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Pyelonephritogenic *Escherichia coli* and Killing of Cultured Human Renal Proximal Tubular Epithelial Cells: Role of Hemolysin in Some Strains

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Acute pyelonephritis, a complication of *Escherichia coli* bacteriuria, must represent a bacterial invasion through the kidney epithelium. To study this process, we overlaid bacterial suspensions onto monolayers of cultured human kidney proximal tubular epithelial cells and measured cytotoxicity by release of lactate dehydrogenase (LDH). Thirty-four isolates cultured from patients with acute pyelonephritis were screened for the ability to cause pyelonephritis in CBA mice by transurethral challenge. The eight most virulent strains (≥70% of mice challenged developed $\geq 10^3$ CFU/g of kidney after 48 h) were selected for study. Each strain displayed mannose-resistant hemagglutination of human O erythrocytes; three strains were phenotypically and genotypically hemolytic. Pyelonephritogenic strains were significantly more cytotoxic (30.1 ± 9.5% LDH release after 18 h) than eight fecal control strains (13.5 ± 11.5% LDH release; $P = 0.0068$). We selected the most cytotoxic strain, CFT073, for further study. Sterile filtrate from this hemolytic strain was significantly more cytotoxic than was the filtrate of the fecal control strain, FN414. Transposon mutagenesis of CFT073 with Tn*phoA* abolished hemolytic activity and cytotoxicity by both whole cells and sterile filtrate. Southern blot analysis revealed that the Tn*phoA* insertion mapped to the *E. coli* chromosomal *hly* determinant within a 12-kilobase *Sal*I restriction fragment. Transformation of a nonhemolytic strain, CPZ005 with plasmid pSF4000, which carries a cloned hemolysin determinant, resulted in highly elevated cytotoxicity. Light micrographs of proximal tubular epithelial cell cultures demonstrated cell damage by pyelonephritogenic strains that was not induced by a fecal strain or the hemolysin-deficient mutant. Results indicate that pyelonephritogenic *E. coli* strains are more frequently cytotoxic for a putative target, that is, human renal tubular epithelium, than are fecal isolates. Hemolysin, in some strains, is apparently responsible for this cytotoxicity.

Bacteriuria is common in various populations throughout the human life span. Infant boys, preschool girls, women of childbearing age, and aged people of both sexes have relatively high incidences and prevalences of bacteriuria (21). Acute pyelonephritis, a bacterial infection of one or both kidneys, is a complication of bacteriuria which often requires hospitalization for treatment and may be accompanied by bacteremia, shock, and death (32). This infection represents an invasion by bacteria that usually have entered the bladder and ascended the ureters to the kidney. To infect kidney parenchyma, they must then traverse the epithelial mucosa. This might be accomplished by going through or between renal epithelial cells, passing the intact or damaged mucosal basement membrane, and finally, reaching the interstitium, therein intensifying an inflammatory response. Although early steps of entry into the lumen of the urinary tract and adherence to the uroepithelium have been well studied, the latter steps are not well understood.

Various factors have been linked epidemiologically to acute pyelonephritis, including P fimbriation, hemolysin production, presence of colicin V plasmids, and expression of certain O serotypes (3, 13, 15, 18, 22, 24, 30, 31). Surveys of patients with acute pyelonephritis have reported that *Escherichia coli* strains isolated from the urine of these patients were much more likely to express P fimbriae and hemolysin than fecal strains of *E. coli* were.

Hemolysin may represent the only polypeptide that is secreted extracellularly by *E. coli* (14) and has been shown to be cytotoxic for a variety of cell types, including erythrocytes (23), fibroblasts (5), granulocytes (10, 20), and other human peripheral leukocytes (4, 6, 7). It appears to exert its effect by eliciting membrane damage by acting as a pore-forming cytolysin (1). By using animal models of peritonitis (41) or a variety of in vitro models (19, 20, 42), genetic studies with cloned hemolysin determinants or naturally occurring plasmids bearing hemolysin genes have implicated this protein in pathogenesis. Surprisingly, only a very few reports have examined the role of this cytotoxin in ascending urinary tract infection (UTI), the most common human infection caused by this organism in developed countries.

We report here that 46% of pyelonephritogenic strains of *E. coli* are hemolytic, and in the most virulent of these hemolytic strains we demonstrated, by transposon mutagenesis, that hemolysin is a cytotoxin for human renal proximal tubule epithelial cells, a putative target cell for pyelonephritogenic bacteria.

MATERIALS AND METHODS

Patients and bacterial strains. While studying antibiotic treatment responses for acute pyelonephritis and other infections caused by gram-negative rods, we collected the

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bacterial strains that cause the clinical syndrome (8, 39). Included in the collection were 61 *E. coli* strains, with one strain each isolated from the blood or urine of 41 women (age, 16 to 97 years) and 20 men (age, 21 to 87 years). Each of these patients was admitted to the University of Maryland Hospital with the clinical syndrome of acute pyelonephritis characterized by bacteriuria of $\geq 10^5$ CFU/ml, pyuria, fever, and no other identified source of infection.

Urine specimens and blood samples from these patients were cultured on EMB agar and Trypticase soy agar (TSA; BBL Microbiology Systems, Cockeysville, Md.) containing 5% sheep blood. Bacteria were determined to the species level by using a numerical taxonomy system (Analytab Products, Plainview, N.Y.). *E. coli* isolates were stored at −70°C in Trypticase soy broth (TSB; BBL) supplemented with 15% (vol/vol) glycerol.

For controls, 27 strains of *E. coli* were isolated from the feces of 20 women (age 20 to 50 years) who had not had a symptomatic urinary tract infection or known bacteriuria within the last 6 months and who had not had diarrhea or received antibiotics in the preceding month. An additional stool isolate, *E. coli* FN414, which has been used as a control strain in a mouse model of acute pyelonephritis (12), was generously provided by Richard Hull (Baylor College of Medicine, Waco, Tex.). *E. coli* WAF270 (strain J198 carrying plasmid pSF4000 encoding alpha-hemolysin) (41) was generously provided by Rodney Welch (University of Wisconsin School of Medicine, Madison).

E. coli strains were cultured from frozen stocks onto TSA. Luria broth was inoculated from the agar culture and incubated overnight at 37°C and was then used to inoculate 100 ml of modified Luria broth (per liter, 10 g of tryptone [Difco], 5 g of yeast extract, 8.5 g of NaCl, 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.2]); the solution was adjusted to 300 mosmol by dilution with a calibrated osmometer (Osmette). Cultures were grown at 37°C with aeration (200 rpm) until the optical density at 550 nm (1-cm path length) equaled 0.9. Bacterial suspensions (approx. 10^9 CFU/ml) were diluted 1,000-fold with fresh modified Luria broth and applied to cell cultures. Viable counts of the inoculum were determined on spread plates. Sterile filtrates were made by centrifuging the 10^9 suspensions (10,000 × *g*, 10 min, 4°C) and filtering the supernatant through 0.2-μm-pore-size cellulose acetate filters (Nalgene). Filtrates were cultured to verify bacteria-free status and were applied directly to cell cultures.

Hemagglutination. *E. coli* isolates were recovered from storage and cultured in Luria broth (5 ml) for 18 h with aeration. Suspensions (1 ml) were centrifuged, and cells were suspended in 0.1 ml of 0.85% NaCl for assay of mannose-resistant hemagglutination (HA) (MRHA). For assay of mannose-sensitive HA (MSHA), cells were grown statically in Luria broth for 48 h and concentrated 10-fold as described above. One drop of bacterial suspension was mixed with 1 drop of a suspension of 3% (vol/vol) human type O erythrocyte in 0.85% NaCl containing 50 mM mannose for MRHA or 1 drop of suspension of 1% (vol/vol) guinea pig erythrocytes in 0.85% NaCl in the presence and absence of 50 mM mannose for MSHA. All HAs were run on rocked ceramic tiles at 23°C. Visible clumping was interpreted as a positive reaction.

Hemolysin assay. For qualitative evaluation of hemolysin production, isolates were inoculated onto 5% sheep blood agar plates. Hemolysis was defined as a distinct zone of clearing around or under isolated bacterial colonies after overnight incubation at 37°C on TSA containing 5% sheep blood.

For quantitative determination of hemolytic titer, *E. coli* strains were grown in modified Luria broth (see formula above) at 37°C with aeration. At various time points, serial twofold dilutions (0.1 ml) were made into 0.85% NaCl into the wells of a 96-well microdilution tray. A 1% sheep erythrocyte suspension in 0.85% NaCl (0.1 ml) was added to each well, and plates were incubated for 24 h at 37°C. Hemolytic titer was defined as the highest dilution in which the supernatant was visibly red due to hemoglobin release.

Plasmid isolation. Plasmid DNA was isolated by alkaline sodium dodecyl sulfate extraction (2) from Luria broth cultures (100 ml) of *E. coli* HB101 carrying pSF4000. DNA was purified by centrifugation to equilibrium in cesium chloride-ethidium bromide density gradients (25). Recombinant plasmids were used to transform competent cells (25) of selected *E. coli* isolates.

Transposon mutagenesis. To provide a necessary antibiotic marker for mating experiments, a nalidixic acid-resistant isolate of *E. coli* CFT073 (*hly*$^+$) was selected by plating approximately 10^9 CFU on Luria agar containing nalidixic acid (50 μg/ml). *E. coli* CFT073 (Nal^r) was mated on nitrocellulose filters (33) with *E. coli* SM10 (lambda *pir*) containing the suicide vector pRT733.1 bearing Tn*phoA* (26), which was generously provided by Ron Taylor (University of Tennessee School of Medicine, Memphis). Transconjugants were selected on nalidixic acid (50 μg/ml) and kanamycin (50 μg/ml) and replica-plated onto TSA containing 5% sheep blood, kanamycin (50 μg/ml), and nalidixic acid (50 μg/ml). Nonhemolytic isolates were selected for further analysis.

DNA hybridization. Dot blots of whole-cell DNA from *E. coli* isolates were prepared by spotting overnight culture lysates onto nitrocellulose filters as described by Maniatis et al. (25). A 1.5-kilobase (kb) *Pst*I-*Bgl*I restriction fragment from within the hemolysin *hlyA* gene sequences of plasmid pSF4000 (41) was isolated by electroelution of the fragment from a preparative 0.7% agarose gel, labeled with [^{32}P]ATP by random primer extension, and used for hybridization under stringent (50% formamide, 65°C wash) hybridization conditions. *E. coli* WAF270 (described above) was used as a positive control. *E. coli* HB101 was used as a negative control.

For Southern hybridization (25), chromosomal DNA (2 μg), which was isolated by the method of Marmur (28), was digested overnight with *Eco*RV or *Sal*I, electrophoresed on a horizontal 0.7% agarose gel, and transferred to nitrocellulose. For a DNA probe specific for *E. coli* hemolysin genes, a 4.6-kb *Eco*RV-*Sma*I restriction fragment (which spans significant portions of *hlyA* and *hlyB*) was isolated and labeled with [α-^{32}P]ATP by nick translation (25). The blot was hybridized under stringent conditions (50% formamide, 65°C wash), washed, dried, and autoradiographed. Plasmid pSF4000, which was digested with *Eco*RV, was used as a positive control.

Mouse model of UTI. A modification of the mouse model of ascending UTI originally described by Hagberg et al. (12) was used for this study, as described previously (17). Female CBA/J mice (weight, 22 to 24 g; Jackson Laboratory, Bar Harbor, Maine) were used for this study. Urine was cultured 24 h prior to challenge, and mice with bacteria present at $>10^2$ CFU/ml were excluded.

A sterile polyethylene catheter (internal diameter, 0.28 mm; outer diameter, 0.61 mm; length, 25 mm) was gently inserted in the bladder through the urethra. A 30-gauge needle attached to a tuberculin syringe containing bacterial

suspension at 2×10^{10} to 4×10^{10} CFU/ml was inserted in the catheter lumen, and 0.05 ml (1×10^9 to 2×10^9 CFU) was slowly infused into the bladder over 30 s. Bacterial suspensions were confirmed for HA prior to inoculation. Pyelonephritogenic strains were not inoculated unless a positive MRHA was demonstrated. Fecal isolates were inoculated only if they were negative for MRHA. Mice were sacrificed 48 h after challenge with an overdose of methoxyflurane. Half of each kidney was weighed, homogenized, and cultured with Levine EMB agar.

Isolation and culture of human renal proximal tubular epithelial cells. Human renal proximal tubular epithelial cells were isolated, cultured, and characterized as described previously (35). Briefly, the cortex of a recently autopsied kidney was minced and digested with a collagenase solution (100 U/ml) in tissue culture medium (Cellgro; Mediatech, Herndon, Va.) supplemented with gentamicin (0.1 mg/ml) and 10 mM HEPES at 37°C in a spinner culture flask (Bellco Glass, Inc., Vineland, N.J.). Tissue fragments were gently pelleted (5 min, 72 × *g*, 23°C), washed three times in phosphate-buffered saline (pH 7.2), and suspended in tissue culture medium (Cellgro supplemented with insulin [2.4 U/ml], 10% inactivated fetal bovine serum, fungizone [1.23 μg/ml], penicillin [100 U/ml], streptomycin [100 μg/ml], and 10 mM HEPES [pH 7.2]); cells were plated at a density of 10^6 live cells per ml of cell culture medium and maintained at 37°C in an atmosphere of 5% CO_2–95% air. Confluent monolayers were observed in 10 to 14 days. Confirmation that these cells represented proximal tubular epithelial cells was made by biochemical characterization and specific lectin-binding analysis as described previously (35). Primary cultures were trypsinized and reseeded at a density of 1×10^5 to 2×10^5 cells per ml of medium onto tissue culture dishes (Miles Laboratories, Inc., Elkhart, Ind.).

Lactate dehydrogenase determination. To assay for bacteria-induced cytotoxicity, release of lactate dehydrogenase (LDH) from epithelial cell cultures was measured. Cell cultures were inoculated with 2 ml of 10^6 bacteria per ml as described above. At intervals over 24 h, samples (0.2 ml) were removed and added to cuvettes (1-cm path length) containing 0.8 ml of modified Hanks balanced salt solution (without phenol red or glucose; pH 7.4) (36). Reactions were initiated by the addition of 5 μl each of 130 mM sodium pyruvate and 20 mM NADH (final concentrations, 0.65 mM pyruvate and 0.1 mM NADH). Assays were run at 25°C, and LDH activity was monitored by following the decrease in A_{340} with a spectrophotometer (Response; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Typical assays ran for 1 min. For estimation of total activity, monolayers were disrupted by the addition of Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 0.20%. After 10 min, samples (0.2 ml) were assayed as described above. LDH release was calculated as a percentage of the total activity. Assays were run in triplicate for each bacterial inoculation (36). For a negative control, 0.2 ml of a bacterial suspension (10^9 bacteria per ml) in the absence of cell culture was assayed. As a positive control, cell cultures were treated with 10 μM ionomycin (Calbiochem-Behring, La Jolla, Calif.) for 24 h at 37°C.

RESULTS

Characterization of *E. coli* strains. Sixty-one *E. coli* strains were isolated from 61 patients with acute pyelonephritis. A total of 46 (75%) strains were $MRHA^+$, 24 (39%) were $MSHA^+$, and 28 (46%) were beta-hemolytic. Of these strains, 34 were arbitrarily selected for screening for the ability to cause pyelonephritis in the CBA mouse model of ascending UTI. We selected for study the eight most virulent strains (Table 1); each of these strains caused pyelonephritis in at least 70% of the mice. All eight of the pyelonephritogenic strains that caused pyelonephritis in both humans and mice demonstrated MRHA of human type O erythrocytes, a phenotypic trait of P-fimbriated strains. Five strains expressed type 1 fimbriae, as assayed by MSHA of guinea pig erythrocytes. Three of the eight strains were hemolytic on blood agar plates and hybridized on dot blots with an *E. coli* hemolysin gene probe; none of the five nonhemolytic strains hybridized on dot blots.

Of the 27 fecal strains tested, 8 (30%) strains were $MRHA^+$, which was significantly less ($P = 0.0001$) than that of the pyelonephritogenic strains; 17 (63%) were $MSHA^+$ (not significantly different); and 2 (7%) were beta-hemolytic, which was significantly less ($P = 0.001$) than that of the 61 pyelonephritogenic strains. All strains were screened for pyelonephritogenicity in the mouse model of UTI. Only one strain caused pyelonephritis in ≥70% of mice, which was the break point demonstrated by our eight selected pyelonephritis strains. We selected the seven least virulent fecal strains; all caused pyelonephritis in ≤20% of mice that were challenged. An additional control, FN414, was used; this is a fecal control strain that was used in the original report of the mouse model we adopted (12); it caused no pyelonephritis in any mice and was the least virulent of our strains. None of these eight fecal strains displayed MRHA or produced hemolysin; one strain gave a positive MSHA reaction.

Cytotoxicity for human renal tubular cells. The eight pyelonephritogenic strains and the eight fecal control strains were inoculated onto cultured human renal proximal tubular cells (Table 1). After only 3 h of incubation, the mean percent LDH for pyelonephritis strains, 3.0 ± 2.4, was significantly higher than that of fecal strains at 1.0 ± 1.1 ($P = 0.0498$). After 18 h of incubation with cultured proximal tubular epithelium, the pyelonephritis strains elicited a mean percent LDH release of 30.1 ± 9.5, which was significantly higher than 13.4 ± 11.5 ($P = 0.0068$), which was the mean for fecal isolates.

Hemolysin. The two pyelonephritis isolates which each consistently caused the release of >40% of tubular epithelial cell LDH were phenotypically hemolytic on blood agar and genotypically $hlyA^+$ by DNA hybridization. Therefore, to study the role of hemolysin in cytotoxicity more thoroughly, we selected the most cytotoxic strain, CFT073, a hemolysin-positive, P-fimbriated pyelonephritogenic strain. As a control, we used FN414, a strain with almost no virulence for mice. Strain CFT073 caused a steady increase in LDH release over time, whereas after 1 h, strain FN414 showed significantly less ($P < 0.001$) cytotoxicity (Fig. 1A).

Sterile filtrates derived from late-exponential-phase cultures (10^9 CFU/ml) of strain CFT073 demonstrated significantly more ($P < 0.0004$) cell killing than did filtrates from the fecal isolate FN414 at all time points after 3 h (Fig. 1B). Cell killing by filtrates derived from a hemolysin-deficient mutant of CFT073 (see below) was not significantly different from that by filtrates derived from the fecal FN414 strain. A dramatic increase in LDH release was noted between 3 and 6 h for the CFT073 filtrate. No additional release was seen at 18 h. However, a significant increase was observed between 18 and 24 h. To demonstrate that these effects observed with whole-cell suspensions (Fig. 1A) were not due to differential growth between strains, growth curves were established by quantitative culture of media sampled directly from tissue

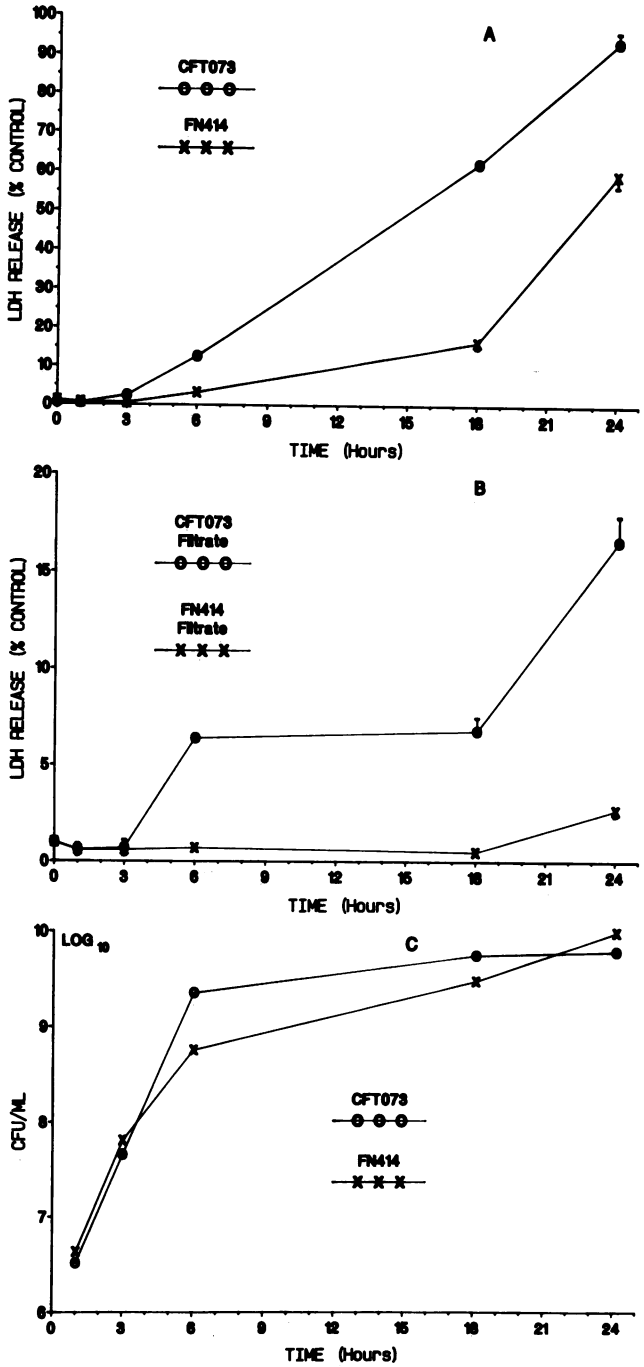

FIG. 1. LDH release by cultured human proximal tubular epithelial cells exposed to *E. coli* strains and culture filtrates. Confluent monolayers of human proximal tubular epithelial cells were overlaid with *E. coli* suspensions (10^6 CFU) or broth filtrates from suspensions of 10^9 bacteria. At various intervals, samples were taken from tissue culture wells, and soluble LDH was determined and expressed as a percentage of enzyme released by treatment of the cell culture with Nonidet P-40. (A) Pyelonephritis isolate CFT073 (○) and fecal isolate FN414 (×). (B) Sterile filtrates of CFT073 (○) and FN414 (×). (C) Samples taken from tissue culture wells were used to determine cfu per milliliter for each bacterial strain at the indicated times. ○, CFT073; ×, FN414.

culture wells containing bacterial suspensions (Fig. 1C). In addition, cytotoxicity was not due to a change in pH or osmolality, as values remained within the physiological range between 0 and 24 h in tissue culture wells containing bacterial suspensions or sterile filtrates.

Hemolysin mutant. We suspected that the hemolysin was responsible for the observed cytotoxicity since hemolytic strains were most cytotoxic, the factor was filterable, and *E. coli* hemolysin has been demonstrated to be cytotoxic for erythrocytes, polymorphonuclear leukocytes, and fibroblasts. To study the effect of specifically inactivating the chromosomal hemolysin determinant by transposon mutagenesis, a nalidixic acid derivative of *E. coli* CFT073 was mutagenized with the suicide vector pRT733.1 carrying Tn*phoA*. Selection of the nalidixic acid-resistant mutant was necessary to provide a marker for mating experiments. Of approximately 1,000 Kan^r Nal^r transposon insertions, 4 were found to be nonhemolytic on sheep blood agar plates. None of the four isolates secreted detectable active hemolysin, as assayed by the hemolytic titer of whole cells. Other strain characteristics remained unchanged, including a strong MRHA pattern and 18 biochemical markers that were originally used for determination of the bacteria to the species level.

One of these four hemolysin-negative mutants, which was characterized as CFT073 *hly*::Tn*phoA*, was arbitrarily selected for further study. A Southern blot of *Eco*RV- or *Sal*I-digested chromosomal DNA isolated from CFT073 and CFT073::Tn*phoA* was hybridized with a 4.6-kb *Eco*RV-*Sma*I, ^{32}P-labeled restriction fragment that was isolated from the hemolysin determinant of plasmid pSF4000 (Fig. 2). Tn*phoA* was found to insert within the 12-kb *Sal*I fragment that carries the 8.2-kb hemolysin determinant (*hlyC*, *hlyA*, *hlyB*, and *hlyD*). The site of insertion was outside the boundaries of a 7-kb *Eco*RV fragment. These data allowed us to map the site of insertion to within *hlyD*, one of the genes encoding the secretory apparatus necessary for the export of an active *hlyA* gene product (14, 42).

The hemolysin-deficient mutant was compared with the wild-type and nalidixic acid derivative of CFT073 for the ability to kill human proximal tubular epithelial cells (Fig. 3). Curves of CFT073 and CFT073 (Nal^r) were not significantly different. Cell killing by the mutant was diminished when compared with killing by both the parental strain CFT073 and the nalidixic acid-resistant derivative ($P < 0.02$ at 1 h; $P < 0.003$ at 3 h and thereafter). The curves were actually parallel after 6 h. Therefore, the only difference between the curves was between 3 and 6 h. This is the time period when hemolysin is being secreted before it is shut off in the late exponential phase. Curves for the hemolysin-negative mutant were similar to those of the control fecal strain FN414.

Light micrographs of cell cultures overlaid with bacterial strains confirmed the results of experiments in which we measured LDH release (Fig. 4). Whereas CFT073 severely damaged the monolayer after 6 h and completely cleared cultured cells from the surface by 18 h, hemolysin-negative CFT073::Tn*phoA* and fecal control FN414 both demonstrated minimal damage to cultured cells at 6 h and failed to clear the monolayer by 18 h.

Recombinant hemolysin. A pyelonephritogenic strain that was nonhemolytic (CPZ005) was transformed with plasmid pSF4000, which encodes the *E. coli* hemolysin (41). LDH release from cultured proximal tubular epithelium was measured after 3 and 18 h (Table 1). Active hemolysin production by *E. coli* CPZ005(pSF4000) resulted in very high cytotoxicity; there was 84.5% LDH release after 18 h as

TABLE 1. Characteristics, pyelonephritogenicity, and cytotoxicity for cultured human proximal tubular epithelium of *E. coli* strains selected for study

Source of isolate and strain	HA		Hemolysin		No. of mice with the following CFU/g of kidney		LDH release (% ± SD of control) at:	
	MRHA[a]	MSHA[b]	Hemolysis on blood agar[c]	*hlyA*$^+$ by DNA hybridization[d]	$<10^3$	$>10^3$	3 h	18 h
Pyelonephritis								
CFT204	+	+	+	+	1	9	2.0 ± 0.1	27.2 ± 0.5
CFT331	+	+	+	+	3	7	3.1 ± 0.5	41.9 ± 1.2
CFT073	+	−	+	+	2	8	8.6 ± 0.1	43.0 ± 0.9
CFT131	+	+	−	−	1	9	1.4 ± 0.0	20.9 ± 0.1
CFT434	+	MR[e]	−	−	1	9	1.9 ± 0.4	23.1 ± 0.2
CFT132	+	−	−	−	2	8	2.1 ± 0.1	19.5 ± 0.4
CFT189	+	−	−	−	3	7	1.5 ± 0.1	27.4 ± 0.4
CPZ005	+	+	−	−	3	7	3.6 ± 0.3	38.1 ± 1.2
Mean							3.0 ± 2.4[f]	30.1 ± 9.5[g]
CPZ005(pSF4000)	−	−	+	+	ND[h]	ND	5.2 ± 0.3	84.5 ± 0.4
Fecal								
FN414	−	−	−	−	10	0	0.3 ± 0.0	17.6 ± 0.7
EFC4	−	−	−	−	9	1	0.0 ± 0.2	6.2 ± 1.0
EFC19	−	−	−	−	9	1	2.7 ± 0.2	38.9 ± 0.6
EFC21	−	−	−	−	8	2	2.4 ± 0.3	12.8 ± 0.6
EFC9	−	−	−	−	8	2	2.0 ± 0.2	8.8 ± 0.9
EFC20	−	−	−	−	12	2	0.4 ± 0.2	12.8 ± 0
EFC1	−	−	−	−	8	2	0.0 ± 0.0	10.1 ± 0.7
EFC24	−	+	−	−	8	2	0.4 ± 0.5	0.0 ± 0.0
Mean							1.0 ± 1.1	13.4 ± 11.5

[a] HA of human type O erythrocytes; not inhibitable by 50 mM mannose.
[b] HA of guinea pig erythrocytes; inhibitable by 50 mM mannose.
[c] Zone of clearing around or under bacterial colony cultured on TSA containing 5% sheep blood.
[d] Positive dot blot hybridization with 1.5-kb *PstI-BglII* restriction fragment from pSF4000.
[e] MR, HA of guinea pig erythrocytes; not inhibitable by 50 mM mannose.
[f] $P < 0.05$ when compared with fecal strains after 3 h of incubation.
[g] $P < 0.01$ when compared with fecal strains after 18 h of incubation.
[h] ND, Not determined.

opposed to 38.1% for the wild-type strain carrying no plasmid. This elevated in vitro cytotoxicity could not be evaluated in vivo since carriage of the hemolysin plasmid pSF4000 repressed P-fimbriae expression, a factor that is critical for the development of pyelonephritis in the mouse model of ascending UTI. Passage of the strain on nonselective medium for up to 18 passages did not reestablish P-fimbriae expression.

DISCUSSION

E. coli is the most common cause of acute pyelonephritis. Although much is known about the fimbriae of uropathogenic *E. coli* strains and the ability to adhere to uroepithelium, little is known of the pathogenicity of this disease. Surveys, including that described herein, have shown that pyelonephritogenic strains are more likely than fecal strains of *E. coli* to possess certain traits. These include P and F fimbriae, hemolysin, aerobactin, and certain O serotypes (3, 13, 15, 18, 22, 24, 30, 31). In this series of experiments, we have begun to determine the mechanisms of pathogenesis of one of these virulence factors, hemolysin, upon what may be a target cell for acute pyelonephritis, the human renal proximal tubular epithelial cell.

Over the last several years, *E. coli* hemolysin has been examined as a putative virulence factor in various extraintestinal infections. Welch et al. (41) first demonstrated that transformation of an avirulent strain with cloned hemolysin genes significantly lowered the 50% lethal dose of the strain in a rat model of peritonitis. Transposon inactivation of the cloned determinant abolished the virulence trait. Hemolysin-negative mutants developed by spontaneous deletion (11, 20), acridine orange mutagenesis (16), curing (34), or transposon mutagenesis (37, 38) of hemolysin-encoding plasmids were used in virulence assays, including intraperitoneal or intravenous injection, measurement of protein exudation and platelet deposition after skin challenge, assay of neutrophil binding of C5a and hexapeptide, and quantitation of leukotriene release from human polymorphonuclear leukocytes, respectively. These reports all support the supposition that hemolysin is a potent virulence factor for various infections caused by *E. coli*.

Ironically, although UTI is the most common infectious disease caused by *E. coli* in developed countries and *E. coli* is the most common organism that causes UTI, few reports have addressed hemolysin as a virulence factor for acute pyelonephritis. Fried and Wong (9) observed that hemolytic strains of *E. coli* could establish pyelonephritis upon intravenous injection, whereas nonhemolytic strains could not. Marre et al. (29) used a rat model to demonstrate that adding back a hemolysin determinant to a spontaneous deletion mutant resulted in increased virulence, as measured by renal bacterial counts. This study and those that have examined

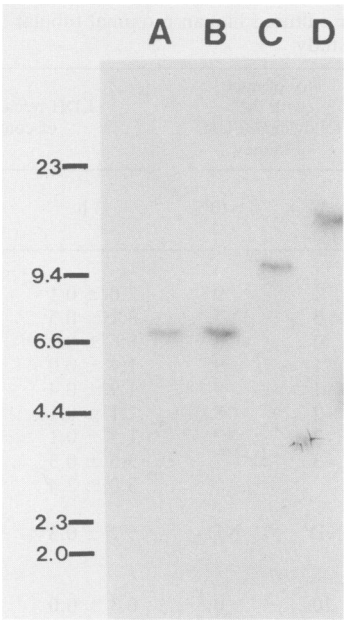

FIG. 2. Southern blot analysis of chromosomal DNA isolated from *E. coli* CFT073 and CFT073::Tn*phoA* hybridized with a hemolysin gene probe. Chromosomal DNA was isolated from *E. coli* CFT073 and a hemolysin-negative Tn*phoA* mutant of CFT073. DNA was digested with *Eco*RV (lanes A and B) or *Sal*I (lanes C and D), electrophoresed on a 0.7% agarose gel, and transferred to nitrocellulose. The blot was hybridized with a 4.6-kb ^{32}P-labeled *Eco*RV-*Sma*I restriction fragment (derived from the hemolysin determinant of pSF4000) and autoradiographed. Lanes B and D, CFT073; lanes A and C, CFT073::Tn*phoA*. Molecular sizes, shown on the left in kilobases, were estimated by comparison with the migration of *Hin*dIII-digested lambda DNA.

other extraintestinal infections have relied on the introduction of cloned hemolysin determinants that are present on high-copy-number plasmids. As shown here for strain CPZ005(pSF4000) (Table 1), an increased gene copy number (that is, the presence of the cloned hemolysin determinants on the multicopy pBR322 derivative) results in increased hemolysin secretion, as noted by an increased hemolytic titer and elevated cytotoxicity. Such recombinant strains may not reflect the true in vivo hemolysin dose that is delivered to the host.

As reported here, in at least one strain, hemolysin appears to be the cytotoxin and was a trait that was present in nearly half of the isolates cultured from the blood or urine of patients with acute pyelonephritis. Cytotoxic activity was found to be secreted, soluble, and filterable (Fig. 1C). A specific hemolysin-negative construction demonstrated significantly reduced toxicity compared with that of its hemolytic-positive parent; the residual cytotoxicity of the mutant was comparable to those of fecal isolates (Fig. 3). Furthermore, cytotoxicity was greatly enhanced when a nonhemolytic isolate was transformed with pSF4000 encoding the alpha-hemolysin cloned from *E. coli* J96, a uropathogenic strain (Table 1). Thus, cytotoxicity, which was mediated by hemolysin in some strains, may damage or kill renal tubular epithelial cells, thus effecting an important step in the pathogenic mechanism of acute pyelonephritis.

Hemolysis by *E. coli* is caused by a 107-kilodalton protein with a number of interesting characteristics (14, 42). In erythrocytes, hemolysis is irreversibly inserted as a monomer into the cytoplasmic membrane bilayer, forming a pore of about 2 nm (1). This water-filled pore allows the efflux of potassium ions and the influx of calcium ions and uncharged molecules of less than 2 nm, with subsequent lysis.

Gene sequences, which are usually on the chromosome, include four genes, *hlyC*, *hlyA*, *hlyB*, and *hlyD*, in the

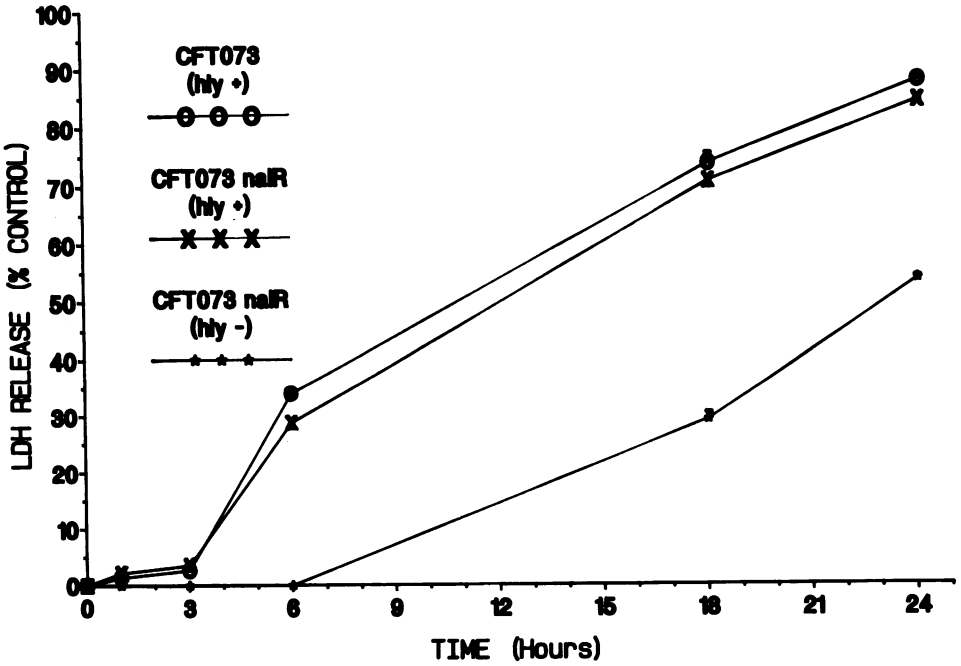

FIG. 3. LDH release by human proximal tubular epithelial cells exposed to *E. coli* CFT073 and a hemolysin-negative derivative. Monolayers were exposed to pyelonephritis isolate CFT073 (○), a nalidixic acid-derivative of CFT073 (×), and a hemolysin-negative transposon mutant of CFT073 Nal^r (CFT073::Tn*phoA*) (*), and LDH release was measured over time as described in the legend to Fig. 1.

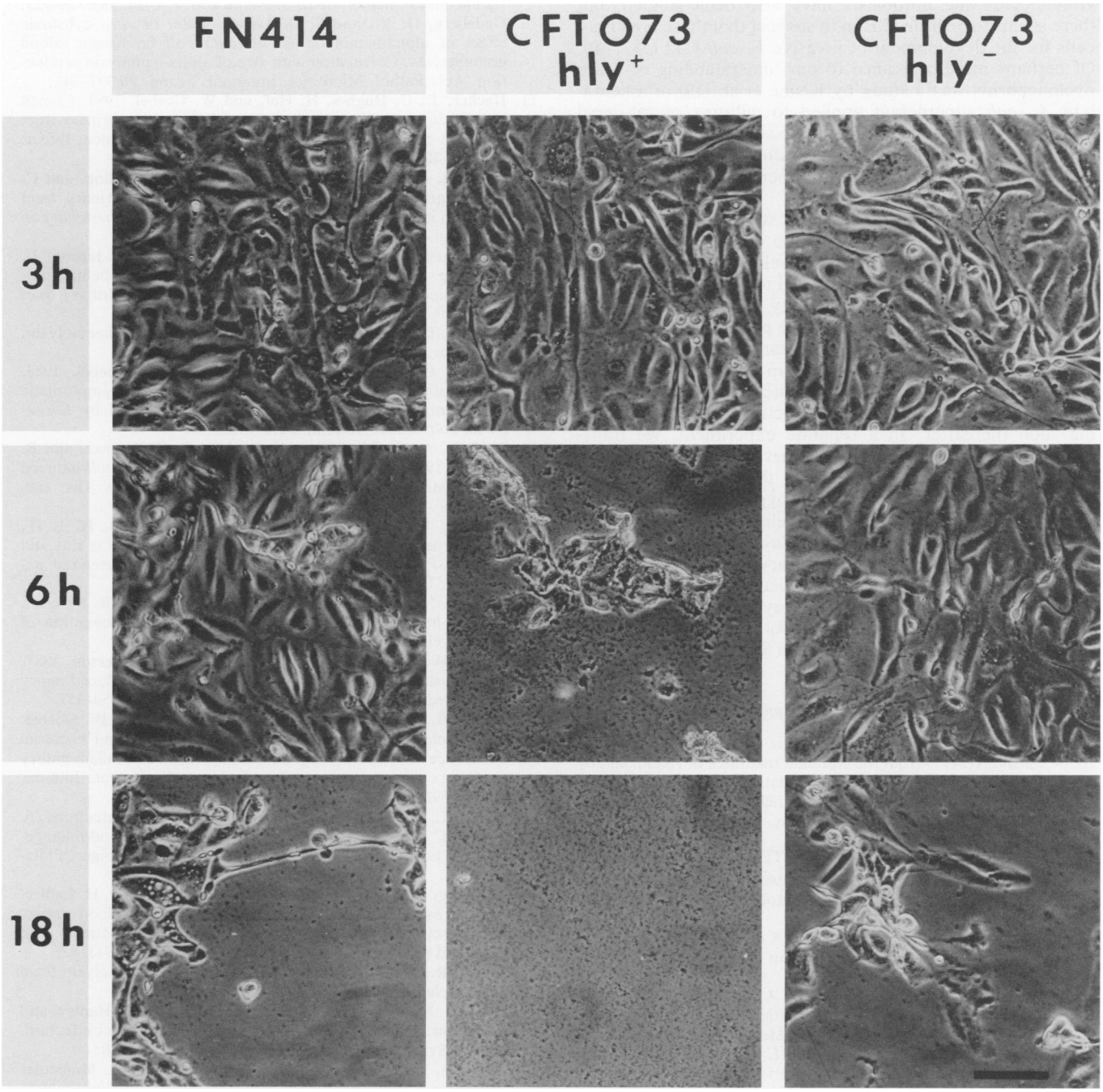

FIG. 4. Phase-contrast light micrographs of human proximal tubular cell monolayers treated with strains of *E. coli*. Monolayers of proximal tubular epithelium were overlaid with suspensions of *E. coli*, as described in the legend to Fig. 1. Representative micrographs were taken at 3, 6, and 18 h after the addition of bacterial suspensions. Left column, fecal control strain FN414; middle column, hemolysin-positive pyelonephritogenic CFT073; right column, hemolysin-negative Tn*phoA* transposon mutant of CFT073 (CFT073::Tn*phoA*). Partial lifting of cells treated with FN414 and hemolysin-negative derivative of CFT073 at 18 h and complete lifting of cells treated with hemolysin-positive CFT073 after 18 h were representative of the entire tissue culture monolayer. Magnification, ×130; bar, 100 μm.

indicated order of transcription (42). The structural gene *hlyA* encodes hemolysin. The *hlyC* product remains intracellular and is necessary for posttranslational activation of the structural polypeptide. The products of *hlyB* and *hlyD* appear to be associated with the inner membrane and are used to transport hemolysin from the cytoplasm through the inner and outer membranes to the exterior (14). One of these secretory genes may have been affected in the hemolysin-deficient mutant of CFT073 described here since the site of Tn*phoA* insertion mapped within the *hlyD* open reading frame.

Although hemolysis was first noted in erythrocytes and although these cells have been those that are most frequently studied, little evidence exists that true hemolysis represents an important feature of the pathogenesis of infection by hemolysin-secreting *E. coli*. The effects of hemolysin on

granulocytes and fibroblasts have also been studied, but there seems to be little reason to suspect that these are target cells for the development of invasive *E. coli* UTI (5–7, 10). Of perhaps more relevance to our understanding of acute pyelonephritis was a study by Keane et al. (19) of a hemolytic *E. coli* supernatant applied to cultures of rat renal proximal tubular epithelial cells. Those investigators found that 36% of these cells were killed after 30 min of exposure and that a 1:1,000 dilution of the supernatant resulted in 3% killing at 30 min.

We have taken this concept one step further to look at what we consider to be at least one of the putative target cells for the development of acute pyelonephritis, the human renal proximal tubular epithelial cell (40). We have demonstrated that the marked cytotoxicity noted at 3 to 6 h of incubation in the hemolysin-positive parent is absent in the hemolysin-negative mutant. This early cytotoxicity coincides with the secretion of hemolysin during the mid-exponential phase of growth, which is switched off during the late exponential phase, with hemolytic activity rapidly diminishing soon thereafter. In a separate experiment, we transformed hemolysin genes into another nonhemolytic pyelonephritogenic strain and found that this hemolysin-producing transformant became hypercytotoxic for the human renal proximal tubular epithelial cells.

We studied the process of bacteria-epithelial cell interaction in in vitro cell cultures of primary human renal tubular epithelium. These cells may be particularly useful in understanding acute pyelonephritis, as they are of human origin, are primary cells (i.e., are not transformed), and may represent an in vivo target cell of invasion for pyelonephritogenic *E. coli*.

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