Antibodies against Hemolysin and Cytotoxic Necrotizing Factor Type 1 (CNF1) Reduce Bladder Inflammation in a Mouse Model of Urinary Tract Infection with Toxigenic Uropathogenic *Escherichia coli*

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Uropathogenic *Escherichia coli* (UPEC) is the leading cause of cystitis. Cytotoxic necrotizing factor 1 (CNF1) and hemolysin (Hly) are toxins made by approximately 50% of UPEC isolates. CNF1 and Hly contribute to the robust inflammatory response in the bladders of mice challenged with UPEC strain CP9. We hypothesized that antibodies against CNF1 and/or Hly would reduce cystitis caused by CP9. To test this theory, we immunized female C3H/HeOuJ mice subcutaneously with a genetically derived Hly toxoid or genetically derived CNF1 toxoid plus sublethal doses of CNF1. We collected serum and observed increasing titers of specific and neutralizing antibodies against Hly or CNF1 over time. We challenged the mice intraurethrally with CP9 and euthanized them 24 h later. We observed 10-fold lower bacterial titers in the urine of Hly-immunized mice than in that of sham-immunized mice but no difference in kidney bacterial titers. Immunized mice also exhibited significantly less cystitis than sham-immunized mice. In CNF1-vaccinated mice, we detected neither a difference in urine or kidney bacterial titers nor a reduction in the severity of cystitis versus that of sham-immunized mice. We then passively administered an anti-CNf1 monoclonal antibody intraperitoneally to female C3H/HeOuJ mice prior to intraurethral challenge with CP9. Upon challenge, we noted no difference in colonization of the urine or kidney; however, cystitis was reduced significantly in mice treated with the anti-CNf1 antibody versus that in the bladders of mice given an isotype control antibody. Taken together, our data demonstrate that antibodies against CNF1 or Hly reduce the bladder pathology caused by UPEC.

Escherichia coli is responsible for 70 to 80% of uncomplicated urinary tract infections (UTIs), which most commonly occur in women (1). Indeed, in 2007, UTIs accounted for 10.5 million ambulatory patient visits (2). The causal *E. coli* strains are called uropathogenic *E. coli* (UPEC), and they express an array of virulence factors that enable them to colonize the urinary tract, invade urothelial cells, and overcome host defenses. These microbial products include specialized adhesive structures (pili and fimbriae) and toxins (3–6). One such toxin, hemolysin (Hly), is expressed by 60% of UPEC strains, while another UPEC toxin, cytotoxic necrotizing factor 1 (CNF1), is made by about 40% of such isolates (7–9). The genes that encode these toxins and other virulence factors are often found clustered on pathogenicity islands (10).

E. coli Hly can form pores in the membranes of a variety of mammalian cell types with subsequent lysis of these cells (11–13). Even at sublytic concentrations, Hly can modulate host cell signaling pathways, modify the host immune response, and cause cell death (14). An operon composed of four genes, *hlyCABD*, is required for the production and secretion of active Hly (15, 16). The *hlyA* gene encodes a 107-kDa heat-labile inactive Hly precursor or protoxin. HlyC is an acyltransferase that activates Hly by fatty acid acylation of two lysine residues at positions 564 and 690 of HlyA (17). The *hlyB* and *hlyD* genes encode inner membrane proteins that are required for Hly secretion by the type I secretion mechanism (18–20). HlyB is an ATP-binding cassette protein that interacts with HlyD, a membrane fusion protein (21). The HlyBD complex interacts with TolC, an outer membrane transport protein that is not specific to Hly export (22, 23). *E. coli* Hly is the prototype for a family of lytic toxins expressed by certain Gram-negative bacteria; these toxins are classified as RTX toxins (for

repeats in toxin) to reflect the conserved calcium binding domain repeats present in these proteins (14).

E. coli CNF1, CNF2, and CNF3 and *Yersinia pseudotuberculosis* CNF_Y are members of the cytotoxic necrotizing factor family of toxins (24–26). They are 110- to 115-kDa proteins that have a conserved catalytic domain composed of a triad of histidine, cysteine, and valine. The CNF toxins deamidate a glutamine residue on the small Rho family GTPases RhoA, Rac1, and Cdc42 (glutamine at position 63 of RhoA or position 61 of Rac and Cdc42) (25, 27–29). Rho family GTPases act as molecular switches in signal transduction pathways that involve components of the cytoskeleton such as actin, myosin, and microtubules (30). Consequently, Rho family GTPases are involved in cell shape, motility,

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or purpose	Reference
UPEC strains		
CP9	O4:H5:K54 <i>hlyA₁ hlyA₂ hlyA₃ cnf1</i>	42
KG68	CP9Δ <i>hlyA₁::cat hlyA₂ hlyA₃ cnf1</i>	54
KG68::pKG76	CP9Δ <i>hlyA₁::cat hlyA₂ hlyA₃ cnf1</i> /pKG76	This study
KG68::pSDBM	CP9Δ <i>hlyA₁::cat hlyA₂ hlyA₃ cnf1</i> /pSDBM	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 Hte</i> [F' <i>proAB lac^qZΔM15 Tn10</i> (Tet ^r) Amy Cam ^r]	Stratagene
M15/pREP4	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
M15/pREP4/pCNF24	CNF1 expression strain	55
M15/pREP4/pCNF24 C866S	CNF1 toxoid expression strain	This study
KG76	HlyA3 expression strain	This study
M15/pREP4/pSDBM	HlyA3 toxoid expression strain	This study
Plasmids		
pQE-30	Amp ^r <i>E. coli</i> expression vector with N-terminal RGS-6XHis	Qiagen
pCNF24	pQE-30::BamHI <i>cnf1</i> KpnI	46
pCNF24C866S	pQE-30::BamHI <i>cnf1</i> KpnI C866S (CNF1 toxoid)	This study
pKG76	pQE-30::BamHI <i>hlyA₃</i> KpnI	This study
pSDBM	pQE-30::BamHI <i>hlyA₃</i> KpnI K564E K690E (HlyA toxoid)	This study

formation of adhesion complexes, endocytosis, cell cycle progression, vesicle trafficking, and apoptosis (25, 30). Deamidation results in constitutive activation of the GTPase with subsequent downstream derangement of the cellular processes listed above. The ultimate phenotype in a cell depends on the cell type and which GTPase is affected. HEp-2 (laryngeal epithelial) cell culture intoxication with CNF1 causes actin stress fiber formation, formation of lamellipodia and filopodia, and multinucleation; CNF1 also induces apoptosis in urothelial cells (31–33). CNF1 is delivered to host cells via outer membrane vesicles (34). However, the mechanism of CNF translocation to the outer membrane has not yet been determined.

Both Hly and CNF1 play a role in inflammation, as demonstrated in mouse and rat models of UTI. Female mice experimentally infected intraurethrally with UPEC isolate CP9 develop cystitis and pyelonephritis (35, 36); purified recombinant CNF1 administered in the same manner also elicits cystitis (37). Moreover, mice infected with isogenic mutants of CP9 that lack *cnf1*, *hlyA₁*, or both genes exhibit significantly less severe cystitis than mice infected with wild-type CP9 (35, 36). Rats experimentally infected with an isogenic UPEC strain that lacks *cnf1* develop less severe prostatitis (38). In all of these studies, wild-type UPEC strain CP9 and its isogenic mutants colonized the urinary tract equivalently. These experiments demonstrate that CNF1 can directly cause inflammation of the bladder and that the lack of CNF1 or Hly production in a UPEC strain attenuates cystitis. Given that up to 60% of all UPEC strains produce Hly and up to 40% express CNF1 (of which 90% also produce Hly), these toxins may be good targets for therapeutic intervention (8–10, 39).

In this study, we sought to determine whether anti-Hly or anti-CNF1 antibodies could reduce the severity of cystitis caused by a toxigenic UPEC strain. On the basis of previous studies of CNF1 and Hly in rodent models, an antibody response directed against either CNF1 or Hly toxin could, in principle, neutralize the available toxin and potentially reduce the severity of UPEC-associated cystitis. In addition, an antibody response directed against viru-

lence factors should not interfere with the functions of commensal *E. coli* strains. Previous studies with anti-Hly vaccines in mouse models demonstrated a reduced mortality rate in a sepsis model and decreased severity of pyelonephritis (40, 41). Here we show that mice actively vaccinated with a recombinant HlyA toxoid or passively immunized with a neutralizing anti-CNF1 monoclonal antibody demonstrated a significant reduction in the severity of cystitis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. CP9 (O4:H5:K54), our wild-type UPEC strain, was isolated from the blood of a patient with bacteremia at the National Institutes of Health (42). CP9 carries the genes required to produce type I pili and CNF1, as well as at least three Hly operons. (Note that the operon that encodes HlyA1 is responsible for >90% of the hemolytic activity of CP9 [35; R. A. Weingarten, C. L. Ventura, and A. D. O'Brien, unpublished data]). Our laboratory previously generated isogenic CP9 strains in which *cnf1* is partially deleted (CP9 *cnf1*) or *hlyA₁* is completely deleted (KG68, referred to here as CP9Δ*hlyA*) (36, 43). Here, each gene (*cnf1*, *hlyA₁*, *hlyA₂*, or *hlyA₃*) was cloned into pQE-30 for complementation and activity studies and for protein expression and purification (35, 36). Plasmid pQE-30 confers ampicillin (Amp) resistance, adds an RGS-6-histidine tag to the amino terminus of the expressed protein, and contains an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T5 promoter. 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 μg/ml Amp. Media for all M15/pREP4 strains were supplemented with 25 μg/ml kanamycin (Kan) to maintain pREP4 and 100 μg/ml Amp to maintain pQE-30. Liquid cultures were inoculated from fresh colonies grown on LB agar and cultivated at 37°C with aeration. For CP9 challenge inocula, liquid cultures were grown without aeration at 37°C for 48 h. After 48 h, the CP9 cultures were harvested by centrifugation (10,000 × g for 10 min at 4°C) and then resuspended in phosphate-buffered saline (PBS) at a concentration of 10⁹ CFU/ml.

Generation of toxoid-expressing clones. Plasmid *phlyA₃* was isolated from KG76 with the QIAprep Spin Miniprep kit (Qiagen) according to

the manufacturer's protocol. The QuikChange Lightning Multi site-directed mutagenesis (SDM) kit (Stratagene, La Jolla, CA), which exploits mutagenic oligonucleotide primers to generate mutations by PCR amplification, was used to generate four mutations in *hlyA*₃ in *phlyA*₃. The targets for SDM were two lysine residues (K564 and K690) that are acylated by HlyC to activate the HlyA protoxin (17), and they were changed to glutamic acid in consecutive mutagenesis reactions with the following mutagenic primers (Integrated DNA Technologies, Coralville, IA). The K564E primer that starts at nucleotide 1669 in the *hlyA*₃ sequence was 5'-CGTGAAAGGAGGCAGTCCGGAGAGTATGAATATATTACCGAGTTAT-3', and the K690E primer was 5'-GAGCAGGAGGTTTCAGTCGAGAGAGAACTGAAAAACGCAA-3' (the mutations are underlined). In brief, long PCR was done with *phlyA*₃ as the template, the two mutagenic primers, and a master mixture that included *Pfu* Fusion DNA polymerase, buffer, and a deoxynucleotide triphosphate mixture. The mixture was cycled in a thermal cycler as follows: 95°C for 2 min; 30 cycles of 95°C for 20 s, 55°C for 30 s, and 65°C for 3 min and 21 s; and a final 65°C extension for 5 min. Similarly, the CNF1 toxoid-encoding plasmid was generated by SDM (QuikChange SDM kit; Stratagene) of pCNF24 (pQE-30::cnf1) to change the catalytic cysteine residue at position 866 to serine with the following primers: forward, 5'-GGAAATCTAAGTGGTTCTACGACAATTGTTGCCCG-3'; reverse, 5'-CGGGCAACAATTGTCGTAGAACCACTTAGATTTC-3' (29). For both protocols, the mutagenesis reaction mixture was treated with DpnI to digest the template DNA. The resultant mutated single-stranded DNA was chemically transformed into *E. coli* XL-10 competent cells, and cells were plated on LB-Amp after 1 h of recovery in the absence of antibiotic. Amp-resistant transformants were screened for the appropriate mutations by sequencing of the target sites. Once the K564E and K690E mutations were confirmed, the entire mutated *hlyA*₃ and *cnf1* genes were 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 sequences were confirmed to be correct, the plasmids were renamed pSDMB (*phlyA*₃ K564E K690E) and pCNF24 C866S. For purification of the HlyA₃ toxoid (here referred to as HlyA toxoid) and the CNF1 toxoid, the mutated plasmids were transformed into chemically competent *E. coli* M15/pREP4 cells, and one Amp- and Kan-resistant colony was selected for further evaluation.

Purification of HlyA toxoid under denaturing conditions. An overnight culture of *E. coli* M15/pREP4/pSDMB was diluted 1:50 into 500 ml of fresh LB-Amp-Kan and grown at 30°C for 2 h 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 $8,000 \times g$ for 15 min at 4°C. Since preliminary studies indicated that denaturing conditions were optimal for HlyA toxoid purification, the cells were resuspended and lysed by sonication in an alkaline phosphate buffer (20 mM phosphate buffer, pH 8.0 [1.8 ml 1 M Na₂HPO₄, 0.2 ml 1 M NaH₂PO₄ per liter], 1 mM phenylmethylsulfonylfluoride [PMSF], 8 M urea). The insoluble portion of the lysate was pelleted by centrifugation at $15,000 \times g$ for 15 min at 4°C, and the supernatant was filtered. The HlyA toxoid was purified by nickel affinity chromatography and stored at -80°C. The concentration of the toxoid was determined by bicinchoninic acid assay (Thermo Scientific) according to the manufacturer's protocol.

Purification of CNF1 toxin and CNF1 toxoid under native conditions. CNF1 from *E. coli* M15/pREP4/pCNF24 and CNF1 toxoid from *E. coli* M15/pREP4/pCNF24 C866S were expressed and purified according to previously described procedures (33, 44), with the following modifications. Protein expression was induced at 30°C instead of 37°C. For nickel affinity chromatography, the proteins were eluted with phosphate buffer (20 mM phosphate buffer [pH 7.4], 0.5 M NaCl, 1 mM phenylmethylsulfonyl fluoride) that contained 50 mM imidazole instead of 250 mM imidazole.

Evaluation of purified proteins. The purified proteins were evaluated for homogeneity by sodium dodecyl sulfate-polyacrylamide gel electro-

phoresis (SDS-PAGE) and Western blot assay. Samples of each protein preparation were denatured in Laemmli sample buffer and separated by denaturing SDS-7.5% PAGE (Bio-Rad Laboratories, Inc., Hercules, CA). Proteins were either stained with Oriole fluorescent gel stain (Bio-Rad) for 30 min at room temperature or transferred to nitrocellulose membranes with the iBlot Dry blotting system (Life Technologies, Carlsbad, CA). The primary antibody was a mouse anti-RGS-His antibody (Qiagen) diluted 1:2,000. The secondary antibody was goat anti-mouse IgG-Alexa Fluor 532 (Life Technologies) diluted 1:15,000. Both the Oriole-stained gel and the Western blot assay were imaged with the ImageQuant LAS 4000 imager (GE Healthcare Life Sciences).

Hemolysis assay. To confirm that pSDMB encodes an inactive toxoid, *phlyA* and pSDMB were expressed in CP9Δ*hlyA* because the entire *hlyCABD* operon is required for activation and secretion. Wild-type strain CP9 was used as a positive control. Overnight bacterial cultures were diluted 1:50 in fresh LB (with Amp for plasmid maintenance as needed) and grown at 37°C for 4 h with aeration. The bacteria were pelleted by centrifugation at $16,100 \times g$ for 5 min, 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, 10% Ca-saline [0.9% NaCl, 10 mM CaCl₂]) were incubated for 1 h at 37°C. Water and culture supernatants from CP9 and CP9Δ*hlyA*(*phlyA*) served as positive controls. Ca-saline and LB served as negative controls. Intact erythrocytes were pelleted at $5,000 \times g$ for 5 min. The supernatants were transferred to a 96-well microtiter plate, and the intensity of the red color imparted by hemoglobin released from lysed erythrocytes was determined by measurement of absorbance at 562 nm with a BioTek ELx800 microplate spectrophotometer (BioTek, Winooski, VT). Absorbance values were normalized by subtracting the average values of the negative-control samples (LB and Ca-saline) from each sample's absorbance.

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 (36) and was modified as follows. HEP-2 cells were cultured in complete Eagle's minimal essential medium (cEMEM; EMEM with Earle's balanced salt solution [Lonza, Allendale, NJ], 10% fetal bovine serum [Mediatech, Inc., Manassas, VA], 2 mM L-glutamine [Life Technologies], 10 μg/ml gentamicin [Quality Biological, Gaithersburg, MD], 10 U/ml penicillin, and 10 μg/ml streptomycin [American Type Culture Collection, Manassas, VA]) at 37°C in a humidified 5% CO₂ environment. Microtiter plates were seeded with 1×10^3 rather than 4×10^3 HEP-2 cells. Twofold serial dilutions of CNF1 or CNF1 toxoid in cEMEM beginning at a concentration of 1 μg of CNF1 or 10 μg of CNF1 toxoid per well were added to individual wells; each sample was assayed in duplicate. Negative-control wells were treated with cEMEM only. The plates were incubated for 72 h at 37°C in 5% CO₂, and then the cells were fixed, stained with Hema-3 stain (Thermo Fisher Scientific), and 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 Imaging Solutions, Sterling Heights, MI). The percentage of multinucleated cells was calculated by enumeration of the cells that contained two or more nuclei per 100 cells in each digital image.

CNF1 LD₅₀ determination. To establish the 50% lethal dose (LD₅₀) of CNF1, we obtained 4- to 5-week-old female C3H/HeOuJ mice (Jackson Laboratory, Bar Harbor, ME) and allowed them to acclimate to the animal housing environment for 1 week. We administered purified CNF1 diluted in sterile saline by subcutaneous (s.c.) injection to groups of five mice at doses of 10, 7, 5, 4, 3, 2, and 1 μg. The mice were observed multiple times daily for 1 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 by inhalational isoflurane overdose and humanely euthanized by cervical

FIG 1 Active and passive vaccine schedules. Vaccine schedules for active HlyA toxoid (Txd) (A), active CNF1 toxoid and toxin (B), and passive NG8 anti-CNf1 (C) vaccination trials. In the active vaccine trials, the prime (P) vaccine was given on day 0 and the boosts (B) were given at 21-day intervals. Mice were challenged (C) with CP9 2 weeks after the last boost, and mice were euthanized and subjected to necropsy (N) 24 h later. For the passive vaccine trials, equivalent doses (D) of NG8, an irrelevant isotype control antibody (IgG2a), or PBS were delivered by i.p. injection 24 and 4 h prior to challenge (C), and then mice were euthanized and necropsied (N) 24 h later. Adj, adjuvant.

dislocation. The LD₅₀ was determined to be 3.2 µg by the Reed-Muench calculation method (45); we did not use probit analysis because we did not have groups that satisfied the requirements.

Mouse model of ascending UTI. All animal procedures were conducted in accordance with reference 43 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 was used (37).

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

Vaccination procedures. (i) **HlyA toxoid immunization protocol** (Fig. 1A). Groups of 15 female C3H/HeOuJ mice were vaccinated by s.c. injection of 25 µg of HlyA toxoid diluted in sterile PBS and emulsified 1:1 (vol/vol) with the adjuvant TiterMax Gold (TiterMax USA, Norcross, GA). The prime vaccination was followed by two or three boosts at 3-week intervals; the first boost contained adjuvant, while the subsequent boosts did not. Groups of 10 mice were sham vaccinated with sterile PBS in the same manner. Blood was collected from each animal by tail venipuncture prior to the first immunization and 1 week before each boost. Each blood sample was allowed to clot at 4°C for 60 min, and then the cells were pelleted by centrifugation at 8,000 × g for 10

min. Serum was recovered and tested for the presence (enzyme-linked immunosorbent assay [ELISA]) and neutralizing capacity (hemolysis neutralization assay) of anti-HlyA antibodies (see below). Once the majority of the mice had neutralizing serum antibodies, they were inoculated intraurethrally with CP9 for 24 h as described above.

(ii) **CNF1 vaccination procedure** (Fig. 1B). Groups of 15 female C3H/HeOuJ mice were vaccinated by s.c. injection of 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 3-week intervals. In the first trial, the first boost had the same composition as the prime vaccination while the second boost consisted of only 250 ng of CNF1. In the second trial, mice were boosted three times with a mixture of 25 µg of CNF1 toxoid and 400 ng of CNF1 (without adjuvant). Previous work by Meysick et al. in our laboratory showed that the use of sublethal doses of CNF1 as immunogens leads to the production of CNF1-neutralizing monoclonal antibodies (46). Groups of 10 control mice were sham vaccinated with PBS. In all of the trials, blood was collected from each mouse and processed to serum before the prime vaccination and 1 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 h as described above.

Passive immunization of mice with mouse anti-CNf1 monoclonal antibody NG8. Anti-CNf1 monoclonal antibody NG8 is a mouse IgG2a antibody that neutralizes CNF1 (44, 46). To test the capacity of NG8 to reduce the bladder pathology associated with CNF1 during UPEC infection, we administered 100 µg of NG8 in sterile saline to 15 mice via intraperitoneal (i.p.) injection. Five mice were given 100 µg of an irrelevant isotype (IgG2a) control antibody (Southern Biotech, Birmingham, AL), and five mice were given sterile PBS (Fig. 1C). For all of the groups, the antibody or PBS was given at 24 and 4 h prior to an intraurethral challenge with CP9.

Assessment of antibody responses. (i) **ELISAs.** Microtiter plates were coated with 100 ng of HlyA toxoid or CNF1 diluted in PBS. Individual or pooled mouse serum or urine samples were serially diluted 10-fold in 1% bovine serum albumin (BSA) in PBS (BSA-PBS). The sample dilutions were applied to the wells in duplicate. The secondary antibody, goat anti-mouse IgG-horseradish peroxidase (Bio-Rad Laboratories), was diluted 1:5,000 in BSA-PBS and incubated for 1 h at room temperature. The substrate 3,3',5,5'-tetramethylbenzidine (Bio-Rad Laboratories) was added to each well, and the reaction was stopped with 1 N sulfuric acid. The absorbance at 450 nm of each well was measured in an ELx800 Microplate Spectrophotometer (BioTek). The positive control was the mouse monoclonal anti-RGS-His antibody (Qiagen), and the negative control was the secondary antibody alone (no primary antibody). The final absorbance of 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 to assess the specific antibody titer.

(ii) **Western blot assays.** Western blot analysis of purified HlyA toxoid or CNF1 was done as described above, with either pooled serum from vaccinated or sham-vaccinated mice diluted 1:500 or pooled urine diluted 1:50 in blocking buffer as the primary antibody and goat anti-mouse IgG-Alexa Fluor 532 (Life Technologies) diluted 1:15,000 in blocking buffer as the secondary antibody.

(iii) **Neutralization assays.** Serum samples from HlyA toxoid-immunized or sham-immunized mice were diluted 1:100 and then serially 2-fold in LB. The diluted serum was mixed 1:4 with CP9 culture supernatant (source of HlyA) and incubated for 2 h at 4°C. The serum-supernatant mixtures were incubated with sheep erythrocytes as described above for the hemolysis assay. The degree of neutralization of native Hly by serum antibodies of vaccinated mice was determined by a graphical comparison of absorbance values of CP9 supernatant-

FIG 2 CP9-mediated colonization and pathology of the urinary tract are the same in young and aged mice. CP9 titers in the kidneys (A), urinary bladders (B), and urine (C) of aged (16- to 18-week-old) mice 24 h after intraurethral inoculation with CP9. Each symbol represents the number of CFU/ml for one mouse, and the bars represent the geometric mean titers of that group of animals. The dotted line is the limit of detection. Data were analyzed by the two-tailed Student *t* test. (D) Mean severity scores for edema, neutrophilic infiltrate, epithelial degeneration and necrosis (damage), and hemorrhage of groups of mice inoculated with 2.5×10^7 CFU (blue), 5×10^7 CFU (red), or PBS (green). Data were analyzed by two-way ANOVA with the Bonferroni multiple-comparison posttest. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.1$. (E) Images of representative H&E-stained urinary bladders from mice in each treatment group. No pathological lesions were evident in the bladders from the PBS-inoculated mouse. Moderate-to-severe cystitis with edema can be seen in the mice inoculated with both doses of CP9. The stars indicate areas of edema, the circles show dense aggregates of neutrophils, and the arrows point to areas of epithelial damage. Magnification, $\times 40$.

treated and vaccinated or sham-vaccinated serum with that of CP9 supernatant alone.

To assess the neutralizing capacity of antiserum from CNF1 toxoid-vaccinated mice, we used a modification of the CNF1-mediated HEP-2 cell multinucleation assay described above. Mouse serum was diluted 1:9 and then serially diluted 5-fold in cMEM. Diluted serum was incubated with 2.5 ng of CNF1, the lowest concentration found to elicit 100% multinucleation of HEP-2 cells, for 2 h 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 serum from vaccinated or sham-vaccinated mice with that in wells that contained cMEM and CNF1 only.

Statistical analyses. Statistical analyses were done with GraphPad Prism v5.01 for Windows (GraphPad Software, La Jolla, CA). Analysis of variance (ANOVA) with the Bonferroni multiple-comparison posttest was used to assess differences in urinary bladder pathology scores between vaccinated and sham-vaccinated animals. Repeated-measures ANOVA with the Bonferroni multiple-comparison posttest was used to assess the statistical significance of differences between the neutralization assay results of the groups. The Student *t* test was used to determine significant differences between geometric mean CP9 titers in the urine and kidneys of vaccinated and sham-vaccinated mice and to assess the statistical significance of differences between the mean absorbance values of individual dilutions in ELISAs of serum from vaccinated and sham-vaccinated mice.

FIG 3 CP9 titers in the urine and urinary bladders of toxoid- and sham-vaccinated (vacc) mice are equivalent. Bacteria were enumerated in the urine and urinary bladders of HlyA-vaccinated and sham-vaccinated mice (A) and of CNF1 toxoid- and toxin-vaccinated and sham-vaccinated mice (B). Each symbol represents one mouse, and the bar is the geometric mean value of each group. The dotted line is the limit of detection. Data were analyzed by the two-tailed Student *t* test.

RESULTS

Aged mice are susceptible to CP9 bladder infection. The major objective of this study was to determine whether immunization with HlyA or CNF1 toxoid could reduce the degree of inflammation in the bladders of mice challenged intraurethrally with UPEC strain CP9, which makes both Hly and CNF1. Since immunized C3H/HeOuJ mice would be 10 weeks older than the 6- to 8-week-old mice used in our previous CP9 infection studies (35–37), we first determined the impact of age on susceptibility to UPEC infection. For that purpose, we evaluated 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 to determine if a larger inoculum is required to establish cystitis in larger mice. A control group was inoculated with PBS to control for potential effects related to catheterization. We observed no difference in the CP9 titers in the kidneys (Fig. 2A), urinary bladders (Fig. 2B), or urine samples (Fig. 2C) of mice in either infection group 24 h after inoculation. Both challenge groups exhibited moderate-to-severe suppurative cystitis with submucosal edema, hemorrhage, and epithelial damage, while there was minimal pathology in the bladders of mice given PBS (Fig. 2D and E). The degree to which the aged mice were colonized by CP9 and the severity of cystitis were comparable to the results of previous studies with 6- to 8-week-old mice (35–37). We concluded from this study that any reduced cystitis noted in vaccinated mice would not be due to a decreased susceptibility of aged mice.

The number of CFU/ml urine can serve as a surrogate for bladder CP9 titers in vaccinated mice. In previous studies, CP9 and isogenic CP9 mutants that lack *hlyA*₁ and/or *cnf1* colonized the urinary bladder and kidneys at equivalent levels (35, 36). Moreover, the organisms were shed in the urine 24 h after inoculation at levels similar to those found in the bladder. In the present immunization studies, all urinary bladders were used for histologic evaluation and were therefore not available for bacterial enumeration. To evaluate whether the urine titer could serve as a proxy for bladder colonization in immunized mice as it could in nonimmune animals, we vaccinated mice with either the HlyA

FIG 4 Active vaccination with HlyA toxoid elicits specific and neutralizing antibodies against HlyA. (A) HlyA toxoid was separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with pooled serum or urine collected from HlyA toxoid-vaccinated ($n = 15$) or sham-vaccinated ($n = 10$) mice at necropsy. (B) The presence of anti-Hly antibodies in preimmune serum ($n = 15$) and terminal serum from vaccinated mice ($n = 10$) was evaluated by ELISA with HlyA toxoid as the capture antigen. Data points represent the mean absorbance value per group, and the bars indicate the standard error of the mean. The dotted line is the limit of detection. (C) The capacity of terminal serum to neutralize Hly activity in CP9 supernatants was measured in an Hly neutralization assay. Each data point represents the mean absorbance value per group, and the error bar is the standard error of the mean. Data were analyzed by the two-tailed Student *t* test. *, $P \leq 0.0001$ at all serum dilutions.

toxoid ($n = 10$) or CNF1 ($n = 8$) toxoid/toxin according to our established protocols (as in trial 2 or 3 for the HlyA toxoid and trial 2 for CNF1 toxoid and toxin; Fig. 1A and B) and then challenged the mice intraurethrally with CP9 on day 77. Urinary bladders and urine samples were collected and processed for bacterial enumeration. We found no differences in CP9 titers between the bladders and urine samples of mice either vaccinated with toxoid or sham vaccinated with PBS (Fig. 3). Thus, vaccination did not impact urine bacterial counts and the urine and bladder bacterial counts were similar to one another. Therefore, we concluded that urine bacterial counts are valid surrogates for bladder bacterial counts for both naive and toxoid-vaccinated mice.

Active vaccination with HlyA toxoid reduces urinary bladder pathology associated with UPEC Hly. We immunized groups of 15 female C3H/HeOuJ mice with either the HlyA toxoid or PBS according to the schedule in Fig. 1A. To monitor the generation of specific antibody responses against HlyA toxoid for each vaccine trial, we analyzed serum samples obtained from mice after toxoid boosts for the presence of anti-HlyA antibodies by

FIG 5 Active vaccination with HlyA toxoid reduces urine and bladder colonization and cystitis severity. The bacteria in the urine and kidneys of vaccinated (circles) and sham-vaccinated (squares) mice from HlyA toxoid vaccine trial 1 (A) and from trials 2 and 3 (B) were enumerated. Each symbol represents one mouse, and the bar is the geometric mean value of a group. The dotted line is the limit of detection. Data were analyzed by the two-tailed Student *t* test. The average urinary bladder histology scores of vaccinated (blue) and sham-vaccinated (red) mice in trial 1 (C) and in trials 2 and 3 (D) were compared. Each bar represents the mean histology score of a group, and the error bar shows the standard error of the mean. Data were analyzed by the two-way ANOVA with the Bonferroni multiple-comparison posttest. *, $P = 0.01$; **, $P = 0.001$.

Western blotting and/or ELISA. By Western blotting, serum and urine collected from HlyA toxoid-vaccinated mice at necropsy in the second vaccine trial showed strong immunoreactivity against Hly with pooled serum and to a lesser extent with pooled urine, while serum and urine samples from sham-vaccinated mice did not react with Hly (Fig. 4A); the results of this trial are representative of the results of all three trials. We used an ELISA in which HlyA toxoid served as the capture antigen to quantify the antibody response against HlyA. Serum samples collected from vaccinated mice on the day of euthanasia demonstrated 10,000-fold higher anti-Hly IgG titers, on average, than those obtained prior to HlyA toxoid vaccination (here called preimmune serum) or serum from sham-vaccinated mice (Fig. 4B). Thus, vaccinated mice developed a specific anti-HlyA toxoid antibody response in all of the trials.

We next evaluated the capacity of these anti-HlyA antibodies to neutralize Hly. For that purpose, we preincubated serial dilutions of serum from vaccinated or sham-vaccinated mice with native Hly present in CP9 culture supernatants and then added these samples to rabbit erythrocytes. In all of the HlyA toxoid vaccine trials, we found that serum from either HlyA-vaccinated or sham-vaccinated mice reduced erythrocyte lysis. However, the hemolytic activity returned to native levels as the dilution of serum from sham-vaccinated mice approached 1:100, while the serum from HlyA toxoid-vaccinated mice continued to neutralize hemolysis beyond a 1:400 dilution (Fig. 4C). At every dilution, sera from vaccinated mice reduced the

hemolytic activity significantly more than did sera from sham-vaccinated mice ($P \leq 0.0001$) (Fig. 4C). We surmised that mice had some prior exposure to Hly and/or that mouse serum contains proteins that can inhibit the activity of Hly. Indeed, Bhakdi et al. found that human serum albumin, low-density lipoprotein, and high-density lipoprotein could protect erythrocytes from purified-Hly-mediated lysis in an *in vitro* assay (47). Nevertheless, we concluded that vaccination of mice with HlyA toxoid elicited a robust serum anti-HlyA antibody titer that neutralized native Hly to a greater extent than did serum from sham-vaccinated mice.

We challenged vaccinated mice with CP9 21 days after the final HlyA toxoid boost. In the first trial, mice received a total of three vaccinations, the first two with adjuvant (the protocol used is described in Fig. 1A). The CP9 titers in the kidneys of vaccinated and sham-vaccinated mice were similar, but the CP9 titers in the urine of vaccinated mice were 1.1 log lower ($P = 0.01$) than those of sham-vaccinated mice (Fig. 5A). In the second and third trials, in which we added a third boost to the vaccine protocol (Fig. 1A), the HlyA toxoid-vaccinated mice exhibited a nearly 10-fold reduction in the mean urine CP9 titer, but the difference was not statistically significant (Fig. 5B). The CP9 titers in the kidneys of both groups of mice were similar in the latter trials as well (Fig. 5B). In summary, vaccination with HlyA toxoid resulted in lower bacterial counts in urine but no differences in kidney bacterial titers.

We also evaluated the degree of cystitis by histopathologic

analysis. At necropsy, urinary bladders were collected, fixed in formalin, processed into tissue sections on glass slides, and stained with H&E. A veterinary pathologist (M. A. Smith) evaluated the slides in a blinded manner and assigned severity scores of 0 to 5 for each of four parameters of cystitis (edema, neutrophilic infiltrate, epithelial damage, and hemorrhage). In the first trial, we observed a statistically significant ($P = 0.01$) reduction in the neutrophilic infiltrate. However, there was less severe pathology in the other three parameters of cystitis, but these reductions were not statistically significant (Fig. 5C). In the second and third trials, in which a third boost was added to the vaccine protocol (Fig. 1A), we saw a statistically significant reduction in all four parameters of cystitis ($P = 0.001$ for edema, neutrophils, and hemorrhage; $P = 0.01$ for epithelial damage) in vaccinated animals (Fig. 5D). Thus, vaccination with HlyA toxoid reduced the cystitis severity in mice subsequently challenged with UPEC strain CP9 in all three trials.

Active vaccination with CNF1 toxoid and CNF1 toxin does not affect urinary bladder pathology associated with UPEC CNF1. We next evaluated the CNF toxin as a vaccine candidate to reduce the severity of CP9 cystitis in mice. In a preliminary study that used a vaccination schedule similar to that used in the first HlyA toxoid vaccine trial (Fig. 1A), we found that the CNF1 toxoid alone did not elicit a neutralizing anti-CNF1 antibody response (data not shown). Thus, we included a sublethal dose of active CNF1 toxin to increase the potential for the generation of neutralizing anti-CNF1 antibodies. As with the HlyA vaccine studies mentioned above, we analyzed serum samples drawn from mice 14 to 20 days after each vaccination for the presence of anti-CNF1 antibodies by Western blotting and ELISA. We observed strong immunoreactivity in the terminal sera from CNF1 toxoid- and toxin-vaccinated mice and no reactivity in the sera from sham-vaccinated mice (Fig. 6A). By ELISA, in which CNF1 toxin served as the capture antigen, we found that terminal sera from vaccinated mice had 1,000-fold higher anti-CNF1 IgG levels than sera from sham-vaccinated mice (Fig. 6B). Thus, vaccination with CNF1 toxoid and toxin elicited an anti-CNF1 antibody response. We next asked whether these serum antibodies had the capacity to neutralize CNF1-mediated HEP-2 cell multinucleation. We preincubated serial dilutions of serum samples from CNF1 toxoid- and toxin-vaccinated or sham-vaccinated mice with CNF1 and then overlaid the samples on HEP-2 cells in a multinucleation assay and determined the percentage of cells that were multinucleated 72 h later. In each CNF1 toxoid and toxin vaccine trial, we found that sera from CNF1-vaccinated mice neutralized the capacity of CNF1 to elicit HEP-2 cell multinucleation at dilutions of 1:50 ($\leq 10\%$ multinucleation) and 1:250 ($< 50\%$ multinucleation), while sera from sham-vaccinated mice did not neutralize CNF1 activity (100% multinucleation) (Fig. 6C). Thus, we surmised that vaccination with CNF1 toxoid and toxin evoked a specific antibody response that neutralized the capacity of CNF1 to cause multinucleation *in vitro*.

Once the mice exhibited a robust neutralizing anti-CNF1 response, we challenged them intraurethrally with CP9. In the first trial, we inoculated mice 14 days after the final boost with a sublethal dose of CNF1 toxin. In the second trial, the boosts contained both CNF1 toxoid and sublethal CNF1 toxin (Fig. 1B), and the mice were challenged 14 days after the third boost. In all of the trials, we euthanized the mice 24 h after a CP9 challenge and collected urine and the left kidney for bacterial enumeration.

FIG 6 Active vaccination with CNF1 toxoid and toxin elicits specific and neutralizing antibodies against CNF1. (A) Western blot assay of CNF1 probed with pooled mouse serum collected on day 65 of trial 1 (CNF1 vaccinated [vacc], $n = 15$; sham vaccinated, $n = 10$). (B) Individual serum samples from vaccinated and sham-vaccinated mice collected on day 65 were compared by ELISA with CNF1 as the capture antigen (CNF1 vaccinated, $n = 15$; sham vaccinated, $n = 10$). Data points represent the mean absorbance values of duplicate samples. The dotted line is the limit of detection. (C) Neutralization of CNF1-mediated multinucleation of HEP-2 cells by serum from mice vaccinated with CNF1 toxoid and toxin (trial 2). Each data point represents the mean percentage of HEP-2 cells in a sample well that were multinucleated after treatment with CNF1-vaccinated mouse serum collected on day 58 of the first vaccine trial study (CNF1 vaccinated, $n = 15$; sham vaccinated, $n = 10$). The bars represent 1 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 two-way ANOVA with the Bonferroni multiple-comparison posttest. For all panels, the data shown are from a single trial that is representative of both trials. **, $P < 0.001$; *, $P = 0.01$.

There were no statistically significant differences between the CP9 titers in the urine or kidneys of CNF1 toxoid- and toxin-vaccinated and sham-vaccinated mice (Fig. 7A and B).

At necropsy, we collected, fixed, and stained the urinary bladders and then assessed the cystitis parameters in a blinded fashion as described above. We observed no statistically significant differences between the histology severity scores of CNF1 toxoid- and toxin-vaccinated and sham-vaccinated mice in any of the trials (Fig. 7C and D). However, the mean severity score for each cystitis parameter was ≤ 2 in the first trial, a value that corresponds to mild cystitis (Fig. 7C), while the mean severity scores were ≤ 3 in the subsequent trials, values that correspond to moderate cystitis. In summary, vaccination with a CNF1 toxoid-CNF1 toxin cock-

FIG 7 Active vaccination with CNF1 toxoid and toxin does not affect urine and bladder colonization or cystitis severity. Bacteria enumerated in the urine and kidneys of CNF1 toxoid- and toxin-vaccinated (vacc, circles) and sham-vaccinated (squares) mice in trials 1 (A) and 2 (B). Each symbol represents one mouse, and the bar is the geometric mean value of each group. The dotted line is the limit of detection. Data were analyzed by the two-tailed Student *t* test. The average urinary bladder histology scores of CNF1-vaccinated (blue) and sham-vaccinated (red) mice in trials 1 (C) and 2 (D) were compared. Each bar represents the mean histology score of a group, and the error bar is the standard error of the mean. Data were analyzed by two-way ANOVA with the Bonferroni multiple-comparison posttest.

tail and challenge with CP9 did not alter urine or kidney bacterial titers and did not reduce cystitis severity.

Passive immunization of mice with anti-CNF1 monoclonal antibody NG8 reduces cystitis severity. Because studies in our laboratory showed that CNF1-expressing UPEC elicited marked-to-severe cystitis in mice, we reasoned that a higher concentration of neutralizing anti-CNF1 antibody than that generated in response to active immunization could reduce the severity of cystitis caused by CP9. To test this hypothesis, we conducted passive-immunization studies with anti-CNF1 monoclonal antibody NG8, which neutralizes CNF1 in *in vitro* assays (44, 46). In multinucleation neutralization assays, we found that 40 ng of NG8 reduced the HEp-2 cell multinucleation caused by 2.5 ng of CNF1 by 50% (not shown). To determine an effective *in vivo* dose of NG8, we first conducted a pilot study in which we passively immunized mice with 10 or 50 μ g of NG8, an irrelevant isotype (IgG2a) control antibody, or PBS by the i.p. route 24 and 4 h prior to an intraurethral challenge with CP9. We observed no significant differences in colonization of the kidneys or urine or in cystitis severity scores among any of the groups (data not shown). However, there was a trend toward reduced severity in the group that received two 50- μ g doses of NG8. Thus, we passively immunized mice with two doses each of 100 μ g of NG8, 100 μ g of an isotype control, or PBS administered by i.p. injection according to the same schedule (Fig. 1C). As in the previous experiments, mice

were euthanized 24 h after a CP9 challenge and urine samples and kidneys were collected for bacterial enumeration. The CP9 titers in the urine samples and kidneys were not significantly different among the groups (Fig. 8A and B). However, we observed statistically significant reductions in all four cystitis parameters ($P = 0.01$ for edema and hemorrhage; $P = 0.05$ for neutrophils and epithelial damage) in the immunized mice versus both the isotype and PBS control groups (Fig. 8C). In summary, we found that passive immunization with two 50- μ g doses of NG8 resulted in a trend toward reduced cystitis, whereas passive immunization with two 100- μ g doses of NG8 caused less severe cystitis in two trials.

DISCUSSION

In this study, we demonstrated that neutralizing antibodies directed against Hly or CNF1 reduced the severity of cystitis caused by UPEC strain CP9. The antibody response, whether elicited by active vaccination with 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 reduced the levels of urinary bladder colonization, a finding that was statistically significant only in certain trials. Both active vaccination with HlyA toxoid and passive immunization with NG8 significantly reduced the severity of cystitis.

Our investigation is the first, to our knowledge, to evaluate

FIG 8 Passive immunization with anti-CNF1 monoclonal antibody NG8 reduces the severity of CP9-mediated cystitis. Bacteria were enumerated in the urine (A) and kidneys (B) of mice passively immunized with 100 μ g of NG8 (circles), 100 μ g of an IgG2a isotype control (squares), or PBS (triangles). Each symbol represents one mouse, and the bar is the geometric mean value of each group (NG8, $n = 30$; IgG2a, $n = 10$; PBS, $n = 10$). The dotted line shows the limit of detection. Data were analyzed by two-way ANOVA with the Bonferroni multiple-comparison posttest. (C) Average urinary bladder histology scores of mice passively immunized twice with 100 μ g of NG8 (blue), 100 μ g of IgG2a (red), or PBS (purple) (NG8, $n = 30$; IgG2a, $n = 10$; PBS, $n = 10$). Each bar represents the mean histology score of a group, and the error bar is the standard error of the mean. Data were analyzed by two-way ANOVA with the Bonferroni multiple-comparison posttest. **, $P = 0.01$; *, $P = 0.05$.

the effects of vaccination with a genetic Hly toxoid on acute cystitis in a mouse model. O'Hanley et al. demonstrated that UPEC strain J96 caused less severe pyelonephritis in BALB/c mice vaccinated with denatured recombinant Hly (chemical toxoid) than in control mice; in their study, J96 titers in the kidneys were not impacted by vaccination (41). In addition, Moriel et al. showed that vaccination of CD-1 mice with recombinant HlyA protected them from a systemic challenge with UPEC strains in a sepsis model (40). In our first HlyA toxoid vaccine trial, C3H/HeOuJ mice that were actively vaccinated developed a neutralizing antibody response that resulted in a reduction in cystitis severity, albeit only significant in the degree of neutrophilic infiltrate. With the addition of a third boost in the second and third trials, mice again developed a neutralizing antibody response; however, in these trials, we observed a reduction in the severity of all of the acute-cystitis parameters. While the addition of the third boost in the second and third trials resulted in less severe cystitis, we did not observe quantifiable differences in the capacity of serum antibodies to neutralize Hly by *in vitro* assay between the first trial and the second two trials. The more effective response in these trials was likely due to a higher titer in the lumen of the urinary bladder itself and/or more functional antibodies (neutralizing) that we could not quantify by our assay method.

Our laboratory previously showed that transurethral inoculation with CP9 Δ hlyA₁ caused a reduction in cystitis severity that was statistically significant only in the parameters of hemorrhage and epithelial damage (35). We now know that CP9 contains three *hly* operons but the *hly*₁ operon is responsible for >90% of the

hemolytic activity associated with the strain (Weingarten et al., unpublished). Our study showed that neutralizing antibodies against HlyA elicited by vaccination reduced CP9-mediated cystitis severity more effectively than did deletion of *hlyA*₁ from CP9, presumably because CP9 Δ hlyA₁ still produces HlyA₂ and HlyA₃ and thus has detectable hemolytic activity (35). Our data support the theory that the antibodies generated against the HlyA toxoid (created from the *hlyA*₃ gene) in this study are cross-reactive against HlyA₁ and possibly against HlyA₂. A further indication of cross-reactivity is that the sera from mice vaccinated with the HlyA toxoid could neutralize the native Hly proteins in the CP9 supernatant, in which the hemolytic activity was previously demonstrated to be caused predominantly by HlyA₁ (35). HlyA₃ is 97% homologous to HlyA₁ and 98% homologous to HlyA₂. In addition, the two lysine residues associated with activation of the HlyA protoxin are conserved (Weingarten et al., unpublished).

In all three HlyA toxoid vaccine trials, the urinary bladders of HlyA-vaccinated mice had CP9 titers lower than those of sham-vaccinated animals, although the difference was statistically significant only 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 Hly destroys some of the uroepithelium, which may permit 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.

This is the first report of the use of a CNF1 toxoid for therapeutic vaccination to reduce the severity of inflammation associ-

ated with UTIs. Munro et al. performed studies in which CNF1 was used as a mucosal adjuvant in BALB/c mice and reported no significant antibody response to CNF1 (48, 49). We surmise that they used an amount of CNF1 as an adjuvant (1 or 10 µg delivered intranasally) that was below the immunogenic threshold because we found that mice vaccinated s.c. with CNF1 toxoid and a sublethal dose of CNF1 toxin developed a 1,000-fold serum anti-CNF1 antibody titer increase. Moreover, the sera from these vaccinated mice were capable of neutralizing CNF1 *in vitro*. However, this titer increase was almost 1 log lower than the increase in the anti-HlyA titer in the HlyA toxoid vaccine trials. Furthermore, vaccination with the CNF1 toxoid alone or in combination with a sublethal dose of active CNF1 did not reduce the severity of cystitis. Yet, mice passively immunized with two 100-µg doses of CNF1-neutralizing monoclonal antibody NG8 exhibited less severe cystitis when challenged with CP9 than did mice given the isotype control antibody. This finding demonstrates a potential therapeutic role for antibodies directed against CNF1 in the reduction of UTI inflammation. One possible reason that active vaccination did not reduce the severity of cystitis is that adequate titers of neutralizing anti-CNF1 antibodies were not present in the urinary tracts of the actively vaccinated mice at the time of intra-urethral 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 blotting analyses. We were unable to determine whether the urine antibodies were capable of neutralizing CNF1 because urine itself was toxic to the HEp-2 cells used in the neutralization-of-multinucleation assay. We did not evaluate the antibody levels in the serum or urine in the passive study, but all of the NG8 that was present likely had the capacity to neutralize CNF1.

Another reason why active vaccination with CNF1 toxoid was ineffective at reducing cystitis caused by CP9 versus 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 in outer membrane vesicles by an as-yet-unknown mechanism (34, 50). CNF1 may be partially shielded from antibodies in the urinary bladder when in these vesicles or when retained intracytoplasmically in the bacterium. Hly has also been reported to be associated with outer membrane vesicles (51). However, Hly may be more accessible to urine antibodies because it is also a secreted toxin. Higher neutralizing anti-CNF1 antibody titers may be needed in the bladder to inactivate CNF1 versus titers of anti-HlyA antibodies; such high levels of neutralizing antibodies may only have been achieved by the administration of concentrated anti-CNF1 monoclonal antibody NG8. Despite 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.

In this study, we demonstrated that antibodies against HlyA raised by active immunization or against CNF1 transferred by passive immunization can reduce the severity of inflammation during acute cystitis caused by UPEC strain CP9. Inflammation is the most likely cause of the clinical signs of UTI, which include painful urination (dysuria), increased frequency of urination, 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, which are estimated to have an annual cost in the billions and affect >7 million women (52, 53), might also reduce the

number of doctor visits and lost work time and productivity. Since 80% of uncomplicated UTIs and half of complicated UTIs are caused by UPEC, up to 60% of UPEC strains produce Hly, and up to 40% produce CNF1 (of which 90% coexpress Hly), immunization against these toxins could have a significant impact (7–9). However, vaccination against either of these toxins did not significantly reduce colonization of the murine urinary tract by CP9 and therefore did not completely prevent cystitis, an ideal goal for a UTI vaccine. Considering that the mice vaccinated with the HlyA toxoid demonstrated a trend toward reduced colonization of the urinary bladder, further optimization of the vaccine protocol may result in greater reductions of cystitis and colonization.

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