INFECTION AND IMMUNITY, Mar. 1991, p. 1153–1161
0019-9567/91/031153-09$02.00/0

Vol. 59, No. 3

Alpha-Hemolysin Contributes to the Pathogenicity of Piliated Digalactoside-Binding *Escherichia coli* in the Kidney: Efficacy of an Alpha-Hemolysin Vaccine in Preventing Renal Injury in the BALB/c Mouse Model of Pyelonephritis

PETER O'HANLEY,[1,2,3]* GUY LALONDE,[2] AND GEON JI[2]

Division of Infectious Diseases, Department of Medicine,[1] and Department of Microbiology and Immunology,[2] Stanford University, Stanford, California 94305, and Veterans Administration Medical Center, Palo Alto, California 94304[3]

Received 14 August 1990/Accepted 7 December 1990

Digalactoside-binding (Gal-Gal) pili and alpha-hemolysin of *Escherichia coli* have been implicated as important virulence determinants in the pathogenesis of human ascending, nonobstructive pyelonephritis. The pathogenic significance of these determinants was evaluated in vitro and in the BALB/c mouse pyelonephritis model by employing wild-type, avirulent laboratory, and genetically defined cosmids, transformants, and recombinant strains. In vitro data suggest that the cytolytic activity of hemolysin is significantly ($P < 0.05$) enhanced among digalactoside-binding strains which agglutinate erythrocytes. The basis of increased hemolysis is related presumably to more efficient delivery of the toxin to target lipid substrate in the host plasma membrane. Intravesicular administration of bacteria that express both digalactoside binding and hemolysin generally resulted in greater mortality and renal parenchymal injury in mice than strains that expressed none or only one of these determinants. Analyses convincingly demonstrate that digalactoside-binding pili are correlated with upper urinary tract colonization and that hemolysin is correlated with septicemia and renal parenchymal damage. These determinants collectively constitute the minimal virulence factors to produce disease in this model. Their efficacy as vaccines for the prevention of pyelonephritis was also assessed. A purified Gal-Gal pilus vaccine prevented ($P < 0.05$) subsequent colonization by a challenge wild-type strain that exhibited homologous pili. The hemolysin vaccine did not abrogate subsequent bacterial renal colonization on challenge, but it did protect ($P < 0.05$) mice which survived challenge from subsequent renal injury compared with those in the saline control group. The combination of these determinants was also protective. The combination of Gal-Gal pili and hemolysin in a vaccine preparation represents a potentially worthwhile strategy for human immunoprophylaxis against pyelonephritis by interdicting several steps in the pathogenesis of a bacterial mucosal infection.

Extensive data on the genetics and structure-function relationships of digalactoside-binding pili have been reported (1, 11, 12, 20, 23, 24, 29, 44). The operon for digalactoside binding encodes at least nine polypeptides that are responsible for regulation, transport, assembly, pilus anchoring, pilus structure, and attachment functions. Major pilins are encoded by *papA* and polymerize to form the bacterial hairlike surface organelles, termed pili or fimbriae. These fibers consist almost entirely of major pilin, but they also contain pilus tip proteins encoded by *papE*, *-F*, and *-G* genes that specifically facilitate and mediate binding to host digalactoside residues. These heteropolymers, especially PapF and -G polypeptides, are considered responsible for bacterial binding to host uromucosal surfaces containing α-D-Gal*p*-(1-4)-β-Gal*p* (Gal-Gal) moieties (23). Bacterial adherence to the uroepithelia is a prerequisite for subsequent urinary tract injury by other microbial determinants (41, 46). The digalactoside-binding piliated phenotype of *Escherichia coli* appears to be crucial for upper urinary tract colonization in humans (11, 12, 30). There are considerable data that also demonstrate the value of digalactoside-binding pilus immunization in the prevention of *E. coli* pyelonephritis in simian and murine urinary tract infection models (30, 34, 36, 39). Specific immunoglobulin G (IgG) antibodies in the urine to the major pilin select for nonpiliated phase variants, thereby enabling the host to rapidly clear digalactoside-binding piliated phenotypes from the urinary tract (29a). Because of their relatively conserved antigenic properties, digalactoside-binding pili are considered important candidates for broadly cross-protective vaccines against human *E. coli* urinary tract infections.

There are extensive data suggesting that alpha-hemolysin contributes to the severity of bacterial infections (6, 8–10, 21, 27, 34, 45, 48, 50). Alpha-hemolysin causes cellular injury by disruption of the plasma membrane by pore formation, and cell death follows if sufficient damage occurs (2–4, 7, 15, 28, 48). It is known (i) that alpha-hemolysin is antigenic in humans and animals (5, 14, 35, 37, 40) and (ii) that antibody can inhibit hemolytic activity in vitro (14, 35). The antigenic topology of alpha-hemolysin has been partially determined recently by the use of rabbit polyclonal and murine monoclonal IgG antibodies (14; 35). Monoclonal antibodies that recognize the amino-terminal 2 to 160 amino acid residues, residues 626 to 726, and residues within the

* Corresponding author.

TABLE 1. Characteristics of bacterial strains

Strain	O serotype	Source	Gal-Gal agglutination[a]	Hly expression[b]	Reference(s)
J96	O4	Pyelonephritis	+	+	30
3669	O2	Pyelonephritis	+	−	34
C1212	O6	Cystitis	+	+	33, 34, 45
B10	O41	Normal stool	−	+	32
B13	O36	Normal stool	+	−	32
B40	O36	Normal stool	+	−	32
A8	O4	Pyelonephritis	+	+	32
A11	O6	Pyelonephritis	+	−	32
A42	O75	Pyelonephritis	+	+	32
A45	O4	Cystitis	+	+	32
A50	O4	Pyelonephritis	+	+	32
A57	O75	Cystitis	+	+	32
HB101		Laboratory	−	−	31
WAF100		pSF4000 cloned into pAYC184 in HB101	−	+	35, 50
GEJ800		11.7-kb *Sal*I fragment of pSF4000 cloned into pBR322 in VCS	−	+	14
3669/WAF	O2	pSF4000 cloned into pAYC184 in 3669	+	+	This study
B13/WAF	O36	pSF4000 cloned into pAYC184 in B13	+	+	This study
B40/WAF	O36	pSF4000 cloned into pACY184 in B40	+	+	This study
B40/AYC	O36	pAYC184 in B40	+	−	This study
HU849		pHU845 in p678-54	+	−	13
pDAL2-10		C1212-derived cosmid in HB101	+	+	22
pDAL2-10A		C1212-derived cosmid in HB101	−	+	22
pDAL2-28		C1212-derived cosmid in HB101	+	−	22

[a] Gal-Gal agglutination, Digalactoside-binding status. +, Positive; −, negative.
[b] Hly expression, Hemolytic plate activity for human, gorilla, monkey, pig, BALB/c mouse, rat, rabbit, goat, sheep, guinea pig, horse, cow, turkey, chicken, cat, and dog erythrocytes. +, Positive for all; −, negative for all.

Ca^{2+}-binding domain (i.e., residues 425 to 892) exhibit in vitro neutralization of hemolytic activity. Yet, the role of alpha-hemolysin antibody to modulate the pathogenic potential of hemolytic strains has not been evaluated in relevant animal models of infection.

This study was undertaken to determine the contribution of alpha-hemolysin in kidney infection among digalactoside-binding isolates and to evaluate the efficacy of alpha-hemolysin immunization to prevent renal injury in the BALB/c mouse model of experimental ascending, nonobstructive *E. coli* pyelonephritis (11, 12, 30).

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains used in this study are listed in Table 1. Wild-type strains include isolates from urine of women with acute pyelonephritis or cystitis and from stools of healthy women. HB101 is an avirulent laboratory *E. coli* K-12 strain (31). A number of recombinant strains were also employed. The HU849 strain has been previously described (13) and is used to produce digalactoside-binding pili for immunization studies. WAF100 is an *E. coli* HB101 strain transformed with pSF4000 (35, 50). pSF4000 is a pAYC184-derivative with a 11.7-kb insert from the chromosome of *E. coli* J96 and is sufficient to encode for the hemolytic phenotype (50). pSF4000 was introduced into strains 3669, B13, and B40 by using a $CaCl_2$ transformation method (25). These strains were designated 3669/WAF, B13/WAF, and B40/WAF, respectively. Also, the pAYC184 vector was introduced into B40, a normal fecal isolate, as a control (B40/AYC). GEJ800 is an *E. coli* VCS257 strain (Stratagene, La Jolla, Calif.) harboring a pBR322 derivative containing the 11.7-kb *Sal*I fragment from pSF4000 (14). It was used to produce large amounts of alpha-hemolysin for immunization studies. pDAL2-10, pDAL2-10A, and pDAL2-28 are cosmids derived from C1212 DNA conferring the digalactoside-binding or hemolytic phenotype or both (22). Transformants and cosmids that exhibit hemolytic activity represent hemolytic-locked variants because they still produce hemolysin despite inhibiting amounts (i.e., ≥3 mM) of iron in culture broth (48). The culture media were supplemented with the following concentrations (in micrograms per milliliter) of antibiotics where appropriate to select for plasmids of recombinant strains, cosmids, and transformants during culture passage: ampicillin, 100; tetracycline, 50; and chloramphenicol, 20. Strains were stored at −70°C in L broth containing 50% glycerol until needed.

Binding assays. (i) MRHA. Mannose-resistant hemagglutination (MRHA) was performed with erythrocytes from a variety of vertebrate species: blood group OP1 humans, gorillas, monkeys, pigs, BALB/c mice, guinea pigs, rats, rabbits, dogs, cows, and sheep. From freshly drawn heparinized blood, erythrocytes were washed three times in 0.1 M phosphate-buffered saline (PBS), pH 7.4, and suspended in a final concentration of 4% (vol/vol) in PBS with 4% (wt/vol) D-mannose. A single colony from the bacterial strains was passed serially three to six times on tryptic soy agar at 37°C for 18 h and then suspended in PBS at a concentration of 10^6 CFU/ml on the basis of optical density. MRHA by bacteria was tested by slide agglutination performed by combining equal volumes of an erythrocyte suspension and the bacterial suspension.

(ii) Hapten inhibition of MRHA. Hapten inhibition of MRHA by carbohydrate receptor analogs was performed by mixing the bacterial suspension successively with each of the following sugars at a final concentration of 2% (wt/vol) in PBS: α-D-Gal*p*-(1-4)-β-D-Gal*p*, α-D-Gal*p*-(1-4)-β-D-Gal*p*-(1-4)-β-D-GlcNAc*p*,α-D-GalNAc*p*-(1-4)-β-D-Gal*p*, α-D-MeGal*p*, α-D-Gal*p*-(1-3)-β-D-Gal, α-D-GalNAc*p*-(1-2)-β-D-Gal*p*, α-D-Fuc*p*-(1-4)-β-D-GlcNAc*p*, β-D-GalNAc*p*-(1-3)-β-D-Gal*p*, β-D-GlcNAc*p*-(1-3)-β-D-Gal*p*-(1-4)-β-D-Glc*p*, β-D-Glc*p*-(1-2)α-D-Man*p*, α-D-Glc*p*-(1-2)-α-Man*p*, α-D-Glc*p*-

(1-2)-β-D-Gal*p*, α-D-Fuc*p*-(1-6)-β-D-Gal*p*, and β-D-Gal*p*-(1-4)-β-D-Glc*p*. After preincubation for 1 h at room temperature, an equal volume of an erythrocyte suspension was added and the mixture was assessed for agglutination.

(iii) Latex agglutination. Latex agglutination was also employed to confirm digalactoside binding by the bacterial strains. Synthetic α-D-Gal*p*-(1-4)-β-D-Gal*p* with a 8-methoxycarbonylactyl glycoside linker arm was adsorbed to latex beads (Chembiomed Ltd., Edmonton, Alberta, Canada) (31). Lactose linked to a 8-methoxycarbonylactyl glycoside was also adsorbed to latex and employed as an agglutination-negative control. Equal volumes of bacteria were mixed with a 1% (vol/vol) latex suspension in PBS and assessed for slide agglutination.

Lytic assays. (i) Tryptic soy agar plates. Tryptic soy agar plates containing 5% PBS-washed erythrocytes from a variety of vertebrate species were employed to screen for bacterial hemolytic activity. Erythrocytes from the following species were included: human, gorilla, monkey, pig, BALB/c mouse, guinea pig, rat, rabbit, dog, sheep, goat, horse, cow, turkey, chicken, and cat. Plates were seeded with bacterial strains and observed for lysis of erythrocytes after 24 h of growth at 37°C. Tests were serially repeated three times.

(ii) Microtiter hemolysin assay. A microtiter hemolysin assay was used to quantitate the hemolytic activity of bacteria and culture supernatants for a variety of vertebrate erythrocytes (5). A single bacterial colony on tryptic soy agar for each strain was inoculated into tubes containing Luria broth and grown 5 to 7 h at 37°C until the optical density was ≥1.0 at a wavelength of 600 nm. Bacteria were removed from the culture supernatants by centrifugation. The supernatant was filtered through 0.4-μm-pore-size membranes and serially diluted twofold in 10 μM $CaCl_2$ assay buffer (5). Hemolytic activity was tested by combining equal volumes (50 μl each) of an erythrocyte suspension (final concentration, 2%) in assay buffer and culture supernatant in microtiter plates. Plates were incubated at 37°C for 2 h, and the hemolytic microtiters were expressed as the reciprocal of the highest dilution at which any hemolysis was observed (5). Controls included $CaCl_2$ lysis buffer and heat-treated (≥80°C for 5 min) culture supernatants from *E. coli* GEJ800 (14) and C1212 (33, 34, 46) strains. All tests were performed in triplicate at three different times.

The ability of bacteria to lyse agglutinated and nonagglutinated erythrocyte species was assessed by microtiter assay. Bacteria from early-exponential-log-phase broth cultures were washed three times in PBS at 4°C and carefully resuspended at a concentration of 10^6 CFU/ml in $CaCl_2$ lysis buffer plus 4% (wt/vol) D-mannose and, in one case, plus 4% Gal-Gal. Bacterial counts were performed by an agar pour technique to confirm the number of bacteria in the starting well. Bacterial suspensions were serially diluted twofold in assay buffer and assessed for hemolytic microtiter as described above. Plates were incubated for 2 h at 37°C and then read. The control included buffer alone. All tests were performed in triplicate at three different times, and the hemolytic microtiter was recorded for each test.

BALB/c mouse model of experimental pyelonephritis. The pathogenic significance of alpha-hemolysin was assessed in the BALB/c mouse model of pyelonephritis with selected clinical isolates, cosmids, and transformants. Twenty- to 28-week-old female BALB/c mice were employed and dehydrated 18 h prior to bacterial challenge. C1212 (10^6, 10^8, and 10^{10} CFU), pDAL2-10 (10^4, 10^8, and 10^{10} CFU), pDAL2-10A (10^8 and 10^{10} CFU), pDAL2-28 (10^8 and 10^{10} CFU), 3669 (10^6, 10^8, and 10^{10} CFU), 3669/WAF (10^8 CFU), B40 (10^8 CFU), B40/WAF (10^8 CFU), B40/AYC (10^8 CFU), and B10 (10^8 CFU) were administered by intravesicular inoculation in 100 μl of PBS via catheter, as described previously, without acute ureteric reflux (30). Also, PBS and the avirulent laboratory HB101 strain were administered intravesicularly as controls. The avirulent HB101 strain was inoculated in doses of 10^8 or 10^{10} CFU/100 μl. No attempt was made to obstruct the urinary tract, and mice were given water ad lib 2 h after inoculation. Mice were observed every 4 to 6 h over the next 48 h after inoculation for death and were then euthanized. The right kidney was excised under sterile conditions and sagittally cut. The cut surface was smeared on MacConkey agar alone or supplemented as required with antibiotics to select for plasmids of the challenge strain during culture passage. The plates were incubated for 24 h at 37°C and then read. The relative bacterial density grading criteria for an ~3-cm^2 smear were as follows: 5+, confluent growth; 4+, nonconfluent growth, but colonies too numerous to count; 3+, >20 CFU; 2+, 11 to 20 CFU; 1+, 1 to 10 CFU; and 0, no growth. Also, in a selected number of mice, the spleen was excised, homogenized, and cultured on MacConkey agar to assess septicemia. Confirmation of challenge strains in renal tissue smear cultures and spleen cultures was assessed by an array of phenotypic assays to identify the appropriate challenge strain from representative colonies of bacteria on isolation plates. These included slide agglutination of bacteria with anti-02, -6, -36, or -41 sera as required (anti-098 serum was used as a control); hemolytic plate activity of bacteria subcultured from original isolation plates on 5% sheep blood-tryptic soy agar alone or with the appropriate antibiotic for plasmid selection of the challenge strain after 24 h of growth at 37°C; and Gal-Gal latex agglutination status of bacteria from original isolation plates. Also, the right kidney was usually processed for light microscopy. Tissue sections were stained with hematoxylin and eosin, and multiple sections from each kidney were graded for histopathology (see below).

Vaccination trials. (i) Digalactoside-binding pili. Digalactoside-binding pili were purified from the HU849 recombinant strain as previously described (31). Protein purity of the pilus preparation was assessed by the presence of a single Coomassie brilliant blue- or silver-stained band (42) at 17.5 kDa after ≥100 and 10 μg of pilus protein, respectively, as estimated by a modified Lowry assay (26) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19). The pilus preparation contained less than 0.02% of contaminating lipopolysaccharide (LPS) as determined by a 2-keto-3-deoxyoctonate assay (49). This level of LPS contamination in ≤100 μg of pilus preparation was too small to elicit an anti-LPS antibody response in vaccinated mice as determined from a previous study (30).

(ii) Alpha-hemolysin. Alpha-hemolysin was purified electrophoretically in denatured form from culture supernatants of the GEJ800 recombinant strain (14). In brief, the GEJ800 filtrate from a 5-h culture in tryptic soy broth grown at 37°C was precipitated with 10% (vol/vol) trichloroacetic acid at 4°C. After gentle mixing overnight, the trichloroacetic acid precipitate was pelleted by centrifugation and resuspended in SDS sample buffer. This mixture was boiled and subjected to SDS-PAGE. The 110-kDa band identified by Coomassie brilliant blue stain was excised. The protein was electroeluted from the gel, dialyzed against H_2O, and stored lyophilized at −20°C until needed. Purity was confirmed by the presence of single peaks when the protein was analyzed by a combination of high-pressure liquid chromatography em-

ploying ion-exchange and reverse-phase columns at a wavelength of 280 nm. These peaks contained only a 110-kDa protein when subjected again to SDS-PAGE analysis.

(iii) LPS. LPS was extracted from *E. coli* J96 by a phenol-water technique (51). Capsular polysaccharides and nucleic acids were separated from LPS by ultracentrifugation. The LPS preparation was dialyzed extensively against H_2O and stored lyophilized at −20°C until needed. The purity of the LPS was confirmed by the presence of LPS bands in SDS-PAGE gels stained with silver (43), the absence of optical absorbance between 200 and 300 nm, and the absence of Coomassie brilliant blue-stained bands in SDS-PAGE gels when 100 μg was analyzed. LPS was stored lyophilized at −20°C until needed.

(iv) Immunization protocols and challenge experiments. Immunization protocols and challenge experiments involved groups of 20- to 22-week-old BALB/c female mice. The mice were immunized with the following preparations: (i) HU849 digalactoside-binding pili, (ii) GEJ800 alpha-hemolysin, (iii) J96 LPS, (iv) HU849 pili plus GEJ800 alpha-hemolysin, and (v) PBS. The bacterial vaccines were composed of 50 μg of each immunogen. The combination vaccine preparation was composed of both 50 μg of pilus and 50 μg of alpha-hemolysin. The immunogens were suspended in PBS, emulsified with equal volumes (50 μl) of complete (for primary) or incomplete (for booster) Freund's adjuvant, and administered by multiple intramuscular injections. All mice received a total of two 100-μl immunizations separated by a 2-week interval. A specific IgG enzyme-linked immunosorbent assay (ELISA) reciprocal titer of ≥1,000 was elicited against the respective immunogen used to vaccinate the mouse at 12 days after the first booster. This titer was determined among randomly selected vaccinated mice in each group. These mice were not used in bacterial challenge studies. The details for determining antibody levels to pilus, alpha-hemolysin, and LPS by ELISA using alkaline-phosphatase conjugates are described elsewhere (14, 31, 34). In brief, each serum sample was assessed in duplicate and recorded as the mean optical density at a wavelength of 490 nm. A mean optical density greater than twice that of equally diluted serum from PBS-immunized mice was considered to indicate the presence of specific antibody. The reciprocal of the last dilution giving a ratio above this threshold was considered the titer.

Two weeks after the first booster immunization, vaccine recipients were challenged with 10^8 CFU of strain J96 in 100 μl of PBS by intravesicular administration, as described previously (30). It should be noted that the bacterial immunogens are homologous to the specific antigens contained in the challenge J96 strain. The bacteria were harvested into PBS from agar plates after 18 h of growth at 37°C. This inoculum represents 100 times the 100% infectious dose for pyelonephritis but does not cause death in this age group of mice when they have been dehydrated for 12 to 18 h prior to bacterial challenge (30, 34, 39). However, with more prolonged dehydration (up to 24 h) and use of bacteria that are derived from one passage from fresh stock, 40% of the mice died as a consequence of septic shock from pyelonephritis. Also, it was determined that serially passed bacteria (≥10 cycles) caused less death (20%) under the same conditions of prolonged dehydration. The basis for the higher mortality rates was not evaluated in the study. Two days later, the mice were exsanguinated under anesthesia and the sera were screened by ELISA for specific antibody to the immunogen used to vaccinate the mice. Also, bacterial colonization of the right kidney by strain J96 was determined by culture on MacConkey agar and assessed for relative colonization density after 24 h of growth at 37°C as described previously (30). Identification of specific J96 growth was confirmed by slide agglutination of representative colonies on agar by anti-J96 O sera (30). Furthermore, the right kidney from each vaccinated mouse was processed for light microscopy. Tissue sections were stained with hematoxylin and eosin stains. Multiple tissue sections from each mouse kidney 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 the renal pelvis to the medulla; 1+, focal inflammation and edema in the renal pelvis; and 0, normal.

Statistics. A two-tailed Fisher exact test was used to determine whether significant differences existed between (i) hemolytic microtiters for MRHA bacteria which agglutinate cells versus bacteria which do not agglutinate cells, (ii) mortality rates or renal injury for cohorts of mice administered bacteria that express Gal-Gal binding and alpha-hemolysin phenotypes versus bacteria that do not express both of these determinants, and (iii) protection against pyelonephritis for cohorts of mice immunized with different bacterial immunogens versus PBS. P values of <0.05 were considered significant.

RESULTS

Enhanced hemolysin-mediated injury by the combination of digalactoside-binding and alpha-hemolysin-producing strains. All MRHA-positive bacterial strains bound digalactoside-coated latex beads and were inhibited only by α-D-Gal*p*-(1-4)-β-D-Gal*p* and α-D-Gal*p*-(1-4)-β-D-Gal*p*-(1-4)-β-D-GlcNAc*p* in hapten inhibition MRHA assays. These results confirm the digalactoside-binding phenotype and are summarized in Table 1. Digalactoside-binding bacteria were able to agglutinate human, gorilla, monkey, pig, and BALB/c mouse erythrocytes in the presence of mannose and the other 11 carbohydrate analogs lacking Gal-Gal (see Materials and Methods). These digalactoside-binding bacteria were not able to hemagglutinate sheep, guinea pig, rat, rabbit, cow, or dog erythrocytes. Table 1 also summarizes the hemolytic plate activity of the bacterial strains against a variety of erythrocytes. If a bacterial strain was capable of lysing any species of erythrocyte in agar plates, it exhibited hemolytic activity against all 16 vertebrate species studied. The hemolytic microtiters of bacterial culture supernatants for human erythrocytes were or ranged from 512 to 1,024 for GEJ800 strain; 128 to 256 for WAF100 strain; 32 for C1212, pDAL2-10, pDAL2-10A, B40/WAF, 3669/WAF, J96, and A8 strains; 16 for B13/WAF, A42, and A50 strains; 8 for B10, A45, and A57 strains; and 0 for 3669, B13, B40, A11, HB101, B40/AYC, HU849, and pDAL2-28 strains.

The ability of digalactoside binding to enhance the hemolytic capacity of bacteria was also assessed by hemolytic microtiter assay. Table 2 summarizes the hemolytic microtiters of bacterial strains against human and bovine erythrocytes in the presence of mannose. Digalactoside-binding strains were able to agglutinate human erythrocytes but not bovine cells. The toxin's ability to lyse human and bovine erythrocytes was equivalent when culture supernatants were screened. Hemolysis was significantly greater ($P < 0.05$) for hemolytic bacteria that bound to the erythrocytes than for those that did not (Table 2). For example, when 4% Gal-Gal was added to human erythrocytes, the C1212 bacteria did

TABLE 2. Enhanced hemolysis of erythrocytes bound by digalactoside-binding and alpha-hemolysin-producing *E. coli* strains

Strain	Plate Hly status[a]	MRHA status[b]	Hemolytic microtiter[c]	
			Culture supernatant	Whole bacteria
J96	+	$HRBC^+$	32	128–256
		$BRBC^-$	32	16–32[d]
GEJ800	+	$HRBC^-$	512–1,024	256–512
		$BRBC^-$	512	256–512
C1212	+	$HRBC^+$	32	128–256
		$BRBC^-$	32	8–16[d]
pDAL2-10	+	$HRBC^+$	32	512–1,024
		$BRBC^-$	32	64[d]
pDAL2-10A	+	$HRBC^-$	32	64
		$BRBC^-$	32	64
A42	+	$HRBC^+$	16	64
		$BRBC^-$	16	8[d]
A45	+	$HRBC^+$	16	128
		$BRBC^-$	16	32[d]
HU849	−	$HRBC^+$	0	0
		$BRBC^-$	0	0
HB101	−	$HRBC^-$	0	0
		$BRBC^-$	0	0
Control buffer		$HRBC^-$	0	0
		$BRBC^-$	0	0

[a] Plate Hly status, Hemolytic capacity of bacteria to lyse vertebrate erythrocytes in agar. +, Positive; −, negative.

[b] MRHA status, Ability of bacteria to agglutinate human or bovine erythrocyte species in the presence of 4% (wt/vol) D-mannose. +, Positive; −, negative; HRBC, human erythrocyte; BRBC, bovine erythrocyte.

[c] Hemolytic microtiter, the titer or range of the reciprocal of the highest dilution at which hemolysis was observed when either culture supernatant or bacteria were employed with human or bovine erythrocytes.

[d] When compared with the hemolysis of human erythrocytes, the hemolytic microtiter for bovine erythrocytes is significantly different ($P < 0.05$) as calculated by a two-tailed Fisher exact test.

not hemagglutinate the human cells. The bacteria exhibited a hemolytic titer of 16 under this condition instead of a titer of 128 to 256 when C1212 bacteria agglutinated human erythrocytes (data not shown). Also, similar enhanced toxin injury was demonstrated by bacterial strains that express the combination of hemagglutinating and hemolytic phenotypes when pig versus dog and monkey versus rabbit erythrocyte species were used (data not shown).

Uropathogenic potential of hemolytic bacteria and hemolysin-locked variants for BALB/c mice. Overall, three sets of comparisons were examined to ascertain the contribution of hemolysin and digalactoside-binding pili to the pathogenesis of experimental murine pyelonephritis. The following comparisons were made: (i) between wild-type strains that expressed hemolysin or did not, (ii) between initially nonhemolytic, wild-type strains before and after they were transformed by incorporating the pSF4000 plasmid that confers the hemolytic phenotype, and (iii) between laboratory strains made to express either, neither, or both hemolysin and digalactoside binding.

E. coli 3669 is a nonhemolytic, digalactoside-binding wild-type strain which readily colonizes mouse kidneys in high numbers. Furthermore, it causes renal inflammation but only minimal parenchymal necrosis. In contrast, *E. coli* C1212, which is digalactoside binding and hemolytic, is a more destructive uropathogen, as suggested by the fact that it causes greater renal damage than the 3669 strain in this model (Table 3). It is important to note that the 3669 strain does not cause death under these conditions of intravesicular bacterial administration. In contrast, the 3669/WAF strain (i.e., the 3669 strain incorporating pSF4000) causes significant mortality when administered intravesicularly (i.e., 19 deaths [63%] of 30 inoculated mice). It is presumed that the intoxication of the mouse kidney by the alpha-hemolysin of this transformant must produce sufficient renal damage to allow for septicemia as indicated by a positive splenic culture and ultimately leads to death. The 3669/WAF strain was cultured from the spleen in four of four mice that died within the first 48 h of 3669/WAF administration and in five of five mice that were sacrificed after 2 days. In contrast, none of the spleens of four mice that were sacrificed at 2 days after intravesicular inoculation of 3669 contained this strain on subsequent splenic culture. Similar results were obtained with the nonhemolytic B40 strain and its hemolytic derivative, B40/WAF. These data support the hypothesis that hemolysin production contributes to uropathogenicity of digalactoside-binding bacteria and to septicemia.

C1212 bacteria readily colonize kidneys in high numbers and cause injury to the renal parenchyma after intravesicular administration of 10^6 to 10^8 bacteria (Table 3). At a higher dose (i.e., 10^{10}), 2 of 15 (13%) mice died within 36 h after intravesicular inoculation. pDAL2-10, a cosmid strain derived from C1212 DNA and expressing both digalactoside binding and hemolysin, produced higher mortality at an equivalent dose of 10^{10} bacteria than the C1212 strain. In addition, the pDAL2-10A strain, which lacks digalactoside binding but produced hemolysin, was unable to readily colonize the kidney. The pDAL2-28 strain, which exhibits digalactoside binding but does not produce hemolysin, was readily capable of colonizing but was unable to injure the kidney. These studies confirm that the minimal number of recognizable uropathogenic *E. coli* determinants in this model includes digalactoside binding for upper urinary tract bacterial colonization and hemolysin for renal injury. The efficacy of digalactoside-binding pilus and alpha-hemolysin vaccines to prevent experimental pyelonephritis was assessed to further test the validity of these propositions and to provide further evidence about the uropathogenic significance of alpha-hemolysin.

Hemolysin vaccine for the prevention of pyelonephritis. Denatured alpha-hemolysin, digalactoside-binding pili, and LPS were prepared as vaccines from strains homologous to the challenge strain. Vaccine efficacy was examined with preparations that had been rigorously assessed for purity. Cohorts of vaccine recipients were challenged under different stringencies with the J96 strain (Table 4). In trial 1, mice were challenged after 24 h of dehydration with bacteria from fresh stock that had grown for 18 h on agar at 37°C. In trial 2, mice were challenged, after 24 h of dehydration, with bacteria obtained from an 18-h growth at 37°C on agar from stock that had been serially passed for 10 cycles. Under these conditions, 40 and 17% of PBS-immunized mice died within 36 h after intravesicular bacterial inoculation in trial 1 and trial 2, respectively. All mice immunized with bacterial antigen preparations were subsequently confirmed to have specific IgG antibodies to the homologous antigen used to vaccinate. The minimum IgG-specific antibody ELISA reciprocal titer for each vaccinated mouse was ≥1,000 2 days after bacterial challenge. Digalactoside-binding pilus vaccine recipients who survived were significantly protected ($P < 0.05$) from subsequent renal colonization by the challenge strain compared with those protected in other groups (Table 4). Furthermore, these mice were protected from subsequent renal injury presumably because of the absence or decreased number of bacteria colonizing the kidney. There were no

TABLE 3. Hemolysin-producing strains have greater pathogenic potential than nonhemolytic strains in the BALB/c mouse model of *E. coli* pyelonephritis

Strain	Virulence factor[a]	Dose (CFU)	Deaths/mice tested (%)	Bacterial colonization in right kidney at 2 days		
				No. positive/total	Avg RCD[b]	Histopathology grade[c]
3669	Gal-Gal$^+$ Hly$^-$	10^{10}	0/12	12/12	4.8	ND
		10^8	0/10	10/10	4.9	2.4
		10^6	0/9	9/9	4.1	ND
3669/WAF	Gal-Gal$^+$ Hly$^+$	10^8	19/30 (63)[d]	11/11	5.0	4.4[d]
C1212	Gal-Gal$^+$ Hly$^+$	10^{10}	2/15 (13)	13/13	4.8	ND
		10^8	0/10	4/4	4.8	4.0[d]
		10^6	0/10	10/10	4.1	ND
pDAL2-10	Gal-Gal$^+$ Hly$^+$	10^{10}	4/5 (80)[d]	1/1	5.0	ND
		10^8	0/10	10/10	5.0	4.0[d,e]
		10^4	0/10	9/10	3.0	ND
pDAL2-10A	Gal-Gal$^-$ Hly$^+$	10^{10}	1/5 (20)	1/4	1.0	ND
		10^8	0/10	2/10	1.0	1.0
pDAL2-28	Gal-Gal$^+$ Hly$^-$	10^{10}	0/10	10/10	5.0	ND
		10^8	0/10	9/10	4.9	0.2
B40	Gal-Gal$^+$ Hly$^-$	10^8	0/28	28/28	2.9	0.3
B40/WAF	Gal-Gal$^+$ Hly$^+$	10^8	32/43 (74)[d]	9/9	3.3	3.0[d]
B40/AYC	Gal-Gal$^+$ Hly$^-$	10^8	0/22	22/22	3.1	0
B10	Gal-Gal$^-$ Hly$^+$	10^8	0/10	0/10	0	0
HB101	Gal-Gal$^-$ Hly$^-$	10^{10}	0/10	0/10	0	ND
		10^8	0/5	0/5	0	0
Control (PBS)			0/16	0/16	0	0

[a] Gal-Gal, Digalactoside-binding status; Hly, hemolysin-producing status; +, positive; −, negative.
[b] RCD, Relative colonization density (see Materials and Methods), expressed as the mean density of the challenge strain colonizing the kidney per total number of mice in the cohort that were colonized by the challenge strain 2 days after intravesicular inoculation.
[c] Histopathology grade (see Materials and Methods) is expressed as the mean grade of histological renal abnormality per total number of mice in the cohort that were colonized by the challenge strain. ND, Not done.
[d] When compared with their isogenic Gal-Gal$^-$ Hly$^+$ or Gal-Gal$^+$ Hly$^-$ phenotypes, the mortality rate or histopathology grade is significantly different ($P < 0.05$) as calculated by a two-tailed Fisher exact test.
[e] Only 5 of 10 infected mouse kidneys were processed for histology.

significant differences in the renal colonization ability of the J96 strain in hemolysin and LPS vaccine recipients who survived compared with that of the buffer control groups. However, hemolysin vaccine recipients had significantly less ($P < 0.05$) renal injury in both trials than LPS and PBS control recipient groups (Table 4). These data confirm that (i) immunization with digalactoside-binding pili decreases subsequent urinary tract colonization and, therefore, pyelonephritis by a homologous piliated strain; (ii) immunization with hemolysin prevents subsequent renal injury but not colonization by a homologous hemolytic challenge strain; and (iii) the combination of digalactoside-binding pilus and hemolysin vaccines was also protective.

DISCUSSION

The pathogenesis of bacterial mucosal infections may be viewed as the culmination of a sequence of events mediated by specific determinants of microbial virulence. Elucidation of these events has been significantly advanced by the use of genetically defined strains that express one or more determinants. For example, this approach has been used by Marr

TABLE 4. Hemolysin vaccine prevents renal injury but not renal colonization in the BALB/c mouse model of *E. coli* pyelonephritis[a]

Immunogen	Trial 1				Trial 2			
	No. dead/total (%)	Bacterial colonization			No. dead/total (%)	Bacterial colonization		
		No. positive/total	RCD[b]	Histopathology grade[c]		No. positive/total	RCD	Histopathology grade
Hly	4/17 (24)	13/13	3.0	0.3[d]	0/16 (0)	15/16	3.8	0.4[d]
Pilus	8/20 (40)	11/12	1.0[d]	0.1[d]	0/12 (0)	7/12	0.9[d]	0.1[d]
LPS	9/20 (45)	11/11	5.0	3.3	1/17 (6)	11/16	5.0	2.6
Hly + pilus	3/15 (20)	9/12	1.0[d]	0[d]	0/19 (0)	7/19	1.0[d]	0[d]
PBS control	8/20 (40)	12/12	4.5	3.8	4/24 (17)	20/20	4.0	3.8

[a] Fresh stock and serially passed stock were used for trial 1 and 2, respectively. Challenge conditions for both trials included 24 h of dehydration prior to challenge. Colonization in the right kidney was checked 2 days after challenge.
[b] RCD, Relative colonization density (see Materials and Methods), expressed as the mean density of J96 strain colonizing the right kidney per total number of mice in the cohort that were colonized by the challenge strain.
[c] Histopathology grade (see Materials and Methods) is expressed as the mean grade of histological renal abnormality per total number of mice in the cohort that were colonized by the J96 strain.
[d] When compared with the PBS control, the relative colonization density or histopathology grade is significantly different ($P < 0.05$) as calculated by a two-tailed Fisher exact test.

and colleagues (27), who have employed transformants and recombinant strains in an ascending rat pyelonephritis model to examine the contribution of serum resistance, hemolysin, and S fimbriae to the pathogenesis of renal infection. They concluded that all three of these determinants contribute to the multifactorial phenomenon of *E. coli* nephropathogenicity in this model (27). We chose to use genetically defined *E. coli* strains that express digalactoside binding and/or hemolysin in the BALB/c mouse model of *E. coli* pyelonephritis instead of a rat model for a number of reasons. First, BALB/c mice express a Gal-Gal receptor analog on uroepithelia with a distribution similar to that exhibited by humans (30), whereas the rat species does not express this carbohydrate moiety on its epithelial cells. It is understandable that digalactoside-binding strains can colonize the urinary tract of BALB/c mice but not rat species (11). Second, the BALB/c mouse model of *E. coli* pyelonephritis is similar to the natural course of infection in humans (12, 30, 41). Third, BALB/c mice can elicit antibodies to digalactoside binding and hemolysin antigens, as do humans (5, 31, 35, 37, 40). We have employed wild-type *E. coli* strains isolated from human urinary tract infections and constructed cosmids and transformants that exhibit digalactoside binding and/or hemolysin in this murine model to clarify their role in the pathogenesis of ascending, nonobstructive pyelonephritis. The findings in this report reconfirm the importance of digalactoside-binding pili for renal colonization under normal conditions of anatomy (11, 12, 30) and extend the role of digalactoside-binding pili to amplify host injury by alpha-hemolysin in the urinary tract.

The enhanced hemolytic activity of digalactoside-binding strains is due presumably to more efficient delivery of hemolysin. This conclusion is supported by our in vitro data that conclusively demonstrate that hemolytic bacterial strains produce greater cytolysis when they bind to erythrocytes than when they are not able to bind to a specific erythrocyte species. The data from studies in BALB/c mice also establish that digalactoside binding and alpha-hemolysin production constitute the minimal number of virulence determinants for *E. coli* strains to produce renal disease in this model of ascending, nonobstructed pyelonephritis. Although they specifically mediate individual pathogenic steps, their sequential effects in combination are required if disease is to occur. For example, strain B40, a digalactoside-binding nonhemolytic isolate, is readily capable of colonizing the BALB/c mouse kidney, but it does not produce any renal parenchymal damage. When the B40 strain is transformed by incorporating the hemolysin operon encoded by pSF4000, it (viz., B40/WAF) produced considerable mortality and renal injury in mice. The mechanism by which this strain and the other uropathogenic strains employed in this model produced disease was not evaluated. We suspect that hemolysin's intrinsic membrane-damaging property is important for cellular injury and tissue damage (4). The possibility also exists that renal parenchymal injury by pyelonephritogenic *E. coli* strains is mediated by the noxious effects of inflammatory cells and their products in the kidneys as observed in other bacterial infections, e.g., suppurative meningitis. There are convincing data that the *E. coli* strains which exhibit digalactoside binding and other adhesins and produce hemolysin can stimulate the release of inflammatory mediators (e.g., leukotriene and histamine) from leukocytes (16–18, 38, 47). It is possible, therefore, that this combination of phenotypes is specifically responsible for inducing an influx of leukocytes in the kidney. This inflammatory response could ultimately damage the renal parenchyma via leukocyte products (e.g., lysosomal enzymes). The combination of digalactoside binding and hemolysin production has been demonstrated in this report to be important to pathogenesis of experimental *E. coli* pyelonephritis in BALB/c mice. This observation does not imply that other virulence determinants cannot produce or do not contribute to disease in the urinary tract or under different conditions of anatomy (e.g., obstruction or severe vesicoureteric reflux).

In principle, the identification of specific microbial determinants that mediate pathogenic events leading to disease guides a rational choice of immunogens to be incorporated into a defined vaccine. In this study, we have examined the efficacy of digalactoside-binding pili and alpha-hemolysin vaccines for the prevention of pyelonephritis in a murine model of pyelonephritis. The conditions of challenge were much more extreme in this study than those previously employed in this model (30, 34, 39). In this study, control mice died within 48 h after intravesicular bacterial administration, whereas previous control mice did not die despite intravesicular inoculation with the same dose (30, 34, 39). The major difference in this study versus previous studies is a more prolonged dehydration period prior to challenge (i.e., 24 versus 18 h). The criteria for pilus and alpha-hemolysin purity were carefully assessed so that protection conferred by immunization could be attributed to the homologous immunogen(s). The digalactoside-binding pilus vaccine conferred protection among mice who survived the bacterial challenge against subsequent colonization by a homologous, piliated parent wild-type strain. Disease presumably did not occur because insufficient numbers of bacteria were present in the kidney to produce damage. In contrast, the alpha-hemolysin vaccine did not protect mice who survived the challenge against subsequent renal colonization by a wild-type pyelonephritogenic *E. coli* strain. However, despite the dense bacterial colonization of the kidney, there was significantly less renal damage in hemolysin-immunized mice than in the control PBS group. The possibility that decreased bacterial parenchymal invasion, renal epithelial damage, and/or inflammation occurs in these mice needs to be evaluated by fine-structure analysis. These studies are currently being investigated. The combination of digalactoside-binding pilus and hemolysin vaccines was also protective against subsequent renal colonization by the challenge strain and injury among mice who survived 2 days after intravesicular bacterial inoculation. The combination vaccine might theoretically provide more prolonged immunity than single-component vaccines by interdicting several steps of the pathogenic sequence and decreasing the emergence of escape mutants that are antigenic variants.

The immunological mechanism by which the digalactoside-binding pilus and hemolysin vaccines conferred protection against pyelonephritis among mice who survived the bacterial challenge after prolonged dehydration was not addressed in this study. It seems likely that specific antibody enters urine from serum or is produced locally in the kidney and interferes with the functions of these virulence factors. From previous studies (34, 39), specific IgG antibody in urine to the major pilin (PapA) of digalactoside-binding pili correlates with protection. In other work by our group (14) and Pellett and colleagues (37), anti-hemolysin IgG antibodies that bind to the amino-terminal amino acids and regions within the domain associated with the toxin's calcium binding prevent lysis of cells. Future studies will determine whether (i) passive immunization of anti-pilus or anti-hemolysin antibodies can protect mice from pyelonephritis before and after challenge and (ii) antibody can directly or indirectly

affect the production of these determinants by bacteria or interfere with their functions in relevant models of infection. These questions notwithstanding, the importance of these determinants in the pathogenesis of pyelonephritis has been conclusively established. Furthermore, a digalactoside-binding pilus plus alpha-hemolysin combination vaccine is an attractive strategy for the immunoprophylaxis of pyelonephritis in patients with anatomically normal urinary tracts. It is clear that the place in therapeutics of this effective vaccine in a relevant animal model versus the conventional use of antibiotics in the prevention or treatment of urinary tract infection can only be answered by appropriately controlled studies in humans.

ACKNOWLEDGMENTS

This work was supported by grants from the Veterans Administration and by the NIH (AI23435).

We thank John Overdeck and Neil Crellin, Department of Statistics, Stanford University, for their statistical advice.

REFERENCES

1. **Båga, M., S. Normark, J. Hardy, P. O'Hanley, D. Lark, O. Olsson, G. Schoolnik, and S. Falkow.** 1984. Nucleotide sequence of the *papA* gene encoding the Pap pilus subunit of human uropathogenic *Escherichia coli*. J. Bacteriol. **157**:330–333.
2. **Benz, R., A. Schmid, W. Wagner, and W. Goebel.** 1989. Pore formation by the *Escherichia coli* hemolysin: evidence for an association-disassociation equilibrium of the pore-forming aggregates. Infect. Immun. **57**:887–895.
3. **Bhakdi, S., N. Mackman, J. M. Nicaud, and I. B. Holland.** 1986. *Escherichia coli* hemolysin may damage target cell membranes by generating transmembrane pores. Infect. Immun. **52**:63–69.
4. **Bhakdi, S., and J. Tranum-Jensen.** 1988. Damage to cell membranes by pore-forming bacterial cytolysins. Prog. Allergy **40**: 1–43.
5. **Bohach, G. A., and I. S. Snyder.** 1985. Chemical and immunological analysis of the complex structure of *Escherichia coli* alpha-hemolysin. J. Bacteriol. **164**:1071–1080.
6. **Cavalieri, S. J., G. A. Bohach, and I. S. Snyder.** 1984. *Escherichia coli* α-hemolysin: characteristics and probable role in pathogenicity. Microbiol. Rev. **48**:326–343.
7. **Cavalieri, S., and I. Snyder.** 1982. Cytotoxic activity of partially purified *Escherichia coli* alpha hemolysin. J. Med. Microbiol. **15**:11–21.
8. **Fry, T., F. Fried, and B. Goven.** 1975. Pathogenesis of pyelonephritis: *Escherichia coli* induced renal ultrastructural changes. Invest. Urol. **13**:47–54.
9. **Hacker, J., H. Hof, L. Emody, and W. Goebel.** 1986. Influence of cloned *Escherichia coli* hemolysin genes, S-fimbriae, and serum resistance on pathogenicity in different animal models. Microb. Pathog. **1**:533–547.
10. **Hacker, J., C. Hughes, H. Hof, and W. Goebel.** 1983. Cloned hemolysin genes from *Escherichia coli* that cause urinary tract infection determine different levels of toxicity in mice. Infect. Immun. **42**:57–63.
11. **Hagberg, L., I. Engberg, R. Freter, J. Lam, S. Olling, and C. Svanborg-Eden.** 1983. Ascending, unobstructed urinary tract infection in mice caused by pyelonephritiogenic *Escherichia coli* of human origin. Infect. Immun. **40**:273–283.
12. **Hagberg, L., R. Hull, S. Hull, S. Falkow, R. Freter, and C. Svanborg-Eden.** 1983. Contribution of adhesion to bacterial persistence in the mouse urinary tract. Infect. Immun. **40**:265–272.
13. **Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow.** 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect. Immun. **33**:933–938.
14. **Ji, G., and P. O'Hanley.** 1990. Epitopes of *Escherichia coli* alpha-hemolysin: identification of monoclonal antibodies that prevent hemolysis. Infect. Immun. **58**:3029–3035.
15. **Jorgensen, S. E., R. F. Hammer, and G. K. Wu.** 1980. Effects of a single hit from the alpha hemolysin produced by *Escherichia coli* on the morphology of sheep erythrocytes. Infect. Immun. **27**:988–994.
16. **König, B., W. König, J. Scheffer, J. Hacker, and W. Goebel.** 1986. Role of *Escherichia coli* alpha-hemolysin and bacterial adherence in infection: requirement for release of inflammatory mediators from granulocytes and mast cells. Infect. Immun. **54**:886–892.
17. **Konig, W., B. Konig, J. Scheffer, J. Hacker, and W. Goebel.** 1989. Role of cloned virulence factors (mannose-resistant hemagglutination, mannose-resistant adhesins) from uropathogenic *Escherichia coli* strains in the release of inflammatory mediators from neutrophils and mast cells. Immunology **67**:401–407.
18. **Konig, W., J. Scheffer, K. Bremm, J. Hacker, and W. Goebel.** 1985. Role of bacterial adherence and toxin production from *Escherichia coli* on leukotriene generation from human polymorphonuclear granulocytes. Int. Arch. Allergy Appl. Immunol. **77**:1118–1120.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **Lindberg, F., B. Lund, L. Johansson, and S. Normark.** 1987. Localization of the receptor binding protein adhesin at the tip of the bacterial pilus. Nature (London) **328**:84–87.
21. **Linggood, M., and P. Ingram.** 1982. The role of alpha-hemolysin in the virulence of *Escherichia coli* for mice. J. Med. Microbiol. **15**:23–36.
22. **Low, D., V. David, D. Lark, G. Schoolnik, and S. Falkow.** 1984. Gene clusters governing the production of hemolysin and mannose-resistant hemagglutination are closely linked in *Escherichia coli* serotype O4 and O6 isolates from urinary tract infections. Infect. Immun. **43**:353–357.
23. **Lund, B., F. Lindberg, B. I. Marklund, and S. Normark.** 1987. The PapG protein is the α-D-galactopyranosyl-(1,4)-β-D-galactopyranose binding adhesin of uropathogenic *Escherichia coli*. Proc. Natl. Acad. Sci. USA **84**:5898–5902.
24. **Lund, B., F. Lindberg, and S. Normark.** 1988. Structure and antigenic properties of the tip-located P pilus proteins of uropathogenic *Escherichia coli*. J. Bacteriol. **170**:1887–1894.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Markwell, M., S. Haas, L. Bieber, and N. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87**:206–210.
27. **Marr, R., J. Hacker, W. Henkel, and W. Goebel.** 1986. Contribution of cloned virulence factors from uropathogenic *Escherichia coli* strains to nephropathogenicity in an experimental rat pyelonephritis model. Infect. Immun. **54**:761–767.
28. **Menestrina, G., N. Mackman, I. B. Holland, and S. Bhakdi.** 1987. *Escherichia coli* hemolysin forms voltage-dependent ion channels in lipid membranes. Biochim. Biophys. Acta. **905**:109–117.
29. **Norgren, M., S. Normark, D. Lark, P. O'Hanley, G. Schoolnik, S. Falkow, C. Svanborg-Eden, M. Baga, and B. E. Uhlin.** 1984. Mutations in *E. coli* cistrons affecting adhesion to human cells do not abolish Pap pili fiber formation. EMBO J. **3**:1159–1165.

29a. **O'Hanley, P.** Unpublished data.

30. **O'Hanley, P., D. Lark, S. Falkow, and G. Schoolnik.** 1985. Molecular basis of *Escherichia coli* colonization of the upper urinary tract in BALB/c mice. J. Clin. Invest. **75**:347–360.
31. **O'Hanley, P., D. Lark, S. Normark, S. Falkow, and G. Schoolnik.** 1983. Mannose-sensitive and Gal-Gal binding *Escherichia coli* pili from recombinant strains. J. Exp. Med. **158**:1713–1719.
32. **O'Hanley, P., D. Low, I. Romero, D. Lark, K. Vosti, S. Falkow, and G. Schoolnik.** 1985. Gal-Gal binding and hemolysin phenotypes and genotypes associated with uropathogenic *Escherichia coli*. N. Engl. J. Med. **313**:414–420.
33. **Ørskov, I., F. Ørskov, and A. Birch-Andersen.** 1980. Comparison of *Escherichia coli* fimbrial antigen F7 with type 1 fimbriae. Infect. Immun. **27**:657–666.

34. **Pecha, B., D. Low, and P. O'Hanley.** 1989. 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 *Escherichia coli* strains. J. Clin. Invest. **83**:2102–2108.
35. **Pellett, S., D. Boehm, I. Snyder, G. Rowe, and R. Welch.** 1990. Characterization of monoclonal antibodies against the *Escherichia coli* hemolysin. Infect. Immun. **58**:822–827.
36. **Roberts, J., K. Hardaway, B. Kaack, E. Fussell, and G. Baskin.** 1984. Prevention of pyelonephritis by immunization with P-fimbriae. J. Urol. **131**:602–607.
37. **Salit, I. E., J. Hanley, L. Clubb, and S. Fanning.** 1988. The human antibody response to uropathogenic *Escherichia coli*: a review. Can. J. Microbiol. **34**:312–318.
38. **Scheffer, J., W. König, J. Hacker, and W. Goebel.** 1985. Bacterial adherence and hemolysin production from *Escherichia coli* induces histamine and leukotriene release from various cells. Infect. Immun. **50**:271–278.
39. **Schmidt, M. A., P. O'Hanley, D. Lark, and G. Schoolnik.** 1988. Synthetic peptides corresponding to protective epitopes of *Escherichia coli* digalactoside-binding pilin prevent infection in a murine pyelonephritis model. Proc. Natl. Acad. Sci. USA **85**:1247–1251.
40. **Seetharama, S., S. J. Cavalieri, and I. S. Snyder.** 1988. Immune response to *Escherichia coli* alpha-hemolysin in patients. J. Clin. Microbiol. **26**:850–856.
41. **Svanborg-Eden, C., and P. deMan.** 1987. Bacterial virulence in urinary tract infection. Infect. Dis. Clin. North Am. **1**:731–750.
42. **Switzer, R., C. Merril, and S. Shifrin.** 1979. A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Anal. Biochem. **98**:231–237.
43. **Tsai, C. M., and C. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
44. **Uhlin, B. E., M. Norgren, M. Baga, and S. Normark.** 1985. Adhesion to human cells by *Escherichia coli* lacking the major subunit of digalactoside-specific pilus-adhesin. Proc. Natl. Acad. Sci. USA **82**:1800–1804.
45. **Van den Bosch, J., L. Emody, and I. Ketyi.** 1982. Virulence of hemolytic strains of *Escherichia coli* in various animal models. FEMS Microbiol. Lett. **13**:427–430.
46. **van den Bosch, J. F., U. Verboom-Sohmer, P. Postma, J. de Graaff, and D. M. MacLaren.** 1980. Mannose-sensitive and mannose-resistant adherence to human uroepithelial cells and urinary virulence of *Escherichia coli*. Infect. Immun. **29**:226–233.
47. **Ventur, Y., J. Scheffer, J. Hacker, W. Goebel, and W. Konig.** 1990. Effects of adhesins from mannose-resistant *Escherichia coli* on mediator release from human lymphocytes, monocytes, and basophils and from polymorphonuclear granulocytes. Infect. Immun. **58**:1500–1508.
48. **Waalwijk, C., D. MacLaren, and J. DeGraff.** 1983. In vivo function of hemolysin in the nephropathogenicity of *Escherichia coli*. Infect. Immun. **42**:245–249.
49. **Waravdekar, V., and L. Saslaw.** 1959. A sensitive method for the estimation of a 2-deoxy sugar with the use of the malonaldehyde thiobarbituric acid reaction. J. Biol. Chem. **234**:1945–1950.
50. **Welch, R., E. Dellinger, B. Minshew, and S. Falkow.** 1981. Hemolysin contributes to virulence of extra-intestinal *E. coli* infections. Nature (London) **294**:664–667.
51. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **5**:90–96.