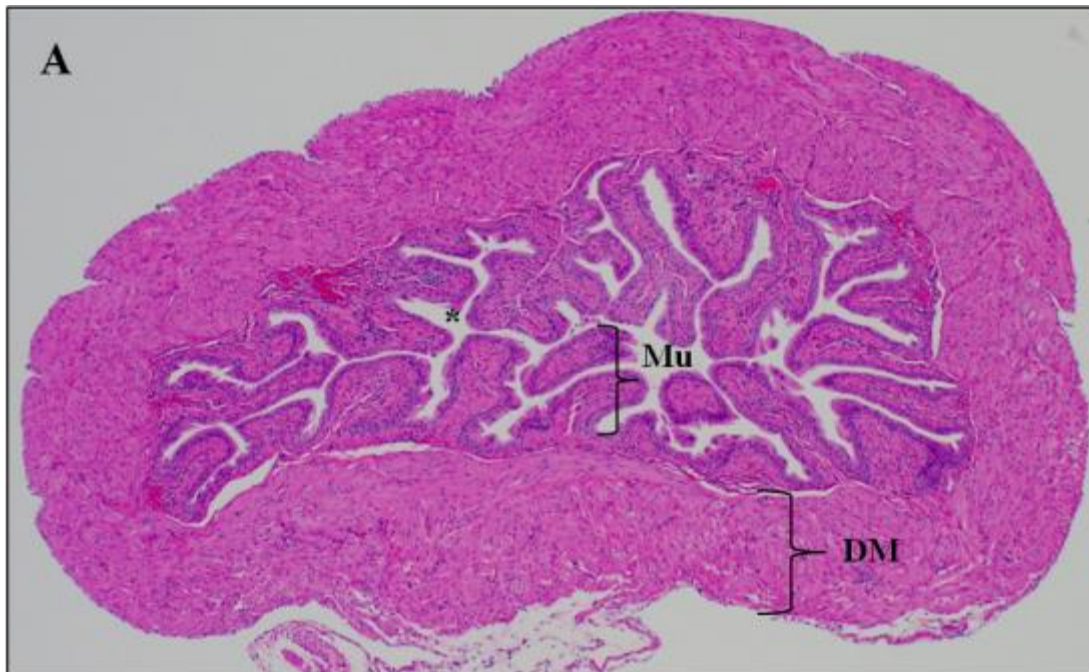
Inflammation in the urinary tract invokes the classic quadrad of *calor*, *dolor*, *rubor*, and *tumor*. The *calor* (fever) and *dolor* (pain) are reflected in the symptoms. The *rubor* (redness and hemorrhage), and *tumor* (swelling) can be seen by histology. Activation of the adaptive immune response against UPEC involves the development of specific IgM, IgG, and IgA antibodies (86; 87). IgG and IgA are present in the urine and act locally to neutralize UPEC (87; 103).

HISTOLOGY OF THE URINARY BLADDER

The histologic architecture of the human and mouse urinary bladder are similar (Figure 3). The bladder is a muscular sac lined by a specialized mucosa. The muscular sac, known as the detrusor muscle, is composed of three layers of smooth muscle that can stretch to accommodate different volumes of urine. The inner lining is the mucosa. The mucosa is composed of 3 or more layers of urothelial cells; these cells were once known as transitional epithelia due to their capacity to change shape from cuboidal to squamous to accommodate bladder expansion. The superficial layer contains large umbrella cells which cover several underlying cells. These cells can stretch and contain apical plaques composed of uroplakin, a substance that makes the bladder impermeable to urine. The lamina propria or submucosa is the layer of loose fibrovascular tissue that contains blood and lymphatic vessels that lies between the muscular layers and mucosa. This simple structure can become remarkably altered by inflammation caused by UPEC infection. In humans, there is a paucity of histopathologic data from acute infections. Biopsy of the urinary bladder during an acute UTI is contraindicated due to the potential for perforation and sepsis. However, data from animal models, especially mice, have revealed the

Figure 3. Histology of the Normal Mouse Urinary Bladder

Panel A. The urinary bladder is composed of smooth muscle called the Detrusor muscle (DM) and a mucosa lining (Mu). The mucosa is highly folded in the relaxed bladder. Urine is stored in the bladder lumen (*). Panel B. The mucosa consists of layers of urothelial cells (U) and the underlying lamina propria (LP) and submucosa (SM). Arrows indicate the layers of the DM. Panel C. The urothelium (U) consists of 2-4 layers of urothelial cells. The most superficial cells, known as umbrella cells (arrowheads), are large with cytoplasm that cover several subjacent urothelial cells. Panel D. The mucosal and muscle layers are stretched thin in the distended bladder. Hematoxylin and eosin. Magnification 40X for A, 200X for B, and 400X for C and D.



pathogenic progression through the inflammatory process during a UTI.

REVIEW OF INFLAMMATION CORRELATED TO CYSTITIS IN MOUSE MODELS OF URINARY TRACT INFECTIONS

During the process of inflammation, there is an acute phase within the first hours to days following UPEC infection which transitions to a chronic phase. A hallmark of acute inflammation is vascular leakage leading to edema (8; 174). One initiator of inflammation in an infected urinary bladder is the interaction of host cell pattern recognition receptors such as toll-like receptor-4 (TLR-4) and the UPEC pathogen-associated molecular patterns such as lipopolysaccharide (LPS). Proinflammatory cytokines and mediators are produced which cause vasodilation and increase vascular leakage and edema. In mice, 24 hours after infection with UPEC, edema forms around the veins in the submucosa and can expand the submucosa more than tenfold. Edema is drained through lymphatic vessels; these vessels are also distended during this phase (Figure 4). The result of fluid loss from the vasculature is to slow blood flow in the area of inflammation in a process called stasis. These changes in the fluid dynamics of the blood flow bring leukocytes from the central flow of the vessel to the vessel wall in contact with endothelial cells in a process called margination (138). Leukocytes in contact with endothelial cells become activated and express adhesion molecules. Endothelial cells express receptors for these adhesion molecules in response to proinflammatory cytokines. The result of expression of such receptors on endothelial cells is attachment of leukocytes to the vessel wall and their ultimate migration through the endothelium into the adjacent tissue matrix in a process called diapedesis (Figure 5). The leukocytes then migrate to the area of inflammation along a trail of chemokines also

Figure 4. Edema of the Submucosa in a Mouse Urinary Bladder

Panel A. Cross section of a mouse urinary bladder with acute cystitis, 24 hours following UPEC infection. Double arrows illustrate the increase in thickness of the submucosa by non-staining edema fluid as compare to the normal mouse bladder in panel B. Panel C. Dilated lymphatic vessel filled with edema fluid (*) in the submucosa which also contains neutrophils (arrow). Hematoxylin and eosin. Magnification 40X for A and B, 200X for C.

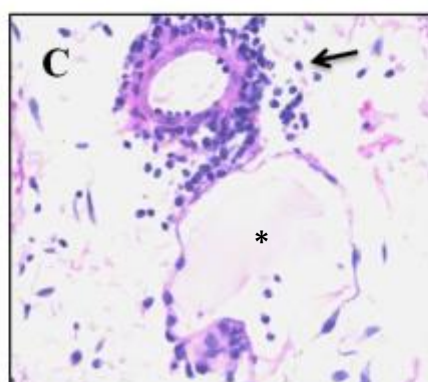
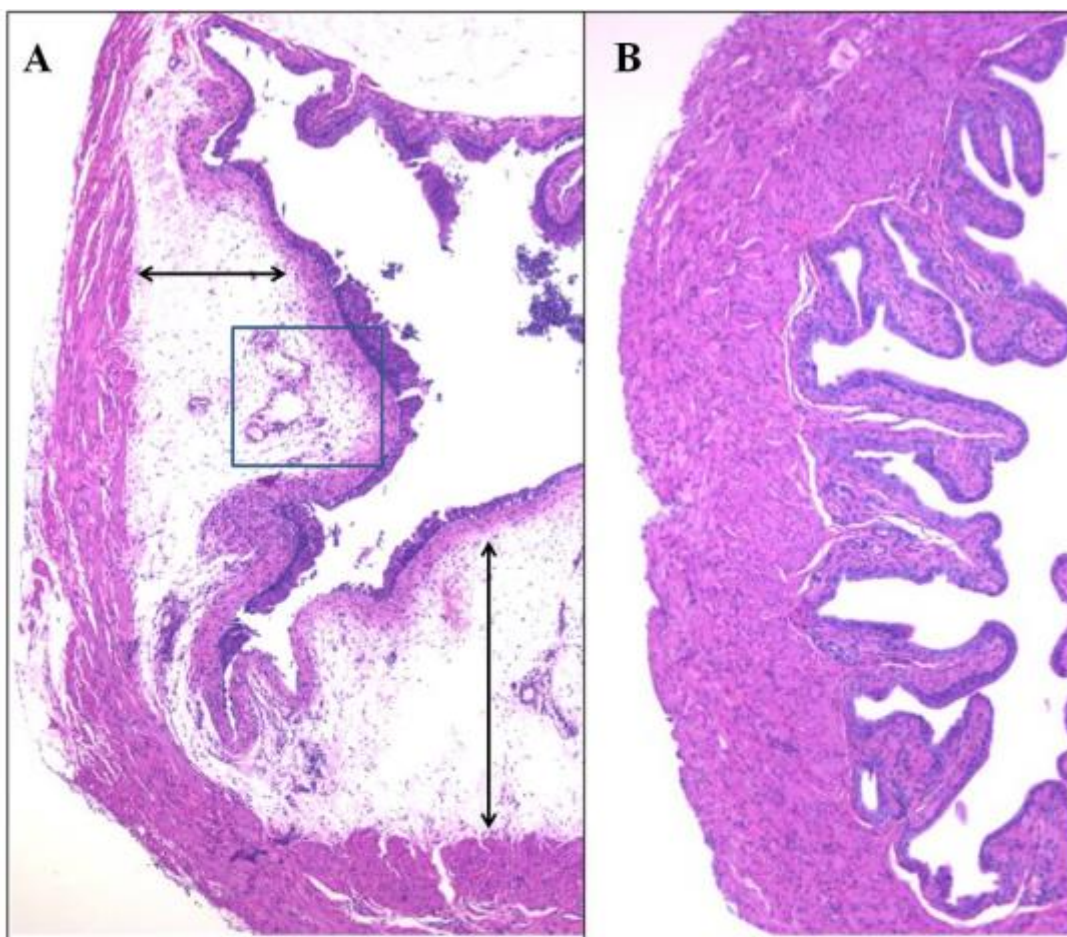
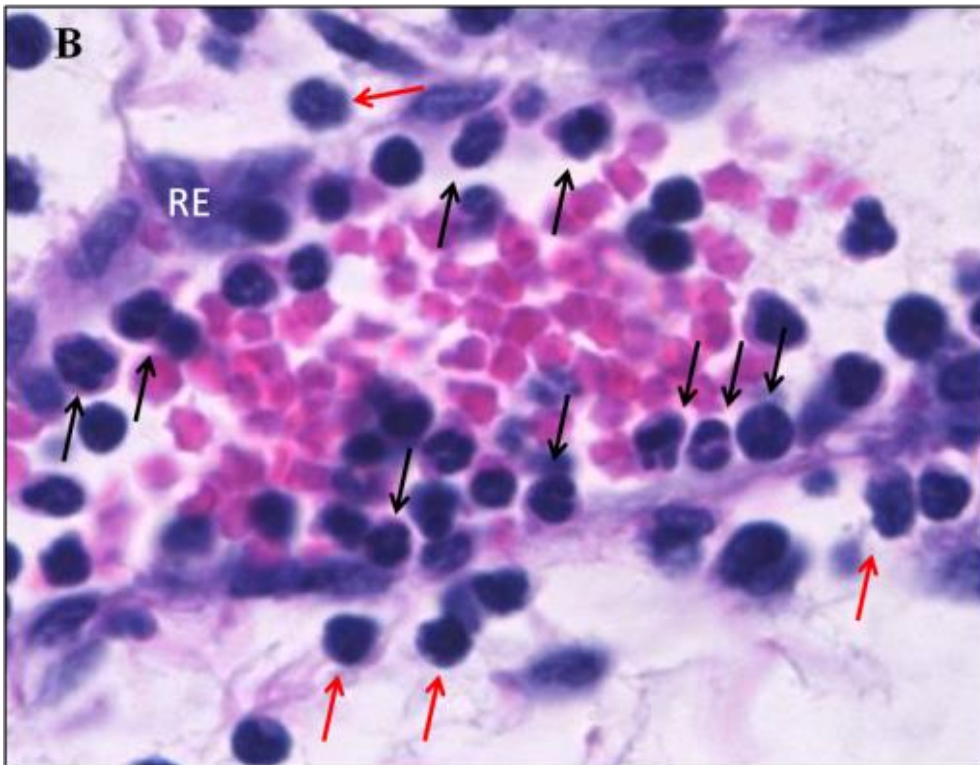
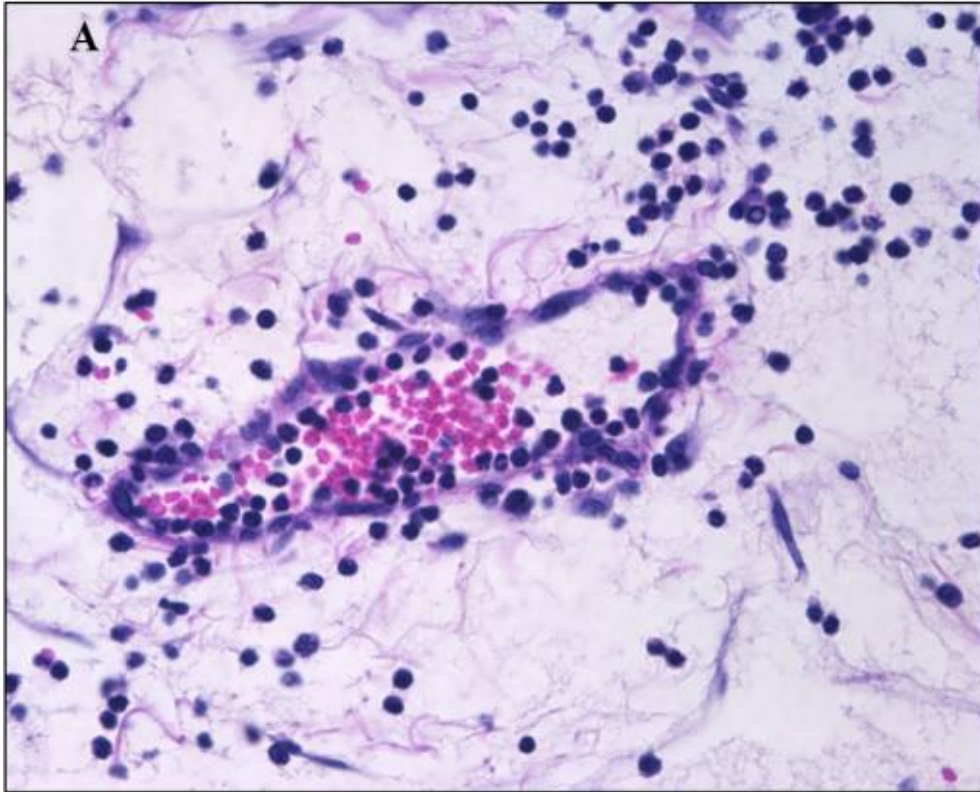


Figure 5. Margination of Neutrophils

Panel A. A vein with marginating neutrophils in the submucosa of a mouse urinary bladder with acute cystitis, 24 hours following UPEC infection. The vein contains erythrocytes and neutrophils at the vessel wall and is surrounded by non-staining edema fluid and extravasated neutrophils. Panel B. Inset of A, black arrows illustrate neutrophils that have adhered to the endothelial lining of the vein. The endothelial cells in the vein are mildly enlarged with increased cytoplasm and large nuclei which is the morphology of reactive endothelium (RE) which is typical in areas of inflammation. The red arrows illustrate neutrophils that have undergone transendothelial migration. Hematoxylin and eosin. Magnification 400X.



produced during inflammation (138). Neutrophils are the first leukocytes to arrive (Figure 6). They migrate into the bladder lumen and are voided in the urine, a phenomenon that results in the clinical sign of pyuria. Neutrophils have an arsenal of antimicrobial compounds stored within cytoplasmic granules. The granules contain preformed proteases, elastases, myeloperoxidase, lysozyme, and NADPH oxidase. Neutrophils can engulf bacteria by phagocytosis and kill them in toxic phagosomes by generation of reactive oxygen and nitrogen species in what is called the respiratory burst. Neutrophils also release granules into the environment; the content of these granules not only kills the microbes but may also cause injury to host tissues in a kind of bystander effect. The combined effect of the host inflammatory response and UPEC virulence factors leads to damage to the cells of the mucosa. In mouse bladders during a UPEC infection, urothelial cells undergo degeneration and necrosis. Dead cells are replaced, a process that results in a compensatory hyperplasia that thickens the mucosa. Degenerating cells become swollen and vacuolated with pale cytoplasm. Necrotic cells are shrunken with hypereosinophilic cytoplasm and have condensed or fragmented nuclei (Figure 7). As the inflammatory process continues for several days, the cellular infiltrate shifts from neutrophils to macrophages and then to lymphocytes and plasma cells as the adaptive immune response begins to mature (Figure 8). If the immune response is successful, and the inciting cause is eliminated, resolution can occur. Inflammatory cells also release anti-inflammatory compounds such as resolvins, protectins, and lipoxin that quell the inflammatory response (7; 195).

Figure 6. Neutrophil Migration

Panel A. Cross section of a mouse urinary bladder with acute cystitis, 24 hours following UPEC infection. Neutrophils (arrow) entering the bladder through veins, inset B. Neutrophils (arrows) migrating through the mucosa between urothelial cells, inset C. Viable and degenerate neutrophils (arrow) accumulated in the lumen of bladder, inset D, the location of the UPEC. Hematoxylin and eosin. Magnification 400X.

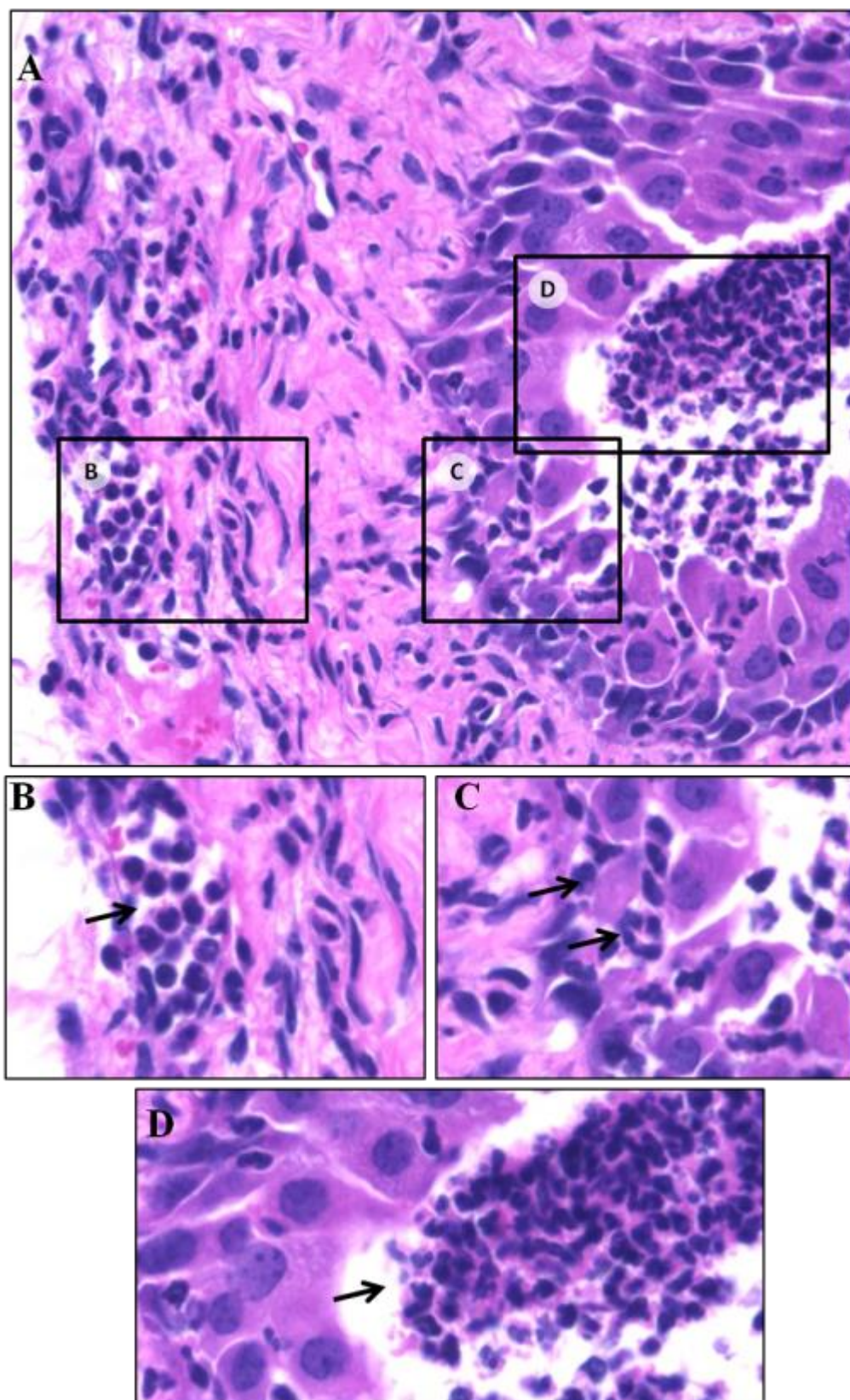


Figure 7. Urothelial Cell Damage

Panel A. Cross section of a mouse urinary bladder with acute cystitis, 24 hours following UPEC infection. The mucosa is thickened and urothelial cells are undergoing degeneration and necrosis. Inset B and C. Necrotic cells are shrunken, hypereosinophilic, and have fragmented nuclei (arrows). Inset D. Degenerating cells are swollen with pale staining cytoplasm and contain robust cytoplasmic vacuoles (arrows). Hematoxylin and eosin. Magnification 400X.

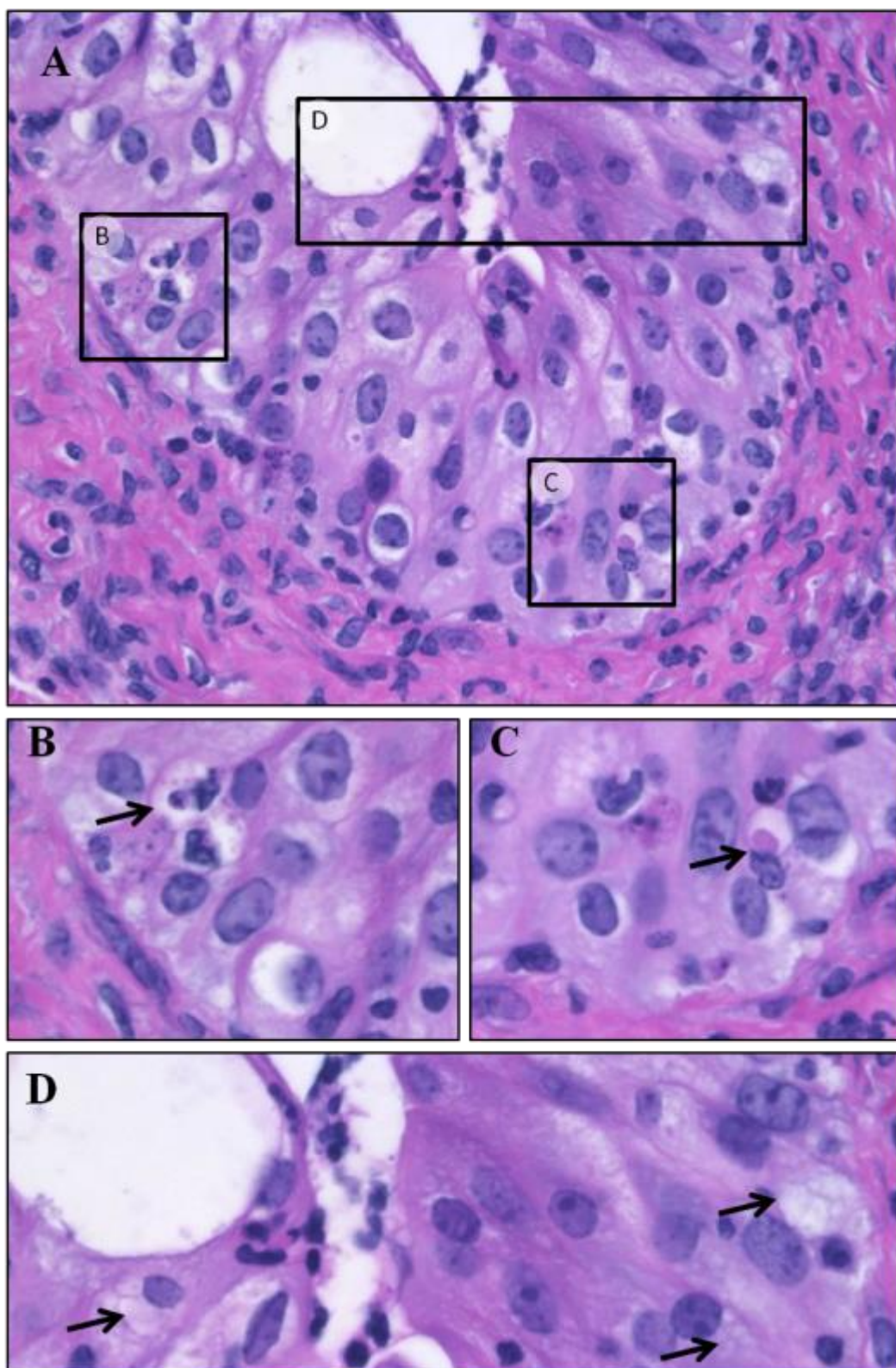
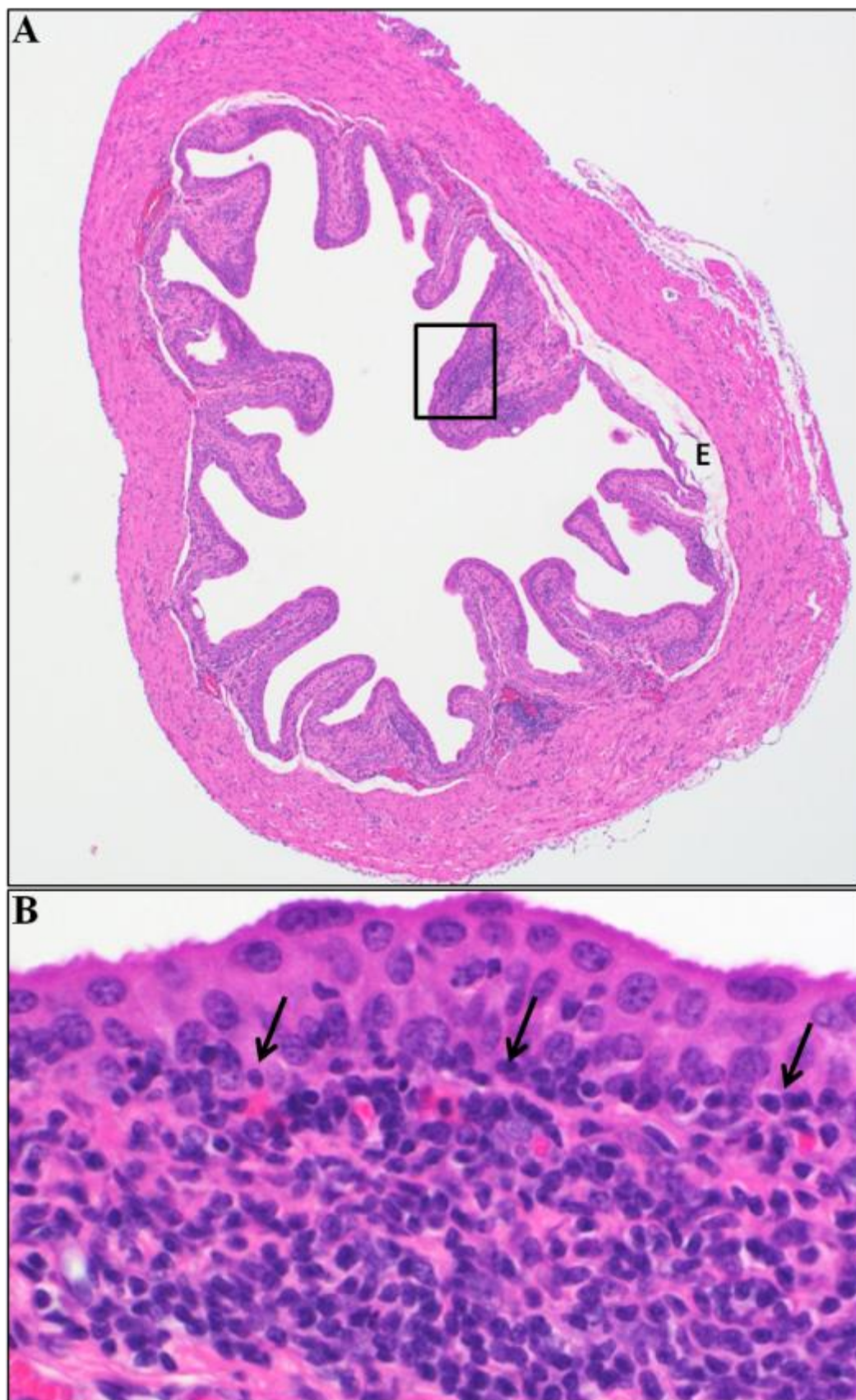


Figure 8. Chronic Cystitis

Panel A. Cross section of a mouse urinary bladder with chronic cystitis, 7 days following UPEC infection. There is mild edema (E) and multifocal aggregates of mononuclear cells (lymphocytes). Inset B. An aggregate of lymphocytes in the submucosa and infiltrating the basal layer of urothelial cells in the mucosa (arrows). Hematoxylin and eosin. Magnification 40X for A and 400X for B.



UPEC VIRULENCE FACTORS

UPEC express an array of virulence factors that enable them to ascend, invade, colonize, and survive in the otherwise inhospitable environment of the urinary tract (Table 2). UPEC flagella enable them to ascend from the urethra to the kidneys against the flow of urine (119). In fact, shearing forces generated by urine flow likely enhance flagellar binding to epithelial cells (215). UPEC possess specialized adhesive structures (pili and fimbria) to attach to the epithelial cells of the urinary tract (139). Once UPEC are attached to uroepithelial cells, the organisms can deliver additional bacterial products to the host cell such as LPS and possibly toxins; these products can, in turn, trigger host responses that lead to intracellular invasion by UPEC and cytokine release by targeted cells (51; 139; 239). In the nutrient poor urinary tract, UPEC utilize secreted factors and associated membrane receptors to scavenge iron from the host (235). UPEC can also use an alternate energy source, short peptides and amino acids, for metabolism (4). Once established in the urinary tract, UPEC deploy strategies to evade and modify the host immune response. UPEC strains invade and hide in epithelial cells, inhibit proinflammatory cytokine production, modify neutrophil function or induce apoptosis, and reduce inflammation (22; 41; 89; 122; 140; 183). Many UPEC also produce toxins such as cytotoxic necrotizing factor 1 (CNF1) and hemolysin (HlyA). The genes for these toxins and other virulence factors are often found on clustered on pathogenicity islands (95). CNF1 and/or HlyA perform a variety of functions from host cell cytoskeletal rearrangement, degeneration, lysis and death, to immune system modulation as described in detail below.

Table 2. Virulence Factors of Uropathogenic *Escherichia coli*

Virulence Factors	Function	Example/effector	References
Flagella	Motility: ascension from distal urethra to bladder and upper urinary tract.	FliC	(119; 215)
Adhesins	Bind to periurethral, bladder, and kidney epithelial cells, erythrocytes, neutrophils, and extracellular matrix components.	Type 1 , P, S, F1c, and Dr Pili	(139)
Capsule	Prevent phagocytosis and complement mediated killing	Various K antigens	(21)
Iron acquisition factors	Appropriate iron from host stores	Siderophores: <i>iro</i> /salmochelin, <i>iut</i> /aerobactin, <i>fyu</i> /yersiniabactin	(235)
Toxins	Cytolysis, immune modulation, inflammation	Hemolysin (HlyA)	(235)
	Cytoskeleton rearrangement, immune modulation, inflammation	Cytotoxic Necrotizing Factor1 (CNF1)	(210)
	Cell degeneration (kidney)	Secreted autotransporter toxin (Sat)	(235)
Evasion of host immune response	Invade bladder epithelial cells	Type-1 pili mediated	(139)
	Reduce inflammatory response	<i>sisA/B</i>	(122)
	Reduce cytokine production	<i>rfa</i> , <i>rfb</i> , <i>surA</i>	(89)
	Modify neutrophil functions	CNF1 mediated	(41)
	Induce neutrophil death	HlyA mediated	(33)

CYTOTOXIC NECROTIZING FACTOR FAMILY

History of Cytotoxic Necrotizing Factor

Cytotoxic Necrotizing Factor (CNF) represents a family of toxins (112). CNF1, CNF2, and CNF3 are produced by *E. coli* and CNF_Y is produced by *Yersinia pseudotuberculosis* (112; 123; 210). The first toxin in this family to be discovered was CNF1 in 1983.

CNF1 was isolated from pathogenic *E. coli* strains obtained from children with enteritis; this toxin caused cytomegaly and multinucleation in HeLa, CHO, and Vero cells and was cytotoxic to HeLa cells (30). CNF1 also causes necrosis when injected into the skin of rabbits and guinea pigs and also systemic hemorrhages and death in intoxicated guinea pigs (29; 30). In 1990, a toxin with similar effects, CNF2, was discovered in *E. coli* strains isolated from calves with diarrhea and a lamb with septicemia (42). CNF2 shares 85% homology with CNF1; CNF2 also has the same catalytic site and similar host cell targets as does CNF1 (112). In 2002, a toxin that could evoke multinucleation in cells was isolated from *Y. pseudotuberculosis* and called CNF_Y. CNF_Y shares the catalytic site and 61% homology with CNF1 (112; 123). Recently, CNF3 was isolated from strains of necrotoxicogenic *E. coli* of sheep and goats (161). CNF3 has the same catalytic site and shares 70% homology with CNF1 (112; 210).

Cytotoxic Necrotizing Factor mode of action

The CNF toxins act by deamidation of small Rho family GTPases (RhoA, Rac1, Cdc42) (61; 68; 192). The activity of CNF_Y is limited to RhoA (82). All share a conserved catalytic domain composed of a histidine, cysteine, and a valine. The

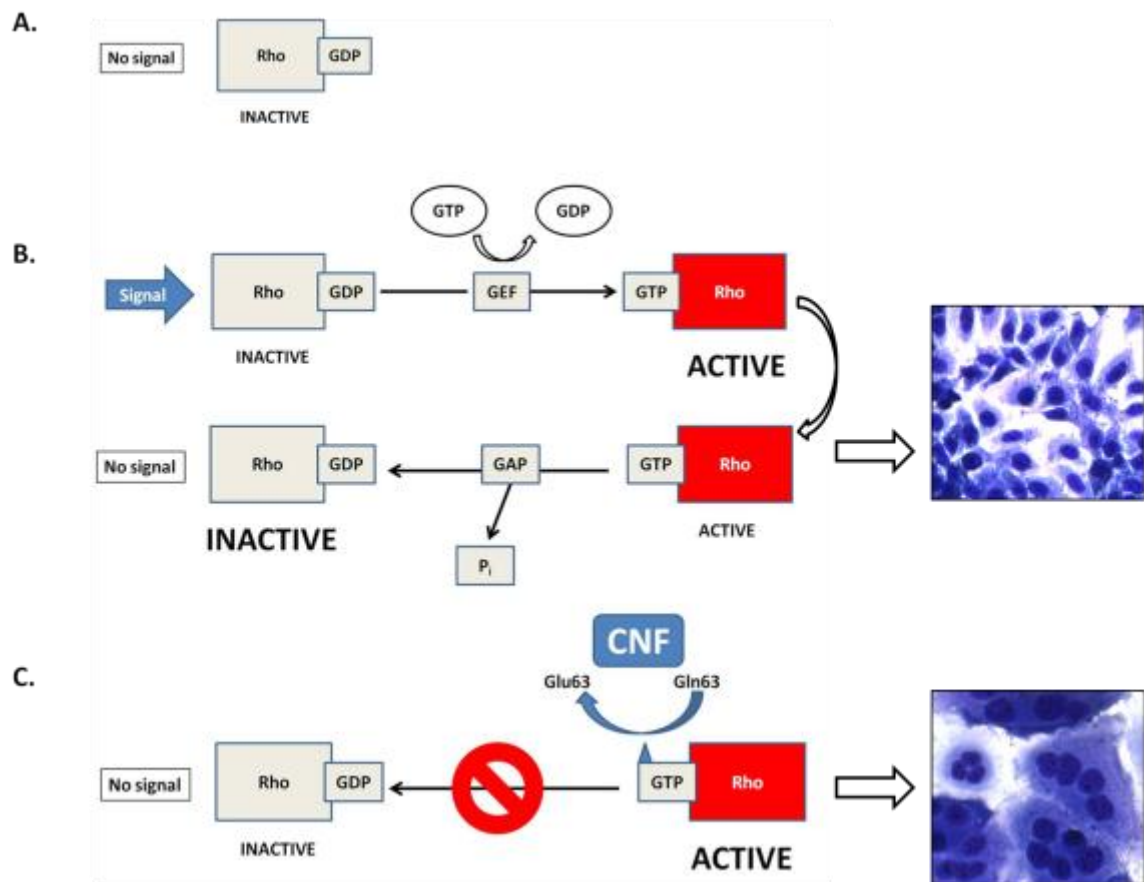
enzymatic function of these toxins is to deamidate a glutamine residue of the GTPase (glutamine at position 63 on RhoA or 61 on Rac1 and Cdc42) (82; 112).

Rho family GTPases act as molecular switches in signal transduction pathways that involve the components of the cytoskeleton, such as actin, myosin, and microtubules (223). Consequently, Rho family GTPases are involved with cell shape, motility, adhesion complexes, endocytosis, cell cycle progression, vesicle trafficking, apoptosis, and transcription (112; 223). Deamidation results in constitutive activation of the GTPase with subsequent downstream derangement of the cellular process listed immediately above (Figure 9). The ultimate phenotype in a cell depends on the cell type and which GTPase is affected. Cell culture intoxication of HEp-2 cells with CNF1 results in actin stress fiber formation, formation of lamellipodia and filopodia, and multinucleation; CNF1 also induces apoptosis in urothelial cells (31; 59; 133).

The CNFs are all 110-115 kDa cytoplasmic proteins. CNF1, CNF3, and CNF_Y are chromosomally-encoded (30; 52; 123). CNF2 is encoded on a transmissible plasmid (162). The toxins are comprised of three domains. The N-terminus contains a receptor binding domain. The central region contains a translocation domain, and the C-terminus contains the catalytic domain (25; 112). CNF1 is delivered to host cells by secretion into outer membrane vesicles (40). However, the mechanism for translocation of CNF to the outer membrane has yet to be elucidated. The receptor for CNF1 and CNF2 is the laminin receptor precursor protein (130). Another receptor was recently reported for CNF1, the Lutheran (Lu) adhesion glycoprotein/basal cell adhesion molecule (BCAM) (170). CNFs are internalized into host cells by receptor-mediated endocytosis (38). The translocation domain inserts into the endosome membrane, and the catalytic domain is

Figure 9. Cytotoxic Necrotizing Factor 1 Mode of Action

A. In the absence of a signal, Rho bound to guanosine diphosphate (GDP) is inactive. B. When stimulated by a signal, guanine exchange factors (GEF) phosphorylate GDP to guanosine triphosphate (GTP), with subsequent activation of Rho. Rho-GTP is then hydrolysed back to inactive Rho-GDP by GTPase activating proteins (GAP). The image shows HEp-2 cells cultured in standard conditions with normal morphology. C. In cells intoxicated by CNF family toxins, the deamidation of glutamine 63 to glutamate blocks the return to the inactive Rho-GDP state leaving the system constitutively active. The image depicts the downstream effect on HEp-2 cells intoxicated with CNF1. The cells are enlarged with multiple enlarged nuclei. Hema-3, Magnification 400X.



cleaved and released into the cytoplasm (112). CNF1 is the best characterized of the toxin family. Although discovered in *E. coli* strains isolated from children with enteritis, CNF1 is more commonly associated with extraintestinal *E. coli* infections such as meningitis, septicemia, and UTIs (25). Surveys of virulence factors in UPEC isolates revealed that *cnf1* is present in 30 to 45 percent of strains (43; 118). Another toxin, hemolysin, is expressed in 60% of UPEC strains (196). Also, nearly 40% of UPEC strains express both CNF1 and hemolysin (118).

HEMOLYSIN AND RTX TOXINS

Hemolysin discovery

Over a century ago, bacteriologists reported the existence of colonic bacilli, then called *Bacillus coli*, that could cause the destruction of red blood cells (hemolysis) (107). Substances that caused such hemolysis would thereafter be called hemolysins. In the 1920s, medical microbiologists noted that isolation of hemolytic strains of *Bacillus coli* was higher from the urine of patients with UTIs (38%) than from feces (11%) (14; 46; 47). By the 1960s, *Bacillus coli* had been renamed *Escherichia coli* and researchers had determined the optimum growth media and culture conditions for these strains to reliably produce hemolysins (201). Through the application of serologic techniques, researchers discovered different types of hemolysins (201; 225). Alpha-hemolysin was the name given to the hemolysin present in culture supernatants or the cell-free secreted hemolysin of *E. coli* (201).

Hemolysin genetics and the RTX family of toxins

In the late 1970s through the 1990s, investigators identified and characterized the genes responsible for hemolysin production and export from the bacterial cells as well as

the general mechanism by which hemolysin disrupted red cells. The genetic determinants for hemolysin are found on both transmissible plasmids and chromosomes (156). However, hemolysin determinants for ExPEC and UPEC are predominantly chromosomal (71; 111). Several investigators determined that multiple genes are necessary for production and secretion of hemolysin. Two genes are required for production of active hemolysin, *hlyA* and *hlyC* (71; 146). The *hlyA* gene encodes a 107 kDa heat-labile inactive hemolysin precursor or protoxin. The protoxin is activated by the action of *hlyC* gene product. In 1994, HlyC was determined to be an acyltransferase that activates hemolysin by fatty acid acylation of two lysine residues at positions 564 and 690 of HlyA (209). Fatty acid acylation is not required for secretion but is necessary for toxin interaction with host cells (208). Two other genes, *hlyB* and *hlyD*, are required for secretion (80; 156; 224). Both genes encode inner membrane proteins. HlyB is an ATP-binding cassette protein that interacts with HlyD, a membrane fusion protein (67; 114). The HlyBD complex interacts with TolC, an outer membrane transport protein that is not specific to hemolysin export (67; 187). This method of secretion is now designated as the type-I secretion system. Sequencing of the hemolysin genes revealed that they are arranged sequentially within a single operon in the order *hlyC*, *hlyA*, *hlyB*, *hlyD* (56; 230). Sequencing also revealed the presence of tandem repeating DNA sequences that code for glycine and aspartate-rich nonapeptides in the C-terminus of HlyA (57). Researchers noted similar conserved homologous tandem repeats, operon organization, and type I secretion systems in other toxins associated with Gram-negative bacteria, including the leukotoxin of *Pasteurella haemolytica* and the adenylate cyclase/hemolysin of *Bordetella pertussis* (117; 228; 236). This latter discovery formed the basis of a new

classification of bacterial toxins termed the RTX or repeats-in-toxin family, for which hemolysin is the prototype (211; 236).

Hemolysin mode of action

Many RTX toxins have a similar mode of action i.e., pore formation with subsequent disruption of host cell membranes. The result of host membrane disruption is often cell lysis or cell death by apoptosis or necrosis (229; 236). Pore-forming and cytolytic effects of hemolysin have been demonstrated in a variety of mammalian cell types (32-34; 97). Pore formation requires a relatively high, i.e., lytic, concentration of HlyA, and the toxin must bind calcium (23; 24). Pore formation by hemolysin was demonstrated by cell and membrane permeability experiments, but neither the exact mechanism of pore formation nor the crystal structure of hemolysin have been fully determined (19; 20; 235). One proposed model for how HlyA functions is as follows: calcium binds to the repeats in the C-terminal domain of HlyA, which, in turn results in conformational changes in the protein that facilitate its adsorption to host membranes (236). The N-terminal domain of HlyA, which is composed of amphipathic α -helices, then inserts into the host cell membrane. The acyl groups in the middle of the hemolysin molecule promote oligomerization with other HlyA molecules. The oligomers then form a pore in the membrane (117; 236). Several studies have found pathogenic effects of HlyA at sublytic concentrations (236). These effects include modulation of host cell signaling pathways, modification of the immune response, and cell death. Many of these cellular responses are similar to those evoked by CNF1 (235).

COMPARISON OF PATHOLOGIC EFFECTS OF HLYA AND CNF1

The mode of action of CNF1 directly affects cell signaling, but sublytic levels of HlyA also cause alterations in these pathways. HlyA can activate MAP kinase signaling indirectly by causing loss of cytosolic potassium (109). Another study demonstrated that HlyA can deactivate the Akt signaling pathway (234). The downstream effects of the Akt pathway involve cell cycle progression, inflammatory responses, and cell survival by inhibition of apoptosis (235). CNF1 positively modulates the Akt pathway to prevent apoptosis and also affects the cell cycle (53; 69; 135). Several studies have shown that CNF1 can either prevent or cause apoptosis, while studies with HlyA generally indicate that it causes apoptosis (60; 90; 91; 110; 133; 135; 183; 234). Like CNF1, HlyA also affects the cytoskeleton. HlyA causes degradation of paxillin, a cytoskeletal component. Degradation of paxillin, in turn, leads to altered cell functions (44). Both CNF1 and HlyA are associated with immune system modulation. Both toxins increase proinflammatory cytokine production such as IL-1 β , IL-6, IL-8, IFN- γ and TNF- α by CNF1 and IL-1b, IL-6, IL-8, and TNF- α by HlyA (54; 70; 221; 235). Both toxins are also associated with increased production of reactive oxygen species in human neutrophils (12; 41; 76; 83). CNF1 increases superoxide anion production in epithelial cells (55). HlyA increases production of proinflammatory lipid mediators, such as leukotriene B₄ (75). CNF1 inhibits neutrophil phagocytosis (41). Another immunomodulatory effect is on natural killer cells; HlyA kills them and CNF1 enhances their function (78; 125). Additionally, both toxins are delivered to host cells on outer membrane vesicles (10; 40). Most of the conclusions cited above about the characteristics of CNF1 and HlyA were derived from studies done with in vitro models.

However, mouse and rat models have also been used to demonstrate a role in inflammation for CNF1 and HlyA.

CNF1 AND HLYA INCREASE INFLAMMATION IN RODENT URINARY TRACT INFECTION MODELS

Mice experimentally infected with UPEC develop cystitis and pyelonephritis as described above. Mice which had purified rCNF1 inoculated into their urinary bladders also develop cystitis (66). Moreover, mice infected with isogenic mutants of UPEC strains that lack *cnf1*, *hlyA*, or both genes exhibit significantly less severe cystitis than mice infected with wild-type UPEC (173; 204). Rats experimentally infected with an isogenic UPEC strain that lacks *cnf1* develop less severe prostatitis (172). In these studies, there was no significant difference in colonization between wild-type UPEC and the isogenic mutants. These experiments demonstrated that CNF1 can directly cause inflammation of the bladder and that the lack of CNF1 or HlyA production in a UPEC strain attenuates cystitis. Given that more than half of all UPEC strains produce HlyA and a third produce CNF1 (most of which also produce HlyA), these toxins may be a good targets for therapeutic intervention (43; 95; 118; 128).

CNF1 AND HLYA AS VACCINE CANDIDATES

The front line treatment for bacterial UTIs is antibiotics (226). Antibiotic resistance is a growing problem. More than 10% of UTIs strains have become multidrug resistant (64; 191). In addition, treatment with antibiotics can destroy commensal populations that normally protect the urinary tract (171). Vaccination against UPEC is recognized as an appealing alternative to antibiotic treatment (103). There have been dozens of UTI vaccine candidates, including some multistrain whole cell vaccines that

have progressed to clinical trials, but UTI research has not yielded an approved candidate (27; 198). Based on the studies on CNF1 and HlyA in rodent models, an antibody response directed against either CNF1 or HlyA toxins could in principle neutralize the available toxin and potentially reduce the severity of UPEC-associated cystitis. In addition, an antibody response directed against virulence factors should not interfere with the functions of commensal *E. coli* strains. Previous studies with an anti-HlyA vaccines using mouse models demonstrated reduced mortality in a sepsis model and decreased severity of pyelonephritis but no evaluation was done of the urinary bladder to examine cystitis (137; 159). CNF1 has not been evaluated as a vaccine candidate. I propose the following hypothesis and specific aims.

HYPOTHESIS

A neutralizing antibody response generated against CNF1 or HlyA will reduce inflammation caused by UPEC infection.

SPECIFIC AIMS

Aim 1. Develop immunogens for both CNF1 and HlyA toxins.

SubAim 1a

Create a toxoid of HlyA by site directed mutagenesis (SDM) that targets the two lysine residues (K564 and K680) required for activation of HlyA from the inactive pro-HlyA. Purify the CNF1 toxoid created by Russo and O'Brien (unpublished data) through SDM that changed the active site cysteine residue (C866) residue, required for GTPase deamidation, to a serine.

SubAim 1b

Determine sublethal doses of active toxins and whether active toxins are necessary for use as vaccine boosts in C3H/HeOuJ mice to enhance the generation of a neutralizing antibody response.

Aim 2. Vaccinate mice with immunogens, challenge with UPEC, and evaluate response.

Immunize C3H/HeOuJ mice with the CNF1 or HlyA toxoids generated in Aim1. Evaluate the antibody response of vaccinated mice for specific antibodies against the toxins by enzyme-linked immunosorbent assay (ELISA) and for the capacity of these antibodies to neutralize the toxins using in vitro assays.

Determine vaccine efficacy at reducing inflammation in the bladder at 24 hours post infection. Process urinary bladders and kidneys for histopathology and bacterial colonization. Assess the urinalysis parameters of hematuria, pyuria, and bacteriuria.

CHAPTER 2: Materials and Methods

BACTERIAL STRAINS, PLASMIDS, AND MEDIA

The strains and plasmids used in this study are listed in Table 3. CP9 (O4:H5:K54), our wild-type UPEC strain, was isolated from the blood of a patient with bacteremia of an unknown source at the National Institutes of Health in the 1980s (184). CP9 encodes the genes required to produce type I pili and CNF1, as well as at least three hemolysin operons. [Note: HlyA1 is responsible for >90% of the hemolytic activity of CP9 ((204); Weingarten, et al., manuscript in preparation).] (184). Our laboratory previously generated isogenic CP9 strains in which *cnf1* is partially deleted (CP9 *cnf1*) or *hlyA1* is completely deleted (KG68, hereafter referred to as CP9 Δ *hlyA*) (1; 173). In addition, each gene (*cnf1*, *hlyA1*, *hlyA2*, and *hlyA3*) was cloned into pQE-30 for complementation and activity studies and for protein expression and purification (173; 204). The plasmid pQE-30 confers ampicillin resistance, adds an RGS-6X-histidine tag to the amino-terminus of the expressed protein, and contains an isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible T5 promoter. For HlyA activity assays, each of the pQE-30 vectors that contains either a *hlyA3* (pKG76, hereafter referred to as *phlyA*) or a *hlyA3* K564E K690E (pSDMB, hereafter referred to as *phlyA* K564E K690E, see below) was electroporated into CP9 Δ *hlyA*. For protein purification, the plasmids were transformed into chemically competent *E. coli* M15pREP4 cells. All bacterial cultures were started from frozen stocks and grown in Luria Bertani (LB) broth or on LB agar. Media for strains that contained pQE-30 vectors were supplemented with 100 μ g/ml ampicillin (amp). Media for all M15pREP4 strains were supplemented with 25 μ g/ml kanamycin (kan) to maintain pREP4 and 100 μ g/ml amp to maintain pQE-30. Liquid cultures were inoculated from fresh colonies grown on LB agar and cultivated at 37°C

Table 3. Bacterial strains and plasmids

Strains and Plasmids	Relevant genotype or purpose	Reference
UPEC Strains		
CP9	Uropathogenic <i>E. coli</i> (O4:H5:K54) <i>hlyA₁, hlyA₂, hlyA₃, cnf₁</i>	(184)
KG68	CP9Δ <i>hlyA₁::cat, hlyA₂, hlyA₃, cnf₁</i>	(203)
KG68::pKG76	CP9Δ <i>hlyA₁::cat, hlyA₂, hlyA₃, cnf₁</i> , pKG76	This study
KG68::pSDMB	CP9Δ <i>hlyA₁::cat, hlyA₂, hlyA₃, cnf₁</i> , pSDMB	This study
<i>E. coli</i> Cloning and Expression Strains		
XL10-Gold	Tet ^R Δ(<i>mcrA</i>)183 Δ(<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i> Hte [F' <i>proAB lacI^q ZAM15 Tn10</i> (Tet ^R) Amy Cam ^R]	Stratagene
M15pREP4	NaI ^s Str ^s Rif ^s Δ <i>lac-ara-gal-mtl</i> F ⁻ <i>recA uvr</i> (pREP4 <i>lacI</i> Kan ^r)	Qiagen
M15pREP4 pCNF24	CNF1 expression strain	(130)
M15pREP4 pCNF24C866S	CNF1 toxoid expression strain	
KG76	M15 pREP4 pKG76	This study
M15pREP4 pSDMB	Production of Hemolysin A toxoid	This study
Plasmids		
pQE-30	Amp ^r , <i>E. coli</i> expression vector with N-terminal RGS-6X His	Qiagen
pCNF24	pQE-30:: <i>BamHIcnf1KpnI</i>	(132)
pCNF24C866S	pQE-30:: <i>BamHIcnf1KpnI</i> C866S (CNF1 toxoid)	This study
pKG76	pQE-30:: <i>BamHIhlyA₃KpnI</i>	This study
pSDMB	pQE-30:: <i>BamHIhlyA₃KpnI</i> K564E, K690E (HlyA toxoid)	This study

with aeration. For CP9 challenge inocula, liquid cultures were grown without aeration at 37°C for 48 hr. After 48 hr, the CP9 cultures were harvested by centrifugation (10,000 x g for 10 minutes at 4°C), then resuspended in PBS at a concentration of 10⁹ CFU/ml.

TOXOID GENERATION

The plasmid *phlyA* was isolated from KG76 with the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's protocol. The Quick Change Lightning Multi[®] site-directed mutagenesis (SDM) kit (Stratagene, La Jolla, CA), which uses mutagenic oligonucleotide primers to generate mutations by PCR amplification, was used to generate four mutations in *hlyA₃* in *phlyA*. The targets for site-directed mutagenesis were two lysine residues (K564 and K690) that are acylated by HlyC to activate the HlyA protoxin (209). Both were changed to glutamic acid in consecutive reactions using the following mutagenic primers (Integrated DNA Technologies, Coralville, IA): the K564E primer starts at nucleotide 1669 in the *hlyA₃* sequence (Figure 13) (5'-CGTGAAAGGAGGCAGTCCGGAG**GAG**TAT GAATATATTACCGAGTTAT-3'), and the K690E primer starts at nucleotide 2047 in the *hlyA₃* sequence (5'-GAGCAGGAGGTTTCAGTCGGAG**GAG**AGAACTGAAAAACGCAA-3'). The mutations are in bold and underlined.

In brief, long PCR was done with *phlyA* as the template, the two mutagenic primers, an enzyme blend that includes *Pfu* Fusion[®] DNA polymerase, buffer, and dNTP mix. The mix was cycled in a thermal cycler as follows: 95°C for 2 minutes; 30 cycles of 95°C for 20 seconds, 55°C for 30 seconds, 65°C for 3 minutes and 21 seconds; and a final 65°C extension for 5 minutes. The parental DNA was digested with *DpnI*. The resultant mutated single stranded DNA was chemically transformed into *E. coli* XL-10 competent

cells, and cells were plated on LB/amp after a 1 hr recovery period in the absence of antibiotic. Amp-resistant transformants were screened for the appropriate mutations by sequencing the target sites. Once the K564E and K690E mutations were confirmed, the entire mutated *hlyA₃* gene was sequenced to ensure that no additional mutations were incorporated. The sequences were compiled and analyzed in Clone Manager v9.0 (Scientific and Educational Software, Cary, NC). Once the compiled sequence was confirmed to be correct, the plasmid was renamed pSDMB. For purification of the HlyA₃ toxoid (hereafter referred to as HlyA toxoid), *phlyA* K564E K690E was transformed into chemically competent *E. coli* M15pREP4 cells, and one amp-resistant colony was selected for further study (see “Toxoid Expression and Purification” below). To confirm that the HlyA toxoid was inactive, the plasmids *phlyA* K564E K690E and *phlyA* were each transformed into electrocompetent CP9Δ*hlyA*, and amp-resistant colonies were selected for further analysis (see “Hemolysis Activity” below). The CNF1 toxoid was previously generated in our laboratory by SDM of pCNF24 (pQE-30::*cnfI*) to change the cysteine 866 (a catalytic site residue) to serine. The resultant toxoid pCNF24 C866S was transformed into *E. coli* M15pREP4 for expression as described above.

TOXOID EXPRESSION AND PURIFICATION

HlyA toxoid purification under denaturing conditions

An overnight culture of *E. coli* M15pREP4 (*phlyA* K564E K690E) was diluted 1:50 into 500 ml fresh LB/amp/kan, and grown at 30°C for 2 hr with aeration. IPTG was added to a final concentration of 1 mM, and the culture was grown at 25°C overnight with aeration. The culture was pelleted by centrifugation at 8000 x g for 15 minutes at 4°C. The cells were resuspended and lysed by sonication in Loading Buffer [20 mM

phosphate buffer, pH 8.0 (1.8 ml 1 M dibasic Na₂HPO₄, 0.2 ml 1 M monobasic NaH₂PO₄ per liter), 1 mM phenylmethanesulfonylfluoride (PMSF), 8 M urea]. The insoluble portion of the lysate was pelleted by centrifugation at 15,000 x g for 15 minutes at 4°C. The HlyA toxoid was purified by nickel affinity chromatography at room temperature with a 5 ml nickel sepharose column (HisTrap HP, GE Life Sciences). The column was equilibrated with Loading Buffer, and then the filtered lysate was applied to the column. The column was washed twice, first with 20 mM phosphate buffer that contained 6 M urea and 25 mM imidazole, then with 20 mM phosphate buffer that contained 4 M urea and 50 mM imidazole. The toxoid was eluted with 20 mM phosphate buffer that contained 4 M urea and 500 mM imidazole. The eluate was dialyzed against 20 mM phosphate buffer that contained 4 M urea with a 10,000 NMWL Slide-A-Lyser dialysis cassette (Thermo Scientific), then aliquoted and stored at -80°C. The concentration of the toxoid was determined by BCA (bicinchoninic acid) assay (Thermo Scientific) according to the manufacturer's protocol.

CNF1 toxin and CNF1 toxoid purification under native conditions

CNF1 from *E. coli* M15pREP4 pCNF24 and CNF1 toxoid from *E. coli* M15pREP4 pCNF24 C866S were expressed and purified according to previously described procedures with the following modifications (72; 133). Protein expression was induced at 30°C instead of 37°C. In the nickel affinity chromatography, the proteins were eluted with a phosphate buffer (20 mM phosphate buffer, pH 7.4, 0.5 M NaCl, 1 mM PMSF) that contained 50 mM imidazole instead of 250 mM imidazole.

Evaluation of purified proteins

The purified proteins (CNF1 toxin, CNF1 and HlyA toxoids) were evaluated for purity by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. Samples of each protein preparation were denatured in Laemmli sample buffer and separated by denaturing SDS-7.5% PAGE (Bio-Rad). Proteins were either stained with Oriole fluorescent gel stain (Bio-Rad) for 30 minutes at room temperature or transferred to nitrocellulose membranes with the iBlot[®] transfer system (Invitrogen). The membranes were blocked in blocking buffer [5% skim milk in phosphate buffered saline (PBS)] for 3 hr at room temperature, then incubated overnight at 4°C with the primary antibody, mouse anti-RGS-His (Qiagen, lot #34610), diluted 1:2000 in the blocking buffer. The membranes were washed with 0.05% Tween 20 in PBS (PBST), then incubated for 1 hr at room temperature with goat anti-mouse IgG-Alexa Fluor[®] 532 (Invitrogen) diluted 1:15,000 in blocking buffer. Both the Oriole-stained gel and the Western Blot were imaged with the Imagequant[™] LAS 4000 imager (GE Life Sciences).

EVALUATION OF HLYA TOXOID ACTIVITY

Hemolysis assay

Overnight cultures of CP9 Δ *hlyA*(*phlyA* K564E K690E) and CP9 Δ *hlyA*(*phlyA*) were diluted 1:50 into fresh LB/amp and grown at 37°C for 4 hr with aeration. The bacteria were pelleted by centrifugation at 16,100 x g for 5 minutes, and the supernatant was collected. Equal volumes of each supernatant and a 10% sheep erythrocyte solution [10% washed sheep erythrocytes (Lampire Biological Laboratories, Pipersville, PA), 80% LB broth, and 10% Ca-saline (0.9% NaCl, 10 mM CaCl₂)] were incubated for 1 hr at 37°C. Water and culture supernatants from CP9 and CP9 Δ *hlyA*(*phlyA*) served as

positive controls. Ca-saline and LB served as negative controls. Intact erythrocytes were pelleted at 5000 x g for 5 minutes. The supernatants were transferred into a 96-well microtiter plate and, the intensity of the red color, imparted by hemoglobin from lysed erythrocytes, was determined by measurement of absorbance at 562 nm with a Biotek ELx800 microplate spectrophotometer (Biotek, Winooski, VT). The final absorbance values were determined by subtracting the averaged values of the negative control samples (LB and Ca-saline) from each sample absorbance. The values were plotted on a bar graph, and the relative hemolytic activity of the isogenic CP9 strains was compared to that of the wild type.

Hemolysin neutralization assay

CP9 culture supernatants were used as the source of hemolysin in this assay, which measures the neutralizing capacity of antisera from mice immunized with HlyA toxoid. CP9 cultures were grown, and supernatants were harvested as described. Mouse antisera were diluted 1:100 in LB, and then they were further diluted serially two-fold in LB. Serum dilutions were mixed 1:4 with CP9 culture supernatant and incubated for 2 hr at 4°C. The serum-supernatant mixtures were then incubated with sheep erythrocytes as described above. Water and CP9 supernatant served as positive controls, while Ca-saline and LB served as negative controls. The remainder of the protocol was conducted as described above. The degree of neutralization of native HlyA by serum antibodies of vaccinated mice was determined by a graphical comparison of absorbance values between CP9 supernatant treated with vaccinated or with sham vaccinated sera to that of CP9 supernatant alone.

EVALUATION OF CNF1 TOXOID ACTIVITY

HEp-2 cell multinucleation assay

An assay that quantifies the degree of multinucleation of HEp-2 cells in response to CNF1 intoxication has been previously described (173) and was modified as follows. Microtiter plates were seeded with 1×10^3 HEp-2 cells rather than 4×10^3 cells. Two-fold serial dilutions of CNF1 or CNF1 toxoid in complete Eagle's minimal essential medium (EMEM) [EMEM with Earle's balanced salt solution (Lonza, Allendale, NJ), 10% fetal bovine serum (Invitrogen-Biosource, Carlsbad, CA), 2 mM L-glutamine (Invitrogen), 10 µg/ml gentamicin, 10 U/ml penicillin, and 10 µg/ml streptomycin] beginning at a concentration of 1 µg per well were added to individual wells. CNF1 toxoid was also evaluated at 10 µg per well. Negative control wells were treated with complete EMEM only. As a positive control, we used a batch of CNF1 that had previously been shown to elicit multinucleation of HEp-2 cells at a known concentration. All samples were run in duplicate. The plates were incubated for 72 hr at 37C in 5% CO₂. Then, the cells were fixed and stained with Hema-3 stain (ThermoFisher Scientific), visualized with an Olympus BX-60 microscope (Olympus, Center Valley, PA), and digital images were captured with a SPOT RT charge-coupled-device digital camera (SPOT™ imagine systems, Michigan, USA). The percentage of multinucleated cells was calculated by enumeration of the number of cells that contained two or more nuclei per 100 cells from each digital image.

CNF1 neutralization assay

We used a modification of the CNF1-mediated HEp-2 cell multinucleation assay to assess the neutralizing capacity of antisera from vaccinated mice (see below). Mouse

sera were diluted 1:9 in complete EMEM, and then serially diluted five-fold in the same medium. Serum dilutions were incubated with 2.5 ng of CNF1, the lowest concentration found to elicit 100% multinucleation of HEp-2 cells, for 2 hr at 37°C. Each reaction mixture was then used to intoxicate HEp-2 cells in the multinucleation assay described above. The degree of neutralization of CNF1 was determined by a comparison of the percentage of multinucleated cells in wells that contained sera from vaccinated, sham-vaccinated, and positive toxin control wells.

Lethal dose fifty percent (LD₅₀) determination for CNF1

To establish the LD₅₀, we obtained 4 to 5-week-old female C3H/HeOuJ mice (Jackson Laboratory, Bar Harbor, ME) that then were acclimated for one week. We administered purified CNF1 by the subcutaneous route to groups of mice in the following doses: 10 µg, 7 µg, 5 µg, 4 µg, 3 µg, 2 µg, and 1 µg. The mice were observed for one week for signs of morbidity and mortality. Ruffled fur, hunched posture, lack of responsiveness to stimuli, lethargy, and labored breathing were indications of morbidity. Mice that exhibited more than two of these signs were anesthetized and humanely euthanized by cervical dislocation. The LD₅₀ was calculated with the Reed-Muench formulation (231) and determined to be 3.2 µg.

MOUSE MODEL OF ASCENDING UTI

All animal procedures were conducted according to the *Guide for the Care and Use of Laboratory Animals* (Eighth Edition, National Academies Press) (1) and were approved by the Institutional Animal Care and Use Committee of the Uniformed Services University of the Health Services. Our previously described mouse model of ascending UTI (173; 204) was used herein with the following modifications. Female C3H/HeOuJ

mice (Jackson Laboratory, Bar Harbor, ME), aged 4 to 5 weeks, were housed in filter-top cages for one week prior to the start of each experiment. Mice had access to food and water *ad libitum*. Mice were placed under general anesthesia with 5% isoflurane in oxygen and maintained at 2.5% isoflurane USP (Clipper Distribution Company LLC, St. Joseph, MO) with a Vet Equip V10 portable anesthesia system (Vet Equip Inc., Pleasanton, CA). A sterile catheter composed of a 2.5 cm length of polyethylene tubing (Becton, Dickinson, Sparks, MD) with a 0.28 mm internal diameter and 0.66 mm external diameter was placed in the urinary bladder through the urethra. The bladder was emptied through the catheter and the urine sample was screened for pre-existing bacteriuria by bacterial enumeration. Mice with pre-existing bacteriuria of greater than 10^2 CFU per ml urine were excluded from the study. The CP9 inoculum was delivered via a 3 cc syringe mounted on an infusion pump (Harvard Apparatus, Holliston, MA) at flow rate 54 μ l per minute (80% force) for 30 seconds. Each bladder was inoculated with 25 μ l of a 10^9 CFU/ml culture (2.5×10^7 CFU CP9) unless noted otherwise. After 24 hr, mice were again anesthetized and catheterized, and urine was collected as described above. Mice were then euthanized by cervical dislocation while under general anesthesia. The left kidney was aseptically removed and homogenized. Homogenized kidneys and urine were serially diluted in PBS and plated on LB agar to enumerate the bacteria. In one experiment, each urinary bladder was homogenized and serial dilutions in PBS were plated on LB agar for bacterial enumeration. Bacterial counts were represented as CFU per ml of urine or organ homogenate.

HISTOLOGIC EVALUATION

The urinary bladder and the right kidney were removed from each mouse; each was placed in 10% neutral buffered formalin (ThermoFisher Scientific) and fixed for a minimum of 24 hr at room temperature. The fixed tissue was processed by ethanol dehydration and embedded in paraffin, then cut into 5 μ m thick sections and stained with hematoxylin and eosin (H&E) (Histoserv, Germantown, MD). The H&E sections of the urinary bladder were evaluated in a blinded manner by a veterinary pathologist (this author) and assigned severity scores for four parameters of cystitis: edema, neutrophilic infiltrate, damage to epithelium (degeneration and necrosis), and hemorrhage. The severity score ranged from 0 to 5 as follows: 0, no lesions; 1, mild; 2, minimal; 3, moderate; 4, marked; and 5, severe.

VACCINATION STRATEGIES

HlyA vaccination procedure

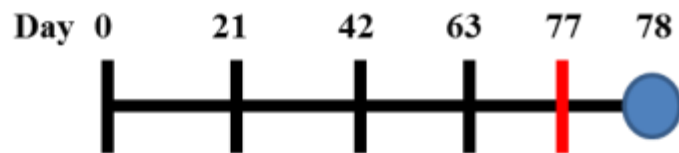
Groups of fifteen female C3H/HeOuJ mice were vaccinated by subcutaneous injection with 25 μ g HlyA toxoid emulsified 1:1 with the adjuvant Titermax Gold[®] (TiterMax USA, Norcross, GA) (Figure 10). The prime vaccination was followed by two boosts at three week intervals; the first boost contained adjuvant while the subsequent boosts did not. In later trials, a third boost without adjuvant was added three weeks after the second boost. Groups of ten mice were sham vaccinated with PBS in the same manner. Blood was collected by tail venipuncture from each animal prior to the first immunization and one week before each boost. Each blood sample was allowed to clot at 4°C for 60 min, then the cells were pelleted by centrifugation at 8,000 x g for 10 min. Serum was recovered and tested for the presence (enzyme-linked immunosorbent assay,

Figure 10. Active and passive vaccine schedules

A. Active HlyA toxoid (txd) vaccine schedules. The prime (P) vaccine was given on day 0 and was followed by boosts (B) at 21 day intervals. Trial 1 differed from trials 2 and 3 by the addition of a third boost (fourth immunization) in the latter two trials. Mice were challenged (C) with CP9 two weeks after the last boost, and mice were euthanized and subjected to necropsy (N) one day later.

B. Active CNF vaccine schedules. Trials 1 and 2 differed by the inclusion of a third boost, a sublethal dose of CNF1 in all of the boosts, and the exclusion of adjuvant from the boosts in Trial 2.

C. Passive immunization schedule for monoclonal antibody (MAb) anti-CNF1 NG8. Equivalent doses of NG8 (D) were delivered by intraperitoneal injection 24 hr and 4 hr prior to challenge. Irrelevant mouse IgG2a was used as an isotype control.

A.

		P	B	B	B	C	N
Trial 1	HlyA txd	25 µg	25 µg	25 µg	-		
Trial 2&3	HlyA txd	25 µg	25 µg	25 µg	25 µg		
	Adjuvant	+	+	-	-		

B.

		P	B	B	B	C	N
Trial 1	CNF1 txd	25 µg	25 µg	-			
	CNF1 toxin	-	-	250 ng			
	Adjuvant	+	+	-	-		
Trial 2	CNF1 txd	25 µg	25 µg	25 µg	25 µg		
	CNF1 toxin	-	400 ng	400 ng	400 ng		
	Adjuvant	+	-	-	-		

C.

		D	D	C	N
Anti-CNF1 NG8		100 µg	100 µg		
Isotype control IgG2a		100 µg	100 µg		
PBS control		100 µl	100 µl		

ELISA) and neutralizing capacity (hemolysis neutralization assay) of anti-HlyA antibodies. Once the majority of the mice had neutralizing serum antibodies, they were inoculated intraurethrally with CP9 for 24 hr as described above.

CNF1 vaccination procedure

Groups of fifteen female C3H/HeOuJ mice were vaccinated by subcutaneous injection with 25 µg of CNF1 toxoid emulsified 1:1 with the adjuvant Titermax Gold®; the first boost contained adjuvant while the subsequent boosts did not. The prime vaccination was followed by two boosts at three week intervals. In the first trial, the first boost was the same as the prime vaccination while the second boost consisted only of 250 ng CNF1. In a subsequent trial, mice were boosted three times with a mixture of 25 µg CNF1 toxoid and 400 ng of CNF1 (without adjuvant). Note: the intraperitoneal (i.p.) LD₅₀ for the lot of CNF1 used in this study was determined to be 3.2 µg, so the amount of active CNF1 used in conjunction with CNF1 toxoid was a sublethal dose (Figure 10). Groups of 10 mice served as controls and were sham vaccinated with PBS. In all trials, blood was collected from each mouse and processed to serum before the prime vaccination and one week prior to each boost to measure the presence (ELISA) and neutralizing capacity (multinucleation assay) of anti-CNF1 antibody levels. Once neutralizing antibody was detected in most of the vaccinated mice, they were challenged intraurethrally with CP9 for 24 hrs as described above.

Passive immunization of mice with monoclonal NG8 anti-CNF1

Monoclonal NG8 anti-CNF1 is a mouse IgG2a antibody that neutralizes CNF1 (72; 132). To test the capacity of NG8 to reduce the bladder pathology associated with CNF1, we administered 100 µg NG8 in sterile saline via i.p. injection to fifteen mice.

Five mice were given 100 µg of an irrelevant isotype (IgG2a) control antibody (Southern Biotech, Birmingham, AL), and five mice were given sterile PBS (Figure 10). For all groups, the antibody or PBS was given at 24 hr and 4 hr prior to intraurethral challenge with CP9.

ASSESSMENT OF ANTIBODY RESPONSE

ELISA

Blood and urine were collected from mice prior to immunization and two weeks after each boost and tested for specific antibody responses by ELISA. Microtiter plates were coated overnight at 4°C with 100 ng of HlyA toxoid or CNF1 diluted in PBS. The plates were blocked for 3 hr at room temperature with 5% bovine serum albumin in PBS. Individual mouse sera or pooled urine was serially diluted ten-fold serial in 1% bovine serum albumin in PBS (BSA-PBS). The dilution samples were applied to the wells in duplicate and incubated overnight at 4°C. The plates were washed 4X with PBST. The secondary antibody, goat anti-mouse IgG-HRP (Bio-Rad), was diluted 1:5000 in BSA-PBS and incubated 1 hr at room temperature. The plates were washed 4X with PBST, then the substrate 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well and incubated for 30 minutes. The reaction was stopped by the addition of an equal volume of 1 N sulfuric acid. The absorbance of each well at 450 nm was measured in an ELx800 Microplate Spectrophotometer (Biotek). The positive control was the mouse monoclonal anti-RGS-His antibody (Qiagen, lot #34610), and the negative control was secondary antibody alone (no primary antibody). The final absorbance for each sample was calculated by subtraction of the average absorbance of the secondary antibody only

as the sample blank. The absorbance of each dilution was plotted against the log of the dilution.

Western blot

To determine the specificity of the antibodies generated against HlyA toxoid or CNF1 toxin/toxoid, purified HlyA or CNF1 was denatured in Laemmli buffer and loaded into several wells of a 7.5% polyacrylamide gel and the samples were subjected to SDS-PAGE, then transferred to nitrocellulose membranes as described above. The membranes were cut into strips that each contained one or two wells of the HlyA or CNF1, then blocked in blocking buffer for 3 hr at room temperature. The strips were incubated overnight at 4°C with either pooled sera from vaccinated or sham-vaccinated mice diluted 1:500 or pooled urine diluted 1:50 in blocking buffer. The positive control for HlyA detection was rabbit polyclonal anti-HlyA (Weingarten et al., manuscript in preparation) diluted 1:5,000 in blocking buffer. For CNF1, the positive control was NG8 (diluted 1:1500). The membranes were washed with PBST, then incubated for 1 hr at room temperature with the secondary antibody goat anti-mouse IgG Alexa Fluor® 532 or goat anti-rabbit IgG Alexa Fluor® 532 (Invitrogen) diluted 1:15,000 in blocking buffer. Each blot was washed 4X with PBST, then imaged with the Imagequant™ LAS 4000 imager (GE Life Sciences).

STATISTICAL ANALYSES

Statistical analyses were done with GraphPad Prism v5.01 for Windows software (GraphPad software, San Diego, CA,). The analysis of variance (ANOVA) with Bonferroni multiple comparisons post-test was applied to assess statistical significance in differences between pathology scores of the urinary bladder between vaccinated and

sham-vaccinated animals. The repeated measures ANOVA with Bonferroni multiple comparisons post-test was also used to assess statistical significance between groups in neutralization assays (hemolysin neutralization and CNF1 neutralization assays). Groups included sera from sham-vaccinated and vaccinated animals, and positive toxin controls. The Student t-test was used to determine significant differences between geometric means of CP9 colonization of urine and kidneys of vaccinated versus sham-vaccinated mice. The Student t-test was also used to assess statistical significance between the mean absorbance values of individual dilutions on ELISA assays of sera from vaccinated and sham-vaccinated mice.

CHAPTER 3: Results

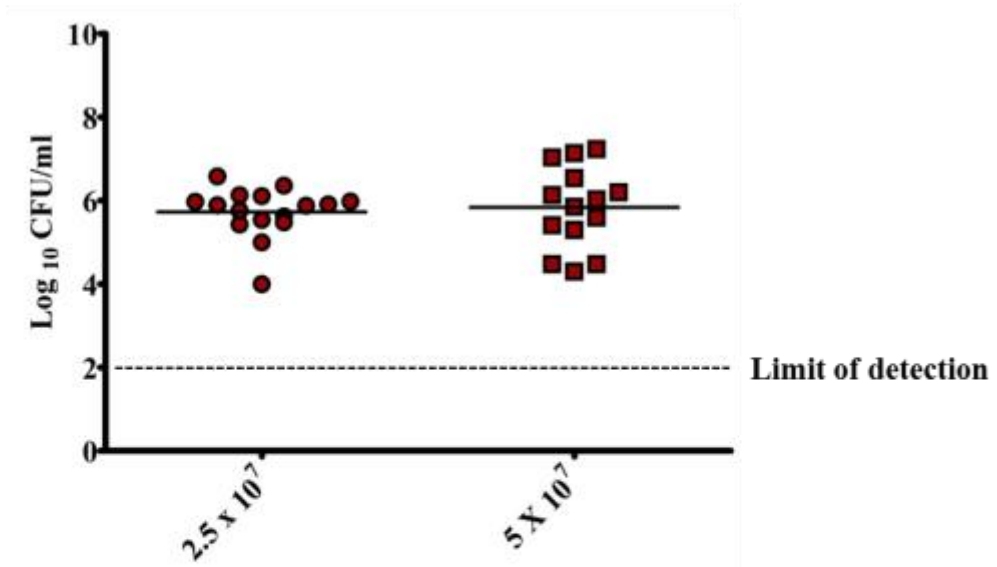
ASSESSMENT OF SUSCEPTIBILITY OF AGED MICE TO CP9 BLADDER INFECTION

The major objective of this study was to determine whether immunization with HlyA or CNF1 toxoids can reduce the degree of inflammation in the bladders of vaccinated mice compared to controls given PBS (sham vaccinated) after intraurethral challenge with the UPEC strain CP9 that makes both HlyA and CNF1. Since immunized C3H/HeOuJ mice would be 10 weeks older than the 6-8 week old mice typically used in CP9 infection studies, we first sought to determine the impact of age on susceptibility to UPEC infection. For that purpose, a pilot experiment was undertaken to evaluate whether 16 to 18 week old C3H/HeOuJ mice (aged mice) were susceptible to UTIs caused by CP9. Two groups of mice were inoculated intraurethrally with CP9. One group of 16 mice was given the dose we planned to use in the vaccine challenge protocol, 2.5×10^7 CFU, and the second group of 16 animals received a higher dose of 5×10^7 CFU. A control group was inoculated with PBS. There was no statistically significant difference in the quantity of bacteria in the kidneys of mice (one left kidney per mouse) in either infection group, 24 hr after inoculation (Figure 11, panel A). There were also no statistically significant differences in the quantity of bacteria in the urinary bladders (Figure 11, panel B) or in the urine samples (Figure 11, panel C) between the two infection groups. Both challenge groups also demonstrated moderate to severe suppurative cystitis with submucosal edema, hemorrhage, and epithelial damage. There was minimal pathology in the bladders of mice given PBS (Figure 12). The degree to which the aged mice were colonized by CP9 and the severity of cystitis was comparable to those results from previous studies with 6 to 8 week old mice (204). We concluded

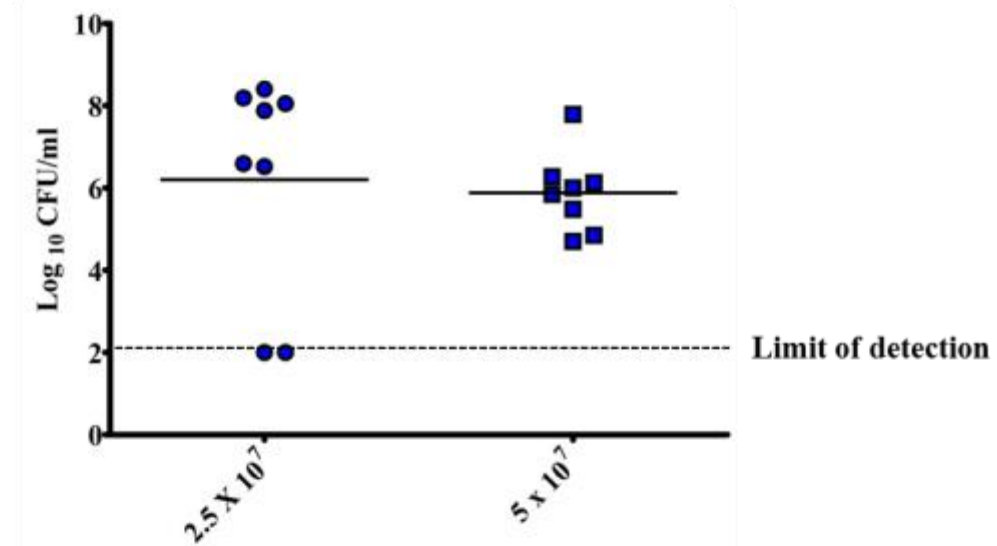
Figure 11. Colonization of the urinary tract of aged mice by UPEC strain CP9

Colony counts of CP9 in the kidney (panel A), urinary bladder (panel B), and urine (panel C) of aged (16-18 weeks old) mice 24 hours after intraurethral inoculation with the bacteria. Each symbol represents the CFU/ml for one mouse, and the bar represents the geometric mean of counts from that group of animals. Data were analyzed by the two tailed Student t-test. There were no significant differences between colonization levels in the kidneys, bladders, or urine, respectively, of mice given either dose of bacteria.

A.



B.



C.

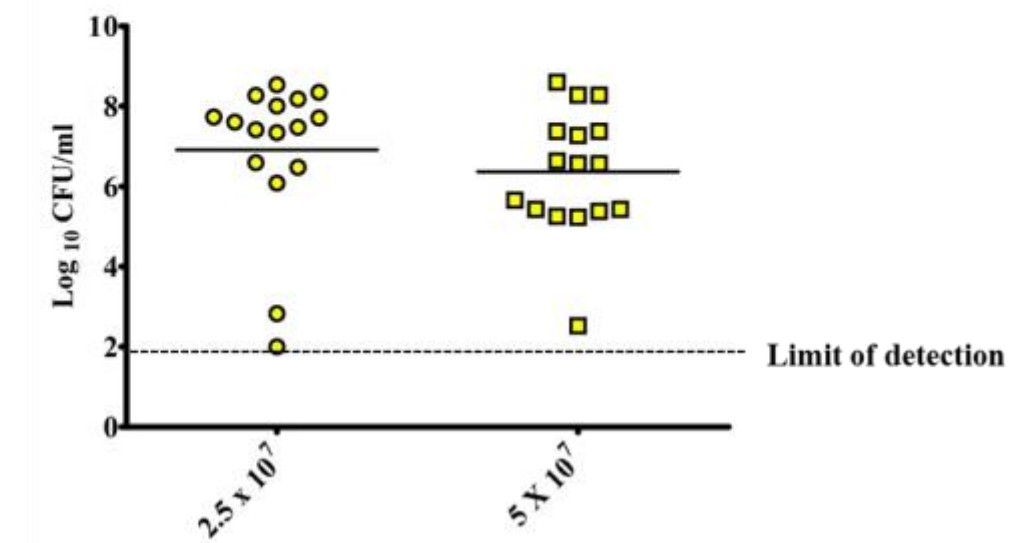
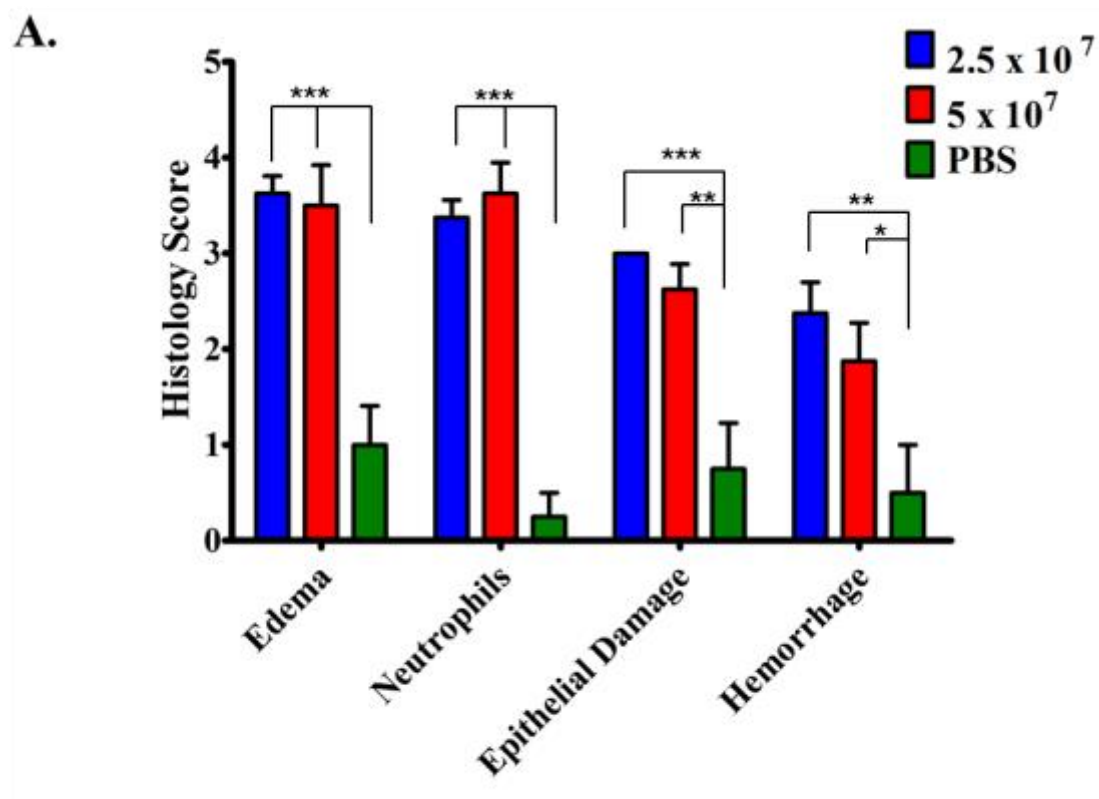


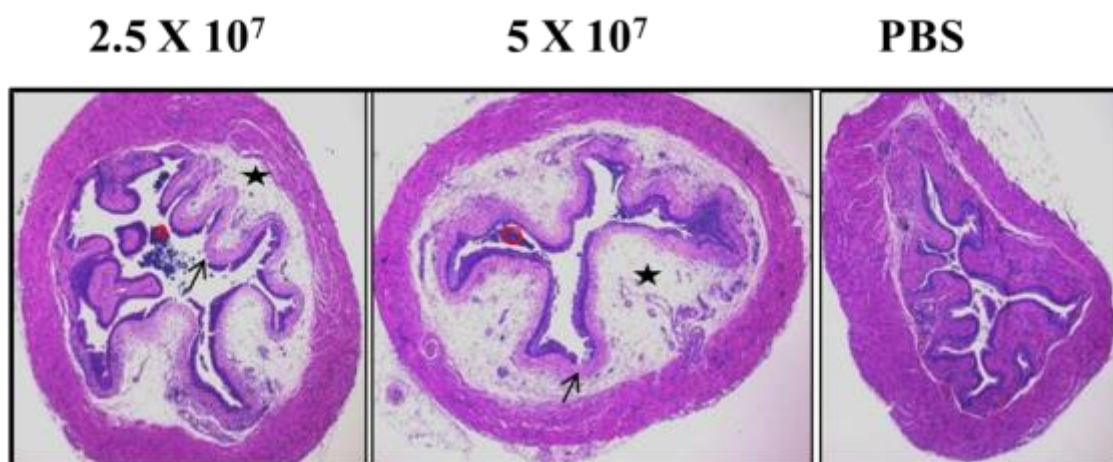
Figure 12. Severity of cystitis in aged mice

A. The mean severity scores of edema, neutrophilic infiltrate, epithelial degeneration and necrosis (damage), and hemorrhage for groups of mice inoculated with 2.5×10^7 CFU (blue), 5×10^7 CFU (red), and PBS (green). Data were analyzed by two-way ANOVA with the Bonferroni multiple comparison post-test. *** = $P < 0.001$; ** = $P < 0.01$, * = $P < 0.1$.

B. Urinary bladders from mice of each treatment group. No pathological lesions are evident in the bladders from the PBS inoculated mouse. Moderate to severe cystitis with edema can be seen in the mice inoculated with CP9. The stars indicate areas of edema, the red circles show dense aggregates of neutrophils, and the black arrows point to areas of epithelial damage. Tissue sections were stained with hematoxylin and eosin. Magnification shown is 40X.



B.



from this study that any reduced cystitis noted in vaccinated mice would not be due to decreased susceptibility of aged mice.

VACCINE STRATEGIES OVERVIEW

We hypothesized that the presence of neutralizing antibodies against HlyA or CNF1 in the sera of mice would serve as correlates of protection to predict the reduction in the severity of cystitis associated with UPEC infection after toxoid vaccination. To test this theory, we employed both active and passive vaccine strategies. To elicit an antibody response against HlyA and CNF1 without toxin-associated morbidity and mortality, we immunized with toxoids that were created by site-directed mutagenesis of the genes that encoded the toxins. In the CNF1 vaccine trial, we also either used sublethal doses of active CNF1 alone for some booster immunizations or we augmented toxoid boosts with sublethal doses of CNF1 (see Figure 10, panel B) to help direct the immune response toward toxin neutralization. Previous work from Meysick et al. in our laboratory showed that the use of sublethal doses of CNF1 as immunogens led to production of some neutralizing CNF1 monoclonal antibodies (136). The sublethal dose of CNF1 used here was determined to be 400 ng based on LD₅₀ studies that estimated that value to be 3.2 µg. The active vaccine protocols were modified based on the results of the initial trials described in the paragraphs below. We also analyzed the capacity of a monoclonal anti-CNF1 antibody (NG8) delivered by intraperitoneal injection to reduce cystitis in passive immunization studies. The different vaccine strategies are depicted schematically in Figure 10. The results of the anti-hemolysin vaccine trials are presented first followed by the active and passive immunization studies against CNF1.

VACCINATION AGAINST HEMOLYSIN

Creation of the HlyA toxoid

We sought to create an enzymatically inactive form of HlyA, or genetically generated toxoid, that could be used to safely immunize animals. For that purpose, we targeted the two lysine residues (K564 and K680) in HlyA that undergo fatty acid acylation by HlyC; acylation of these two amino acids is required for the HlyA protoxin to become active (209). Indeed, previous studies by others showed that the mutation of these lysine residues results in complete inactivation of the hemolytic activity of the toxin but does not affect its secretion from the bacterium (168; 209). Based on these published data, we introduced a total of four A to G point mutations at positions 1690, 1692, 2068, and 2070 in *hlyA₃* (Figure 13) by site-directed mutagenesis so that two positively-charged lysine residues would be altered to two negatively-charged glutamic acid residues in the expressed protein (K564E; K690E). The plasmid that contained the gene for the HlyA toxoid, *phlyA* K564E K690E, was then transformed into CP9 Δ *hlyA*. This strain contains *hlyC*, *hlyB*, and *hlyD*, the products of which are necessary for HlyA activation and export. The HlyA toxoid was present in the supernatant of the CP9 Δ *hlyA* (*phlyA* K564E K690E) transformant as assessed by Western blot analysis of the ethanol-precipitated supernatant (Figure 14, panel A). The supernatant from CP9 Δ *hlyA* (*phlyA* K564E K690E) also had markedly reduced hemolytic activity compared to the supernatant from CP9 as determined in a hemolysis assay (Figure 14, panel B). Thus, we created a toxoid version of HlyA that demonstrated markedly reduced activity in hemolysis assays. We next sought to create working stocks of the HlyA toxoid for use in vaccination trials.

Figure 13. Sequence of the HlyA toxoid gene

The *hlyA₃ toxoid* sequence is 3075 base pairs. Four mutations were introduced into *hlyA₃* by site-directed mutagenesis. Adenine residues at positions 1690, 1692, 2060, and 2070 (bolded and underlined in the lines with arrows) were targeted for mutation and changed to guanine.

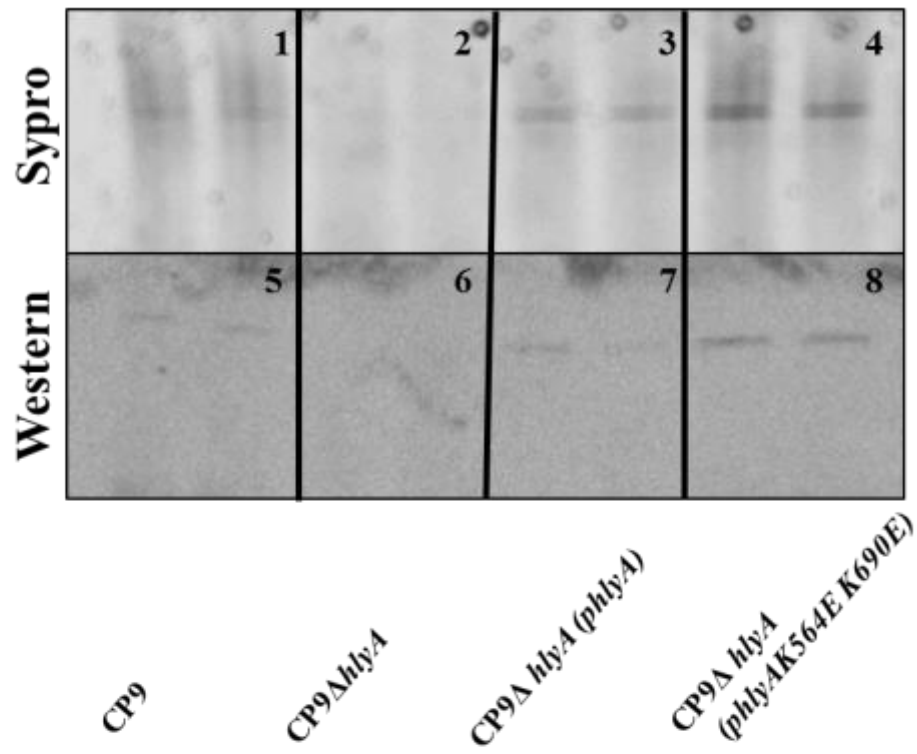
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 cattaattaatgaaatcagcaaaatcatttcagctgcaggttaattttgatgttaagaggaaaga
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 aactttgacagcatcagcataa-3'

Figure 14. HlyA toxoid activity

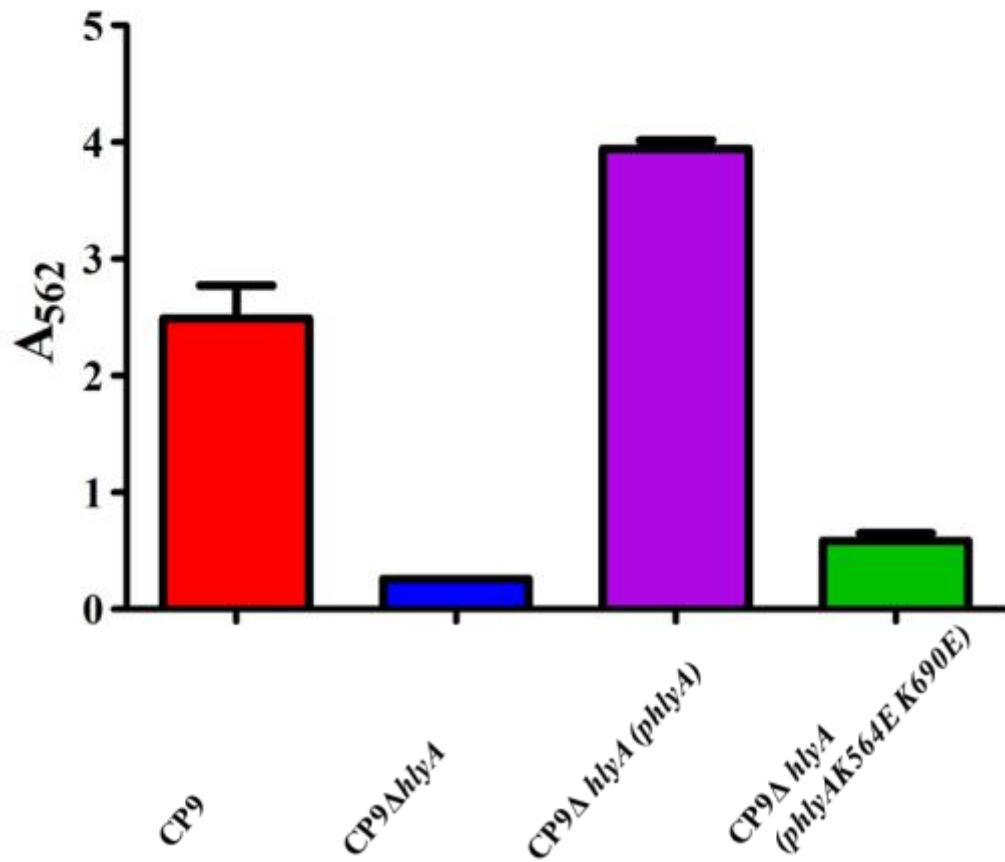
A. Protein stain (Sypro; sections 1-4) and Western blot (sections 5-8) analyses of ethanol-precipitated supernatants from broth cultures of CP9, CP9 Δ *hlyA*, and complemented strains. Each sample was run in duplicate. A band for secreted HlyA toxoid is evident on the immunoblot (compare sections 4 and 8) of the supernatant from the CP9 Δ *hlyA*(*phlyA* K564E K690E). A band of about the same molecular weight (size marker not shown) is in supernatants from CP9 and CP9 Δ *hlyA* (*phlyA*) and likely represents HlyA.

B. Hemolysis assay results on culture supernatants from CP9, CP9 Δ *hlyA*, and complemented strains. Each bar represents the mean of absorbance values from samples prepared in triplicate. The bars represent one standard error of the mean. The HlyA toxoid from CP9 Δ *hlyA*(*phlyA* K564E K690E) was 77% less activity than the native hemolysin of CP9 and 85% less than the recombinant hemolysin produced by CP9 Δ *hlyA* (*phlyA*).

A.



B.

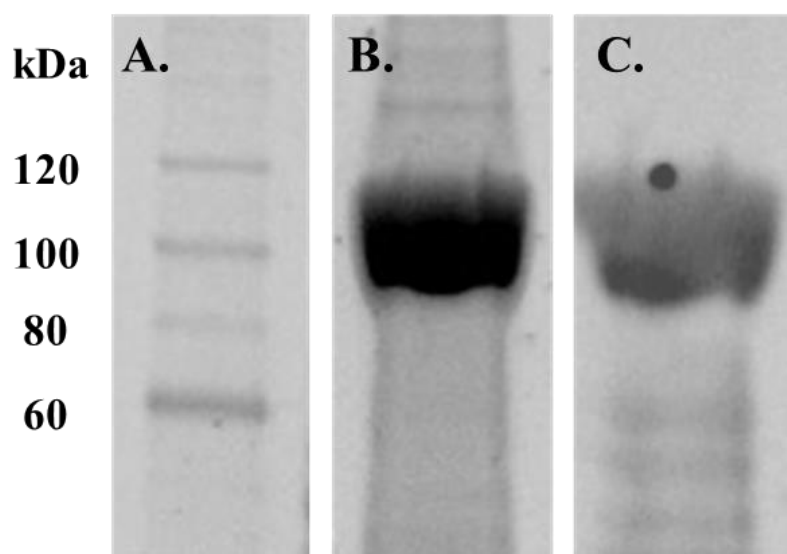


Purification of the HlyA toxoid

The HlyA toxoid was generated so as to express a 6X-Histidine tag on the N-terminus of the molecule. Therefore, to purify the toxoid, phlyA K564E K690E was first transformed into the *E. coli* expression strain M15pREP4. The toxoid was then expressed from M15pREP4 (phlyA K564E K690E), and a cell lysate that contained the toxoid was passed over a nickel affinity chromatography column under native conditions. However, these initial attempts to purify the toxoid were not successful; the toxoid was not significantly enriched compared to protein contaminants (not shown). Subsequently, the purification protocol was changed such that the toxoid was first denatured in a buffer that contained 8M urea. In that procedure, the urea concentration in the column buffer was reduced in steps to 4 M prior to elution of the toxoid from the column. The elution fractions were then combined and dialyzed against a phosphate buffer that contained 4M urea to yield a stock of HlyA toxoid at a concentration of 800 µg protein per ml. That material was assessed for purity by SDS-PAGE electrophoresis and Western blot (Figure 15). There were a few impurities in the eluted material as can be seen in the protein-stained gel (see Figure 15, panel B), but the most prominent protein band appeared to be the HlyA toxoid (compare panels B and C, Figure 15). In sum, we used a protein denaturing protocol to purify and create a working stock of HlyA toxoid that we then used to initiate the HlyA vaccine trials outlined in Figure 10. We then sought to follow the response to the vaccine by evaluation of the anti-HlyA antibody response in vaccinated mice.

Figure 15. Evaluation of the purity of the HlyA toxoid

Oriole stain and Western blot analysis of HlyA toxoid purification product. The proteins in the elution fractions that contained the HlyA toxoid were separated by SDS-PAGE electrophoresis in duplicate. The bands on one gel that contained the size markers (A) and the pooled elution fractions (B) were visualized by an Oriole protein stain (B). Proteins in the second gel were transferred to a nitrocellulose membrane and probed with mouse anti-RGS monoclonal antibody (C) that reacts with the RGS-tag on the HlyA toxoid. There is a prominent band in (C) that is in the same position as that seen on the Oriole stain (at about 100kDa). A few less intense bands of lower molecular weight that may represent some low level of degradation of the HlyA toxoid are also evident.



Specific antibody responses in sera of HlyA toxoid-vaccinated mice

To monitor the generation of specific antibody responses to vaccination against the HlyA toxoid for each vaccine trial, serum samples were obtained from mice after toxoid boosts and then analyzed for the presence of anti-HlyA antibodies by Western blot and/or ELISA. In the first trial, pooled sera and urine from vaccinated mice, collected 18 days after the second boost (day 60 after initiation of vaccination), demonstrated immunoreactivity against the HlyA toxoid band on the blot membrane (Figure 16, panel A). In the second trial, pooled sera from vaccinated mice demonstrated immunoreactivity against the HlyA toxoid band on the blot membrane as early as 20 days after the first boost, day 40 after initiation of vaccination (Figure 16, panel B). Moreover, pooled sera and urine collected 15 days after a third boost (day 78 after initiation of vaccination) showed strong immunoreactivity in samples of HlyA toxoid-vaccinated mice. As anticipated, there was no reactivity with the sera or urine samples from sham-vaccinated mice (Figure 16, panel C). In the third trial, we used an ELISA, in which HlyA toxoid served as the capture antigen, to better quantify the antibody response against HlyA. Serum samples from vaccinated mice that were collected on the day of euthanasia demonstrated 10,000-fold higher anti-HlyA IgG titers on average than did sera from mice obtained prior to HlyA toxoid vaccination (hereafter called pre-immune sera) or sera from sham-vaccinated mice (Figure 17). In all trials, vaccinated mice developed a specific anti-HlyA toxoid antibody response. We next sought to evaluate the capacity of these antibodies to neutralize hemolysin.

Figure 16. Detection of an anti-HlyA response in serum or urine of mice by Western blot analyses at two time points in the HlyA vaccination schedule

A. Western blot of HlyA toxoid probed with sera and urine collected at day 60 from vaccination initiation from mice in trial 1. The secondary antibody was goat anti-mouse IgG. Eighteen days after the second boost with HlyA toxoid, Western blot analyses with pooled sera and urine from vaccinated mice (n= 15) as the probe demonstrated specific anti-HlyA immunoreactivity. There was no immunoreactivity with the HlyA toxoid band of pooled sera or urine from sham-vaccinated mice (n= 10).

B. Western blot of HlyA toxoid probed with sera collected at day 40 from vaccination initiation from mice in trial 2. The secondary antibody was goat anti-mouse IgG. Twenty days after the first boost with HlyA toxoid and adjuvant, Western blot analyses with pooled sera from vaccinated mice (n= 15) as the probe demonstrated specific anti-HlyA immunoreactivity. There was no immunoreactivity with the HlyA toxoid band of pooled pre-immune sera (n=15) or pooled sera from sham-vaccinated mice (n= 10).

C. Western blot of HlyA toxoid probed with probed with sera collected at the conclusion of the study (day 78 from vaccination trial 2 initiation). The secondary antibody was goat anti-mouse IgG. Western blots probed with either pooled sera or urine from vaccinated mice (n=15) demonstrated anti-HlyA immunoreactivity while neither pooled sera or urine from sham-vaccinated mice (n=10) were immunoreactive with the HlyA toxoid band.

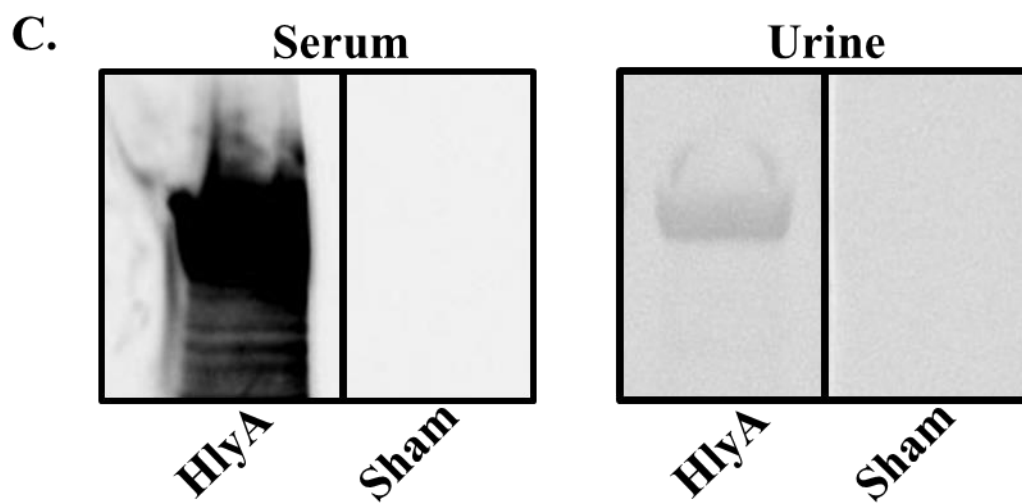
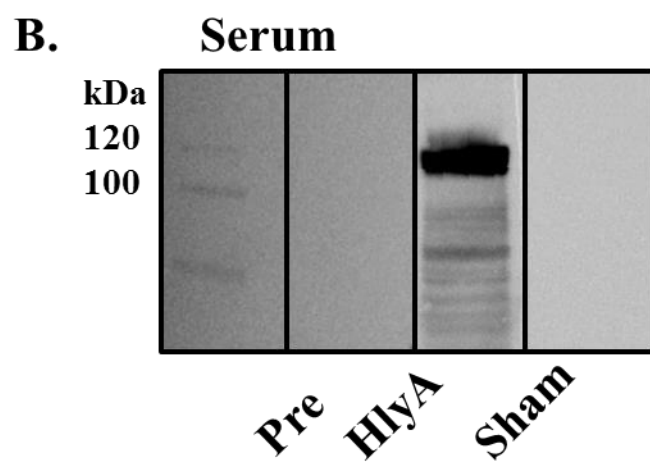
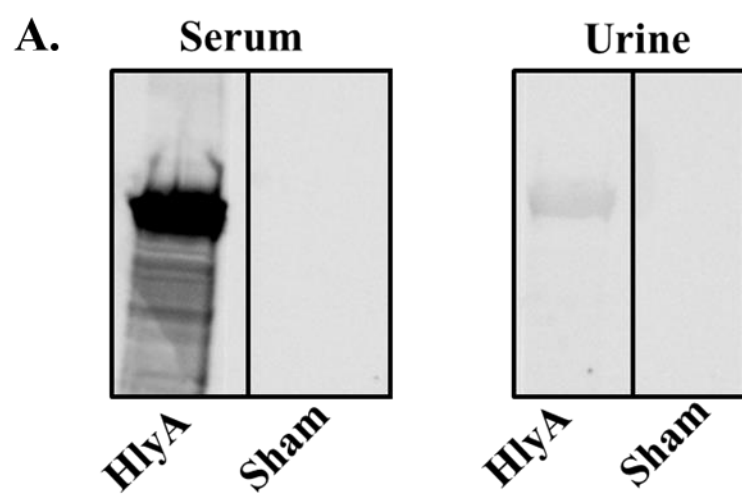
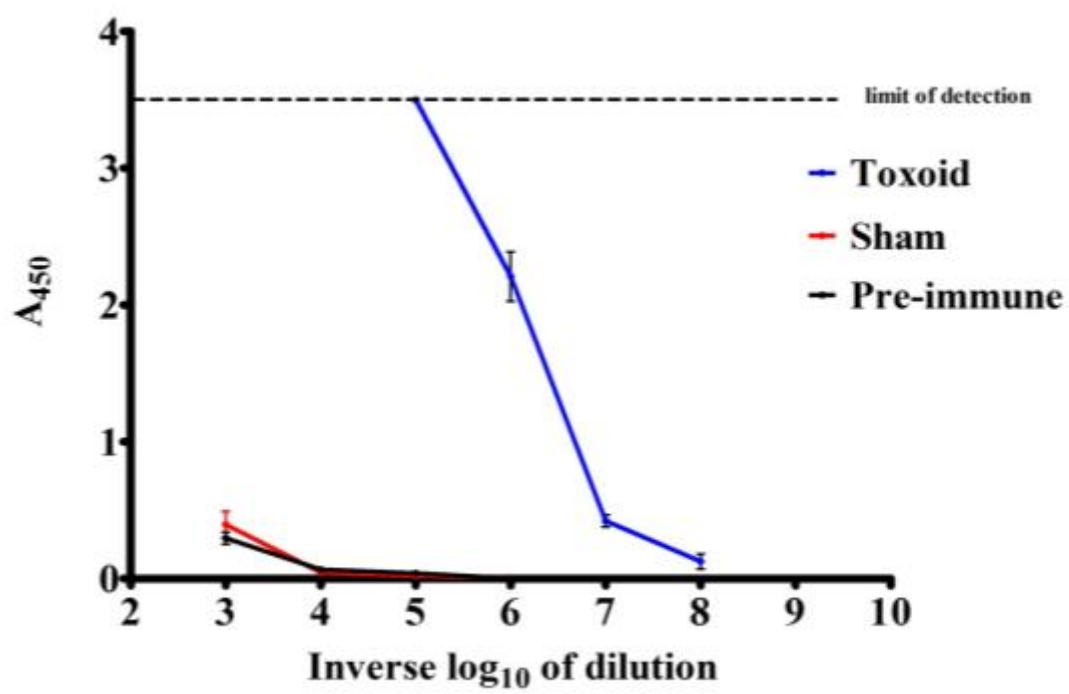


Figure 17. Anti-HlyA titers in sera of HlyA toxoid-vaccinated mice compared to controls

Pre-immune sera from HlyA-vaccinated mice and terminal sera from both HlyA-vaccinated and sham-vaccinated mice were compared by ELISA with HlyA toxoid as the capture antigen. The secondary antibody was goat anti-mouse IgG. Data points represent the mean absorbance value per group (pre-immune group n= 15, toxoid-vaccinated group n=15; and sham-vaccinated group n=10). The bars indicate the standard error of the mean. Vaccinated mice demonstrated 10,000-fold higher anti-HlyA titers than either the pre-immune sera or sera from sham-vaccinated mice. There was no significant difference in anti-HlyA titers between the pre-immune sera and sera from sham-vaccinated mice.



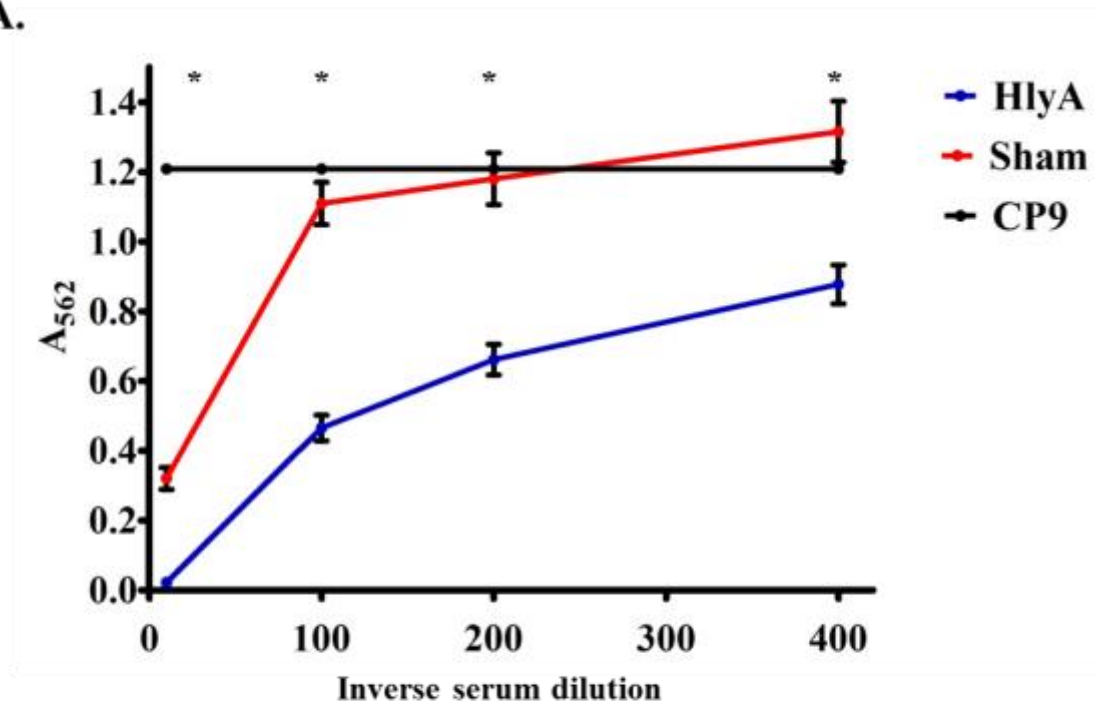
Hemolysin neutralization activity in sera from vaccinated mice

To ascertain whether the specific antibody response to the HlyA toxoid could neutralize hemolysin, serial dilutions of sera from vaccinated or sham-vaccinated mice were incubated with native hemolysin contained in the supernatant of CP9 cultures. After incubation of these mixtures, samples were added to an erythrocyte solution in a hemolysis assay. In all of the HlyA toxoid vaccine trials, we found that sera from either the HlyA-vaccinated or sham-vaccinated mice reduced the hemolytic activity of hemolysin in CP9 supernatant. However, the hemolytic activity returned to native levels as the dilution of sera from sham-vaccinated mice approached 100-fold. In contrast, the sera from vaccinated mice reduced hemolytic activity and did not return to native levels until the 400-fold dilution (Figure 18). In addition, at every dilution, the sera from vaccinated mice reduced the hemolytic activity of hemolysin in CP9 supernatant significantly more than did the sera of sham-vaccinated mice (Figure 18). We surmised that mice had some prior exposure to hemolysin or that mouse serum contains proteins that can inhibit the activity of hemolysin based on the findings of anti-HlyA activity in the sera from sham-vaccinated mice as evaluated by hemolysin neutralization assay. Bhakdi et al. found that human serum albumin, low-density lipoprotein, and high-density lipoprotein could protect erythrocytes from hemolysis following exposure to purified hemolysin with an in vitro assay (18). Nevertheless, we concluded that vaccination of mice with HlyA toxoid resulted in production of a higher anti-HlyA titer by ELISA than sham-vaccinated controls and one that could neutralize native HlyA in a hemolysis assay to a greater extent than did sera from sham-vaccinated mice. We next challenged vaccinated mice with CP9.

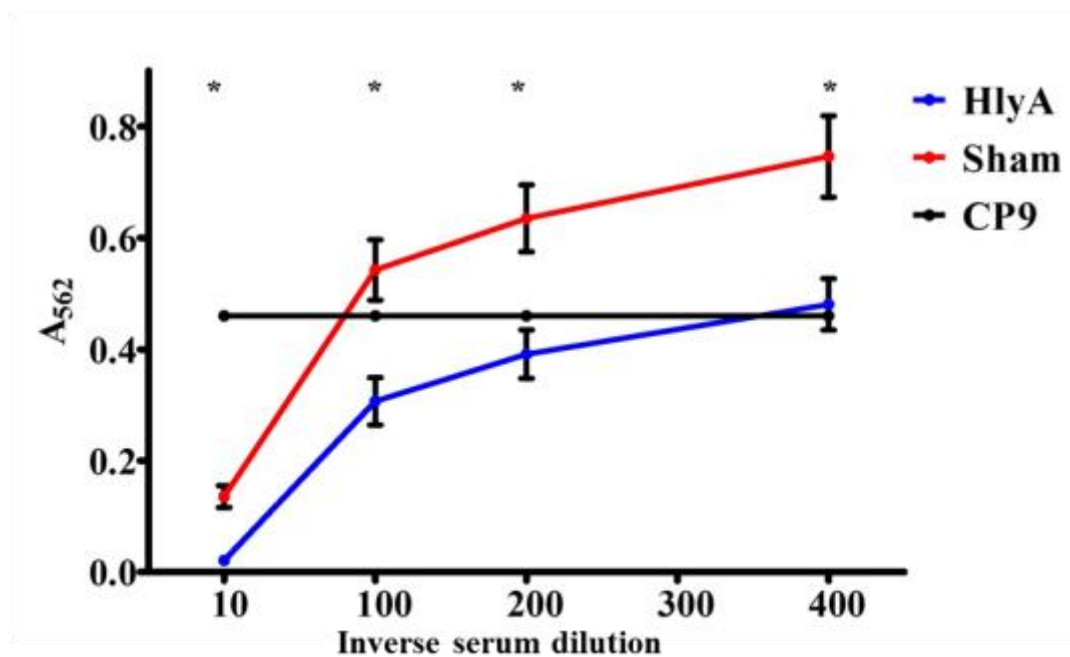
Figure 18. Hemolysin neutralization assays on sera from mice obtained in two HlyA toxoid vaccine trials.

Comparison of neutralizing capacity of sera from HlyA-vaccinated and sham-vaccinated mice. Data points represent the mean absorbance value per group, and the bars are the standard error of the mean. Data were analyzed by the two-tailed Student t-test. Terminal sera from both toxoid-vaccinated (n=15) and sham-vaccinated mice (n=10) from the second (panel A) and third (panel B) vaccine trials were collected on day 78 from vaccine trial initiation. In both trials, sera from the toxoid-vaccinated (blue) and sham-vaccinated (red) mice significantly reduced hemolytic activity of CP9 (black). Sera from sham-vaccinated mice returned to CP9 levels at the 100-fold dilution, whereas sera from vaccinated mice continued to reduce hemolysis until the 400-fold dilution. There was statistically significantly ($* = P \leq 0.0001$) less hemolytic activity when CP9 supernatant was treated with sera from vaccinated mice compared to sham-vaccinated mice at all dilutions.

A.



B.



CP9 colonization of mice following challenge

The challenge occurred 21 days after the final HlyA toxoid boost. In the first trial, mice received a total of three vaccinations, the first two with adjuvant (Figure 10). Mice were sacrificed 24 hours after CP9 challenge, and urine and the left kidney were collected for bacterial enumeration. There was no statistically significant difference in colonization of the kidneys between vaccinated and sham-vaccinated mice (Figure 19). However, there was a statistically significant reduction in the level of CP9 isolated from the urine of vaccinated mice compared to sham-vaccinated mice. In the second and third trial, in which a third boost was added to the vaccine protocol (Figure 10), there was a nearly ten-fold reduction in CP9 in the urine but the difference was not statistically significant (Figure 20). There was again no statistically significant difference in kidney colonization between study groups (Figure 20). In summary, vaccination with HlyA toxoid resulted in lower bacterial counts in the urine, but that reduction was only statistically significant in the first trial. There were no differences in kidney colonization in any trial. We next evaluated the degree of cystitis by histopathology.

Histopathologic evaluation of the urinary bladders from mice in HlyA toxoid vaccine trials

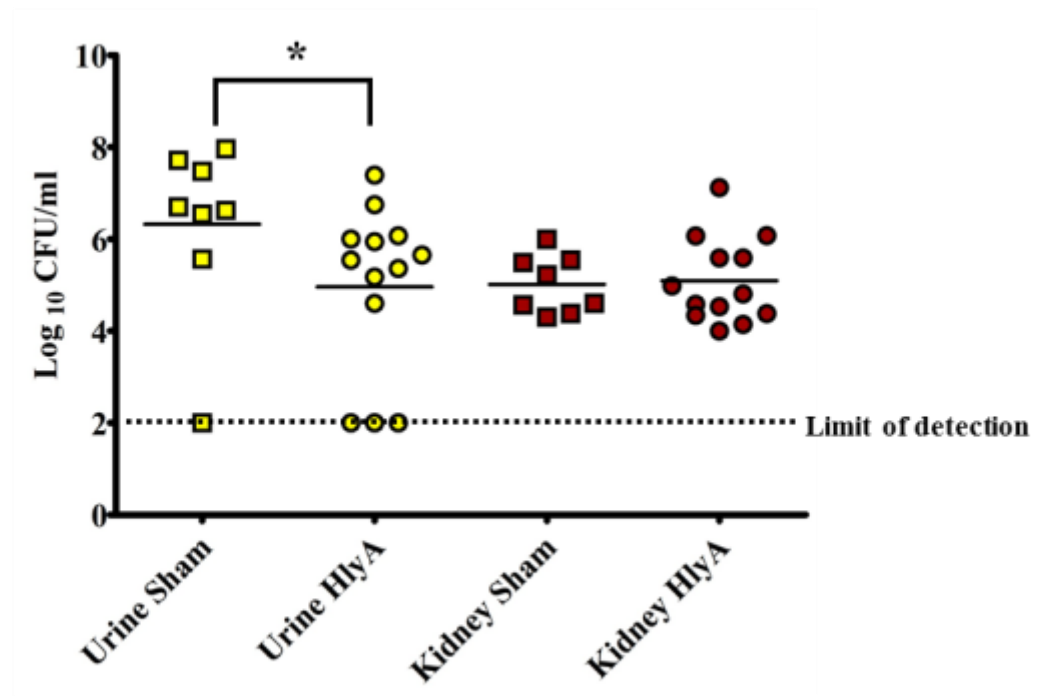
At necropsy, urinary bladders were collected and fixed in formalin and processed into tissue sections on glass slides. The sections on the slides were then stained with hematoxylin and eosin and the slide identities masked. I evaluated the slides and assigned severity scores from 0 to 5 for each of four parameters of cystitis (edema, neutrophilic infiltrate, epithelial damage, and hemorrhage). In the first trial, there was reduced pathology in all four parameters of cystitis in HlyA-vaccinated mice, but only the difference in neutrophils was statistically significant (Figure 19). In the second and third

Figure 19. Levels of CP9 in urine samples and kidneys and histology severity score for bladders of mice in HlyA toxoid vaccine trial 1

A. Bacteria enumerated from the urine and a kidney of HlyA-vaccinated (circles) and sham-vaccinated mice (squares). Each symbol represents one mouse, and the bar is the geometric mean for each group. Data were analyzed by the two tailed Student t-test. The geometric mean of bacteria in the urine of HlyA-vaccinated mice was significantly (*, $P=0.011$) lower than in the urine of sham-vaccinated mice. There was no statistically significant difference in kidney colonization levels between HlyA-vaccinated and sham-vaccinated mice.

B. Average histology scores between HlyA-vaccinated (blue) and sham-vaccinated (red) mice. The bars represent the mean histology score for each group, and the error bar is the standard error of the mean. Data were analyzed by the two way ANOVA with the Bonferroni multiple comparison post-test. The pathology was less severe in the HlyA-vaccinated group but was only significant (*, $P=0.01$) in the neutrophilic infiltrate category.

A.



B.

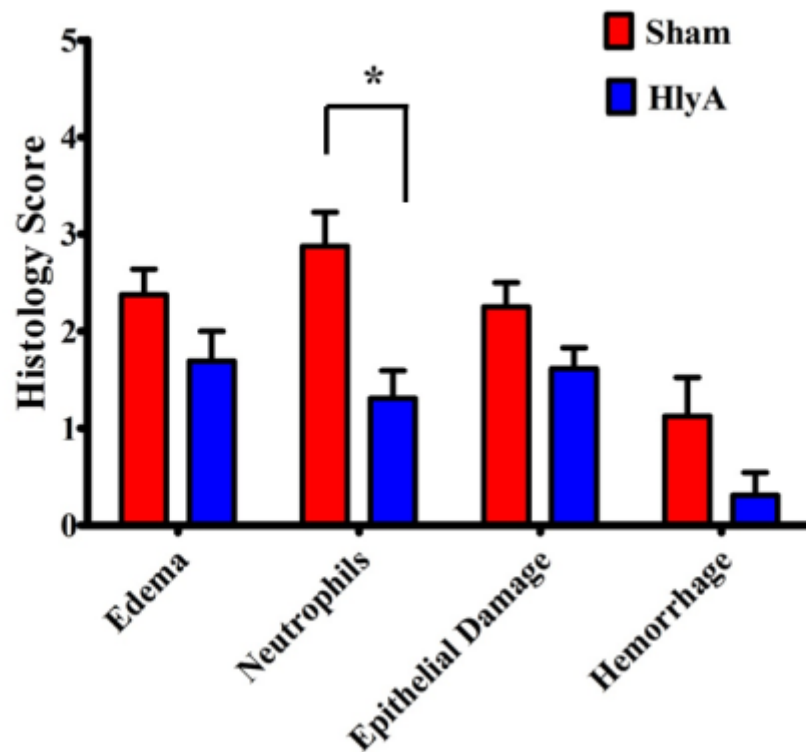


Figure 20. Levels of CP9 in urine samples and kidneys of mice in HlyA toxoid vaccine trials 2 and 3

Bacteria enumerated from the urine and kidney of HlyA-vaccinated (circles) and sham-vaccinated mice (squares). Each symbol represents one mouse, and the bar is the geometric mean for each group. Data were analyzed by the two tailed Student t-test. The geometric mean of bacteria in the urine was lower in the HlyA-vaccinated mice compared to the sham-vaccinated mice, however, the difference was not statistically significant ($P > 0.05$). There were no significant differences in levels of bacteria in the kidneys between HlyA-vaccinated versus sham-vaccinated mice

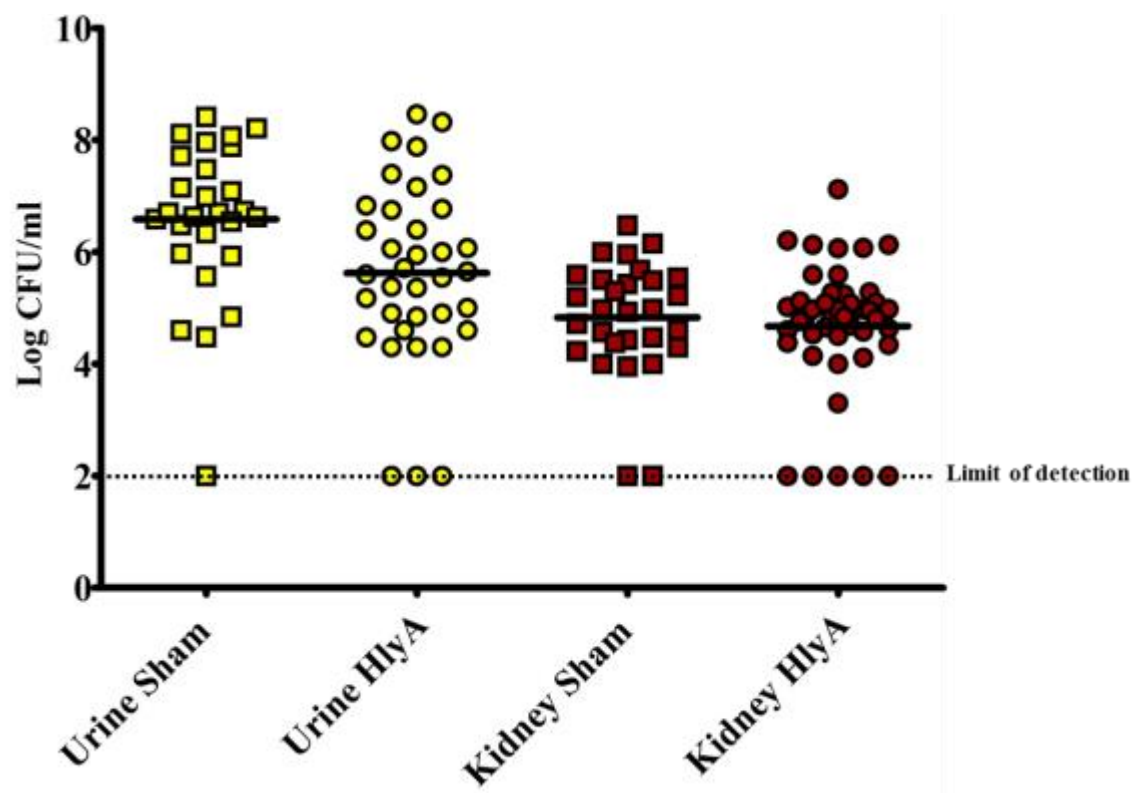
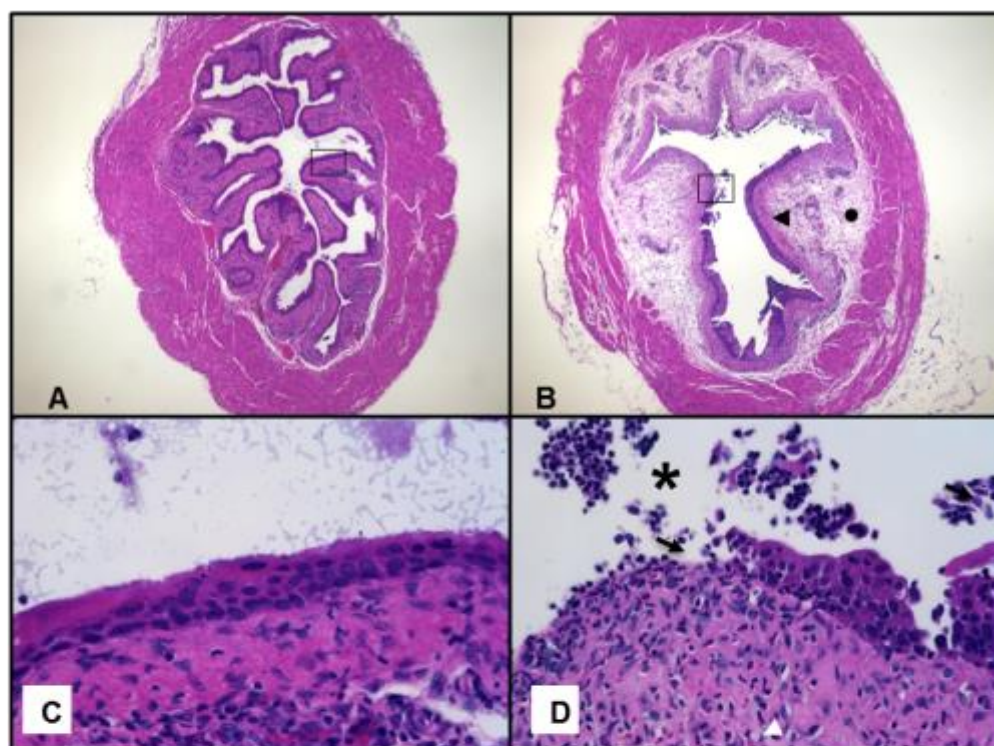


Figure 21. Histology and severity scores of bladders from mice in HlyA vaccine trials 2 and 3

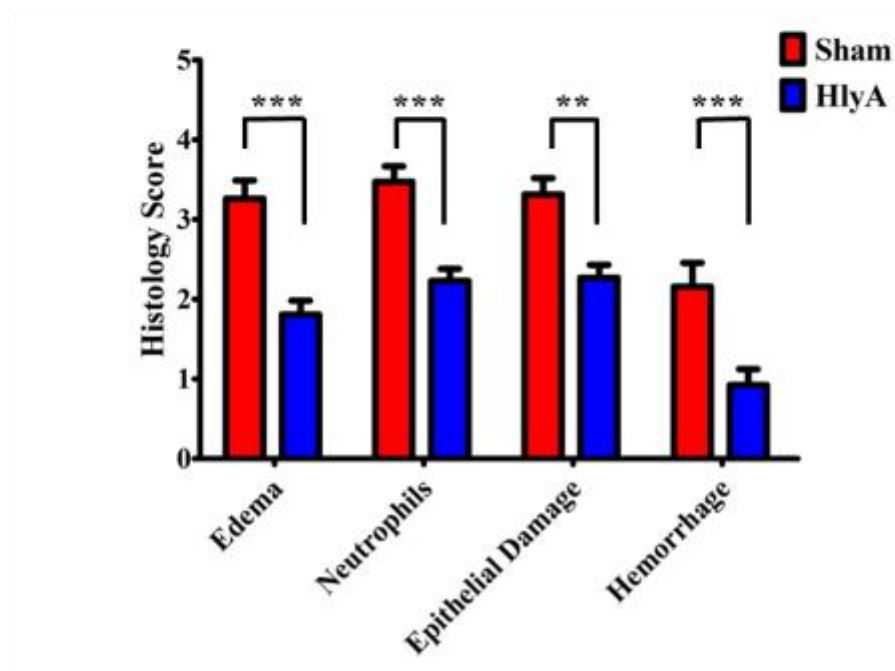
A. Urinary bladders from HlyA-vaccinated (A, C) and sham-vaccinated (B, D) mice challenged with CP9. Panels A, B: 40X, panels C, D: 400X. The symbols demonstrate lesions of cystitis; edema: ●; neutrophils: *; epithelial damage: ←; and hemorrhage: white ◀.

B. Average histology scores between sham-vaccinated (red) and HlyA-vaccinated (blue) mice. The bars represent the mean histology score for each group and the error bar is the standard error of the mean. Data were analyzed by the two way ANOVA with the Bonferroni multiple comparison post-test. The pathology was significantly less severe (***, $P=0.001$; **, $P=0.01$) in the vaccinated group in all four parameters of cystitis.

A.



B.



trial, in which a third boost was added to the vaccine protocol (Figure 10), there was a significant reduction in all four parameters of cystitis in vaccinated animals (Figure 21). Thus, vaccination with HlyA toxoid resulted in decreased cystitis in mice subsequently challenged with UPEC strain CP9 in all three trials. We next used a similar approach to evaluate the response of mice vaccinated against CNF1.

VACCINATION AGAINST CNF1

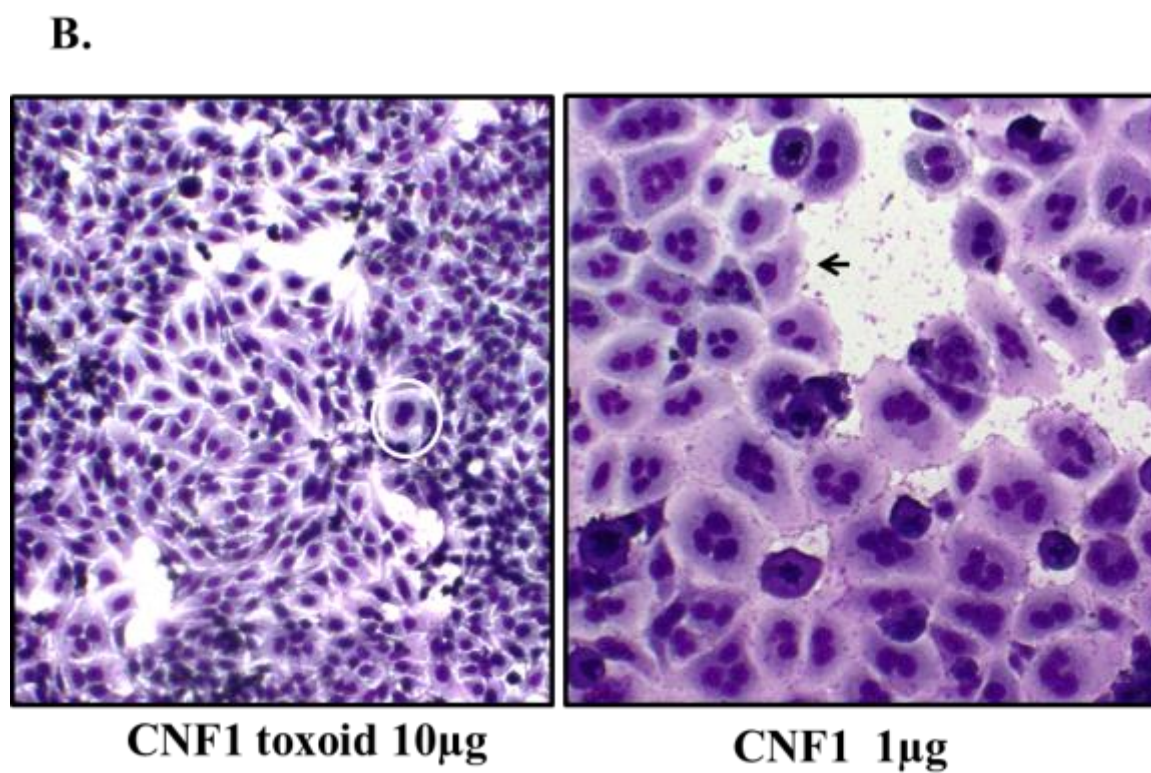
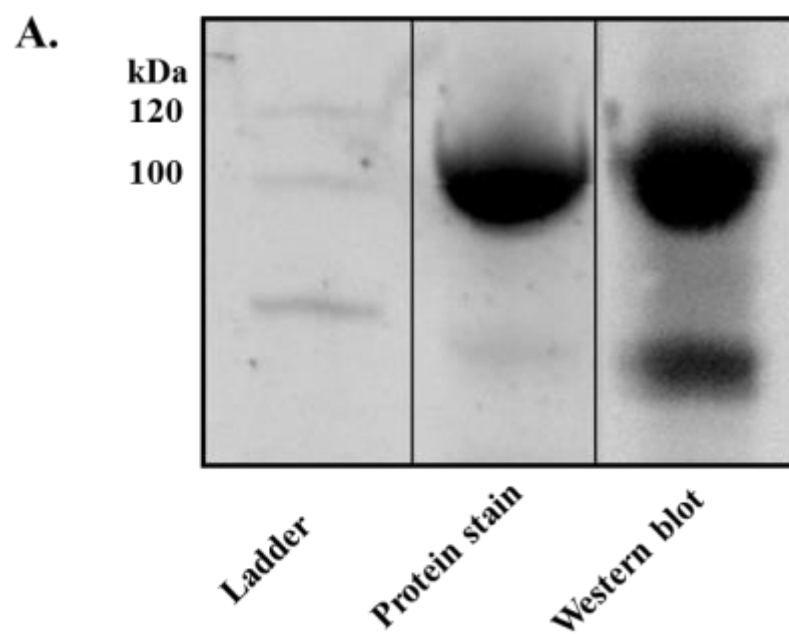
Expression and purification of the CNF1 toxoid

Previously in our laboratory, Russo and O'Brien (unpublished data) used site-directed mutagenesis to alter the *cnf1* gene in pCNF24 (pQE-30::*cnf1*) so that when expressed the CNF1 protein would be inactive. The target for creation of that CNF1 toxoid was the cysteine residue at position 866 in the protein. This amino acid is one of three critical amino acids (the other two are a histidine at position 881 and a valine at position 833) in the catalytic domain of CNF1 that is required for the enzymatic activity of the toxin (193), i.e. deamidation of a glutamine residue of several small GTPases (112). This approach was originally used by Schmidt et al. to create a CNF1 toxoid (193). Like the HlyA toxoid, the CNF1 toxoid was generated so as to express a 6X-Histidine tag on the N-terminus of the molecule. The toxoid-containing plasmid pCNF24 C866S was transformed into the *E. coli* expression strain M15pREP4. The toxoid was then expressed from the transformant, and cell lysates that contained the toxoid were passed over a nickel affinity chromatography column. The elution fractions were combined to yield a stock of CNF1 toxoid at a concentration of 300 µg protein per ml. That material was assessed for purity by SDS-PAGE electrophoresis and Western blot (Figure 22).

Figure 22. CNF1 toxoid purification results and evaluation of activity

A. Oriole stain and Western blot analysis of the CNF1 toxoid purification product. The proteins in the elution fractions that contained the CNF1 toxoid were separated by SDS-PAGE electrophoresis in duplicate. The bands on one gel that contained the size markers (ladder) and the pooled elution fractions were visualized by an Oriole stain (protein stain). Proteins in the second gel were transferred to a nitrocellulose membrane and probed with anti-CNF1 monoclonal antibody NG8 (Western blot). There is a prominent band visible in both the oriole stain and Western blot that is about 100kDa. There are also a few less intense bands of lower molecular weight that likely represent some low level of degradation of the CNF1 toxoid.

B. HEp-2 cells following a 72 hr incubation with either 10 μ g of CNF1 toxoid or 1 μ g of CNF1. The cells incubated with 10 μ g of CNF1 toxoid demonstrate rare multinucleation (within white circle). The cells incubated with CNF1 are enlarged with 2-4 enlarged nuclei. The rare mononuclear cells are also enlarged with an enlarged nucleus (black arrow). Cell culture wells were stained with Hema-3. Magnification shown is 100X.



The resulting stock contained a few impurities in the eluted material as can be seen in Figure 22, panel A, but the most prominent protein band appeared to be the CNF1 toxoid. The active CNF1 was purified from pCNF24 and characterized in the same manner. After the CNF1 toxoid was purified, we characterized it as described below.

Analysis of the CNF1 toxoid

To determine whether the CNF1 toxoid was indeed inactive, we assessed the capacity of the toxoid to induce multinucleation, the phenotype of CNF1 intoxication for HEp-2 cells. We incubated HEp-2 cells that had been seeded on 96-well plates with two-fold serial dilutions of both CNF1 beginning at 1 μ g and the CNF1 toxoid beginning at 10 μ g. Rare HEp-2 cells incubated with the CNF1 toxoid demonstrated multinucleation (Figure 22, panel B). However, the HEp-2 cells incubated with CNF1 demonstrated nearly 100% multinucleation until the dilution resulted in a concentration of CNF1 below 1.25 ng per well. We concluded that the CNF1 toxoid was inactive as it failed to cause multinucleation of HEp-2 cells at a concentration of 10 μ g per well. We next sought to evaluate the CNF toxoid as an immunogen in vaccine trials in mice. As with the HlyA vaccine studies mentioned above, we first evaluated the capacity of the CNF1 toxoid to elicit an anti-CNF1 response.

Specific antibody responses in CNF1-vaccinated mice

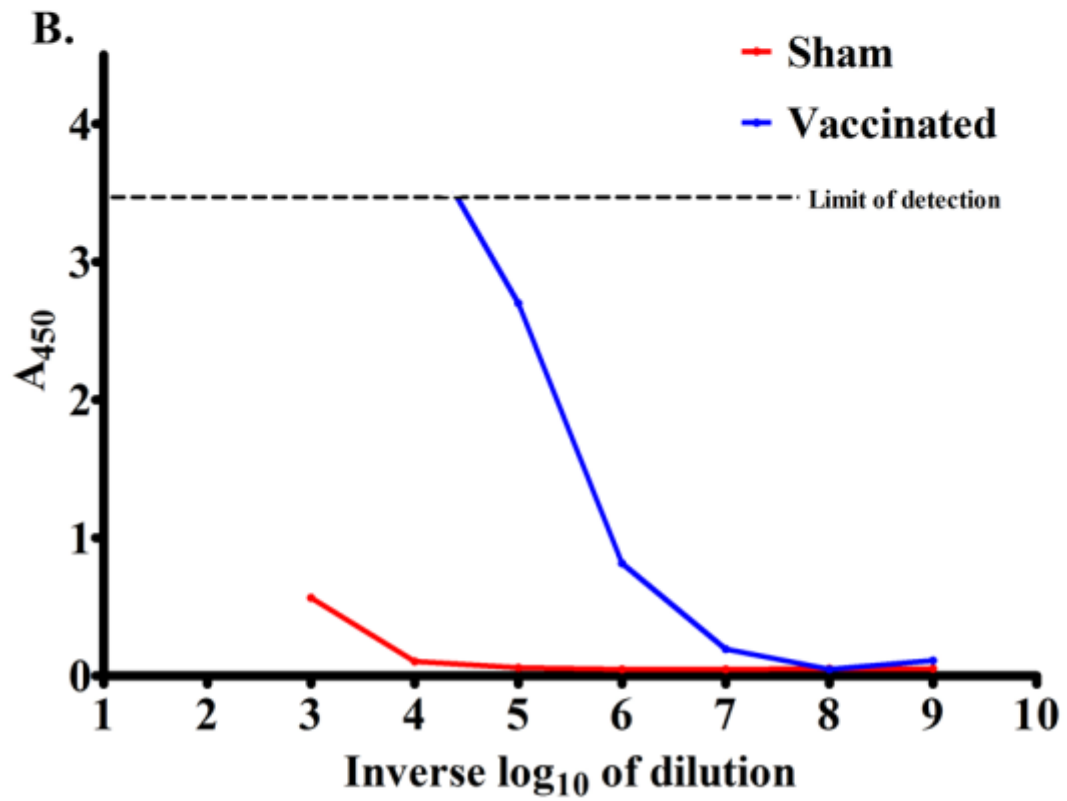
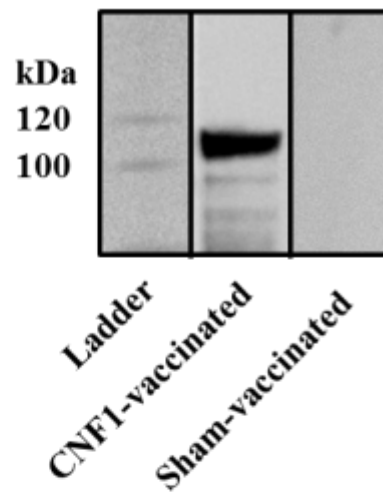
To assess the specific antibody response to vaccination against the CNF1 toxoid (boosted with sublethal doses of CNF1), serum samples were drawn from mice 14 to 20 days after each vaccination and analyzed for the presence of anti-CNF1 antibodies by Western blot and ELISA. In the first trial, strong immunoreactivity was evident in the sera of CNF1 toxoid-vaccinated mice at the time of challenge with CP9, while there was

Figure 23. Detection of a specific anti-CNF1 response by Western blot and ELISA analyses of sera from toxoid vaccinated mice

A. Western blot of CNF1 probed with pooled mouse sera collected day 65 from vaccine trial initiation in the first trial (Toxoid group n=15; Sham group n=10). The secondary antibody was goat anti-mouse IgG. There was strong immunoreactivity to the CNF1 band when probed with sera collected from CNF1-vaccinated mice at day 65 and no reactivity from the sera of sham-vaccinated mice.

B. Sera from both CNF1-vaccinated and sham-vaccinated mice collected on day 65 were compared by ELISA with CNF1 as the capture antigen (toxoid group n=15; sham group n=10). The secondary antibody was goat anti-mouse IgG. Data points represent the mean absorbance value of duplicate samples. CNF1-vaccinated mice demonstrated 1000-fold higher anti-CNF1 titers than the sera from sham-vaccinated mice.

A.



no reactivity with the sera from sham-vaccinated mice (Figure 23). By ELISA, in which CNF1 toxin served as the capture antigen, sera from vaccinated mice, collected on the day of sacrifice in the first trial, demonstrated 1000-fold higher anti-CNF1 IgG levels than did sera from sham-vaccinated mice (Figure 23). Thus, vaccination with CNF1 toxoid/CNF1 resulted in an anti-CNF1 antibody response. We next sought to evaluate whether that antibody response could neutralize CNF1 activity.

Multinucleation neutralization capacity of serum from vaccinated mice

To determine whether the sera from immunized mice could neutralize CNF1, five-fold serial dilutions (beginning at 1:10) of serum samples from CNF1-vaccinated or sham-vaccinated mice were incubated with CNF1. The CNF1 and serum mixtures were then incubated with HEp-2 cells in a multinucleation assay, and the percentage of cells that demonstrated the multinucleated phenotype was determined for each serum dilution/toxin mixture. In the first CNF1 toxoid vaccination trial in which the mice had received the prime vaccine and two boosts (see Figure 10), individual serum samples were obtained from mice on day 65 after the prime vaccination. We found that HEp-2 cells treated with CNF1 mixed with various serum dilutions from CNF1-vaccinated mice had an average level of multinucleation of less than 50 percent of cells at the 1:250 dilution of immune serum and below, and, indeed, less than 10 percent of cells displayed multinucleation at the 1:10 dilution of such serum (Figure 24). However, HEp-2 cells treated with CNF1 mixed with serum dilutions from sham-vaccinated mice showed the same levels of multinucleation as did cells treated with CNF1 alone, regardless of sham serum dilution. In the second trial, mice received the prime vaccine and two boosts, and the serum samples were collected on day 58 following the prime vaccination. HEp-2

Figure 24. Neutralization of CNF1-mediated multinucleation of HEp-2 cells by sera from mice vaccinated with CNF1 toxoid (trial 1)

Each data point represents the mean percentage of HEp-2 cells in a sample well that demonstrated the multinucleation phenotype after being treated with a mixture of CNF1 and the inverse dilution of mouse serum collected on day 65 after the initiation of the first vaccine trial (toxoid group n=15; sham group n=10). The bars represent one standard error of the mean. The dashed line represents the activity of CNF1 alone. The black line designates the 50% multinucleation point. Data were analyzed by the two way ANOVA with the Bonferroni multiple comparison post-test. The degree of multinucleation of HEp-2 cells was significantly lower (**, $P < 0.001$; *, $P = 0.01$) in cells treated with a mixture of CNF1 and sera from CNF1-vaccinated mice compared to a mixture of CNF1 and sera from sham-vaccinated mice.

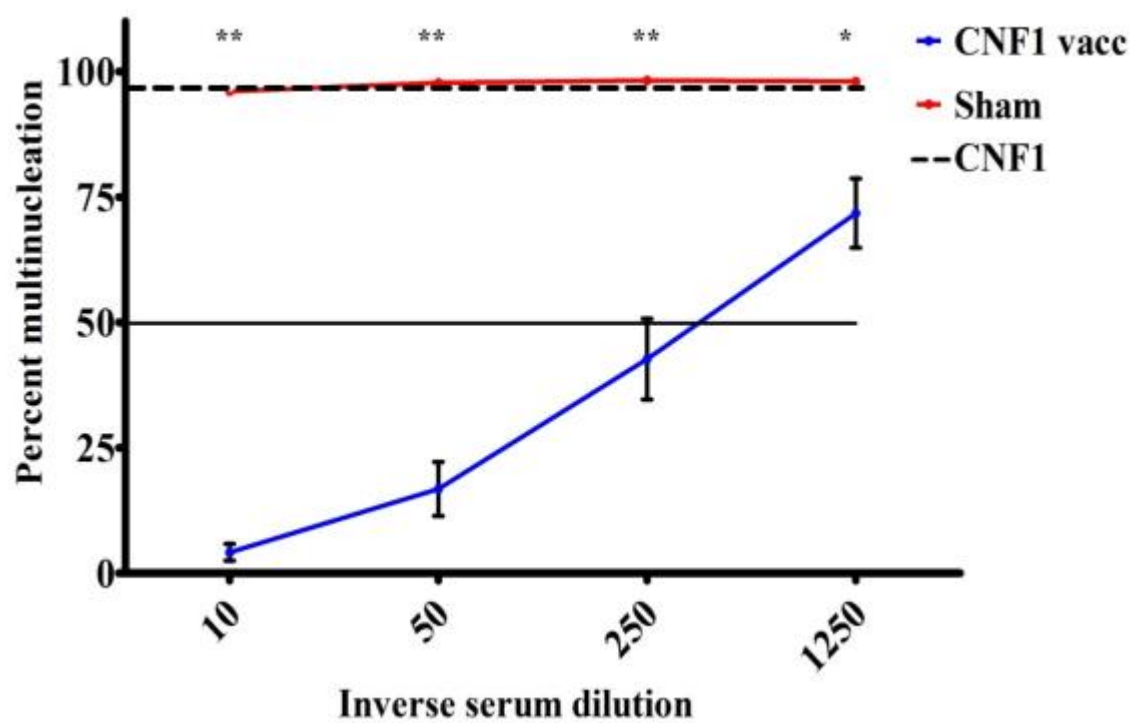
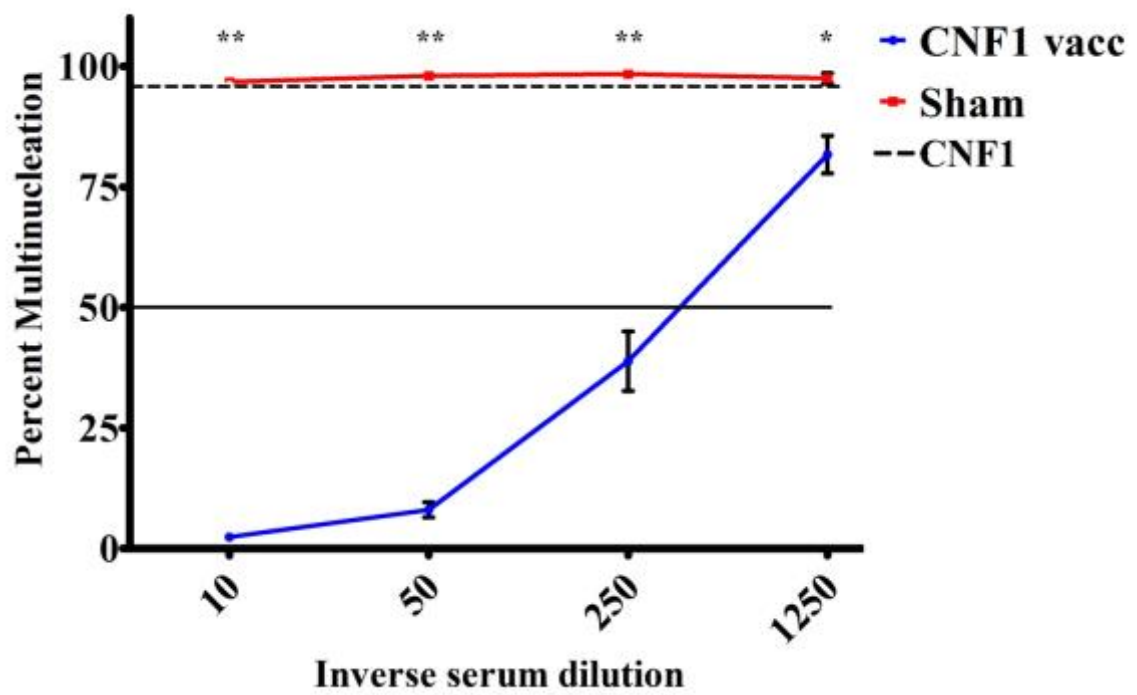


Figure 25. Neutralization of CNF1-mediated multinucleation of HEp-2 cells by sera from mice vaccinated with CNF1 toxoid (trial 2)

Each data point represents the mean percentage of HEp-2 cells in a sample well that demonstrated the multinucleation phenotype after being treated with a mixture of CNF1 and the inverse dilution of mouse serum collected on day 58 after the initiation of the first vaccine trial study (toxoid group n=15; sham group n=10). The bars represent one standard error of the mean. The dashed line represents the activity of CNF1 alone. The black line designates the 50% multinucleation point. Data were analyzed by the two way ANOVA with the Bonferroni multiple comparison post-test. The degree of multinucleation of HEp-2 cells was significantly lower (**, $P < 0.001$; *, $P = 0.01$) in cells treated with a mixture of CNF1 and sera from CNF1-vaccinated mice compared to a mixture of CNF1 and sera from sham-vaccinated mice.



cells treated with CNF1 plus serum dilutions from these CNF1-vaccinated mice demonstrated multinucleation in less than 50 percent of cells at the 1:250 dilution of immune serum and below, and less than 10 percent multinucleation in the 1:50 dilution of such serum and below (Figure 25). As in the first trial, HEp-2 cells treated with CNF1 mixed with serum dilutions from sham-vaccinated mice showed the same levels of multinucleation as cells treated with CNF1 alone. Therefore we surmise that vaccination with CNF1 toxoid/CNF1 evoked an antibody response that could neutralize the multinucleating activity of CNF1 in vitro.

CP9 colonization of mice following challenge

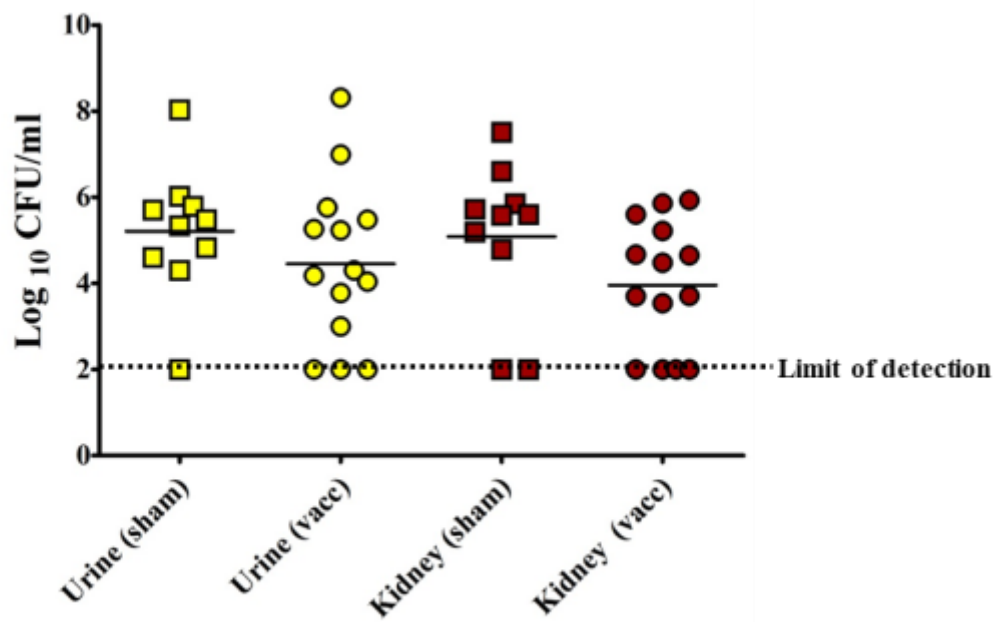
We next challenged the mice with CP9. In the first trial, mice were challenged 35 days after the final boost. Mice were sacrificed 24 hours after CP9 challenge, and urine and the left kidney were collected for bacterial enumeration. There were no statically significant differences between CNF1 vaccinated and sham-vaccinated mice in urine bacterial counts or levels of CP9 in the kidneys (Figure 26). In the second trial, in which a third boost was added to the vaccine protocol, the boosts were modified to contain both CNF1 and CNF1 toxoid without adjuvant (Figure 10). Mice were challenged 18 days after the third boost, and there were again no statistically significant differences between CNF1-vaccinated and sham-vaccinated mice in urine bacterial counts. There were also no statistically significant differences in kidney colonization between the two groups (Figure 27).

Figure 26. Levels of CP9 in urine samples and kidneys and histology severity score for bladders of mice in CNF1 toxoid vaccine trial 1

A. Bacteria enumerated from the urine and kidney of vaccinated (circles) and sham-vaccinated mice (squares). Each symbol represents one mouse, and the bar is the geometric mean for each group. Data were analyzed by the two-tailed Student t-test. There were no significant differences in urine bacterial counts in CNF1-vaccinated versus sham-vaccinated mice. There were also no significant differences in kidney colonization levels in CNF1-vaccinated versus sham-vaccinated mice.

B. Average histology scores between sham-vaccinated (red) mice and CNF1-vaccinated (blue). The bars represent the mean histology score for each group, and the error bar is the standard error of the mean. Data were analyzed by the two way ANOVA with the Bonferroni multiple comparison post-test. There were no significant differences in histology severity scores in any parameter of cystitis between CNF1-vaccinated and sham-vaccinated mice.

A.



B.

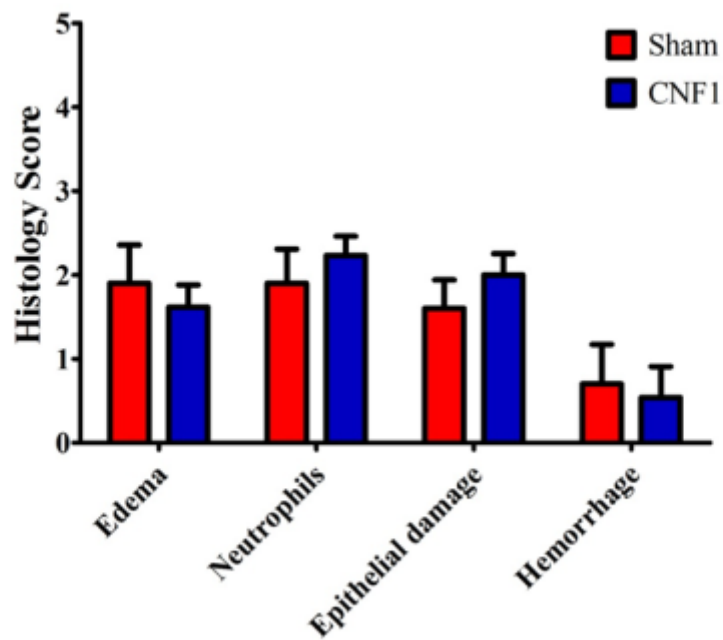
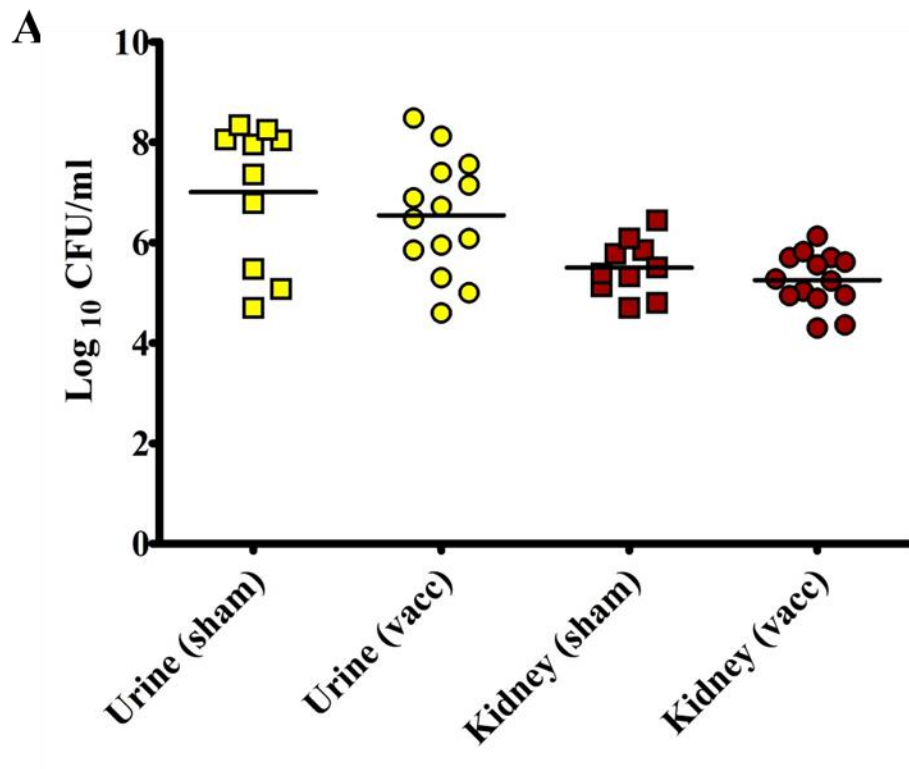


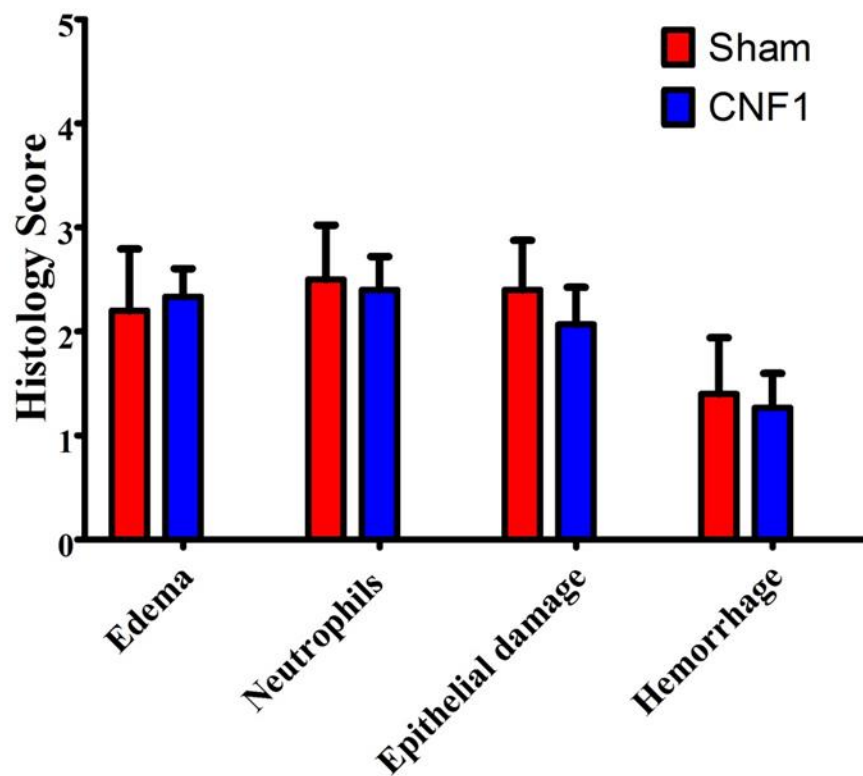
Figure 27. Levels of CP9 in urine samples and kidneys and histology severity score for bladders of mice in the second CNF1 toxoid vaccine trial.

A. Bacteria enumerated from the urine and kidney of vaccinated (circles) and sham-vaccinated mice (squares). Each symbol represents one mouse, and the bar is the geometric mean for each group. Data were analyzed by the two-tailed Student t-test. There were no significant differences in urine bacterial counts in CNF1-vaccinated versus sham-vaccinated mice. There were also no significant differences in kidney colonization in CNF1-vaccinated versus sham-vaccinated mice.

B. Average histology scores between sham-vaccinated (red) mice and CNF1-vaccinated (blue). The bars represent the mean histology score for each group, and the error bar is the standard error of the mean. Data were analyzed by the two way ANOVA with the Bonferroni multiple comparison post-test. There were no significant differences in histology severity scores in any parameter of cystitis between CNF1-vaccinated and sham-vaccinated mice.



B.



In summary, we found that, in both vaccine trials, vaccination with CNF1 toxoid/CNF1 did not affect the urine bacterial counts between CNF1-vaccinated or sham-vaccinated mice. There were also no differences in kidney colonization between CNF1-vaccinated or sham-vaccinated mice. We next evaluated the degree of cystitis by histopathology.

Histopathologic evaluation of the urinary bladders of mice vaccinated against CNF1

At necropsy, urinary bladders were collected, fixed, processed into stained tissue sections on glass slides, and graded by me in a blinded fashion as described in the HlyA vaccine experiments. In the first trial (Figure 26), there were no statistically significant differences in histology severity scores between CNF1-vaccinated and sham-vaccinated mice. In addition, the mean severity score for each parameter of cystitis was 2 or below, a value that corresponds to mild cystitis. In the subsequent trial (Figure 27), there were again no statistically significant differences in severity scores between CNF1-vaccinated and sham-vaccinated mice. In this experiment, the mean severity scores were 3 and below, values that correspond to moderate cystitis. Thus, there were no statistically significant differences in the degree of cystitis between CNF1-vaccinated or sham-vaccinated mice in either trial. We next sought to determine whether cystitis could be reduced by passively immunizing mice with a neutralizing anti-CNF1 monoclonal antibody.

Passive immunization of mice with anti-CNF1 monoclonal antibody NG8

To assess whether neutralizing antibodies against CNF1 if given in high enough concentrations could reduce the severity of cystitis caused by CP9, we conducted passive immunization studies with the anti-CNF1 monoclonal antibody NG8. This antibody can neutralize CNF1 in in-vitro assays (72; 132). In multinucleation neutralization assays, we

found that 40 ng of NG8 reduced multinucleation of HEp-2 cells intoxicated with 2.5 ng of CNF1 by 50 percent (not shown). To determine an effective in vivo dose of NG8, we first conducted a pilot study in which we passively immunized mice with two doses of 10 µg or 50 µg of NG8 by the intraperitoneal route at 24 hr and again at 4 hr prior to challenge with CP9. We then did additional experiments in which we immunized mice with two doses of 100 µg of NG8 administered by the intraperitoneal route according to the same schedule (Figure 10).

CP9 colonization of mice following challenge

As in the previous experiments, mice were sacrificed 24 hours after CP9 challenge and urine and kidneys were collected for bacterial enumeration. In the pilot study, there were no statistically significant differences among the NG8-immunized mice, the isotype (IgG2a), and the PBS control groups in urine bacterial counts. There were also no statistically significant differences in levels of CP9 in the kidney (Figure 28). Similarly, in the subsequent trials in which two 100 µg doses of NG8 were administered to mice before CP9 infection, there were no statistically significant differences among NG8-immunized and the two control groups of mice in urine bacterial counts or in levels of CP9 in the kidneys (Figure 29). In summary, in all the passive experiments, there were no statistically significant differences in levels of CP9 in the urine of passively immunized mice compared to the control groups. There were also no statistically significant differences in levels of CP9 in the kidney among the study groups. We next evaluated the degree of cystitis by histopathology.

Figure 28. Levels of CP9 in urine samples and kidneys from mice passively immunized with two different doses of NG8.

Bacteria enumerated from the urine and kidney of mice passively immunized with 10 μ g of NG8 (circles), 50 μ g of NG8 (squares), the isotype control, 50 μ g of IgG2a, (triangle), or with PBS (downward triangle). Each symbol represents one mouse, and the bar is the geometric mean for each group (n=5 for each group). Data were analyzed by the two way ANOVA with the Bonferroni multiple comparison post-test. There were no significant differences in urine bacterial counts (Panel A) in mice passively immunized with NG8 versus the two control groups. There were also no significant differences in kidney colonization levels (Panel B) in mice passively immunized with NG8 versus the two control groups.

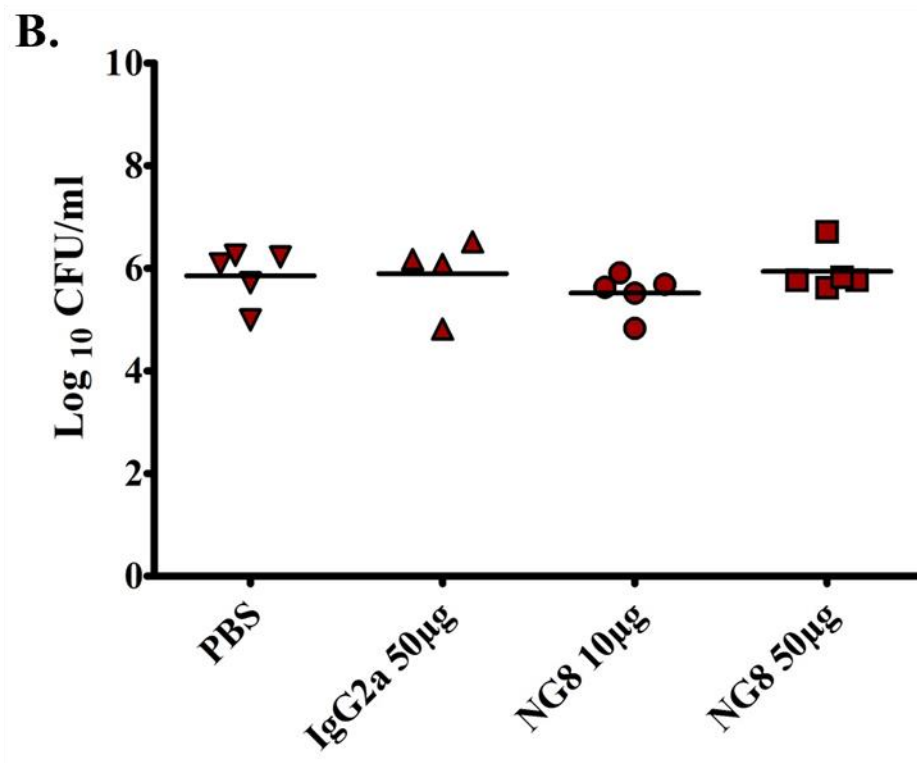
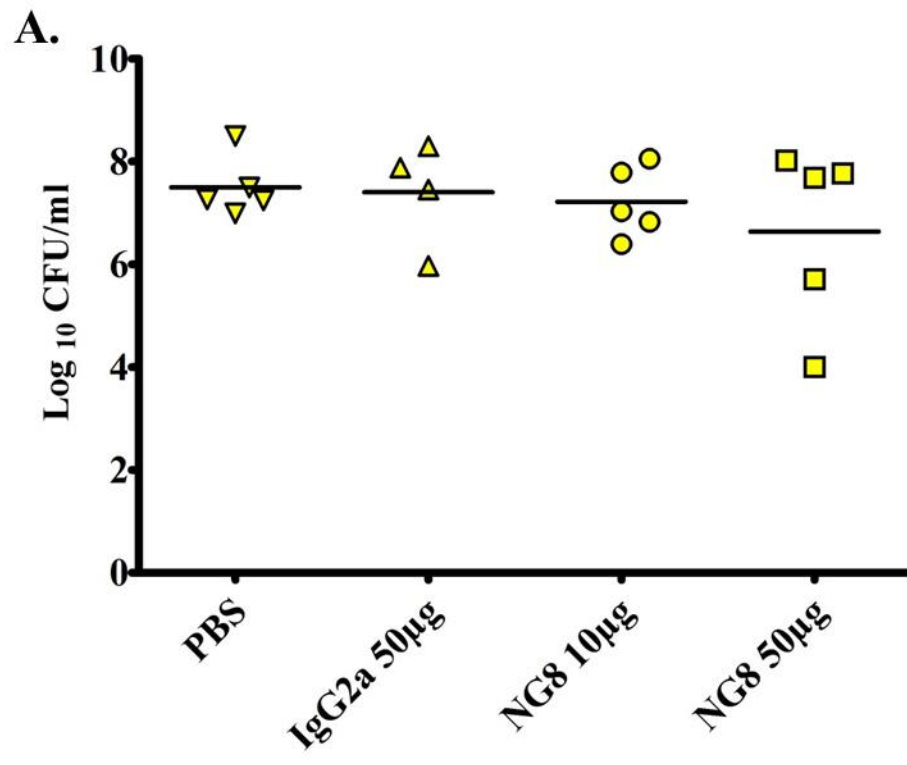
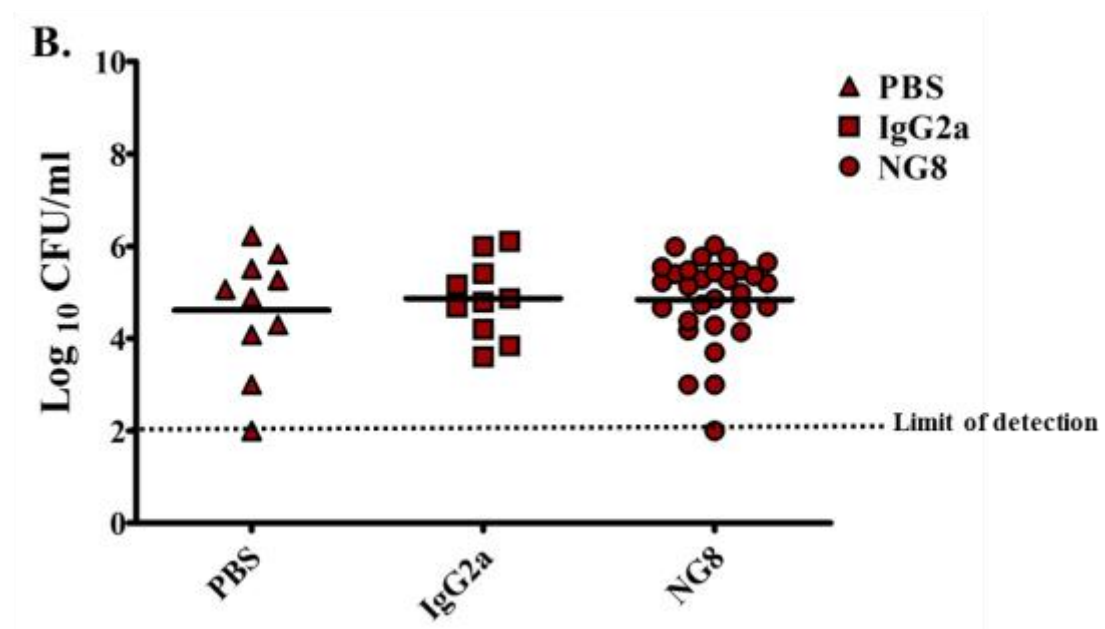
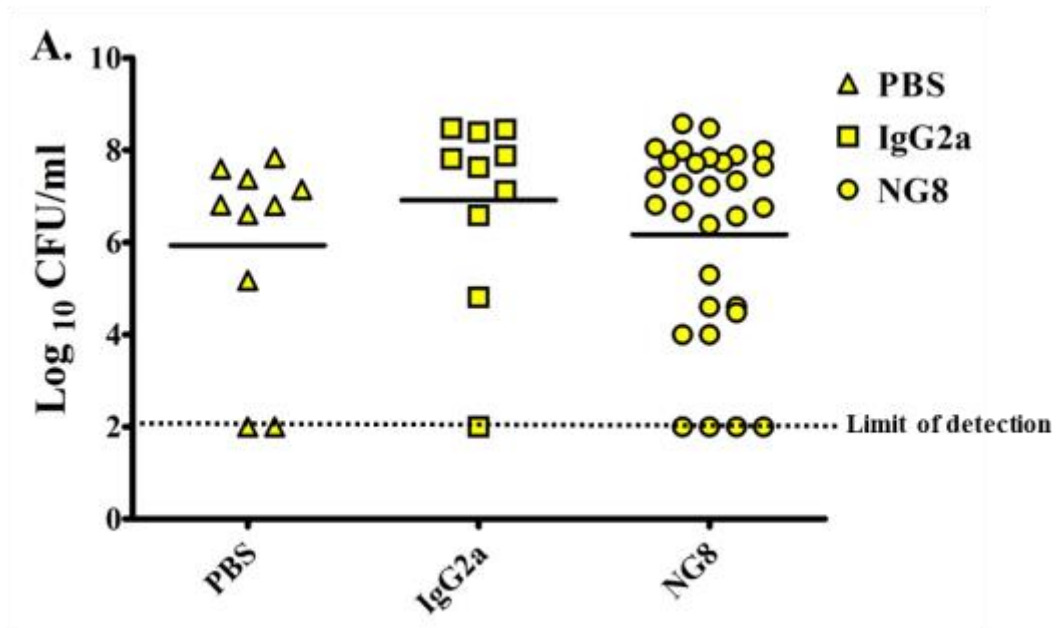


Figure 29. Levels of CP9 in urine samples and kidneys from mice passively immunized with two 100µg doses of NG8.

Bacteria enumerated from the urine and kidney of mice passively immunized with 100 µg of NG8 (circles), the isotype control, 100 µg of IgG2a (squares), or with PBS (triangle). Each symbol represents one mouse, and the bar is the geometric mean for each group (NG8 group n=30, IgG2a group n=10, PBS group n=10). Data were analyzed by the two way ANOVA with the Bonferroni multiple comparison post-test. There were no significant differences in urine bacterial counts (Panel A) in mice passively immunized with NG8 versus the two control groups. There were also no significant differences in kidney colonization levels (Panel B) in mice passively immunized with NG8 versus the two control groups.



Histopathologic evaluation of the urinary bladders of passively immunized mice

At necropsy, urinary bladders were collected, fixed, processed into stained tissue sections on glass slides, and graded by me in a blinded fashion as described for the previous experiments. In the pilot study (Figure 30), mice that were immunized with two 50 µg doses of NG8 demonstrated lower average severity scores in the parameters of edema and hemorrhage when compared to the isotype and PBS control groups, but these differences were not statistically significant. There were no other statistically significant differences in the histology severity score among the NG8-immunized mice and the control groups. In the subsequent studies in which mice were immunized with two 100 µg doses of NG8 (Figure 31), there were statistically significant reductions in all four parameters of cystitis in the immunized mice as compared to both the isotype and PBS control groups. Thus, we found that passive immunization with two doses of 50 µg of NG8 resulted in a trend toward reduced cystitis, whereas, passive immunization with two doses of 100 µg of NG8 resulted in a statistically significant reduction in cystitis in two trials. Finally, we sought to validate the use of urine bacterial counts as a surrogate for urinary bladder colonization in immunized mice.

Validation that CFU/ml in urine can serve as a surrogate for bladder colonization levels in vaccinated mice

In previous studies, CP9 and isogenic CP9 mutants that lack *hlyA₁* and a complete *cnfI* gene all demonstrated the capacity to colonize the urinary bladder and kidneys at equivalent levels (173; 204). Moreover, the organisms were shed in the urine 24 hr after inoculation at levels similar to those found in the bladder. In the current immunization studies, all urinary bladders were used for histologic evaluation and were not available

Figure 30. Histology severity score for bladders of mice from mice passively immunized with different doses of NG8.

Average histology scores among mice passively immunized with 10 μ g of NG8 (light blue), 50 μ g of NG8 (dark blue), the isotype control, 50 μ g of IgG2a, (red), or with PBS (purple). The bars represent the mean histology score for each group, and the error bar is the standard error of the mean. Data were analyzed by the two way ANOVA with the Bonferroni multiple comparison post-test. There were no significant differences in histology scores among any group. However, the mice immunized with 50 μ g of NG8 demonstrated less pathology in the parameters of edema and hemorrhage as compared to the control groups.

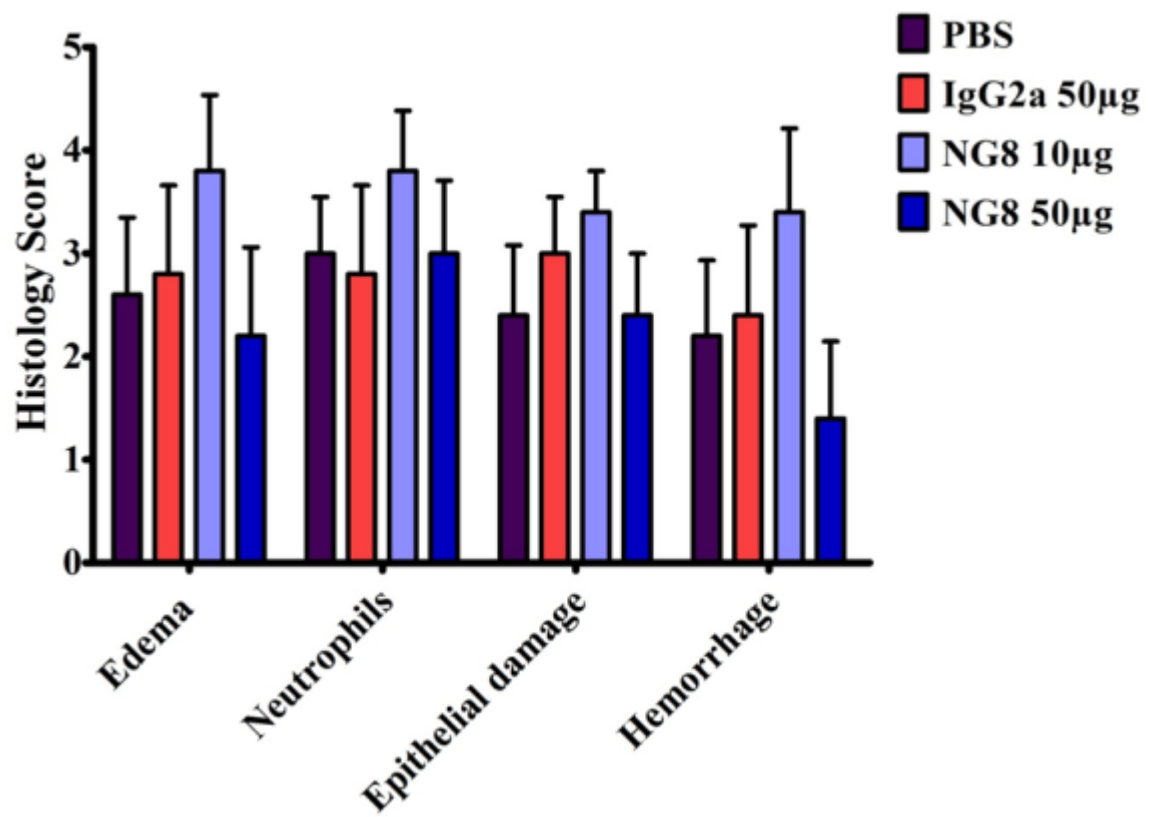
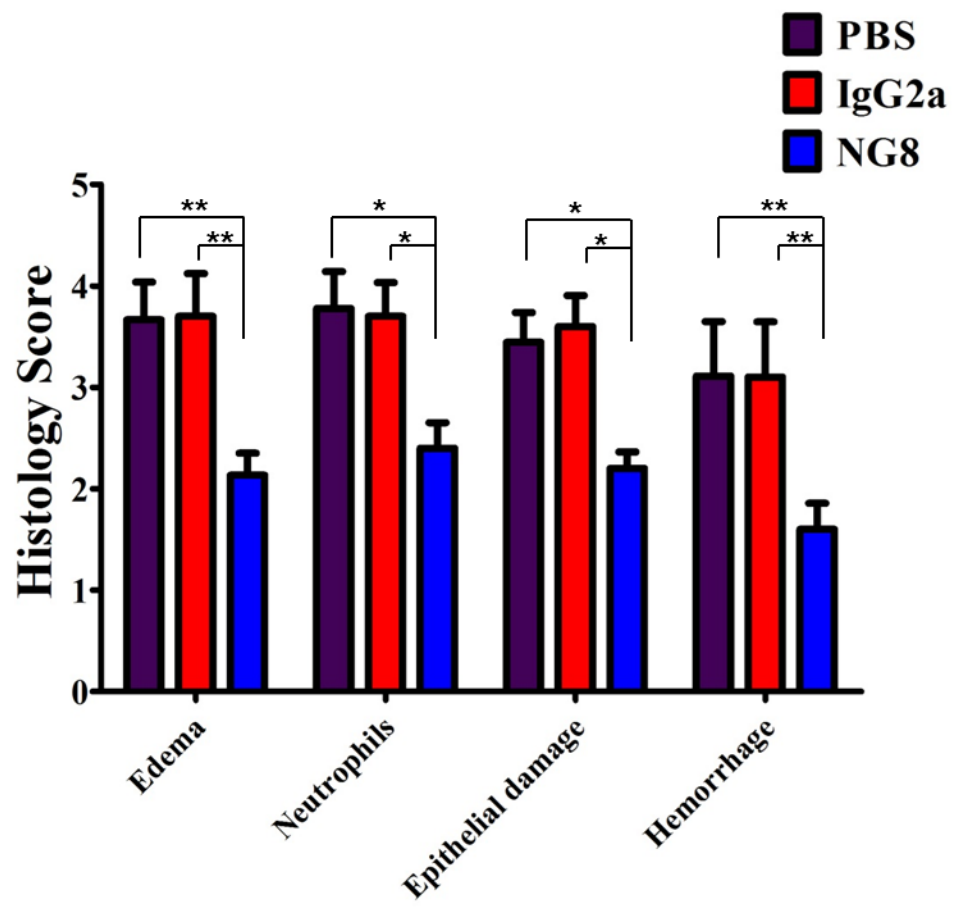


Figure 31. Histology severity score for bladders of mice from mice passively immunized with two 100µg doses of NG8.

Average histology scores between mice passively immunized with 100 µg of NG8 (blue) and 2 control groups, the isotype control, 100 µg of IgG2a, (red), and PBS (purple) (NG8 group n=30, IgG2a group n=10, PBS group n=10). The bars represent the mean histology score for each group, and the error bar is one standard error of the mean. Data were analyzed by the two way ANOVA with the Bonferroni multiple comparison post-test. The pathology was significantly less severe (**, $P=0.01$; *, $P=0.05$) in the NG8-immunized group in all four parameters of cystitis when compared to the control groups.



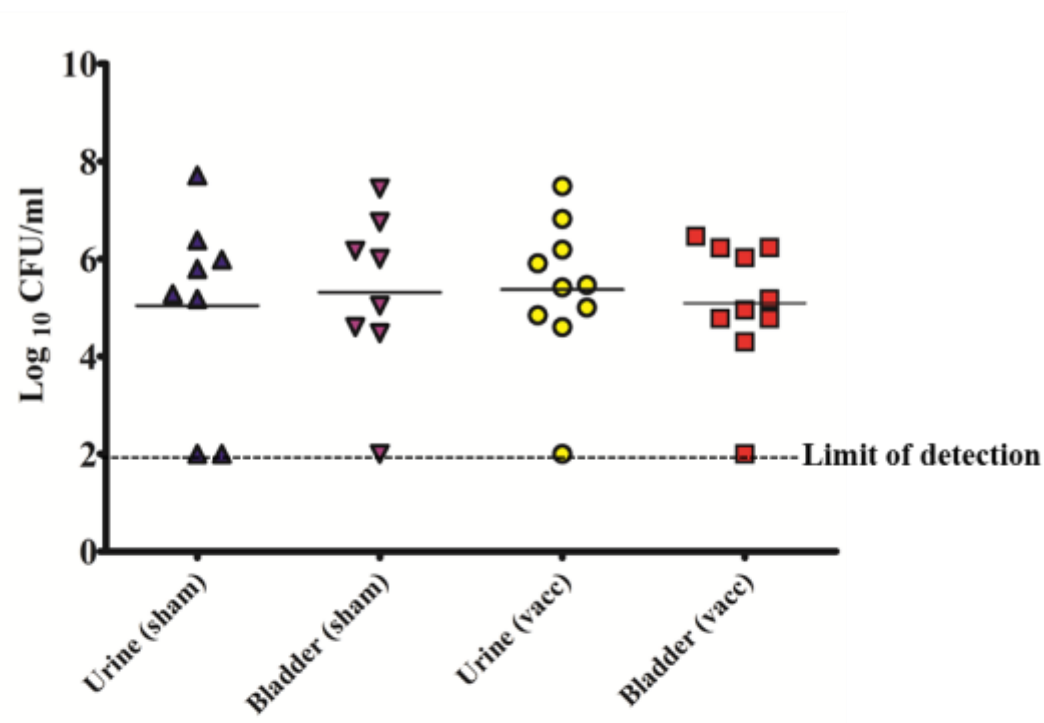
for determination of colonization. To evaluate whether the quantification of bacteria in the urine could serve as a proxy for bladder colonization in immunized mice as it could in non-immune animals, an experiment was done to determine whether the quantity of bacteria in the urine of vaccinated mice challenged with CP9 was comparable to colonization levels in the urinary bladder. For that purpose, mice were vaccinated with either the HlyA toxoid (n=10) or CNF1 (n=8) toxoid/toxin according to our established protocols (as in trials 2 or 3 for the HlyA toxoid or trial 2 for CNF1 toxoid/toxin; see Figure 10), then challenged with CP9. Urinary bladders and urine were collected for bacterial enumeration. We found no statistically significant differences between colonization levels of CP9 in the bladders and levels of bacteria in the urine of mice that were vaccinated with either toxoid. There were also no statistically significant differences in colonization levels in the urine or in the bladders between vaccinated and sham-vaccinated mice (Figure 32). Thus vaccination did not impact urine bacterial counts, and the urine counts were proportional to CP9 counts in the bladder. Therefore, we concluded that urine bacterial counts are valid surrogates for bladder bacterial counts as applied to both naïve and toxoid vaccinated mice in this study.

Figure 32. Comparison between levels of CP9 in the bladder and the urine in toxoid-vaccinated mice and between toxoid-vaccinated and sham-vaccinated mice.

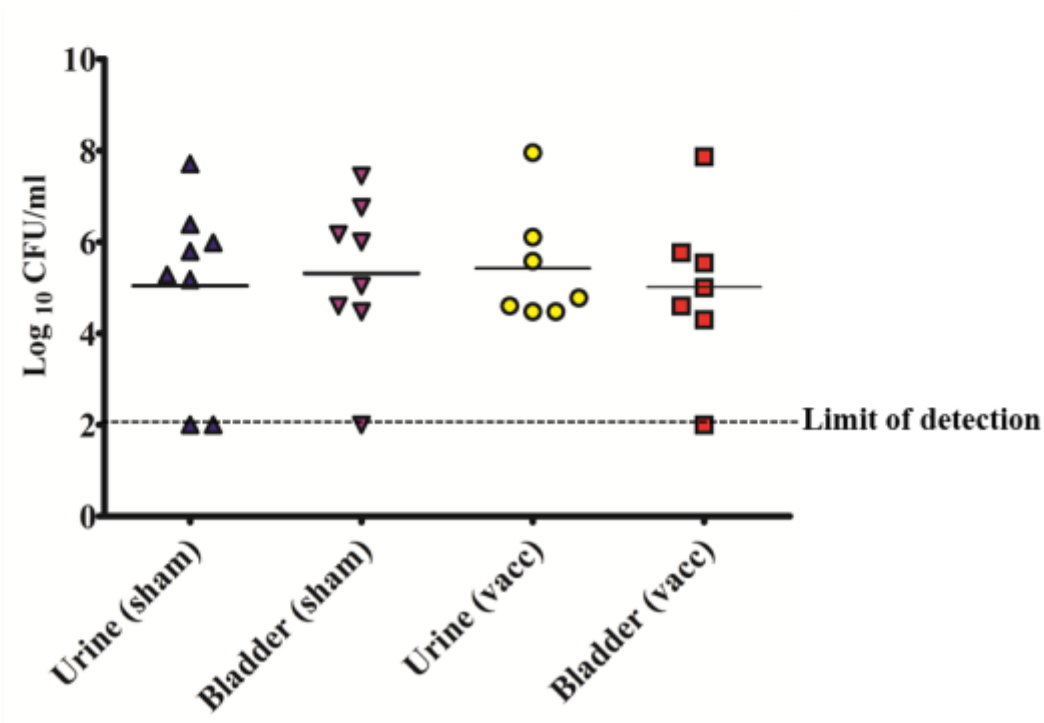
A. Bacteria enumerated from the urine and urinary bladder of HlyA-vaccinated and sham-vaccinated mice. Each symbol represents one mouse, and the bar is the geometric mean for each group. Data were analyzed by the two-tailed Student t-test. There was no significant difference between urine bacterial counts (yellow circles) and bladder colonization levels (red squares) in HlyA-vaccinated mice. There was also no significant difference between urine bacterial counts in HlyA-vaccinated mice (yellow circles) compared to sham-vaccinated mice (blue triangles) or bladder colonization levels in HlyA-vaccinated mice (red squares) compared to sham-vaccinated mice (purple inverted triangles).

B. Bacteria enumerated from the urine and urinary bladder of CNF1-vaccinated and sham-vaccinated mice. Each symbol represents one mouse, and the bar is the geometric mean for each group. Data were analyzed by the two-tailed Student t-test. There was no significant difference between urine bacterial counts (yellow circles) and bladder colonization levels (red squares) in CNF1-vaccinated mice. There was also no significant difference between urine bacterial counts in CNF1-vaccinated mice (yellow circles) compared to sham-vaccinated mice (blue triangles) or bladder colonization levels in CNF1-vaccinated mice (red squares) compared to sham-vaccinated mice (purple inverted triangles).

A.



B.



CHAPTER 4: Discussion

SUMMARY OF MAJOR FINDINGS

In this study, we demonstrated that neutralizing antibodies directed against HlyA or CNF1 reduced the severity of cystitis following challenge with UPEC strain CP9. C3H/HeOuJ mice that were vaccinated against HlyA toxoid or CNF1 toxoid/CNF1 developed a 10,000-fold or 1,000-fold increase in anti-toxin serum IgG titers as assessed by ELISA, respectively. Moreover, the antibody response against each toxin was capable of neutralizing that toxin as measured by in vitro assays. The antibody response, whether elicited by active vaccination CNF1 toxoid or passive immunization with anti-CNF1 monoclonal antibody NG8, did not affect colonization of the urinary tract. However, active vaccination with the HlyA toxoid resulted in lower levels of colonization of the urinary bladder, but this finding was not statistically significant in all trials. Both active vaccination against HlyA and passive immunization with NG8 resulted in a significant reduction in the severity of cystitis. However, active immunization against CNF1 did not reduce the severity of cystitis.

VACCINATION AGAINST HLYA

O'Hanley, et al. demonstrated that vaccination of BALB/c mice with a denatured recombinant hemolysin had no effect on colonization of the kidneys but did result in less severe pyelonephritis than in control mice upon challenge with UPEC strain J96 (159). Moriel et al. showed that vaccination of CD1 mice with recombinant hemolysin protected mice challenged with UPEC strains in a sepsis model (137). Our study is the first, to our knowledge, to evaluate the effects of vaccination with a hemolysin toxoid on acute cystitis in a mouse model. In the first vaccine trial, C3H/HeOuJ mice that were actively

vaccinated with HlyA toxoid developed a neutralizing antibody response that resulted in a reduction in cystitis, albeit only significant in the degree of neutrophilic infiltrate. With the addition of a third boost in the second trial, mice again developed a neutralizing antibody response; however, there was a significant reduction in severity in all the parameters of acute cystitis following challenge. These results were repeated in a third trial. While the addition of the third boost in the second and third trial resulted in less severe cystitis, we did not demonstrate quantifiable differences in the capacity of serum antibodies to neutralize hemolysin by in vitro assay between the first and the second two trials. The more effective response in these trials was likely due to either a higher titer in the lumen of urinary bladder itself and/or more functional antibodies (neutralizing) that we could not quantify by our assay method. Smith et al. showed that bladder infection with a CP9 mutant that lacked the *hlyA₁* gene resulted in less severe cystitis that was statistically significant only in the parameters of hemorrhage and epithelial damage (204). We now know that UPEC strain CP9 contains three HlyA operons but HlyA₁ is responsible for >90% of the hemolytic activity associated with the strain [Weingarten, et al., manuscript in preparation]. Our study showed that an anti-HlyA antibody response due to vaccination resulted in a greater reduction in cystitis than was demonstrated by Smith et al. in bladders infected with the CP9 Δ *hlyA₁* mutant. This difference in the two studies may reflect the fact that antibodies generated against the HlyA toxoid (created from the *hlyA₃* gene) are cross reactive against HlyA₁, the specific hemolysin that is responsible for >90% of the hemolytic activity of CP9 (204) and possibly HlyA₂. Conversely, HlyA₂ and HlyA₃ can still be produced by a CP9 Δ *hlyA₁*. A further indication of cross reactivity is that the sera from mice vaccinated with the HlyA toxoid

could neutralize the native hemolysins in the CP9 supernatant, in which the hemolytic activity was previously demonstrated to be predominantly caused by HlyA1 (204).

In all three HlyA toxoid vaccine trials, the urinary bladders of mice were colonized to lower levels by CP9 in HlyA-vaccinated mice compared to the sham-vaccinated animals, though the level was only statistically significantly lower in the first trial. This trend suggests that anti-HlyA antibodies in the bladder may interfere with some aspect of CP9 colonization. One explanation for this observation may be that hemolysin destroys some of the uroepithelium which, in turn, permits infection of the next layer of cells in the bladder. Perhaps anti-HlyA antibodies block the damage and limit the site of colonization to the first layer of cells. Exploration of this idea or other scenarios to explain these findings represent a possible future direction of this study.

IMMUNIZATION AGAINST CNF1

This is the first report of the use of a CNF1 toxoid as a therapeutic vaccination for UTI. Munroe, et al. published studies in which CNF1 was used as an adjuvant and reported no significant antibody response to CNF1 (141; 142). We surmise that they used an amount of CNF1 for their purposes as an adjuvant (1 µg or 10 µg delivered intranasally) that was below the immunogenic threshold because we found that mice vaccinated with CNF1 toxoid with the addition of toxin developed a 1000-fold rise in serum anti-CNF1 antibody titer. Moreover, the sera from these vaccinated mice were capable of neutralizing CNF1 when evaluated with an in-vitro assay. However, this rise in titer was almost a log lower than the rise in the anti-HlyA titer in the HlyA toxoid vaccine trials. Furthermore, vaccination with the CNF1 toxoid even in combination with a sublethal dose active CNF1 did not result in a reduction of cystitis after challenge with

CP9. Yet, mice passively immunized with two 100 µg doses of the CNF1-neutralizing monoclonal antibody (NG8) did exhibit a less severe cystitis when challenged with CP9 than did mice that were given the control antibody. This finding demonstrates the potential therapeutic role for antibodies directed against CNF1 in reduction of UTI inflammation. One possible reason that active vaccination did not reduce the severity of cystitis is that there were inadequate titers of neutralizing anti-CNF1 antibodies in the urinary tract of the actively vaccinated mice at the time of intraurethral challenge with CP9. Anti-CNF1 antibodies were present in the urine of actively immunized mice but to a lesser extent than found in serum, as estimated by Western blot analyses. We were not able to determine whether the urine antibodies were capable of neutralizing CNF1 because urine itself was toxic to the cells (HEp-2) used in the neutralization of multinucleation assay. We did not evaluate the levels of antibody in the serum or urine in the passive study, but all the NG8 that was present was likely capable of neutralizing CNF1. Another explanation as to why active vaccination with CNF1 toxoid was ineffective at reducing cystitis caused by CP9 as compared to HlyA toxoid vaccination is that CNF1 may be less accessible to antibodies in the urinary bladder than is HlyA. CNF1 is a cytoplasmic protein that is exported to host cells on outer membrane vesicles by an as yet unknown mechanism (40; 115). CNF1 may be partially shielded from antibodies in the urinary bladder when on the surface of these vesicles or when retained intracytoplasmically in the bacterium. Hemolysin may be more accessible to urine antibodies because it is a secreted toxin. Higher titers of neutralizing anti-CNF1 antibodies may need to be present in the bladder to inactivate CNF1 than HlyA; such high levels of neutralizing antibodies may only have been achieved by administration of

concentrated NG8 anti-CNF1 monoclonal antibody. In spite of the problems with the active CNF1 vaccine trial, immunization against either HlyA or CNF1 could play a role in a UPEC vaccine as envisioned below.

HLyA AND CNF1 IN FUTURE UPEC VACCINES

In this study, we demonstrated that antibodies raised against HlyA or CNF1, either by active or passive immunization can reduce the severity of inflammation during acute cystitis following challenge with a UPEC strain. Inflammation is the most likely cause of the clinical signs of UTI, including painful urination (dysuria), increased frequency urinate, and the presence of pus (pyuria), and blood (hematuria) in the urine. A vaccine that reduces inflammation and the related pain and discomfort associated with uncomplicated UTIs might also reduce the number of doctor visits and lost work time and productivity, which are estimated to have an annual cost in the billions and affect over 7 million women (63; 73). Since 80 percent of uncomplicated UTIs and half of complicated UTIs are caused by UPEC and up to 60% of UPEC produce hemolysin and up to 45% produce CNF1 of which 90 percent coexpress hemolysin, immunization against these toxins could have a significant impact (43; 118; 196). In addition, passive NG8 therapy (or more likely a human anti-CNF1 neutralizing monoclonal antibody) could be useful in hospitalized patients, especially in conjunction with urinary catheterization. However, in this study, vaccination against either of these toxins did not significantly reduce colonization of the urinary tract and therefore did not completely prevent cystitis, an ideal goal for a UTI vaccine. Considering that the murine subjects vaccinated with HlyA toxoid demonstrated a trend toward reduced colonization of the urinary bladder, further optimization of the vaccine protocol may result in greater

reductions in cystitis and colonization. Future research should focus on optimization of the vaccine protocol, but should also seek to better understand the mechanisms of protective immunity in the urinary bladder.

FUTURE DIRECTIONS OF RESEARCH INTO CNF1 AND HLYA TOXOID VACCINATION

To increase efficacy of the toxoid vaccines, the titer of functional anti-toxin antibodies in the lumen of the urinary bladder should be increased. However, the mechanism of antibody translocation from the serum into the urine or from local sites of production is not understood at this time. In passive immunization studies, as in the anti-CNF1 monoclonal antibody (NG8) experiment in this report and in previously reported studies (214), one can speculate that antibodies delivered to the peritoneum of mice can diffuse into the bloodstream and then entered the urine in the kidney. However, there are typically low concentrations of antibodies in the urine of healthy individuals. Nevertheless, Johansson et al. demonstrated that antibody titers against recombinant cholera toxin in tissue (lung, vagina, and small intestine) of mucosally vaccinated mice were higher than the corresponding serum titers and that tissue titers were low in passively immunized mice (94). These results suggested to those authors that the antibodies in tissue were predominantly produced at the site (94). The study described here did not evaluate the antibody responses to toxin in different parts of the urinary tract. An examination of the kinetics of the antibody response in the kidney and urinary bladder after vaccination with toxoids might help direct the design of future immunization protocols. We also did not measure serum or secretory IgA responses to the toxins. It is possible that that antibody class may be of more importance in protecting against toxins in the bladder than are IgG anti-toxin antibodies. Nevertheless, we still contend that the

most critical aspect of the protective immune response against HlyA and CNF1 is the presence of neutralizing antibodies of sufficient titer at the site of infection regardless of the antibody class or subclass.

Other areas of future investigation as related to optimization of immunization with HlyA and CNF1 toxoids are to try alternative routes of vaccination and adjuvants. In both the HlyA and CNF1 toxoid trials, the antigen was delivered subcutaneously and with the adjuvant Titermax gold® in the prime vaccination and the first boost in the HlyA trials and the first vaccination in the CNF1 trial (Figure 10). The use of this route and adjuvant led to the generation of a strong serum and lesser urine antibody response. A potential modification to the vaccine protocols could be to target local production of antibodies by mucosal immunization. The mucosal routes of immunization routes could include intranasal, sublingual, or intravaginal. Studies in mice, primates, and humans showed multi-fold titer elevation in the urine after administration of subunit or whole cell UPEC vaccines with varying degrees of protection by the intranasal (28; 188), sublingual (124), and vaginal (218; 219) routes. A change in route would necessitate a change in adjuvant. Cholera toxin has been used successfully to raise urine antibody titers in mucosal UTI vaccines in mice and humans (28; 181; 188).

Another modification to each toxoid vaccine protocol could be to evaluate cystitis at a later time point after CP9 challenge. The protocols for the HlyA toxoid and the CNF1 toxoid/toxin vaccination schemes evaluated efficacy in reducing inflammation in acute cystitis. Mice were sacrificed 24 hours after challenge with CP9. Vaccination with the HlyA toxoid had little effect and CNF1 toxoid/toxin had no effect on colonization at this time point. However, differences in bacterial clearance due to the presence of

neutralizing antibody may be demonstrated if the mice were evaluated days after challenge.

Another area of study could be to combine the toxoids into a multivalent UPEC vaccine, if active vaccination with CNF1 toxoid/toxin can be improved so as to achieve protection against cystitis severity. One advantage to such a vaccine is that CNF1 and HlyA are both virulence factors and a protective response would spare untoward effects on commensal strains. Additional UPEC virulence factors could also be included as vaccine components. Recently, Brumbraugh et al. used a bioinformatics technique to identify potential UPEC virulence factors that could be used as vaccine antigens without affecting commensal strains (28). They identified an iron receptor, FyuA, that when used as an immunogen reduced colonization in the kidney of BALB/c mice (28). As UTI vaccine research continues, other virulence-associated factors may be identified that could be incorporated into a multi-component UPEC vaccine.

Beyond UTIs, the CNF1 toxoids could play a role in vaccines directed against pathogenic intestinal and other extraintestinal *E. coli* in humans and animals (169). CNF1 was originally obtained from pathogenic *E. coli* strains from children with enteritis. CNF1 has also been discovered to contribute to invasion of the central nervous system through the blood brain barrier in neonatal meningitis (108; 227). Hemolysin is a member of a family of the RTX family of toxins, some which could also be created in toxoid versions to reduce associated morbidity (117). One such member, the leukotoxin (LtxA) of *Aggregatibacter actinomycetemcomitans*, is associated with periodontal disease (LAP, localized aggressive periodontitis) in humans (101). A toxoid version of LtxA, delivered orally, may be effective as a vaccine for LAP.

In this study, we demonstrated the principle that antibodies directed against HlyA by active vaccination or CNF1 by passive immunization can reduce the severity of acute cystitis caused by a UPEC strain. With further optimization of vaccination protocols, these toxoids may be used as a component of a multivalent vaccine to prevent UTI infection and/or reduce signs and symptoms caused by UPEC strains. Ultimately, a UTI vaccine may be given to individuals who suffer from repeated bladder infections that impact the quality of their lives.

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