

Gal-Gal Pili Vaccines Prevent Pyelonephritis by Piliated *Escherichia coli* in a Murine Model

Single-component Gal-Gal Pili Vaccines Prevent Pyelonephritis by Homologous and Heterologous Piliated *E. coli* Strains

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Abstract

The initial pathogenic step in nonobstructive *Escherichia coli* pyelonephritis usually involves the binding of a bacterial adhesin with host uroepithelial glycoprotein receptors containing the D-Gal p α 1 \rightarrow 4 D-Gal p β 1 (Gal-Gal) moiety. In this study, groups of mice were immunized with Gal-Gal pili and challenged 2 wk later intravesicularly with *E. coli* strains expressing homologous or heterologous pili. 63 of 129 pili-immunized mice (49%) were protected from subsequent *E. coli* renal colonization compared with 5 of 85 control mice (6%). Among mice that had *E. coli* cultured from their right kidney, control mice had greater bacterial colony counts than pili-immunized animals ($P < 0.05$). Light microscopic examination of kidneys demonstrated less histopathology among pili immunized mice than among control mice ($P < 0.05$). Protection correlated with the presence of specific IgG antibodies in the urine and serum that bind to the major pilin structural polypeptide and not to the Gal-Gal pili tip adhesin per se. These results support the concept that immunization with a bacterial surface-coat constituent can prevent mucosal infection by interfering with colonization. Also Gal-Gal pili of *E. coli* represent a suitable candidate for immunoprophylaxis against pyelonephritis.

Introduction

The Gal-Gal binding pili phenotype of piliated *Escherichia coli* correlates with uropathogenicity because it is a critical ligand for upper urinary tract epithelial colonization in the anatomically normal urinary tract (1–5). The gene for Gal-Gal binding encodes for a major pilin protein (pap A), assembly and transporter proteins, and three other pilus tip proteins, one of which (pap G) constitutes the putative adhesin (6–8). Major pilin (pap A) subunits and pilus tip proteins polymerize and appear under the electron microscope as hairlike appendages (pili) extending from the bacterial cell membrane. Intact pili consist almost entirely of the major pilin (pap A) polypeptide. The fibers of polymerized major pilin facilitate binding by the tip adhesive protein to receptors on host epithelial sur-

faces. The receptors for Gal-Gal binding are contained in neutral glycolipids of the globoseries including globotetraosylceramide and trihexosylceramide. They contain D Gal p α 1 \rightarrow 4 D Gal p β 1 (Gal-Gal), which is the receptor for this bacterial ligand (4, 9). For this reason, they are termed Gal-Gal pili. Also, the fiber of polymerized major pilin and its subunits bind nonspecifically to a variety of substances because of their hydrophobic nature. Therefore, these surface organelles possess many features that promote bacterial mucosal colonization.

Hull et al. (10) and Low et al. (11) isolated chromosomal genes from three pyelonephritis strains of *E. coli* that encode for four distinct major pilins (pap A) associated with Gal-Gal binding. These genes have been cloned into nonpiliated *E. coli* strains to produce four different Gal-Gal pili recombinant strains. Recent genetic, protein, and serologic studies have confirmed striking similarities among the major pilin (pap A) proteins of Gal-Gal binding strains, suggesting that there are a limited number of major pilin (pap A) types (O'Hanley, P., G. Muralidhar, H. Rugo, and D. Low, unpublished observations). In previous work, mice that were immunized with purified Gal-Gal pili from a recombinant strain were protected from subsequent pyelonephritis after being challenged intravesicularly with bacteria that express homologous Gal-Gal pili (12). In another study (13), mice were immunized with synthetic peptides that correspond to linear sequence fragments within the NH₂-terminal and the immunodominant epitope of the major pilin (pap A) polypeptide from the same recombinant strain used in the earlier study. These vaccinated mice were protected from subsequent pyelonephritis by bacteria that express homologous pili (13). In this report, we have extended our original observations and demonstrated here that Gal-Gal pili vaccines from one of four different Gal-Gal pili types confer protection against pyelonephritis by *E. coli* exhibiting homologous and heterologous Gal-Gal pili. We conclude that Gal-Gal pili might be important immunogens for the prevention of *E. coli* pyelonephritis in humans. Also, the possibility exists that there are protective clonal epitopes within the major pilin polypeptide of Gal-Gal pili.

Methods

Bacterial strains and growth conditions

E. coli pyelonephritis isolates: J96 (O4,K6), 3669 (O2,K5), and C1212 (O6,K2), were used in mice challenge studies. The Gal-Gal binding phenotype was expressed by these piliated strains after three 18-h serial passages on trypticase soy agar (TSA)¹ (Difco Laboratories, Detroit,

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1. Abbreviations used in this paper: CFU, colony-forming units; RBC, red blood cells; TSA, trypticase soy agar; TSM buffer, 0.05 M Tris-HCl, pH 7.0, 1.5 M NaCl, and 1.0 M MgCl₂.

Table 1. Functional Properties and Relationships of Gal-Gal Pili Recombinant Strains and Parent Strains

Parent strain*	Pilus operons	O and K antigens	Properties		
			Production of		
			Hemolysin	Col V [‡]	Serum resistance
J96	MS, [§] Gal-Gal	O4, K6	+	+	+
3669	MS, Gal-Gal	O2, K5	-	+	+
C1212	MS, two types of Gal-Gal	O6, K2	+	+	+

Recombinant Gal-Gal pili strain	Construction from the DNA parent strain	O and K antigens	Properties		
			Production of		
			Hemolysin	Col V	Serum resistance
HU849	J96	K12	-	-	-
200A	3669	K12	-	-	-
201B	C1212	K12	-	-	-
210B	C1212	K12	-	-	-

* Parent strains were isolated from the urine of women with symptomatic pyelonephritis. [§] MS, mannose-binding pili. [‡] Col V, Colicin V. ^{||} Gal-Gal, digalactoside binding pili.

MI). The bacteria were grown on TSA at 37°C, harvested into 0.1 M PBS, pH 7.4, and verified for Gal-Gal binding before being inoculated into the bladder by slide hemagglutination using 5% human erythrocytes (RBC) of the P₁ blood group in PBS with 4% (wt/vol) D-mannose (Sigma Chemical Co., St. Louis, MO) and by slide agglutination of latex particles that had synthetic Gal-Gal bound to their surface (Chembiomed, Ltd., Edmonton, Alberta, Canada) (14). The number of colony-forming units (CFU) of these bacteria per milliliter PBS was determined by OD at 600 nm (wavelength of light path = 1 cm) and confirmed by a standard laboratory agar pour technique. Recombinant *E. coli* isolates: HU849, 200A, and 201B and 210B, are strains expressing the Gal-Gal pili from J96, 3669, and C1212 isolates, respectively. Their construction has been previously described (10, 11). Briefly, bacteriophage lambda transfecting particles carrying recombinant cosmid molecules with portions of J96, 3669, or C1212 genomes that encode for Gal-Gal binding were used to transduce the nonpilated *E. coli* strain HB101. Clones that conferred Gal-Gal binding were then identified by hemagglutination assays. The respective genomes were subcloned into the vector pACYC184. These hybrid plasmids were separately transformed into the nonpilated, minicell-producing *E. coli* K12 strain p678-54. Table 1 summarizes the properties and relationships of the Gal-Gal pili recombinants and their parent strains.

Pili purification

Gal-Gal pili from recombinant strains were purified from organisms grown on TSA for 18 h at 37°C by a modification of the method of Brinton (15). Bacteria were harvested into ice-cold 0.005 M Tris-HCl buffer, pH 8.3, homogenized at 4,000 rpm in an Omnimixer (Dupont Instruments-Sorvall Biomedical Division, Newport, CT), and the sheared bacteria removed by centrifugation at 12,000 g for 30 min. Pilus filaments were precipitated by the addition of 10% (vol/vol) of 0.05 M Tris-HCl, pH 7.0, 1.5 M NaCl, and 1.0 M MgCl₂ (TSM buffer) with continuous stirring at 4°C for at least 12 h. The pellet was collected after centrifugation at 12,000 g for 30 min and resolubilized in Tris-HCl buffer. Insoluble contaminants were removed by successive rounds (more than or equal to six cycles) of precipitation with TSM and resolubilization in Tris-HCl buffer. Pili were then dialyzed against distilled water and purity assessed by electron microscopy, SDS-PAGE (14), and Western immunoblotting (13, 16). Preparations were judged to be pure by the following criteria: (a) absence of particulate debris by

electron microscopy, (b) the presence of a single protein band after 10 µg and ≥ 100 µg of pilus protein as estimated by weight (see below) were subjected to SDS-PAGE, and (c) the presence of a single band on radiographs corresponding to the respective major pilin polypeptide after 10 µg of pilus protein were subjected to SDS-PAGE and Western immunoblotting. Immunoblotting used pilus-specific rabbit hyperimmune sera diluted 1:200 in PBS (14). A single protein band was observed in gels stained with silver (17) or Coomassie brilliant blue after 10 or ≥ 100 µg of pilus protein were subjected, respectively, to SDS-PAGE. The pilus protein corresponded to the respective major pilin (pap A) polypeptide in each of the four preparations. The relative molecular weights were determined in SDS-PAGE gels for each major pilin (pap A) polypeptide as follows: 17.5 kD for HU849 pili, 19 kD for 200A pili, 21 kD for 201B pili, and 17 kD for 210B pili. These data suggest that the purified pili preparations consist almost entirely of their major pilin (pap A) polypeptide and are free of additional proteins that might induce antibody production. Purified pili preparations were lyophilized for storage at room temperature.

Murine pyelonephritis model, vaccination trials

28-wk-old, specific pathogen-free female BALB/c mice were used. Groups of mice were immunized subcutaneously with the four pili preparations. Pili vaccines consisted of 100 µg of protein estimated by weighing 30 mg of purified, lyophilized pili that were then suspended in PBS to a final concentration of 100 µg per 100 µl. The pili were emulsified in equal volumes of CFA and administered by multiple injections. Also, groups of mice were immunized subcutaneously with emulsified PBS-Freund's adjuvant as a control.

2 wk after immunization, the mice were challenged intravesicularly with 10⁸ CFU of bacteria in 100 µl as described previously (12, 13). No attempt was made to obstruct the urinary tract and inoculation of 100 µl into the bladder did not cause acute ureteric reflux. 2 d later, mice were killed by prolonged ether administration. 250–500 µl of blood was extracted by cardiac puncture, allowed to clot, and centrifuged at 3,000 g for 5 min. The serum was collected and stored at -20°C until assayed for antipili antibody classes as described below. An attempt was made to collect urine from each mouse for determining pili antibody levels. The urine was stored at -20°C until assayed. The right kidney was excised under sterile conditions and sectioned sagittally with a sterile scalpel. The cut surfaces were smeared on MacConkey agar (Difco Laboratories) plates and incubated for 18 h at 37°C. Speci-

mens were assessed for renal bacterial colonization density per 3-cm² area by the following relative grading criteria: 5+ (confluent growth; i.e. > 90% of area); 4+ (not confluent growth but colonies too numerous to count); 3+ (> 20 CFU); 2+ (11–20 CFU); 1+ (1–10 CFU); 0 (no growth). The relative renal bacterial colonization density scale was compared with the total number of CFU per whole minced right kidney in a selected number of additional control mice administered intravesicularly J96 or 3669 strains. There were no significant differences between a particular relative colonization grade and the total number of CFU per kidney of J96- and 3669-infected mice. A minimum of three mice infected with either J96 or 3669 strains were evaluated in these comparisons. The following preliminary comparisons could be made: 5+ was equivalent to a mean of 3×10^3 CFU/kidney; 4+ was equivalent to a mean of 1×10^3 CFU/kidney; 3+ was equivalent to a mean of 2×10^2 kidney; 1+ was equivalent to a mean of 10 bacteria/kidney; and 0 grade corresponded to no growth in kidneys. The 2+ grade could not be evaluated in these preliminary studies. Renal bacterial isolates were verified as the inoculation strain by slide agglutination with somatic O-antigen antisera. A portion of the right kidney was also fixed in 10% formalin and processed for light microscopy. Sections were subsequently stained with hematoxylin and eosin stain. Randomly selected sections were also assessed for the presence of bacteria in renal tissue by immunohistochemical staining with somatic O-antigen antisera as described by Sternberger (18) or by Gram stain. The slides were graded for histopathology by the following scale: 5+ (diffuse tissue necrosis and inflammatory cell infiltrate extending from the pelvis to the cortex); 4+ (extensive segmental inflammation and microabscesses from the pelvis to the cortex); 3+ (focal inflammation and microabscesses from the pelvis to the medulla); 2+ (focal inflammation and edema from renal pelvis to the medulla); 1+ (focal inflammation and edema in the renal pelvis); and 0 (normal).

Evaluation of serum and urine antibodies in immunized mice

Pili-specific antibodies in serum and urine of immunized mice that were challenged intravesicularly with *E. coli* were assessed by ELISA and Western immunoblotting techniques. ELISA was used to detect and quantitate specific pili antibody classes. The Western immunoblotting technique was used to assess the specificity of serum IgG antibodies against Gal-Gal pili gene products.

(a) *ELISA.* Presence of pili-specific IgG, IgA, and IgM was assessed in serum and urine by ELISA (12, 14). Polystyrene microtiter wells were coated with 100 ng of pili from each of the preparations in 100 μ l of 0.1 M sodium carbonate buffer, pH 9.6, for 24 h at 37°C. Wells were then washed three times with PBS containing 0.01% Brij-35 (Sigma Chemical Co.) (PBS-Brij). 100- μ l dilutions of serum and urine were added to the wells and allowed to bind to antigen for 3 h at 37°C. The range of serum titers⁻¹ included the following dilutions in PBS: 500, 1,000, 5,000, and 10,000. Urine was assessed only at a titer⁻¹ of 20. The wells were then washed with PBS-Brij. 100 μ l of alkaline phosphatase-conjugated rabbit anti-mouse IgM, IgG, or IgA (Tago, Inc., Burlingame, CA) diluted 1:50,000 in PBS was added to wells and allowed to incubate for 1 h at 37°C before washing with PBS-Brij. 100 μ l of alkaline phosphatase substrate (Sigma Chemical Co.) at a concentration of 1 mg/ml in 1.0 M diethanolamine buffer, pH 9.8, was added to each well and the enzymatic color reaction allowed to proceed 15 min before being stopped with 50 μ l of 1.0 NaOH. Nonsensitized wells, bovine albumin-sensitized wells, and sensitized wells exposed only to the secondary antibody served as negative controls. Absorbance was read at 405 nm in an ELISA autoreader (Dynatech Laboratories, Torrance, CA). Each sample was assessed in duplicate or triplicate and recorded as the mean OD. A mean value greater than two times the negative controls was considered to indicate presence of specific antibody.

(b) *Western immunoblotting.* The specificity of serum IgG pili antibodies to bind Gal-Gal pilus gene products was assessed by Western immunoblotting in a selected number of mice immunized with homologous pili that were purified from recombinant strains. Proteins de-

rived from single-TSA agar grown colonies of each of the four Gal-Gal pili recombinant strains were electrophoretically transferred from SDS-PAGE gels to nitrocellulose (16, 19). Serum was diluted 1:100 in PBS and was allowed to bind to denatured pilus gene products contained in 0.1 \times 5.0 cm nitrocellulose strips corresponding to a lane in which one colony had been subjected to SDS-PAGE. Bound antibody was detected with ¹²⁵I-labeled protein A, after 18–24-h autoradiographic exposure as described by Burnette (20). Sera of mice immunized with PBS-Freund's adjuvant were used as controls.

Statistics

The chi-square (χ^2) test was used to compare the differences in results between control and pili-immunized mice.

Results

The efficacy of Gal-Gal pili vaccines to prevent *E. coli* pyelonephritis was assessed in a murine experimental pyelonephritis model. The characteristics of the model are as follows: (a) it simulates an ascending mode of infection; (b) the relevant uroepithelial surfaces exhibit Gal-Gal receptors in a manner very similar to human uroepithelium; and (c) it does not entail obstruction to urine flow or traumatic manipulation of urinary tract tissue (12).

Vaccine efficacy of recombinant Gal-Gal pili for the prevention of renal colonization and pyelonephritis

Four different Gal-Gal pili were prepared as vaccines from recombinant strains: HU849, 200A, 201B, and 210B. The pili preparations were scrutinized for the presence of protein by SDS-PAGE and Western immunoblotting. The pili preparations consisted essentially of their respective major pilin polypeptide. No other proteins were detected by these techniques. Although there is contaminating LPS from the *E. coli* K12 strain P678-54 in the pili preparations, the percent by weight of LPS in lots of pili preparation as judged from considerable experience is < 0.01 as determined by a 2-keto-3-deoxyoctonate assay (21). Also, LPS is not a protective immunogen for mice in this experimental pyelonephritis model (12).

100 μ g of purified pili from each recombinant strain or PBS were emulsified in CFA and administered by subcutaneous injections to 28-wk-old, female BALB/c mice. 2 wk later, the mice were challenged with 10⁸ CFU of homologous or heterologous Gal-Gal pilated uropathogenic *E. coli* strains to assess pili vaccine efficacy. 2 d after bladder inoculation, mice were killed because at this time, maximal renal culture density and histopathological abnormalities occur in this experimental infection model (12). Table II summarizes pili vaccine efficacy to prevent renal colonization and pyelonephritis by uropathogenic *E. coli* strains that express either homologous or heterologous Gal-Gal pili.

(a) *Renal colonization density.* 129 mice immunized with Gal-Gal pili and 85 control mice were evaluated for the density of specific renal *E. coli* growth from smears of sagittal right kidney sections 2 d after challenge. 80 of 85 (94%) right kidneys from control mice were culture positive for the infecting organism as determined by slide agglutination assays employing specific somatic O-antigen antisera. 59 of 85 (69%) control mice had heavy growth (i.e., $\geq 4+$), which is equivalent to $\geq 10^3$ CFU/kidney as determined in preliminary studies. The density of bacterial growth from kidney sections of control mice inoculated with 3669 strain was less than that observed in mice administered J96 and C1212 strains. The basis for this

Table II. Vaccine Efficacy of Recombinant Gal-Gal Pili Types against Pyelonephritis by Homologous (Ho) and Heterologous (He) Piliated Uropathogenic *E. coli*

Immunogen	Total no. mice/group	Challenge strain (Ho/He Pili)*	Mean relative renal colonization density (\pm SD) [‡]	P value [§]	Mean histopathology grade (\pm SD)
Saline	28	J96 (Control)	3.6 \pm 1.7		3.2 \pm 0.04
Saline	31	3669 (Control)	3.1 \pm 1.8		2.1 \pm 0.02
Saline	26	C1212 (Control)	4.4 \pm 1.0		3.2 \pm 0.02
HU849 Pili	16	J96 (Ho)	0.6 \pm 0.8	<0.001	0.4 \pm 0.25
HU849 Pili	6	3669 (He)	0.8 \pm 1.5	<0.01	0.5 \pm 0.96
HU849 Pili	7	C1212 (He)	0.9 \pm 0.8	<0.001	0.4 \pm 0.15
200A Pili	11	J96 (He)	2.2 \pm 1.7	>0.1	1.5 \pm 1.36
200A Pili	13	3669 (Ho)	0.9 \pm 1.3	<0.05	0.7 \pm 0.24
200A Pili	9	C1212 (He)	1.2 \pm 1.1	<0.01	0.8 \pm 0.32
201B Pili	12	J96 (He)	0.8 \pm 0.9	<0.01	0.3 \pm 0.30
201B Pili	13	3669 (He)	0.7 \pm 0.9	<0.01	0.2 \pm 0.12
201B Pili	14	C1212 (Ho)	1.1 \pm 1.4	<0.01	0.6 \pm 0.24
210B Pili	14	J96 (He)	0.2 \pm 0.4	<0.001	0.4 \pm 0.12
210B Pili	7	3669 (He)	0.6 \pm 0.5	<0.01	0.4 \pm 0.14
210B Pili	7	C1212 (Ho)	1.9 \pm 1.4	>0.05	0.7 \pm 1.11

* Ho/He Pili refers to whether the specific challenge strain expresses homologous Gal-Gal pili or heterologous Gal-Gal pili when compared with the pilus immunogen. [‡] Assessed by culture 2 d after challenge dose (10^8 CFU/30-wk-old female BALB/c mouse); relative renal colonization density for all 214 mice is expressed as the mean (\pm SD) of the specific *E. coli* strain cultured from the kidney smear per immunogen and challenge group (see Methods). [§] P value is determined by the Chi-squared test by comparing the mean colonization density of control and experimental cohorts challenged with the same *E. coli* strain. $P < 0.05$ was considered significant. ^{||} Assessed by light microscopy 2 d after challenge dose; mean renal histology grade is expressed as the mean (\pm SD) among at least half the mice per group (see Methods).

discrepancy is not due to quantitative differences of Gal-Gal pili expression among these strains as determined by electron microscopy or single colony SDS-PAGE analysis. It is known, however, that the 3669 strain does not invade the renal parenchyma of BALB/c mice (Pecha, B., and P. O'Hanley, manuscript submitted for publication). This may account for the differences in renal colonization density between 3669-infected and J96- and C1212-infected mice, because these latter two strains do invade renal tissue. Also, the presence of additional unknown adhesins might be expressed by J96 and C1212 strains to account for their relative higher renal bacterial counts than kidneys infected with 3669 strain.

The majority of Gal-Gal pili vaccinated mice were protected from subsequent heavy renal colonization by challenge strains. 63 of 129 (49%) pili-immunized mice had sterile renal cultures. Among the remaining 66 pili-immunized mice, renal colonization by the infecting strains was significantly less than control mice. Mice that were immunized with HU849 pili and 210B pili were protected ($P < 0.01$) from subsequent renal colonization by J96, 3669, and C1212 challenge strains. HU849 pili and 201B pili immunizations protected mice from homologous piliated wild-type challenge by J96 and C1212 strains, respectively. Furthermore, these immunizations were cross-protective against challenge by the corresponding parent strains and the heterologous Gal-Gal piliated 3669 strain. Mice that were immunized with 200A pili and 210B pili were not completely protected from the three wild-type strains. Mice that were immunized with 200A pili were susceptible to subsequent renal colonization by the J96 strain. 200 A Gal-Gal pili are serologically heterologous to the Gal-Gal pili of the J96 strain (16) and this might account for this failure of immunoprophylaxis. 200A pili immunization did afford protection ($P < 0.05$) against subsequent heavy renal growth after challenge

by the homologous piliated 3669 strain and the heterologous piliated C1212 strain. 210B pili are homologous to one of the two expressed Gal-Gal pili types of the C1212 strain (11); yet, mice that were immunized with 210B pili were susceptible to subsequent renal colonization by C1212 strain challenge. Protection might not be afforded by this pili immunization because the majority of Gal-Gal pili expressed by C1212 bacteria in the urinary tract are of a different type (i.e., 201B pili). The proportion of C1212 bacteria expressing 210B pili is usually $< 10\%$ (11). Vaccine recipients of 210B pili were protected from subsequent *E. coli* renal colonization when challenged with heterologous piliated J96 and 3669 strains.

(b) *Protection against pyelonephritis by Gal-Gal pili immunization.* These studies also sought to distinguish renal colonization as determined by culture from pyelonephritis as determined by light microscopy. Control mice had histological evidence of pyelonephritis 2 d after intravesicular inoculation with the three uropathogenic strains. Microscopic examinations of renal tissue from all three groups of infected control mice revealed PMN cells within tubules of the medulla and/or renal pelvis. In addition, there was more intense renal injury observed in mice infected with hemolytic J96 and C1212 strains. Parenchymal necrosis, microabscess formation, and bacterial invasion into the parenchyma were observed in mice infected with hemolytic J96 and C1212 strains. The renal histopathology was less severe in mice infected with the nonhemolytic strain 3669. The abnormalities found in mice infected with the 3669 strain consisted primarily of inflammatory exudate in the renal pelvis without evidence of bacterial invasion. Mice immunized with pili were protected ($P < 0.02$) from renal damage by challenge strains with the exception of mice immunized with 200A pili and challenged with the J96 strain. The benefit of pili immunization to prevent pyelonephritis

Table III. Immunogenicity of Gal-Gal Pili and Correlations with Protection against Renal Colonization and Pyelonephritis by Homologous (Ho) and Heterologous (He) Piliated *E. coli* Strains

Immunogen	Total no. mice/group	Challenge strain (Ho/He pili)*	Serum anti-pilus IgG ELISA titer ^{-1‡}		Protection [§]
			Mean (±SD)	Interquartile range	
Saline	28	J96 (Control)	Neg.	Neg.	–
Saline	31	3669 (Control)	Neg.	Neg.	–
Saline	26	C1212 (Control)	Neg.	Neg.	–
HU849 Pili	16	J96 (Ho pili)	6,231±2,722	5,000–10,000	+
HU849 Pili	6	3669 (He pili)	7,500±2,500	5,000–10,000	+
HU849 Pili	7	C1212 (He pili)	5,143±3,603	5,000–10,000	+
200A Pili	11	J96 (He pili)	3,364±3,471	1,000–5,000	–
200A Pili	13	3669 (Ho pili)	2,000±2,542	1,000–5,000	+
200A Pili	9	C1212 (He pili)	5,375±3,903	1,000–5,000	+
201B Pili	12	J96 (He pili)	1,542±1,561	500–1,000	+
201B Pili	13	3669 (He pili)	2,615±3,335	500–1,000	+
201B Pili	14	C1212 (Ho pili)	2,107±2,633	1,000–5,000	+
210B Pili	14	J96 (He pili)	4,000±3,601	5,000–10,000	+
210B Pili	7	3669 (He pili)	6,143±4,454	5,000–10,000	+
210B Pili	7	C1212 (Ho pili)	8,571±3,499	≥10,000	–

* Challenge strain (Ho/He pili) refers to the *E. coli* strain used in the challenge and whether the pili of the challenge strain was homologous (Ho) or heterologous (He) to the pili immunogen for a group of mice. ‡ Results are expressed as the mean and the interquartile range of the reciprocal of the greatest serum dilution positive for ELISA. The following serum dilutions in PBS included: 1/100 (minimum), 1/500, 1/1,000, 1/5,000, 1/10,000 (maximum). Negative titers (Neg.) indicate OD values less than two times the background reading using serum dilutions of 1/100. The interquartile range is the interval that constituted 50% of the positive titers among mice that received a particular immunogen.

§ Protection against subsequent renal colonization and pyelonephritis by challenge *E. coli* strains in groups of mice is expressed as occurring (+) or not (–). Groups of pili-vaccinated mice that were protected from subsequent challenge by a homologous (Ho) or heterologous (He) piliated strain had renal colonization density and renal histopathology scale values significantly different ($P \leq 0.05$) than control challenged mice (see Methods).

correlates with decreased colonization ability of challenge strains.

Correlation of pili antibody with protection. The presence of homologous Gal-Gal pili antibody of IgG, IgA, and IgM classes in the serum and urine was assessed in pili vaccine recipients by ELISA. No IgM or IgA pili antibody was detected in serum or urine. Only pili antibody of the IgG class was detected in the clinical samples of pili vaccine recipients. The four Gal-Gal pili vaccines produced high serum titers⁻¹ of specific IgG antibodies in all immunized mice (Table III). The titers⁻¹ in HU849 pili and 210B pili-immunized mice were usually $\geq 5,000$; the titers⁻¹ in 200A pili and 201B pili-immunized mice were usually $\geq 1,000$. The apparent differences in the immunogenicity of these pili were not investigated in this study. A minimum specific pili serum IgG antibody titer⁻¹ of 500 was associated with protection against subsequent *E. coli* renal colonization in 10 of 12 pili vaccine groups when compared to control groups that had no detectable specific pili antibody (data not shown). Despite high serum specific pili titers⁻¹ in 200A pili and 210B pili vaccine recipients, these groups of mice were uniformly not protected from renal *E. coli* colonization subsequently challenged with J96 and C1212 strains, respectively.

Attempts to collect urine from each mouse were not completely successful. A total of 20 urine samples from control mice and a total of 79 urine samples from pili vaccine recipients were obtained for analyses. None of the control mice had detectable pili-specific IgG antibody in their urine. 36 of 79

urine samples (46%) from the pili vaccine recipients contained specific pili IgG antibodies when assessed at a dilution of 1:20 in PBS. We suspect that there was a variety of reasons why no specific pili urinary antibody was detectable in the remaining 43 of the 79 pili-vaccinated mice (54%) despite the presence of high specific pili antibody titer in their serum. They include antibody degradation before and until the time of assay, ongoing urinary tract infection, which led to more rapid degradation of specific antibody, and/or the dilution factors associated with urine excretion or the addition of PBS to the collected urine sample. Sufficient volumes of urine could not be obtained to screen for specific pili antibody at more concentrated levels. However, the presence of specific pili IgG antibody at a dilution of 1:20 in the urine correlated under these assay conditions with protection in 28 of 31 mice (90%) that were protected by pili vaccination.

The specificity of murine serum pili IgG antibodies to bind to proteins expressed by Gal-Gal pili recombinant strains was assessed by Western immunoblotting. The sera of 32 pili vaccinated mice and 8 control mice were screened. A minimum of six sera with specific ELISA titers⁻¹ of $\geq 1,000$ from mice immunized with a particular pili preparation were screened. When the sera of pili immunized mice were analyzed by immunoblotting to proteins expressed by the homologous recombinant strain from which the pili had been purified, only the respective major pilin polypeptide was detected (data not shown). These data corroborate that the pili preparations consist almost entirely of their major pilin polypeptide and that

the protection conferred by pili vaccines can be confidently attributed to this homologous immunogen, as has been previously shown (13).

Discussion

The ability to colonize the uroepithelial mucosa is a prerequisite for uropathogenic *E. coli* strains to infect the urinary tract. The majority of pyelonephritogenic strains express Gal-Gal pili (1–5). These bacterial surface organelles facilitate binding to globoside present in the uroepithelium. Their ability to bind to Gal-Gal moieties is not inhibited by uromodulin or the Tamm-Horsfall glycoprotein (12). Uromodulin in urine is a natural host factor that prevents the common mannose-binding bacteria to colonize the anatomically normal urinary tract (22). After sufficient multiplication on the renal mucosa by Gal-Gal piliated *E. coli*, the infectious inoculum can cause tissue damage that is mediated by other microbial virulence determinants, e.g., hemolysin. Therefore, the pathogenesis of nonobstructive, ascending-tract *E. coli* pyelonephritis may be viewed as the culmination of a sequence of events mediated by specific determinants of microbial virulence. Although interference with any step in the pathogenic sequence could conceivably prevent infection, the critical nature of the renal mucosal binding event by pyelonephritogenic *E. coli* makes it an attractive choice for intervention by immunoprophylaxis. The efficacy of purified Gal-Gal pili vaccines to prevent *E. coli* pyelonephritis by homologous piliated strains has been demonstrated in the BALB/c mouse and primate experimental pyelonephritis models (12, 23). Furthermore, protection against experimental murine *E. coli* pyelonephritis by a homologous piliated strain has been conferred by synthetic major pilin (pap A) peptide vaccines (13). The protective epitopes for this major pilin corresponded to linear sequences within: (a) the NH₂-terminal that is highly conserved among Gal-Gal major pilins and poorly immunogenic and (b) type-specific major antigenic domain that resides immediately after the intradisulfide bridge. The studies reported here demonstrate the broadly protective nature of Gal-Gal pili immunization, specifically the major pilins associated with Gal-Gal binding, in preventing *E. coli* pyelonephritis by homologous and heterologous piliated strains in a murine model.

The criteria for pili purity were carefully assessed so that protection conferred by a pili vaccine could be attributed to the homologous immunogen. Based on electron microscopy and SDS-PAGE analyses, the pili preparations consisted almost entirely of major pilin (pap A) polymers. The results of Western immunoblots of pili-vaccinated mice demonstrate conclusively that immunization with the purified pili from recombinant strains produces specific IgG antibody in serum to the homologous major pilin (pap A) polypeptide. There were no detectable antibodies in serum to the homologous accessory proteins of the Gal-Gal pilus gene in pili vaccine recipients as determined by our test conditions. The significance of this for immunoprophylaxis is that protection against experimental pyelonephritis can be afforded by antibodies that bind to the major pilin structural component (pap A) and not the Gal-Gal adhesin per se. The results from this study corroborate our earlier findings that protective epitopes exist within the polymerized pap A gene product of the Gal-Gal pilus operon (13) and they can be used as vaccines to prevent *E. coli* pyelonephritis.

Gal-Gal pili vaccines conferred protection against subsequent renal colonization by homologous and heterologous piliated *E. coli* strains in the majority of mice used in the pyelonephritis model. The capacity of pili vaccines to prevent or to decrease renal colonization by pyelonephritogenic *E. coli* strains correlated with protection against renal damage. These observations further support the pathogenic significance of Gal-Gal pili mediated uroepithelial colonization in pyelonephritis. In only 2 of 12 challenge trials, Gal-Gal pili vaccines failed to prevent subsequent *E. coli* renal colonization in mice when compared with control groups. These vaccine failures were not attributable to the lack of elicited specific pili antibody production. Purified Gal-Gal pili were highly immunogenic in mice with high titers produced within 2 wk after primary immunization against the homologous major pilin (pap A) polypeptide alone. The serological basis for the two vaccine failures can be explained by the heterologous nature of the Gal-Gal major pilins expressed by challenge strains and the elicited pili antibody in immunized mice. Therefore, these considerations indicate that there is more than one clonal protective epitope among Gal-Gal pili and/or major pilin. The significance of this for immunoprophylaxis is that efficacious whole Gal-Gal pili vaccines must comprise a number of pili types to be broadly protective against *E. coli* pyelonephritis. However, recent findings suggest that the serodiversity of the major pilins (pap A) associated with Gal-Gal binding is limited and the majority of types is comprised by the four Gal-Gal pili used in this study (Rugo, H., P. O'Hanley, G. Muralidhar, and D. Low, unpublished observations). Therefore, an efficacious vaccine combination of purified Gal-Gal pili might be formulated and practical in that only a limited number of pili types need to be included for broad immunoprophylactic effect.

The mechanism whereby the Gal-Gal pili vaccines confer protection was not addressed in this study. We speculate that vaccine efficacy relates to the binding of specific IgG antibody in the urine to the Gal-Gal piliated bacteria, thereby interfering with the mucosal colonization process. It is not known if urinary-specific pili IgG antibody selects for non-Gal-Gal pili phase variants or subtly effects pilus function as a result of steric hindrance or the agglutination of pilus filaments. Specific pili IgG antibody does not prevent *in vitro* bacterial adherence to Gal-Gal receptor (O'Hanley, P., unpublished observations). Therefore, this report suggests that the benefit of Gal-Gal pili immunization is not directly related to the interference of bacterial adherence to the receptor but to interference with bacterial colonization ability.

The findings in this report suggest that Gal-Gal pili are attractive candidates for the immunoprophylaxis of *E. coli* pyelonephritis in humans without anatomical abnormalities of the urinary tract. There are theoretical concerns that immunization with whole Gal-Gal pili might elicit greater antigenic diversity among these organelles by antibody selection. This could lead to circumvention of effective host immunity. In addition, this may account for susceptibility in selected individuals to recurrent episodes of pyelonephritis. It could be argued that the use of major pilin subunit vaccines that correspond to linear domains that are highly conserved among Gal-Gal pili, are not normally immunogenic within the native molecule, are structurally essential, and specify protective epitopes are more likely to produce long-term immunity against *E. coli* pyelonephritis, as has been suggested previously (13). The therapeutic value of Gal-Gal pili vaccines to prevent acute

and possibly recurrent pyelonephritis will only be critically assessed by appropriately controlled studies in humans.

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