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Research Article

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Molecular Basis of *Escherichia coli* Colonization of the Upper Urinary Tract in BALB/c Mice

Gal-Gal Pili Immunization Prevents *Escherichia coli* Pyelonephritis in the BALB/c Mouse Model of Human Pyelonephritis

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Abstract

Most human pyelonephritis *Escherichia coli* isolates express both mannose (MS)- and globoside (Gal-Gal)-binding pili. An ascending *E. coli* urinary tract infection model was established in the 16-wk-old female BALB/c mouse to compare the pathogenic significance of MS and Gal-Gal pili and their efficacy as vaccines for the prevention of pyelonephritis. The distribution and density of pilus receptor compounds in urogenital tissues and as soluble compounds in urine were determined with antibodies to the synthetic receptor analogues, α D-Gal(1 \rightarrow 4) β D-Gal and α D-Man(1 \rightarrow 2) α D-Man. Both carbohydrates were detected in vagina, bladder, ureter, and renal pelvis epithelium and in collecting duct and tubular cells. A pilus receptor compound also was detected in urine. It competitively inhibited the binding capacity of MS pili and was found to be physically, chemically, and immunologically related to Tamm-Horsfall uromucoid. Infectivity and invasiveness were quantitatively and histologically characterized for four *E. coli* strains: J96, a human pyelonephritis strain that expresses both MS and Gal-Gal pili; two recombinant strains prepared from J96 chromosomal DNA encoding MS pili or Gal-Gal pili; and the nonpilated K12 recipient. Intravesicular administration of J96 (10^6 colony-forming units [CFU]) resulted in renal colonization and invasion in each of nine mice. The Gal-Gal clone (10^6 CFU) colonized the kidneys in each of 10 mice but did not invade. In contrast, the MS clone (10^6 CFU) did not colonize renal epithelium or invade. This effect was superceded when larger doses ($\geq 10^{10}$ CFU) of the MS clone were administered in volumes that cause acute vesicoureteric reflux. The efficacy was determined of vaccines composed of pure MS or Gal-Gal pili or the lipopolysaccharide containing O somatic antigen of the challenge strain, J96. The Gal-Gal pilus vaccine blocked renal colonization in 19 of 22 mice and renal invasion in 10 of 11 mice. Gal-Gal pili may be useful immunogens for the prevention of pyelonephritis in anatomically normal urinary tracts.

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Introduction

Uropathogenic *Escherichia coli* colonize epithelial surfaces by elaborating proteinaceous appendages termed pili (or fimbriae) that bind receptor carbohydrates (1-3). Two major classes of chromosomally encoded pili have been defined functionally by their receptor specificities and implicated in the pathogenesis of cystitis and pyelonephritis (4). Type 1 or common pili bind Tamm-Horsfall uromucoid (5), a mannose-containing glycoprotein in the urine of placental animals, and attach to African green monkey kidney cells (6). They are also termed mannose-sensitive (MS)¹ because D-mannose and α -methyl-D-mannopyranoside inhibit their agglutination of guinea pig erythrocytes (7). Most human pyelonephritis *E. coli* isolates also express pili that bind neutral glycolipids of the globoseries including globotetraosylceramide and trihexosylceramide (8). These can be extracted from human uroepithelial cells (9) and are antigens of the human P blood-group system (10). They contain the moiety, α D-Gal(1 \rightarrow 4) β D-Gal, and a synthetic analogue of this disaccharide (Syn Gal-Gal) inhibits hemagglutination. Pili with this binding specificity have been termed P fimbriae, PAP pili (pyelonephritis-associated pili), or Gal-Gal pili.

MS and Gal-Gal pili are simultaneously expressed by most pyelonephritis strains (11). Therefore, the pathogenic significance of each may be difficult to assess with clinical isolates. The molecular cloning of putative virulence determinants and the use of the recombinants in appropriate models of human disease can provide convincing evidence for their role in pathogenesis. This strategy was pursued by Hull et al. (12), who cloned from strain J96, a human pyelonephritis *E. coli* isolate, two distinct chromosomal fragments that separately encode MS or Gal-Gal pili. When harbored in a nonpilated derivative of *E. coli* K-12, the two recombinant strains, SH48 and HU849, were found to express MS or Gal-Gal pili, respectively, and to exhibit collectively the binding repertoire of the clinical isolate. When purified to homogeneity (13) the pili possessed (a) the binding specificity of the recombinant strain from which they were prepared, (b) partial N-terminal and C-terminal sequence homology, and (c) <5% shared antigenicity.

The studies reported here have employed pure pilus proteins, the clinical isolate and both recombinants in a murine pyelonephritis model to determine the pathogenic significance of

1. Abbreviations used in this paper: CFU, colony-forming unit; ELISA, enzyme-linked immunosorbent assay; Gal-Gal, globoside; LPS, lipopolysaccharide; MS, mannose-sensitive; PBSA, phosphate-buffered saline with bovine serum albumin; Syn Gal-Gal, synthetic analogue of globoside; Syn Man-Man, synthetic analogue of mannose; TSA, trypticase soy agar.

MS and Gal-Gal pili and their efficacy as vaccines. The distribution and density of pilus receptor compounds in tissue and their presence as soluble factors in urine were determined with antireceptor antibodies to elucidate the molecular basis for renal colonization. Moreover, renal colonization and invasion were systematically distinguished. The results indicate that (a) Gal-Gal pili are critical determinants of renal colonization in the absence of acute vesicoureteral reflux, (b) colonization and invasion are distinct pathogenic events mediated by separate microbial molecules, and (c) vaccines that block colonization can also prevent invasion.

Methods

Bacterial strains and growth conditions. *E. coli* J96 (04, K6), a human pyelonephritis isolate, is hemolytic, colicin V-positive, motile, and resistant to the bactericidal action of normal serum (11). Simultaneous expression of MS and Gal-Gal pili by J96 was accomplished by three serial single colony passages on trypticase soy agar (TSA, Difco Laboratories, Detroit, MI) grown for 18 h at 37°C followed by static overnight growth in trypticase soy broth at 37°C.

A cosmid gene library was prepared from J96 DNA (11). In brief, bacteriophage λ -transducing particles carrying recombinant cosmid molecules with portions of the J96 genome were used to transduce the nonpilated *E. coli* K-12 strain HB101. Clones that conferred MS or Gal-Gal hemagglutination were identified. The respective genomes were subcloned into the vector pACYC184. The Gal-Gal and MS derivatives contained 11.1 kilobase pairs (kb) and 11.5 kb of the original J96 chromosomal DNA, respectively. These hybrid plasmids were separately transformed into the nonpilated minicell, producing *E. coli* K12 strain P678-54. The resulting recombinant strains SH48 and HU849 and their pili were functionally, chemically, and serologically characterized (13, and our Table I) and found to mediate MS and Gal-Gal hemagglutination, respectively. Both recombinants expressed approximately the same number of pilus filaments per cell as determined by electron microscopy and inhibition enzyme-linked immunosorbent assay (ELISA) (see pertinent sections below), but neither was hemolytic, motile, nor colicin V-positive. Thus, the resulting recombinant strains

differed only with regard to the binding specificity of their pili, except that SH48 was made resistant to chloramphenicol and HU849 resistant to tetracycline. The two recombinants and their pili were employed in a murine pyelonephritis model (see later section). P678-54 was used as a nonpilated negative control.

The number of colony-forming units (CFU) per milliliter of trypticase soy broth was estimated by comparing the optical density at 620 nm (1-cm light path) to the CFU of serially diluted cultures on TSA.

Pili purification. MS pili from strain SH48 and Gal-Gal pili from strain HU849 were purified from organisms grown on TSA for 24 h at 37°C by a modification of the method of Brinton (14). The organisms were harvested into ice-cold 0.005 M Tris-HCl buffer, pH 8.3, and homogenized (4,000 rpm) for 30 min at 4°C in a Sorvall Omnimixer (Dupont Instruments-Sorvall Biomedical Div., Newton, CT), and the sheared bacteria were removed by centrifugation at 12,000 g for 30 min. Pilus filaments were precipitated in 0.05 M Tris-HCl buffer, pH 7.0, containing 0.15 M NaCl by the addition of MgCl₂ to 0.1 M (TSM buffer) and collected by centrifugation at 12,000 g for 45 min and the pellet was dissolved in Tris-HCl buffer, pH 8.3. Insoluble contaminants were removed by centrifugation at 12,000 g for 60 min, and the pili were precipitated from the supernatant in TSM buffer, pH 7.0. After six successive cycles of solubilization and crystallization by exposure to Tris-HCl buffer, pH 8.3 and TSM buffer, 7.0, respectively, the pili were dialyzed against water and their purity was assessed by electron microscopy, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE), amino-terminal amino acid sequence analysis, and for lipopolysaccharide (LPS) content. Their functional properties (Table I) were ascertained by the method of O'Hanley et al. (13).

Electron microscopy. Preparations of pili were negatively stained with 2% (wt/vol) aqueous uranyl acetate on copper grids coated with Formvar and carbon.

SDS PAGE. Pilus protein (10–50 μ g) was assessed by SDS PAGE according to the method of Laemmli (15). Because MS pili do not enter the stacking gel under these conditions, SH48 pili were depolymerized before electrophoresis by the addition of HCl, pH 1.8, according to the method of McMichael and Ou (16). The gels were stained with Coomassie Brilliant Blue R250 (Sigma Chemical Co., St. Louis, MO) or silver-stained (17, 18) for the detection of contaminating LPS.

Table I. Functional, Serologic, and Chemical Properties of *E. coli* Strains and Isolated Pili

Strain	Pilus operon	Source	Antibiotic resistance	Agglutinating properties			
				Erythrocyte species			Carbohydrate binding§
				Human	Guinea pig		
HA*/HAI‡	HA/HAI	Syn Gal-Gal	Syn Man-Man				
J96	MS + Gal-Gal	Human pyelonephritis	None	+/Syn Gal-Gal	+/ α MM	+	+
SH48	MS	Recombinant (J96 DNA)	Tetracycline	-/-	+/ α MM	-	+
HU849	Gal-Gal	Recombinant (J96 DNA)	Chloramphenicol	+/Syn Gal-Gal	-/-	+	-
P678-54	None	K12-derived host	None	-/-	-/-	-	-
Pili	N-terminal sequence [¶]		Shared antigenicity [¶]				
	1	25					
SH48	APTIPQGQGVKVTENGTVVDAPCSIS		<5%	-/-	+/ α MM	-	+
HU849	AATTVNGGTVHFKEVVNAACAVID		<5%	+/Syn Gal-Gal	-/-	+	-

* Hemagglutination (HA) of human or guinea pig erythrocytes: + positive; - negative. ‡ Hapten inhibition (HAI) of hemagglutination by Syn Gal-Gal or α -methyl-mannoside (α MM) according to the method of O'Hanley et al. (13). § Agglutination of latex beads adsorbed with Syn Gal-Gal or Syn Man-Man (13): + positive; - negative. ¶ After O'Hanley et al. (13). Underlined residues are conserved. † Determined by a two-step competitive ELISA (13).

Amino-terminal amino acid sequence analysis. Pili from strains SH48 and HU849 were subjected to automated Edman degradation (Table I) with a Beckman 890C liquid-phase sequencer (Beckman Instruments, Palo Alto, CA) using a 0.1 M Quadrol program. Each amino acid phenylthiohydantoin derivative was identified and quantitated by reverse-phase high-pressure liquid chromatography and confirmed by gas-liquid chromatography and/or thin-layer chromatography.

LPS analysis. In addition to SDS PAGE detection of LPS (as discussed above), the 2-keto-3-deoxyoctonate content of 500–1,000- μ g pilus protein samples was quantitatively determined according to a modification of the method of Waravdekar and Saslaw (19) by relating their optical density at 548 nm to standard curves derived from LPS prepared by the Westphal phenol-extraction technique (20) from *E. coli* strains P678-54 and J96.

Somatic O antigen preparation. Somatic O antigen was prepared, as described elsewhere (21), from strains J96 and P678-54 to determine the efficacy of a non-pilus, LPS-containing, cell envelope vaccine and for the production of antisera for the immunoperoxidase localization of bacteria in renal tissue. Bacteria were grown overnight on TSA at 37°C, suspended in phosphate-buffered saline, pH 7.4 (PBS), and boiled for 30 min. The heat-killed bacteria were collected by centrifugation and washed extensively in PBS.

LPS preparation. LPS was purified to detect antibody to LPS in vaccine recipients. It was extracted from strains J96, HU849, and P678-54, separated from capsular polysaccharides (22) and nucleic acids by ultracentrifugation (23), dialyzed against water, and assessed for purity by ultraviolet spectrophotometry, Lowry protein assay (24), and SDS PAGE. In 1-mg samples no ultraviolet absorption occurred between 200 and 300 nm (1-cm light path) and no protein was detected by the Lowry assay. When 100- μ g samples were analyzed by Coomassie Blue-stained SDS PAGE, no protein bands were noted. In contrast, when 40- μ g samples were subjected to SDS PAGE and silver-stained for LPS (18), characteristic LPS banding was observed. On the basis of these studies the LPS preparations were judged to be free of nucleic acid and protein.

Antibody production in rabbits. Antibody to HU849 and SH48 pili was prepared in female New Zealand white rabbits to determine their antigenic relatedness (13, and our Table I) and to detect soluble pilus receptor compounds in murine urine by an inhibition ELISA (see later section). Preimmune sera were obtained. 50 μ g of each pilus protein in 1 ml of PBS was emulsified with an equal volume of complete Freund's adjuvant and administered by multiple subcutaneous and intramuscular injections. Booster injections in Freund's incomplete adjuvant were administered 6 wk later. 10 d thereafter, the animals were bled by cardiac puncture and the sera were filter-sterilized, heated to 65°C for 30 min, and stored at -20°C.

Antibody to the J96 and P678-54 somatic O antigens (discussed previously) was elicited with 10⁸ heat-killed bacteria as described. Antibody to the J96 somatic O antigen was assessed for specificity by slide agglutination (12). 04, but not 02, 06, 08, 018ab, or 075, *E. coli* strains were agglutinated by J96 somatic O antibody.

Synthetic disaccharide pilus receptor compounds and antireceptor antibody. The chemical synthesis of the disaccharides α D-Gal(1 \rightarrow 4) β D-Gal (Syn Gal-Gal) and α D-Man(1 \rightarrow 2) α D-Man (Syn Man-Man) as their 8-(methoxycarbonyl)octyl glycosides was performed according to the method of Lemieux (25) by Chembiomed Ltd. (Edmonton, Alberta, Canada). Their structures were confirmed by examination of their ¹H and ¹³C nuclear magnetic resonance spectra. These compounds were coupled to bovine serum albumin (BSA) (26) and administered to San Juan rabbits in Freund's complete adjuvant as previously reported (27). Monospecific antibody to Syn Man-Man and Syn Gal-Gal was prepared from hyperimmune sera by affinity chromatography with the appropriate synthetic disaccharide immunoabsorbent. The bound antibodies were eluted in 1% (vol/vol) ammonium hydroxide at 4°C and the 280-nm absorbing eluate was neutralized with saturated KH₂PO₄. The antibody was dialyzed against PBS, concentrated by ultrafiltration and filter-sterilized. Electrophoresis on cellulose acetate membranes in

barbital buffer, pH 8.6, revealed a single band with the same mobility as purified rabbit IgG.

The specificity of the antibodies to Syn Gal-Gal and Syn Man-Man was determined by solid-phase inhibition radioimmunoassays. The specificity of anti-Syn Gal-Gal was tested with natural glycosphingolipids (Supelco, Inc., Bellefonte, PA): (a) globotetraosylceramide (globoside, the P antigen of the P blood group system) and globotriosylceramide (Fabry trihexosylceramide, the P^k antigen of the P blood group system), which contain α D-Gal(1 \rightarrow 4) β D-Gal as an internal moiety or in the terminal, nonreducing position, respectively; and (b) glucocerebroside and the monosialoganglioside, G_{M1}, and the disialoganglioside, G_{D1a}, which lack the α D-Gal(1 \rightarrow 4) β D-Gal moiety. In addition, the Syn Gal-Gal antibody specificity was tested with galactose, glucose, and four synthetic disaccharides other than Syn Gal-Gal (Chembiomed Ltd.)—Syn Man-Man, α D-Gal(1 \rightarrow 4) β D-Glu, α D-Gal NAc(1 \rightarrow 4) β D-Gal, and α D-Gal(1 \rightarrow 3) β D-Gal. The Syn Man-Man antibody specificity was determined with mannose, methyl-D-mannopyranoside, fucose, glucose, galactose and, in addition to Syn Man-Man, with the synthetic disaccharides (Chembiomed Ltd.)—Syn Gal-Gal and α D-Man(1 \rightarrow 6) α D-Man.

The tissue-typing reagents (as discussed below) were composed of the monospecific antibody (0.5 mg/ml) and BSA (10 mg/ml) in PBS.

Tissue specimens. BALB/c mice were ether anesthetized. Tissues were taken from the introitus, vagina, bladder, ureter, renal pelvis and kidney. The tissue biopsies were fixed in buffered formalin and processed for paraffin-embedded serial sections.

Immunohistochemical localization of α D-Man(1 \rightarrow 2) α D-Man and α D-Gal(1 \rightarrow 4) β D Gal in BALB/c urogenital tissue. Immunohistochemical staining (avidin-biotin-peroxidase complex) was performed on formalin-fixed paraffin sections, which had been dewaxed through xylol, cleared with graded alcohols, and mounted on glass slides. The slides were flooded with normal goat serum (DAKO Accurate Chemical Corp., Hicksville, NY) diluted 1:10 in PBS with 1% (wt/vol) BSA (PBSA) for 30 min to reduce nonspecific binding of antisera. Excess serum was removed by blotting and the sections were then incubated for 1 h at room temperature with either rabbit anti-Syn Gal-Gal or anti-Syn Man-Man (diluted 1:50 in PBSA). The slides were washed in PBS and the sections were then incubated for 30 min at room temperature with biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA). After the slides were repeatedly washed in PBS, the Vectastain avidin-biotin-peroxidase complex (Vector Laboratories) reagent was applied for 60 min at room temperature and then removed by washing in PBS. These sections were developed for 5 min at room temperature in 0.01% (vol/vol) hydrogen peroxide and 0.05% (wt/vol) diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) in 0.05 M Tris buffer, pH 7.2. The slides were thoroughly washed in distilled water, hematoxylin-counterstained, and mounted. Sections were examined under the light microscope and the brown color reaction product was graded: 3+ intense, 2+ moderate, 1+ weak, and 0 negative. The specificity of the immunohistochemical assay was determined by showing that the staining reaction was abolished by (a) absorbing the Syn Man-Man or Syn Gal-Gal antisera with the homologous (10% wt/vol) hapten and (b) substituting the biotinylated antibody and the avidin-biotin-peroxidase complex reagent with PBSA. The negative control was normal rabbit serum substituted for the primary antisera.

Pilus-receptor compounds in BALB/c mouse urine. The urine of 16-wk-old BALB/c female mice without antibodies in serum to MS and Gal-Gal pili was examined for the presence of soluble pilus receptor compounds. The urine of 5–10 mice was pooled, dialyzed against water with 1,000-mol-wt cutoff membranes (Spectropor, Spectrum Medical Inc., Los Angeles, CA), concentrated, and eluted in 0.1 M sodium phosphate buffer, pH 6.0, containing 0.15 M NaCl from a TSK G3000SW, 7.5-mm \times 60-cm, high-pressure liquid chromatography-steric exclusion column (Varian Instruments, Palo Alto, CA). The fractions were monitored by their absorbance at 280 nm, and those under peaks were pooled and their protein concentrations were adjusted to 2 mg/ml.

Soluble pilus-receptor compounds were sought by determining the capacity of urine and urine chromatography fractions to inhibit the binding of MS and Gal-Gal pili to their respective synthetic receptor analogues. Serial twofold dilutions of urine or urine fractions were incubated with an equal volume of pili (50 µg/ml) in PBS, pH 6.0, in PBSA precoated 1.5-ml plastic microfuge tubes (Beckman Instruments) at 38°C for 3 h followed by overnight incubation at 4°C with constant tumbling. The tubes were centrifuged in a Beckman model B microfuge for 5 min and 100-µl supernatant aliquots added to microtiter wells that had been previously sensitized with either Syn Man-Man or Syn Gal-Gal BSA conjugates (1 µg in 0.1 M sodium carbonate buffer, pH 9.6, for 12 h at 37°C). After 3 h at 37°C, the wells were washed with 0.15 M NaCl containing 0.05% (vol/vol) Brij-35 (NaCl-Brij, Sigma Chemical Co.). 100 µl of MS or Gal-Gal pili antiserum, diluted 1:10,000 and 1:30,000 (13), respectively, with PBSA with 0.05% (vol/vol) Brij were added to each well. The plates were incubated for 3 h at 37°C and then washed three times with NaCl-Brij. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) diluted 1:1000 in PBS-Brij was added to all wells and the plates incubated for 1 h at 37°C and then washed three times with NaCl-Brij. 1 mg/ml of *p*-nitrophenyl phosphate (Sigma) in 0.1 M diethanolamine buffer, pH 9.8, was added to each well and the reaction allowed to proceed for 30 min at 37°C. The reaction was stopped by the addition of 2 N NaOH and the absorbance determined at 405 nm with a MR 580 Micro ELISA Auto-Reader (Dynatech, Torrance, CA). Negative controls included unsensitized wells and sensitized wells that were exposed only to second antibody. A positive control consisting of 0.2% (wt/vol) BSA in PBS was incubated with pili instead of urine. The assays were performed in triplicate and the results recorded as the mean.

Identification of a MS pili-binding urine factor. 200-µg samples from fractions of the urine chromatograph were prepared for SDS PAGE (15) as described above. The gels (separating gel, T = 10.0; stacking gel, T = 2.5) were stained for protein with Coomassie Blue and for carbohydrate by the periodic acid-Schiff method (28). In addition, the transblotting technique of Towbin et al. (29) was used to determine if any of the urine protein bands resolved by SDS PAGE were bound by goat anti-human Tamm-Horsfall protein (Cappel Laboratories).

A murine model of E. coli pyelonephritis. 14- and 28-wk-old female BALB/c mice were obtained from the established mouse colony of Dr. Robert Kallman and the late Dr. Henry Kaplan at this institution. The mice are reared to be specific pathogen-free with a defined gastrointestinal flora by standard procedures, including cesarean delivery in a germ-free environment and followed by a nonpathogenic, defined bacterial cocktail *per ora* at 4 wk of age (30). The kidneys were sterile and the urine was not colonized by aerobic gram-negative bacteria before inoculation (explained below).

J96 (10⁵, 10⁶, and 10⁸ CFU), HU849 (10⁶ CFU), SH48 (10⁶, 10⁸, 10¹⁰, and 10¹² CFU) and P678-54 (10⁶ and 10¹² CFU) were administered by urethral, intravesicular catheterization. The bacteria were grown overnight in trypticase soy broth for 18 h at 37°C, their concentrations (colony-forming units per inoculation volume) adjusted with sterile trypticase soy broth and their putative functional attributes confirmed (Table 1). The mice were anesthetized with ether, and a French No. 2 Fogarty catheter (V. Mueller, Inc., Irvine, CA) with the tip cut off was inserted into the urethral meatus and then advanced until a characteristic "pop" indicated its entry into the bladder. Then, the inoculum was injected and the catheter removed. No attempt was made to obstruct the urinary tract. Acute ureteric reflux with different inoculum volumes was sought by administering the inoculum plus methylene blue to control animals of the same age and then immediately examining the ureters and renal pelvis of autopsied animals for bluish discoloration. The inoculum volume for 10⁵ and 10⁶ CFU was 100 µl and did not cause ureteric reflux. However, reflux was observed when 10⁸, 10¹⁰, and 10¹² CFU were administered in a 250-µl volume.

2 d later, the mice were sacrificed by prolonged ether anesthesia. Urine and renal tissue were processed as follows: the suprapubic abdominal area was compressed to express urine which was collected from the urethral meatus with a sterile 10-µl loop and inoculated onto ~0.5 cm² area of TSA or TSA supplemented with antibiotics (Table 1). The plates were incubated for 18–24 h at 37°C and read. Specimens were assessed for colony-forming unit density per ~0.5 cm² area by the following relative grading criteria: 5+ (confluent growth, >90% of the area); 4+ (not confluent growth but too numerous to count); 3+ (>20 CFU); 2+ (≤20 CFU); 1+ (≤3 CFU); and 0 (no growth).

Urine and renal isolates were identified as follows: J96, a gram-negative rod that grows on TSA without added antibiotics and is agglutinated by J96 somatic O antigen antisera; SH48, a gram-negative rod that grows on chloramphenicol-supplemented TSA, agglutinated by rabbit antisera to SH48 pili, and exhibits MS, but not Gal-Gal, agglutination of latex particles coated with synthetic receptor analogues (Table 1); HU849, a gram-negative rod that grows on tetracycline-supplemented TSA, is agglutinated by rabbit antisera to HU849 pili, and exhibits Gal-Gal, but not MS, agglutination of latex particles coated with synthetic receptor analogues; P678-54, a gram-negative rod that grows on TSA, does not exhibit MS and Gal-Gal agglutination of latex particles (Table 1), and is not agglutinated by SH48 or HU849 pili antisera.

The kidneys were sterilely excised and sagittally sectioned through the midpelvis. The cut surface was streaked on TSA, the plates were incubated for 18–24 h at 37°C, and the colonies on a ~2-cm² area impression streak were assessed as described above. Renal tissue was processed for light microscopy. The sections were stained by hematoxylin and eosin and Giemsa stains. In addition, renal tissues from mice challenged with J96 were subjected to immunoperoxidase staining with J96 or P678-54 somatic O antigen antisera (diluted 1:100 in PBS) as described by Sternberger (31). The specificity of the J96 and P678-54 immunoperoxidase-staining reactions was confirmed by determining that none of the serologic reagents other than the J96 and P678-54 somatic O antigen antisera reacted with J96 or P678-54, HU849, and SH48, respectively. Renal invasion was defined by the presence of the immunoperoxidase brown reaction product within tissue and by the local accumulation of polymorphonuclear leukocytes, indicating the development of parenchymal microabscesses.

Immunization protocols and challenge experiments. Groups of 16-wk-old BALB/c mice were immunized with five separate vaccines: (a) pure SH48 MS pili, (b) pure HU849 Gal-Gal pili, (c) J96 somatic O antigen, (d) P678-54 somatic O antigen, and (e) PBS (1 ml). The immunogens were emulsified in equal volumes of complete Freund's adjuvant and administered by multiple subcutaneous and intramuscular injections. The pili vaccines were composed of 50 µg of protein estimated by weighing 10 mg of purified, lyophilized pili, which were then suspended in PBS to a final concentration of 50 µg/ml. The somatic O antigen vaccines were composed of 10⁸ heat-killed bacteria in 1 ml of PBS.

2 wk after immunization the vaccine recipients were challenged with 10⁶ CFU of J96 in 100 µl by intraurethral catheterization (as described above). 2 d later the mice were exsanguinated and the sera were processed as described above. Urine collection from each mouse was attempted and assessed for specific J96 growth. Renal colonization was determined by culture and renal invasion by light microscopy and immunoperoxidase staining as previously described.

ELISA for LPS and pili antibodies. The sera of vaccine recipients and normal mice were assessed for IgG antibodies to specific LPS or pili by a direct ELISA (32). The wells of disposable polystyrene "U" microtiter plates were sensitized with 100 µl of a 1 µg/ml solution of LPS or pilus protein in 0.1 M sodium carbonate buffer, pH 9.6, for 12 h at room temperature. Wells were washed three times with NaCl-Brij. Serially diluted sera in PBSA-Brij were added and the plates were incubated for 3 h and then washed. Alkaline phosphatase-conjugated rabbit anti-mouse IgG (Miles Research Products, Bethesda, MD)

diluted 1:500 in PBS-Brij was then added to each well and incubated 1 h at 37°C. The plates were washed three times with NaCl-Brij and processed as described previously. Negative controls consisted of non-sensitized wells and sensitized wells that were exposed only to second antibody. The absorbance was determined at 405 nm on the ELISA auto-reader. Each sample was assessed in triplicate and recorded as the mean.

Pili-specific antibody was sought in the urine of 28-wk-old BALB/c mouse vaccine recipients, inasmuch as the urine volumes at this age are sufficient for immunologic analysis. Four controls were immunized with saline emulsified with complete Freund's adjuvant, and seven mice were immunized with 100 µg of purified Gal-Gal pili in Freund's adjuvant. 2 wk later, urine and blood were obtained for immunologic analysis and the mice were challenged with 10⁸ CFU of J96. Gal-Gal pili antibody in serum and in urine diluted 1:10, 1:20, and 1:40 in PBSA-Brij was determined by ELISA with alkaline phosphatase-conjugated rabbit anti-mouse IgG, IgM, and IgA.

Protein concentration and protein standards. Protein concentration was determined by weight on an analytical balance if the sample was >10 mg or by the Lowry assay (24) using BSA as a standard. SDS PAGE protein standards were purchased from Bethesda Research Laboratories (Bethesda, MD).

Statistics. Fisher's exact test, Chi-square (χ^2) test, and Student's *t* test were used.

Results

The relative efficacy of MS and Gal-Gal pili vaccines was assessed in a murine pyelonephritis model. The 16-wk-old BALB/c mouse was selected for these studies because its immune system is mature and the bladder epithelial cells of this species reportedly contain globotetraosylceramide and bind Gal-Gal pili (8). The characteristics of the model are as follows: (a) it simulates an ascending infection mode, and (b) it does not entail obstruction to urine flow or traumatic manipulation of tissue. In addition, relevant uroepithelial surfaces should exhibit MS and Gal-Gal pilus-receptor compounds. This was assessed with an indirect immunoperoxidase assay.

Pilus-receptor compounds—immunoperoxidase localization in genitourinary tissues. The pyelonephritogenic *E. coli* strain used in this study and the recombinant strains derived from it express pili that bind Syn Gal-Gal or Syn Man-Man (13). These synthetic receptor analogues were used as immunogens and the affinity-purified, monospecific rabbit antibodies bound the same synthetic receptors as the recombinant clones (discussed above). These antibodies were employed in an immunocytochemical assay with paraffin sections of female BALB/c mouse urogenital tissues to determine the distribution and relative density of MS and Gal-Gal pilus receptor carbohydrates. The specificity of these antibodies was determined with a competitive RIA (data not shown). Of the natural and synthetic compounds tested (see Methods), anti-Syn Gal-Gal bound only Syn Gal-Gal and the neutral glycosphingolipid, triaosylceramide, which specifies the P^k antigen of the P blood group system and contains α D-Gal(1→4) β D-Gal in the terminal, nonreducing position. Of particular interest, it did not bind globoside, which specifies the P antigen of the P blood-group system and contains the digalactoside moiety in an internal position. Anti-Syn Man-Man bound only Syn Man-Man but not monosaccharides or the closely related dimannoside α D-Man(1→6) α D-Man. It is concluded that the Syn Gal-Gal

antibody is specific for compounds with a nonreducing α D-Gal(1→4) β D-Gal moiety, particularly the P^k antigen, and that the Syn Man-Man antibody is specific for compounds that contain α D-Man(1→2) α D-Man, particularly the asparagine-linked "high-mannose" oligosaccharides of glycoproteins.

The specificity of the immunohistochemical assay with these reagents was confirmed by the complete inhibition of tissue staining by absorption with the homologous compound. In addition, no brown reaction product was detected when PBS or normal rabbit serum was substituted for the primary antibody.

Both antibodies bound to urogenital mucosa and a brown reaction product was easily detected. Representative sections of the Gal-Gal pilus-receptor analogue distribution in BALB/c mouse vesical and renal tissues are depicted in Figs. 1 and 2. Diffuse, intense cytoplasmic staining of collecting tubular and convoluted distal tubular epithelial cells is noted. Similarly, bladder epithelial surfaces are strongly positive. The lamina propria and vascular structures are negative.

Table II summarizes the tissue distribution and relative density of the MS and Gal-Gal pilus-receptor compounds. Both were detected on the epithelial surfaces of the vagina, bladder, ureter, renal pelvis, and kidney including the collecting tubules. This established a molecular basis for the possible importance of both MS and Gal-Gal pili in the pathogenesis of pyelonephritis. In contrast, they were not detected in glomeruli, and the loop of Henle cells lacked the Gal-Gal receptor analogue.

Pilus-receptor compounds in urine. The fixation methods used in the immunoperoxidase assay cannot preserve surface mucins and other soluble factors in urine that might bind pili. Therefore, pilus-receptor compounds were sought in pooled BALB/c mouse urine. BSA-conjugated MS and Gal-Gal pilus-receptor analogues were employed in a solid-phase competitive binding assay with pure MS or Gal-Gal pili and serially diluted urine. Specific pili antibody was used to determine quantitatively the receptor-bound pilus antigen at each urine dilution. A titrable urine factor inhibited the binding of MS pili to its receptor analogue (Fig. 3). In contrast, no urine factor blocked the receptor binding capacity of Gal-Gal pili.

The urine compound that bound MS pili was partially characterized by molecular sieve chromatography. It eluted in the void volume from a high-pressure liquid chromatography-steric exclusion TSK G3000SW column (Fig. 4). When analyzed by SDS PAGE, this peak contained a 87,000-mol-wt protein, which was stained by the periodic acid-Schiff reaction for glycoprotein. When electrophoretically transferred to nitrocellulose paper, the protein was bound by goat anti-human uromucoid at 1:1000 dilution in PBS. This urine factor is therefore physically, chemically, and antigenically identical to the Tamm-Horsfall protein.

The BALB/c mouse pyelonephritis model—parameters for determining vaccine efficacy. The BALB/c mouse model was examined with four *E. coli* strains to assess the pathogenic significance of MS and Gal-Gal pili and to establish conditions for determining the efficacy of pilus vaccines for the prevention of pyelonephritis. The following strains were employed (Table I): J96, a hemolytic, serum resistant, colicin V-positive human pyelonephritis isolate that simultaneously expresses Gal-Gal and MS pili; SH48 and HU849, recombinant strains prepared

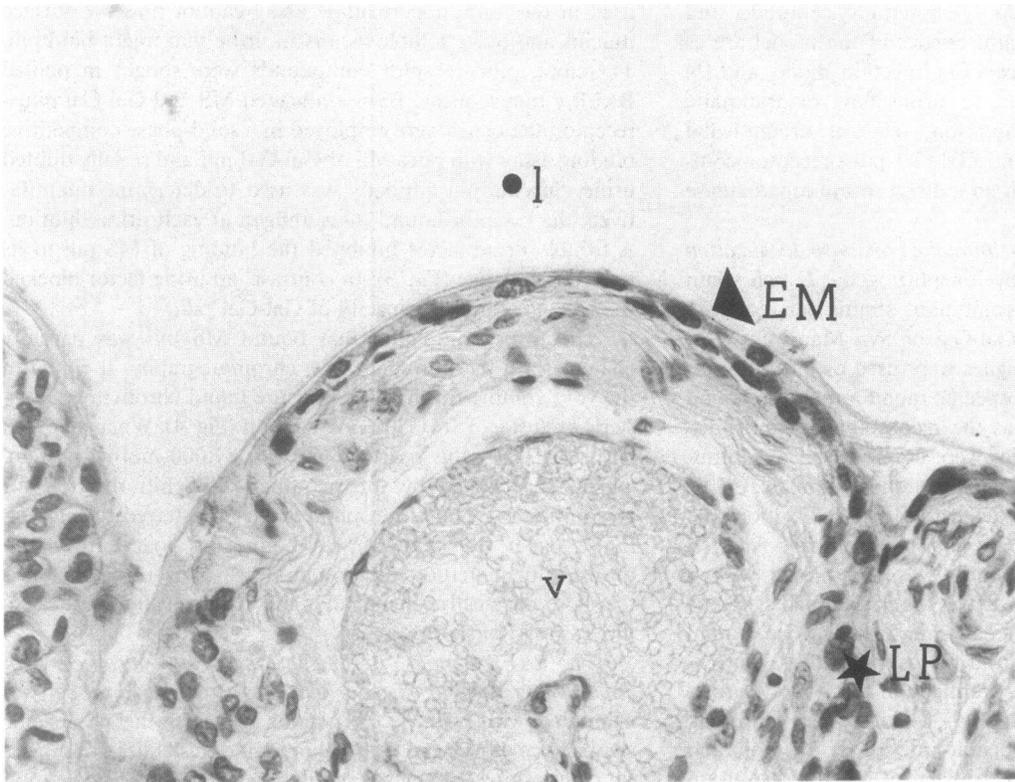
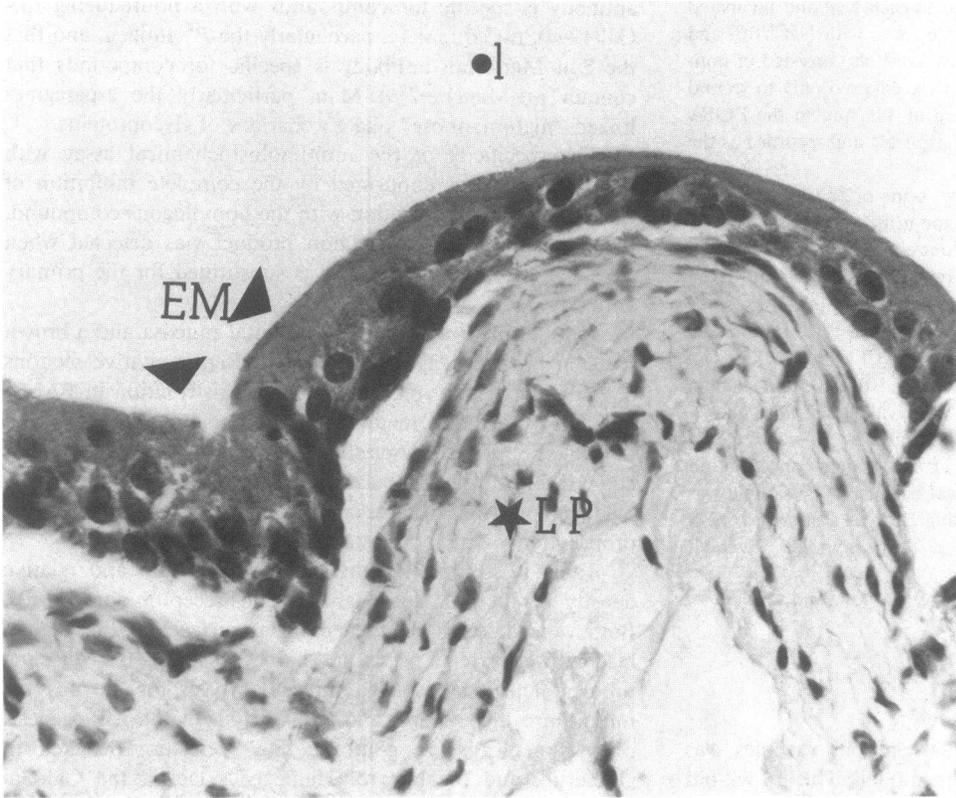


Figure 1. (Top) Immunohistologic localization of α D-Man(1 \rightarrow 2) α D-Man (see text) in the BALB/c mouse bladder ($\times 400$). Immunoperoxidase staining of the bladder epithelial mucosa (EM) is intense. No staining of the lamina propria (LP \star) is evident. (Bottom) Mouse

bladder tissue processed as a negative control did not employ monospecific antibody to α D-Man(1 \rightarrow 2) α D-Man. No immunoperoxidase reaction product is apparent. \bullet l, lumen; v, vein.

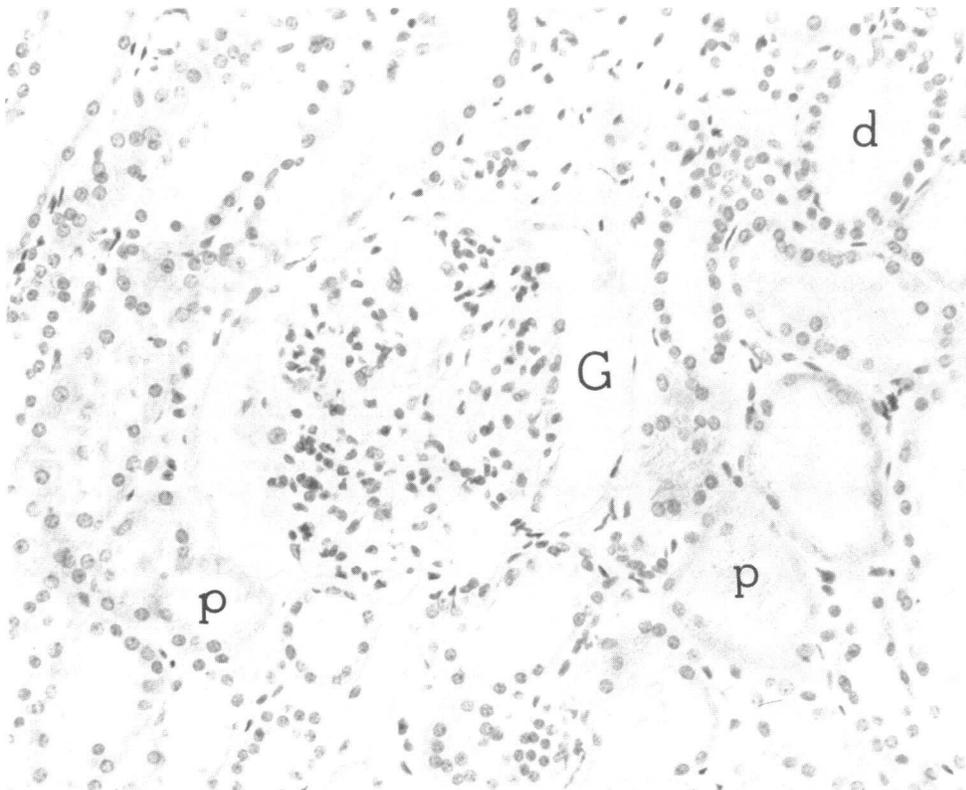
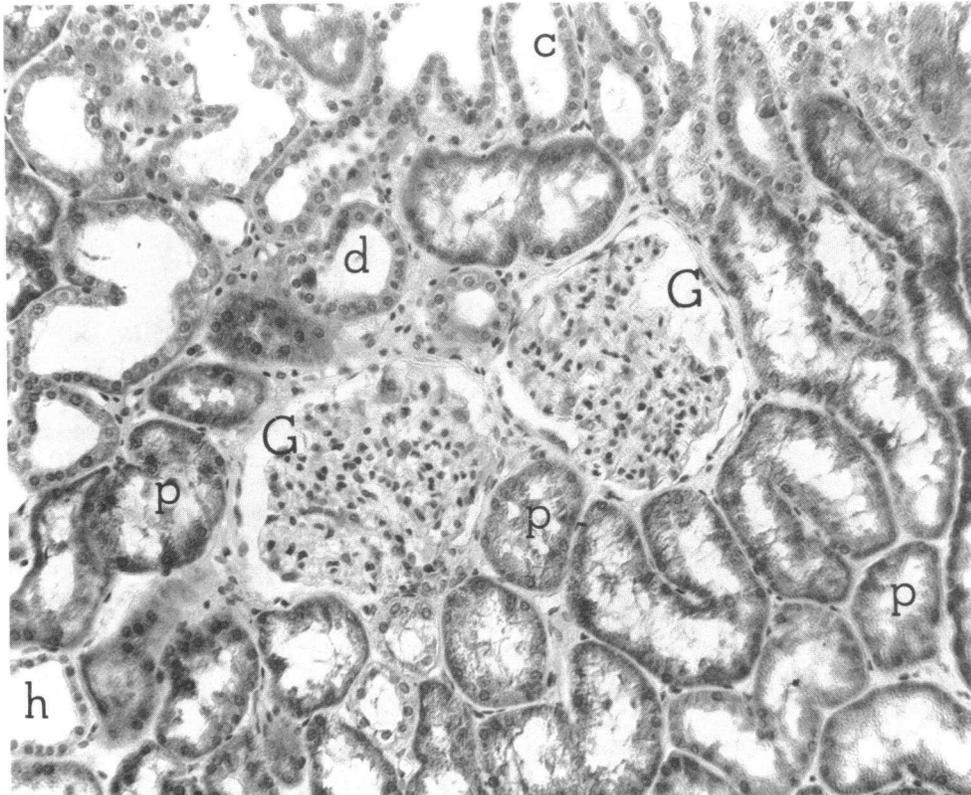


Figure 2. (Top) Immunohistologic localization of α D-Gal(1 \rightarrow 4) β D-Gal (see text) in the BALB/c mouse kidney (\times 190). Immunoperoxidase staining of proximal tubular cells (*p*), distal convoluted tubular cells (*d*), and collecting tubular cells (*c*) is intense. No staining of

glomeruli (*G*) or loop of Henle cells (*h*) is evident. (Bottom) Mouse kidney tissue processed as a negative control did not employ monospecific antibody to α D-Gal(1 \rightarrow 4) β D-Gal (\times 250). No immunoperoxidase reaction product is apparent.

Table II. Immunoperoxidase Detection of Syn Man-Man and Syn Gal-Gal Pilus Receptors in BALB/c Mouse Urogenital Tissue

Tissue/cell	Intensity of pilus disaccharide receptor*	
	Syn Man-Man‡	Syn Gal-Gal§
Vagina/squamous epithelium	+3	+3
Bladder/transitional epithelium	+3	+2
Lamina propria		
Fibroblasts	0	0
Smooth muscle	+2	+2
Ureter/transitional epithelium	+3	+2
Renal pelvis/transitional epithelium	+3	+2
Kidney		
Glomerulus	0	0
Proximal tubular cells	+2	+2
Loop of Henle cells	+1	0/+1
Collecting ducts	+3	+3

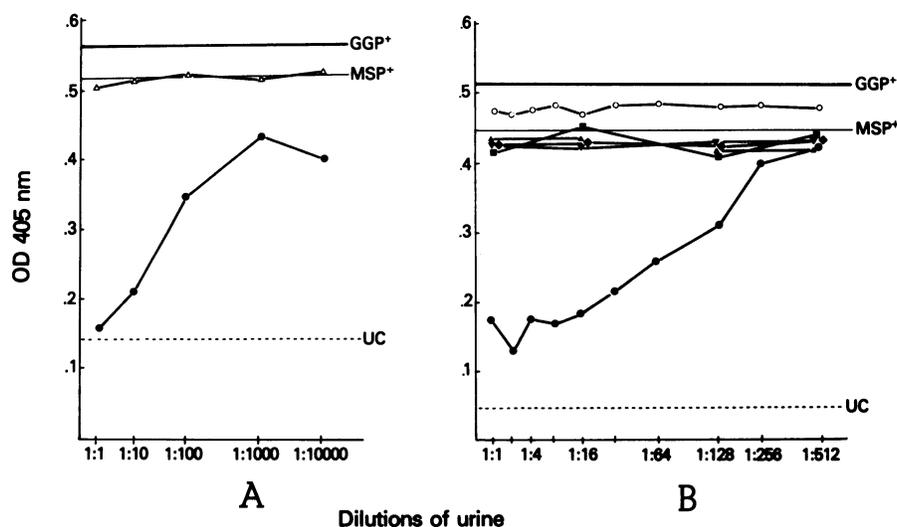
* Intensity of pilus disaccharide receptor—graded 3+ (intense brown) to 0 (no brown reaction product).

‡ Mannose pilus receptor was assessed by monospecific, affinity-purified rabbit antibody to synthetic α D-Man(1 \rightarrow 2) α D-Man.

§ Gal-Gal pilus receptor was assessed by monospecific, affinity-purified rabbit antibody to synthetic α D-Gal(1 \rightarrow 4) β D-Gal.

^{||} The distribution of pilus receptors in the lamina propria of the bladder were the same throughout the urogenital tract.

from J96 DNA that express MS or Gal-Gal pili, respectively, but are not hemolytic, colicin V-positive, or motile; and P678-54, a nonpiliated K12 recipient. The strains were administered to the bladder by intraurethral catheterization. Urine and renal colonization were determined by culture. Renal invasion was



inhibition assay. Only peak II fractions (●) inhibited receptor binding by MSP⁺; fractions under peaks I (▲), III (◆), IV (▼), and V (■) were inactive. No urine fraction, including peak II (○) inhibited receptor binding by GGP⁺. Each data point represents the average of three or more determinations.

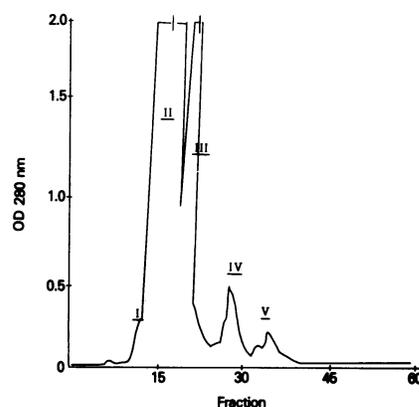


Figure 4. Molecular sieve chromatography of BALB/c mouse urine protein. Fractions (1.0 ml) were eluted from a HPLC-TSK G3000 SW column (7.5 mm \times 60 cm) in 0.1 M PBS, pH 6.0, at a flow rate of 0.5 ml/min. Fractions under peaks I–V were employed in the ELISA inhibition assay for pilus-receptor compounds in BALB/c mouse urine. Peak II, the MS pilus-binding fraction (Fig. 3 B), was analyzed by SDS PAGE (15) and found to contain a periodic acid-Schiff-positive 87,000-mol-wt protein, which was bound by goat anti-human uromucoid (Tamm–Horsfall protein) when subjected to Western blotting (29).

operationally defined as follows: (a) a positive immunoperoxidase stain for O antigen in renal parenchyma and (b) light microscopy evidence for the focal accumulation of bacteria and polymorphonuclear leukocytes. These criteria appear to exclude the possibility that bacteria or bacterial antigen might appear to be in renal tissue but instead be in tubular lumina as a result of tubulocalyceal backflow, because (a) ureteric reflux did not occur when inocula of 10^6 organisms in 100 μ l were instilled in the bladder and (b) no immunologic or morphologic evidence of invasion, as defined above, was found with the recombinant strains or the nonpiliated control (discussed below).

J96 (10^6 CFU) colonized the urine and kidneys in all mice (Table III). In doses of 10^6 and 10^8 CFU, focal parenchymal

Figure 3. (A) Mannose-sensitive pili (MSP⁺) and Gal-Gal pili (GGP⁺) receptor analogues in BALB/c mouse urine. Inhibition of binding by MSP⁺ and GGP⁺ for their respective solid-phase synthetic receptors by 10-fold dilutions of BALB/c mouse urine was assessed by ELISA (see text). A titrable urine factor prevented receptor binding by MSP⁺ (●). No urine factor prevented receptor binding by GGP⁺ (Δ). Urine control (uc) (----): absorbance of urine diluted 1:1 in PBS, pH 6.0, without pilus antigens. MSP⁺ (—) and GGP⁺ (—): absorbance of bound MSP⁺ or GGP⁺ detected by specific antibody in the absence of BALB/c mouse urine. (B) Urine fractions responsible for MSP⁺ receptor-binding inhibition. Fractions under peaks resolved by molecular sieve chromatography (Fig. 4) were assessed by the ELISA

Table III. BALB/c Mouse Urinary Tract Infection Model

Strain	Pilus operon(s)	Inoculum	Colonization				
			Urine		Right kidney		Renal invasion right kidney‡
			No. +/total	RCD*	No. +/total	RCD*	
			<i>n/n</i>		<i>n/n</i>	<i>n/n</i>	
J96	MS§ and Gal-Gal	10 ⁵	4/7	3.2	5/7	2.3	ND†
		10 ⁶	8/8	5.0	9/9	4.4	9/9
		10 ⁸	5/5	4.8	5/5	4.3	5/5
HU849	Gal-Gal	10 ⁶	8/9	4.2	10/10	3.6	0/10
SH48	MS	10 ⁶	4/8	1.4	0/8	0	0/8
		10 ⁸	ND	ND	0/7	0	ND
		10 ¹⁰	ND	ND	5/7	1.1	ND
		10 ¹²	ND	ND	6/6	3.5	0/3
P678-54	None	10 ⁶	0/11	0	0/11	0	0/5
		10 ¹²	0/5	0	0/5	0	ND

* Relative colonization density (RCD) is expressed as the mean of the particular strain colonizing the urine or kidney per total number of inoculated mice. ‡ Renal invasion—assessed by light microscopy. § MS, Mannose-sensitive agglutination. || Gal-Gal, α D-Gal(1 \rightarrow 4) β D-Gal agglutination. † ND, not done.

concentrations of bacteria indicative of renal invasion were found in each of the 14 autopsied mice (Fig. 5). HU849 (10⁶ CFU) did not invade, although it colonized the urine in 9 of

10 mice and the kidneys in 10 of 10 mice (Table III). In contrast, SH48 (10⁶ CFU) colonized the urine in four of eight mice but did not colonize or invade the kidneys. P678-54 did

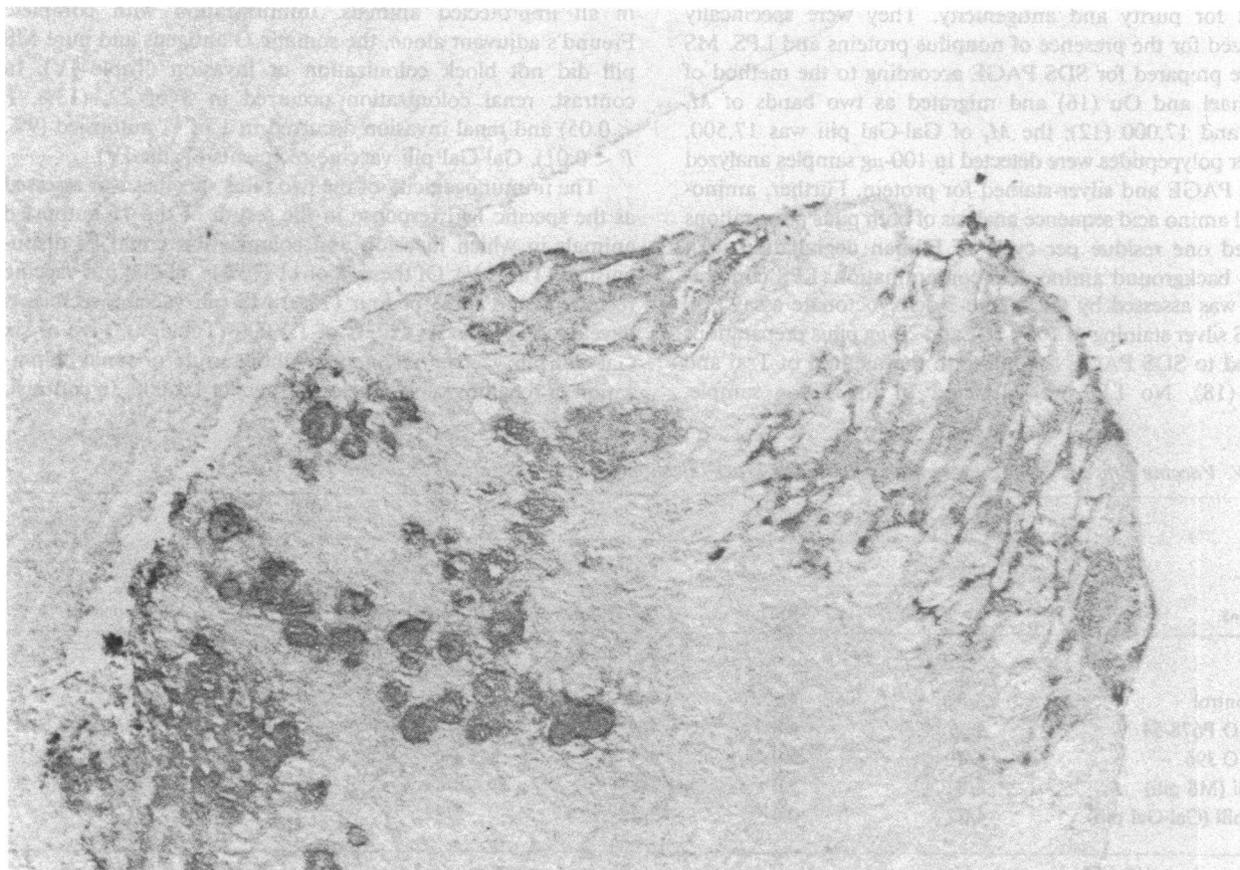


Figure 5. Renal invasion by *E. coli* J96 of the BALB/c mouse kidney. Renal tissue was obtained 2 d after intravesicular inoculation of 10⁶ CFU and prepared for microscopy by immunoperoxidase

staining with anti-O4 antiserum. A dark reaction product within the renal papilla represents J96 bacteria or its O antigen within tissue. (\times 40).

not colonize the BALB/c mouse urinary tract. These data suggest that Gal-Gal pili bind epithelial cell receptor molecules and thereby promote renal colonization. Conversely, the binding capacity of MS pili for mannose-containing receptor compounds on renal epithelium (Table II) may be inhibited in vivo by the Tamm-Horsfall glycoprotein in urine. To characterize this phenomenon in greater detail, increasingly larger inocula were administered in an attempt to supercede this inhibitory effect. SH48 (10^8 , 10^{10} , and 10^{12} CFU) were administered in 250 μ l. This volume resulted in acute ureteric reflux. Renal colonization occurred only with inocula of 10^{10} and 10^{12} CFU (Table III), and the colonization density was correlated with the challenge dose.

These studies also sought to distinguish renal invasion and colonization. Recombinant strains HU849 and SH48 colonized renal epithelium at challenge doses of 10^6 and 10^{10} CFU, respectively (Table III), but did not invade. In contrast, J96 (10^6 CFU) caused renal colonization and invasion. These data indicate that colonization and invasion are distinct pathogenic events probably mediated by separate microbial molecules. The efficacy of Gal-Gal and MS pili pyelonephritis vaccines was determined to test the validity of this proposition and to provide further evidence about the pathogenic significance of these pilus proteins.

Gal-Gal and MS pili vaccines for the prevention of pyelonephritis. MS and Gal-Gal pili were prepared as vaccines from recombinant strains SH48 and HU849. Vaccine efficacy was examined with pilus preparations that had been rigorously assessed for purity and antigenicity. They were specifically scrutinized for the presence of nonpilus proteins and LPS. MS pili were prepared for SDS PAGE according to the method of McMichael and Ou (16) and migrated as two bands of M_r 19,000 and 17,000 (12); the M_r of Gal-Gal pili was 17,500. No other polypeptides were detected in 100- μ g samples analyzed by SDS PAGE and silver-stained for protein. Further, amino-terminal amino acid sequence analysis of both pilus preparations generated one residue per cycle of Edman degradation with <0.01% background amino acid contamination. LPS contamination was assessed by the 2-keto-3-deoxyoctonate assay (19) and LPS silver staining of 100-, 50-, and 20- μ g pilus preparations subjected to SDS PAGE according to the method of Tsai and Frasch (18). No LPS was detected in the 20- μ g sample.

Inasmuch as the sensitivity of this assay was determined empirically to be ~ 10 ng of LPS, the percent by weight of contaminating LPS was judged to be <0.01. Nonetheless, it may not be possible to extract all contaminating LPS from gram-negative bacterial surface proteins, including pili, with aqueous solvents (33). Further, small amounts of protein-bound LPS may elicit an immune response. Therefore, LPS containing somatic O antigens were also prepared as vaccines (see below) and their efficacy was compared with pili. The antigenic cross-reactivity of the MS and Gal-Gal pili vaccines was assessed by a two-step competitive ELISA with antisera to each pilus preparation. Less than 5% shared antigenicity was detected (13). Finally, 100- μ g of each pilus preparation was electrophoresed in SDS PAGE slab gels and electrophoretically transferred to nitrocellulose paper according to Swanson's et al. (34) modification of Towbin's (29) method. The pilus bands were bound only by the homologous pilus antisera, diluted 1:1000 in PBS. Therefore, these considerations indicate that the protection conferred by a Gal-Gal or MS pilus vaccine can be confidently attributed to the homologous immunogen.

50 μ g of purified Gal-Gal or MS pili or somatic O antigen (prepared from heat-killed J96 or P678-54 organisms) or PBS were emulsified in complete Freund's adjuvant and administered by intramuscular and subcutaneous injections to 16-wk-old BALB/c mice. 2 wk later the mice were challenged with 10^6 CFU of strain J96 by intravesicular inoculation to assess vaccine efficacy. This inoculum is ~ 100 -fold the ID_{50} and 2-d postchallenge results in J96 renal colonization and invasion in all unprotected animals. Immunization with complete Freund's adjuvant alone, the somatic O antigens and pure MS pili did not block colonization or invasion (Table IV). In contrast, renal colonization occurred in 3 of 22, (13%, $P < 0.05$) and renal invasion occurred in 1 of 11 autopsied (9%, $P < 0.01$), Gal-Gal pili vaccine recipients (Table IV).

The immunogenicity of the two pilus vaccines was assessed as the specific IgG response in the serum of the 15 autopsied animals in which invasion and colonization could be distinguished (Table V). Of these, 8 of 11 (73%) Gal-Gal pili vaccine recipients and three of four (75%) MS pili vaccine recipients developed specific IgG titers of 1:1,000 (Table V). Five of six Gal-Gal pili vaccine recipients without urine or renal colonization or renal invasion developed titers of 1:1,000. In contrast,

Table IV. Vaccine Efficacy for the Prevention of Urinary Tract Infection by *E. coli* Strain J96*

Immunogen‡	J96 Colonization§					J96 renal invasion of right kidney¶
	Urine		Right kidney	Left kidney		
	No. +/total	RCD	No. +/total	No. +/total	RCD	
	<i>n/n</i>		<i>n/n</i>	<i>n/n</i>		<i>n/n</i>
Buffer control	8/8	4.6	8/8	8/8	4.2	4/4
Somatic O P678-54	4/4	4.8	4/4	4/4	4.0	4/4
Somatic O J96	4/4	5.0	4/4	4/4	4.5	4/4
SH48 pili (MS pili)	8/8	5.0	8/8	8/8	5.0	4/4
HU849 pili (Gal-Gal pili)	4/22	0.6	3/22	2/22	0.3	1/11

* Challenge dose: 10^6 CFU by urethral intravesicular catheterization 2 wk after immunization. ‡ Administered to 16-wk-old BALB/c mice in complete Freund's adjuvant by subcutaneous and intramuscular injections. § Assessed by culture 2 d after challenge dose; relative colonization density (RCD) is expressed as the mean of J96 colonizing the urine or the kidney per total number of inoculated mice or the total number of kidneys examined, respectively. ¶ Assessed by light microscopy.

Table V. Vaccine Efficacy of Gal-Gal and MS pili in Autopsied BALB/c Mice: Correlation with Specific IgG Antibody in Serum*

Vaccine recipient	J96 Colonization‡		J96 renal invasion§	Gal-Gal and MS pili serum ELISA titer
	Urine	Kidney		
C1	+	+	+	<10
C2	+	+	+	<10
C3	+	+	+	<10
C4	+	+	+	<10
Gal-Gal 1	+	-	-	1,000
Gal-Gal 2	-	-	-	1,000
Gal-Gal 3	-	+	-	100
Gal-Gal 4	-	+	+	100
Gal-Gal 5	-	-	-	1,000
Gal-Gal 6	-	-	-	1,000
Gal-Gal 7	-	-	-	1,000
Gal-Gal 8	-	-	-	100
Gal-Gal 9	-	-	-	1,000
Gal-Gal 10	+	-	-	1,000
Gal-Gal 11	+	-	-	10,000
MS 1	+	+	+	1,000
MS 2	+	+	+	1,000
MS 3	+	+	+	1,000
MS 4	+	+	+	100

* 16-wk-old BALB/c mice were immunized with normal saline plus Freund's adjuvant (recipients C1-C4), Gal-Gal pili (50 µg) plus Freund's adjuvant (recipients Gal-Gal 1-11) or MS pili (50 µg) plus Freund's adjuvant (recipients MS 1-4) and then challenged by intravesicular administration with 10⁶ CFU of *E. coli* strain J96. 2 d later blood and urine were obtained and the kidneys were assessed for colonization and invasion.

‡ Assessed by culture as described in Methods; no growth and culture-positive for *E. coli* strain J96 are denoted by + and -, respectively.

§ Assessed by immunoperoxidase staining with J96 somatic O antigen antisera and light microscopy as described in Methods; invasion is denoted by + and absence of invasion by -.

^{||} Reciprocal of the greatest serum dilution positive by ELISA for the homologous pilus antigen. The controls' sera (C1-C4) were tested separately with Gal-Gal and MS pili.

both autopsied Gal-Gal pili recipients with renal colonization or invasion developed titers of only 1:100 (Table V). The titers of all O antigen recipients were >1:1,000 (data not shown).

IgG, IgM, and IgA Gal-Gal pili antibody also was sought in the serum and urine of 28-wk-old BALB/c mouse vaccine recipients and correlated with protection to the intravesicular administration of 10⁸ CFU of strain J96 (Table VI). Urine and renal colonization occurred in each of four controls vaccinated with saline in complete Freund's adjuvant. In contrast, no urine or renal colonization occurred in seven Gal-Gal pili vaccine recipients, and in each IgG and IgM antibody titers in serum were 1:10,000 and 1:1,000, respectively. Three vaccine recipients developed urine IgG pili antibody titers of 1:10. No IgA pili antibody was detected in serum or urine.

Discussion

E. coli isolated from endogenous infections of the urinary tract usually originate in the colon (35, 36). From the stool, uro-

pathogenic strains colonize the vaginal introitus and periurethral region (37). Colonization of uroepithelium may ensue, leading to bacteriuria. Symptoms arise when invasion of mucosa, cell death, and inflammation occur in bladder or kidney. Cystitis and pyelonephritis, therefore, may be viewed as a culmination of a sequence of events mediated by specific determinants of microbial virulence. It follows that uropathogenic *E. coli* appear to comprise a pathogenic phenotype: they usually belong to a restricted number of O and K antigen serogroups (35, 38); they are resistant to the bactericidal action of normal human serum (39, 40); they secrete hemolysin (41, 42) and produce colicin V (43, 44); and they attach to uroepithelial cells in vitro (45-47).

Mucosal colonization is the in vivo equivalent of epithelial cell adherence and appears to precede and be a prerequisite for subsequent pathogenic events (as discussed above). It is mediated by bacterial adhesions and the epithelial cell surface molecules to which they bind. The studies reported here examine how the binding specificity of Gal-Gal and MS pili of known purity and the location and density of their respective receptor molecules determine the colonizing capacity of a bacterium. In addition, evidence is provided for the efficacy of a Gal-Gal pilus pyelonephritis vaccine.

The murine pyelonephritis model employed in these studies was designed to imitate infection in the anatomically normal human urinary tract. The characteristics of this model and a model recently reported by Hagberg et al. (48) are as follows: (a) bacteria are introduced into the bladder by urethral catheterization, thereby simulating an ascending infection mode; (b) the inoculum volume required to cause infection by the clinical isolate does not lead to ureteric reflux; (c) urine flow is not obstructed nor renal tissue traumatized; and (d) transformants are used that harbor recombinant plasmids specifying only MS or Gal-Gal pili. Although many animal species have been proposed as urinary tract infection models (49), the BALB/c mouse was chosen for these studies because globoseries glycolipids are present in the human and mouse kidney and contain αD-Gal(1→4)βD-Gal, the receptor moiety for Gal-Gal binding. Moreover, human *E. coli* pyelonephritis isolates bind mouse, but not rat, uroepithelial cells (47). In contrast to other urinary tract infection models (49), including Hagberg's et al. (48), this study determined the distribution and relative density of Gal-Gal and MS pilus receptor compounds on uroepithelial surfaces and as soluble factors in urine, systematically distinguished renal colonization and invasion by microbiologic and immunohistochemical criteria, and compared the efficacy of pyelonephritis vaccines composed of MS or Gal-Gal pili that had been purified from recombinant strains and chemically and serologically characterized (13).

The molecular basis for the binding of pili to uroepithelium was assessed with antireceptor antibodies by a technique previously employed to localize the Lewis blood group antigens in tissues (25-27). Syn Man-Man and Syn Gal-Gal were chemically synthesized and found to bind MS and Gal-Gal pili preparations, respectively (13, our Table I). These were used as immunogens and immunoadsorbents. The resulting affinity purified Syn Gal-Gal antibody bound αD-Gal(1→4)βD-Gal in the terminal, nonreducing position of trihexosylceramide (the P^k blood group antigen), but not other common glycosphingolipids. The affinity-purified Syn Man-Man antibody bound αD-Man(1→2)αD-Man (but not mannose or αD-

Table VI. Vaccine Efficacy of Gal-Gal Pili in 28-wk-old BALB/c Mice: Correlation with Specific IgG, IgA, and IgM Antibody in Serum and Urine*

Vaccine recipient	J96 Colonization‡		Gal-Gal pili antibody titer§					
	Urine	Kidney	Serum			Urine		
			IgG	IgM	IgA	IgG	IgM	IgA
CI	+	+	<100	<100	<100	<10	<10	<10
CII	+	+	<100	<100	<100	<10	<10	<10
CIII	+	+	<100	<100	<100	<10	ND	ND
CIV	+	+	<100	<100	<100	<10	ND	<10
Gal-Gal 23	-	-	10,000	100	100	<10	<10	<10
Gal-Gal 24	-	-	10,000	1,000	<100	10	<10	<10
Gal-Gal 25	-	-	10,000	1,000	<100	10	<10	<10
Gal-Gal 26	-	-	10,000	1,000	<100	10	<10	<10
Gal-Gal 27	-	-	10,000	1,000	<100	<10	<10	<10
Gal-Gal 28	-	-	10,000	1,000	<100	<10	<10	<10
Gal-Gal 29	-	-	10,000	1,000	<100	<10	<10	<10

* 28-wk-old mice were immunized with normal saline plus Freund's adjuvant (recipients CI-CIV) or Gal-Gal pili (100 µg) plus Freund's adjuvant (recipients Gal-Gal 23-29). 2 wk later urine and blood were obtained for immunologic analysis and the mice were challenged with 10⁸ CFU of *E. coli* strain J96. 2 d thereafter the mice were sacrificed, and urine and renal colonization was determined. ‡ Assessed by culture as described in Methods; no growth and culture-positive for *E. coli* strain J96 are denoted by + and -, respectively. § Reciprocal of the greatest serum or urine dilution positive for Gal-Gal pili by ELISA with rabbit anti-mouse IgG, IgM, and IgA as second antibody. || ND, not determined.

Man(1→6)αD-Man), a moiety of the "high-mannose," asparagine-linked oligosaccharides of glycoproteins (50). These reagents were employed to detect pilus-receptor compounds in BALB/c mouse tissues (Table II), and their specificity for their respective antigens in tissue was further confirmed by (a) the complete inhibition of the immunoperoxidase reaction by the soluble homologous synthetic disaccharide, (b) the absence of immunoperoxidase staining when normal rabbit IgG was substituted for the specific immune IgG, (c) the presence in single tissue sections of both immunoperoxidase positive and negative cell types (Table II, Figs. 1 and 2), and (d) the total absence of the Syn Gal-Gal antigen in rat uroepithelium (Lark, D., unpublished observation). Intense or moderate (3+ and 2+) staining by antibodies to Syn Man-Man and Syn Gal-Gal was found in vaginal squamous epithelium, and may explain why vaginal colonization often precedes bacteriuria (37), in the transitional epithelium of the bladder, ureter, and renal pelvis, and in the columnar epithelium of the collecting tubules. Relevant epithelial surfaces therefore exhibit pilus receptor analogues for both MS and Gal-Gal pili. The significance of these findings in the BALB/c mouse was strengthened by the observation of Lark (personal communication) that human uroepithelial tissues also contain MS and Gal-Gal pilus receptor carbohydrates.

Soluble receptor compounds were also sought in pooled BALB/c mouse urine. No urine factor bound Gal-Gal pili (Fig. 2). However, a titrable factor in urine bound MS pili and was found to be chemically and immunologically identical to the Tamm-Horsfall glycoprotein (5). This compound is produced by cells of the ascending limb of the loop of Henle and the distal convoluted tubule and secreted into tubular urine. There it may entrap MS pilated *E. coli* (5) and block their capacity to bind mannose-containing cell surface glycoconjugates.

The significance of these findings was examined in the BALB/c mouse model by comparing the infectivity and inva-

siveness of four *E. coli* strains: J96, a virulent human pyelonephritis isolate that simultaneously expresses MS and Gal-Gal pili; SH48 and HU849, two recombinant strains that express MS and Gal-Gal pili, respectively, but not other virulence determinants including hemolysin, colicin V, and motility; and P678-54, the nonpilated K12 recipient. J96 (10⁶ CFU) colonized the urine and renal pelvis of all mice and invaded renal parenchyma (Table III). The Gal-Gal pili recombinant (10⁶ CFU) colonized urine and renal pelvis but did not invade, indicating that colonization and invasion are distinct pathogenic events. The MS pili recombinant (10⁶ CFU) neither colonized nor invaded renal pelvis. However, when 10¹⁰ and 10¹² CFU were administered in a volume that resulted in acute ureteric reflux, the MS pili recombinant colonized the renal pelvis in 11 of 13 animals. These results (a) indicate that Gal-Gal pili promote renal colonization by binding epithelial cell globoseries glycolipids and (b) provide an explanation for the prevalence of Gal-Gal-piliated *E. coli* among pyelonephritis isolates (9). In contrast, although MS pili can effect bladder bacteriuria (51, our Table III), they do not mediate renal colonization when inocula < 10⁸ CFU are administered and acute ureteric reflux does not ensue. Under these conditions the binding capacity of MS pili for cell surface receptor molecules may be attenuated by the Tamm-Horsfall protein (discussed above). However, this effect can be overcome when larger inocula are administered in volumes that exceed the bladder's capacity. This result is consistent with the findings of Lomberg et al. (50) that MS-piliated strains that do not express Gal-Gal pili are significant causes of pyelonephritis only in patients with vesicoureteral reflux.

The relative pathogenic role of MS and Gal-Gal pili was also assessed by determining their efficacy as vaccines for the prevention of pyelonephritis. Because other bacterial constituents including O (51), K (52), and H (53) antigens have been tested as pyelonephritis vaccines and may confer protection, the criteria for pilus purity was carefully defined (as discussed

above) and LPS containing O somatic antigens were administered to control animals. The Gal-Gal pilus vaccine alone conferred significant protection against both renal colonization and invasion (Table IV). Renal colonization and/or invasion occurred in two of the three autopsied Gal-Gal pili vaccine recipients with specific antibody titers < 1:1000 (Table V), indicating that the immune response in serum might be roughly correlated with the efficacy of this vaccine. These results corroborate those of Roberts et al. (54), who recently reported that a Gal-Gal pilus vaccine prepared from a clinical isolate prevented pyelonephritis in monkeys. In contrast, Silverblatt and Cohen (55) reported the efficacy of a MS pili pyelonephritis vaccine in a rat model subjected to transient ligation of the ureters. Their results may be a consequence of (a) the murine species chosen for study, in that rat uroepithelia lack globoseries glycolipids (unpublished observation) and/or (b) the obstruction to urine flow.

The mechanism by which the Gal-Gal pilus vaccine conferred protection was not addressed in this study. It seems likely that specific antibody either enters urine from serum or is produced locally and binds pili in situ. The former possibility is supported by our observation that three of seven vaccine recipients had detectable Gal-Gal pili IgG antibody in urine (Table VI) and by the reported efficacy of passive immunization with MS pili antisera in the rat pyelonephritis model of Silverblatt and Cohen (55). Future studies will determine if efficacy is the result of a direct effect on pilus receptor-binding function or an indirect consequence of a change in the surface properties of the organism. This question notwithstanding, the importance of globoseries glycolipid adhesins in the pathogenesis of pyelonephritis is confirmed by four separate observations: (a) Gal-Gal pili are expressed by most pyelonephritis isolates (8, 9); (b) renal uroepithelium, but not urine, contains Gal-Gal pilus receptor compounds (Table II, Fig. 2); (c) Gal-Gal, but not MS, pilated recombinants colonize renal epithelium in the absence of acute vesicoureteral reflux (Table III); and (d) Gal-Gal, but not MS, pilus vaccines prevent pyelonephritis in the unobstructed urinary tract (Table IV).

These results indicate that Gal-Gal pili are attractive candidates for the immunoprophylaxis of pyelonephritis in patients without anatomic abnormalities of the urinary tract. However, these studies (Table III) and those of others (48, 50) also indicate that MS pili may promote bladder bacteriuria in normals and renal colonization in patients with vesicoureteral reflux. Therefore, it is clear that the place in therapeutics of Gal-Gal or MS pilus vaccines versus the conventional use of prophylactic antibiotics in the prevention of urinary tract infections can only be answered by appropriately controlled studies in humans.

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