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Contribution of *Proteus mirabilis* Urease to Persistence, Urolithiasis, and Acute Pyelonephritis in a Mouse Model of Ascending Urinary Tract Infection

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***Proteus mirabilis*, a significant cause of bacteriuria and acute pyelonephritis in humans, produces urease. This high-molecular-weight, multimeric, cytoplasmic enzyme hydrolyzes urea to ammonia and carbon dioxide. To assess the role of urease in colonization, urolithiasis, and acute pyelonephritis in an animal model of ascending urinary tract infection, we compared a uropathogenic strain of *P. mirabilis* with its isogenic urease-negative mutant, containing an insertion mutation within *ureC*, the gene encoding the large subunit of the enzyme. Mice challenged transurethrally with the parent strain developed significant bacteriuria and urinary stones. The urease-negative mutant had a 50% infective dose of 2.7×10^9 CFU, a value more than 1,000-fold greater than that of the parent strain (2.2×10^6 CFU). The urease-positive parent strain reached significantly higher concentrations and persisted significantly longer in the bladder and kidney than did the mutant. Indeed, in the kidney, the parent strain increased in concentration while the mutant concentration fell so that, by 1 week, the parent strain concentration was 10^6 times that of the mutant. Similarly, the urease-positive parent produced significantly more severe renal pathology than the mutant. The initial abnormalities were in and around the pelvis and consisted of acute inflammation and epithelial necrosis. By 1 week, pyelitis was more severe, crystals were seen in the pelvis, and acute pyelonephritis, with acute interstitial inflammation, tubular epithelial cell necrosis, and in some cases abscesses, had developed. By 2 weeks, more animals had renal abscesses and radial bands of fibrosis. We conclude that the urease of *P. mirabilis* is a critical virulence determinant for colonization, urolithiasis, and severe acute pyelonephritis.**

Proteus mirabilis is an important etiologic agent of urinary tract infection in humans (27, 30). The complications of infection, which can occur in catheterized and noncatheterized patients, include development of urolithiasis (25), urinary tract obstruction (25), obstruction of urinary catheters (23), pyelonephritis (27, 30), and bacteremia (30). This cascade of serious complications is thought to be mediated in part by urease, a high-molecular-weight, multimeric, cytosolic, nickel metalloenzyme (13, 14, 22). The enzyme promotes alkalinization of urine by catalyzing the hydrolysis of urea to ammonia and carbon dioxide (8). Elevated pH, in turn, induces development of struvite stones by precipitating normally soluble polyvalent ions (8).

To determine the contribution of urease to the uropathogenicity of *P. mirabilis*, we evaluated the outcome of experimental challenge with a urease-positive strain of *P. mirabilis* and its isogenic urease-negative mutant. We have previously reported the construction of a urease-negative mutant of a human urinary tract isolate of *P. mirabilis* by homologous recombination with cloned urease determinants (12). The gene *ureC*, which encodes the large subunit of the enzyme, was used as a target for gene disruption, rendering the bacterium unable to synthesize a functional urease. When assayed 2 days after transurethral challenge of mice, the urease-negative mutant was cleared from the urinary tract more rapidly than the urease-positive parent strain (12).

The aim of the present series of experiments was to evaluate, over time, the role of urease in the ability of *P. mirabilis* to persist, elicit stone formation, and cause acute pyelonephritis. Studies were conducted over a 2-week period in a mouse model of ascending urinary tract infection.

MATERIALS AND METHODS

Bacterial strains. *P. mirabilis* HI4320 and its isogenic mutant, derived by specific chromosomal mutation with homologous recombination (12), were used for animal challenge. The parent strain, a human urinary tract isolate, is urease positive, hemolytic, motile, fimbriated (12, 19), and susceptible to ampicillin (MIC, 2 μg/ml). The mutant, a cointegrate of suicide vector pBDJ102 (containing a partial copy of *ureC* with an additional deletion in the interior of the gene), is identical to the parent strain in all respects except that it is urease negative and resistant to ampicillin (MIC, ≥256 μg/ml).

Inoculum preparation. Inocula for mouse challenges were prepared from overnight growth on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) for the urease-positive parent or Trypticase soy agar plus 200 μg of ampicillin per ml for the urease-negative mutant. Cells were harvested from the plates, washed three times, and finally suspended in phosphate-buffered saline (pH 7.2; PBS) to a concentration of 2×10^{10} to 4×10^{10} CFU/ml and to an optical density corresponding to the desired concentration. Inocula were diluted 10-fold in PBS as required for some experiments. Inocula were enumerated by the spread plate technique by using 10-fold dilutions on Luria agar containing

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2% (wt/vol) agar and 0.5% (vol/vol) glycerol to prevent swarming (3). Viable counts were recorded as CFU per milliliter of inocula.

Mouse model. A murine model of ascending urinary tract infection was used to evaluate the uropathogenicity of the parent and isogenic mutant strains of *P. mirabilis*. This model, a modification of the procedure of Hagberg et al. (9), was previously used by us to assess the uropathogenicity of *Escherichia coli* (21), *P. mirabilis* (12), and *Providencia stuartii* (10) strains. Six- to eight-week-old female CBA/J mice (Jackson Laboratories, Bar Harbor, Maine) were used. Prior to challenge, spontaneously voided urine was collected in a sterile petri dish and bacteriuric mice were discarded. Mice were challenged while anesthetized with methoxyflurane (Metofane; Pitman-Moore, Inc., Washington Crossing, N.J.) by inserting a polyethylene catheter (2.5-cm long; outer diameter, 0.61 mm; Clay Adams, Parsippany, N.J.) into the bladder through the urethra and infusing 0.05 ml of 2×10^{10} to 4×10^{10} CFU/ml over 30 s into the bladder. Tenfold dilutions of this inoculum were also used for challenge in 50% infectious dose (ID_{50}) studies. Previous studies have documented that this procedure does not induce vesicoureteral reflux of the inoculum. The urethral catheter was removed immediately after challenge, and mice were cared for by the normal routine. Mice were inspected daily to monitor morbidity and mortality.

To collect kidney and urinary tract specimens, mice were sacrificed by an overdose of methoxyflurane at 2 days, 1 week, or 2 weeks postchallenge. The bladder and both kidneys were removed aseptically and inspected for evidence of urolithiasis. The bladder and a portion of each kidney were separately weighed and homogenized in glass blenders (Knotes, Vineland, N.J.). Tissue homogenates from each kidney were cultured quantitatively by the spread plate technique on Luria agar (2) containing 2% agar and 0.5% glycerol (3). Viable counts were recorded as CFU per gram of specimen. The ID_{50} for each strain was calculated by the method of Reed and Muench (26) from results at 1 week after challenge with log dilutions by using the ratio of the number of mice with kidney counts of $\geq 10^3$ CFU/g to the total number of mice challenged at each inoculum dilution. The Dienes phenomenon (6) was used to assist in determining whether mouse isolates and the inoculum were the same strain. By this procedure, different isolates are inoculated onto Trypticase soy agar (BBL) plates and allowed to grow and swarm. A line of demarcation develops at the swarm interfaces of different strains, whereas the interfaces of the same strain converge.

Pathologic evaluation. Half of each kidney cut longitudinally was preserved in 10% formalin (pH 7.2), embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically. The severity of renal pathology was determined by a semiquantitative score of kidney damage by using the following categories: 0, no abnormality; 1+, mild pyelitis with infiltration of low to moderate numbers of neutrophils in the pelvic cavity, necrosis of individual epithelial cells, and intact uroepithelium; 2+, moderate pyelitis with diffuse damage to the uroepithelium with infiltration of moderate numbers of neutrophils in the uroepithelium and in the parenchyma adjacent to the pelvic cavity; 3+, severe pyelitis with moderate inflammation in the parenchyma adjacent to the pelvis with moderate damage to the uroepithelium (which initially is disrupted because of necrosis and ulceration or later is thickened by regenerative hyperplasia); 4+, acute pyelonephritis involving <50% of the kidney; and 5+, severe acute pyelonephritis with extensive (>50%) damage.

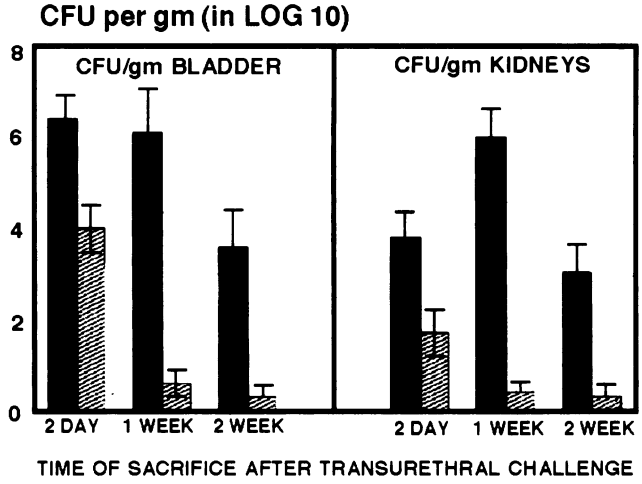

FIG. 1. Quantitative bacterial counts in the bladders and kidneys of mice challenged transurethrally with *P. mirabilis* HI4320 (solid bars) or its urease-negative isogenic mutant (hatched bars). Bars represent the geometric means (± standard deviations) for all mice in a group (n = 20 mice per group, except at 2 days, where n = 18).

Urolithiasis. Mouse bladders were inspected macroscopically at the time of sacrifice for the presence of crystalline deposits. One crystalline deposit, recovered from the bladder of a mouse sacrificed 1 week after challenge with 10^9 CFU of the parent strain, was analyzed during electron microscopy by energy-dispersive x-ray microanalysis (11). The presence of urinary crystals was also noted during microscopic examination of kidney sections.

Statistics. Results from mice challenged with the urease-positive parent or the urease-negative mutant were compared by Student's t test or Fisher's exact test. Renal scores were compared by the Mann-Whitney U test.

RESULTS

Infectivity. CBA mice were sacrificed 2 days, 1 week, or 2 weeks after transurethral challenge with 10^9 CFU of the urease-positive parent strain HI4320 of *P. mirabilis* or its urease-negative mutant. Quantitative cultures of bladder homogenates (Fig. 1) demonstrated colonization by the parent strain at 2 days to 2 weeks after challenge. Although colonization declined by more than 2 logs by 2 weeks postchallenge, a significant level of colonization persisted (geometric mean, 4×10^3 CFU/g). Mortality, a frequent consequence of challenge with the parent strain, was time dependent (0 of 18 dead at 2 days, 5 of 20 [25%] dead at 1 week, 7 of 20 [35%] dead at 2 weeks). Significantly lower numbers of the mutant strain colonized the bladder at 2 days postchallenge. The numbers of bacteria declined rapidly over the 2-week observation period. Mortality was not observed in mice challenged with the mutant strain.

Kidney colonization by the parent strain was significantly greater than colonization by the mutant strain at each time interval and peaked 1 week after challenge ($P \leq 0.002$ compared with the mutant). The urease-negative mutant did not effectively colonize the kidney in these experiments. On the basis of these results, further studies were undertaken to determine the ID_{50} at 1 week postchallenge.

The ID_{50}s for the parent and mutant strains were calcu-

lated by the method of Reed and Muench (26) by using quantitative counts of kidney homogenates obtained from mice sacrificed 1 week after challenge. Ratios of the number of mice with kidney counts of $\geq 10^3$ CFU/g to the total number of mice challenged were determined. These ratios were 18/20 for mice challenged with 10^9 CFU/ml, 13/16 for mice challenged with 10^7 CFU/ml, and 5/10 for mice challenged with 10^5 CFU/ml. The ID_{50} for the parent strain calculated from those ratios was 2.2×10^6 organisms. The ID_{50} for the mutant strain was $>2.7 \times 10^9$ organisms. Therefore, by this index, the uropathogenicity of the parent strain was ≥1,000-fold greater than that of the mutant strain in the mouse model.

Kidney pathology. Renal pathology results are summarized in Fig. 2 and illustrated in Fig. 3 to 7. Lesions in the kidneys were significantly ($P < 0.01$, Mann-Whitney U test) more severe at each time point in mice challenged with the parent strain HI4320 than in mice challenged with the isogenic urease-negative mutant. In general, the damage to the kidneys of mice infected with the urease-negative mutant was usually mild and limited to the pelvic epithelium, whereas severe pyelonephritis developed in mice infected with the urease-positive parent strain.

At 2 days postchallenge, the kidneys of mice infected with the urease-positive parent strain had mild to moderate numbers of neutrophils in the renal pelvic lumen as well as within the uroepithelium and immediately subepithelial. Focal areas of uroepithelium exhibited necrosis. Occasionally, the collecting ducts within the distal papillae contained low numbers of neutrophils. In contrast, kidneys from mice infected with the urease-negative mutant strain showed low numbers of neutrophils within the pelvic space with no uroepithelial or subepithelial infiltration or damage to the uroepithelium. Urinary crystals were not demonstrated in either group at 2 days postinfection.

At 1 week postchallenge, the pathologic findings in the kidneys were more severe, especially in mice challenged with the parent strain (Fig. 2). Twenty mice challenged with the urease-positive parent strain showed diffuse moderate to severe pyelitis, necrosis of the uroepithelium, and moderate numbers of neutrophils in the renal pelvic cavity. In five of these mice (25%), pyelonephritis was severe, with ulceration of the uroepithelium, acute necrosis of the tubular epithelium in the adjacent medulla and papilla, marked congestion of blood vessels, and intense infiltration of neutrophils (Fig. 3 and 4). The lumen of the pelvis was dilated and contained necrotic epithelial cells, moderate numbers of neutrophils, bacteria, and crystals (Fig. 4). Mice with severe pyelitis showed necrosis and thickening of the uroepithelium accompanied by moderate neutrophil infiltration (Fig. 5). Two of these mice died, and each showed extensive acute necrosis and abscess of the kidney parenchyma.

By contrast, when mice were challenged with the urease-negative mutant and examined at 1 week postchallenge, 15 of 20 animals were found to have mild to moderate pyelitis; none had severe pyelonephritis. Eight of the 15 mice showed mild necrosis of individual uroepithelial cells, and some had mild erosion of the uroepithelium (Fig. 6). Cortical abscesses, tubular necrosis, crystal formation, and parenchymal necrosis did not occur.

At 2 weeks postchallenge with the parent strain, 6 of the 19 (32%) mice examined histologically, including three mice which died, had severe renal damage including infiltration of numerous neutrophils and macrophages accompanied by fibrosis, replacing tubules in localized or extensive areas of the medulla and cortex (Fig. 7). Some mice had radial bands

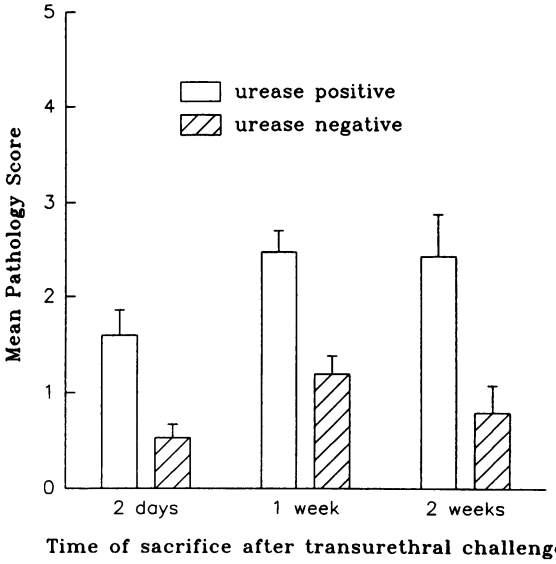

FIG. 2. Severity of histopathologic lesions in the kidneys of mice at 2 days, 1 week, and 2 weeks after transurethral challenge with 10^9 CFU of the parent strain HI4320 of *P. mirabilis* and its urease-negative mutant. The criteria for the semiquantitative scores of renal pathology are described in Materials and Methods. Error bars represent standard errors of the mean.

of fibrosis, multiple abscesses in the renal parenchyma, and acute necrosis adjacent to the pelvis and extending through the cortex. There were bacterial colonies, moderate numbers of neutrophils and struvite crystals in the lumen of the pelvis. Eight mice showed mild to moderate pyelitis. There were no renal lesions in five mice (25%).

In comparison, the kidneys of mice challenged with the urease-negative mutant showed mild pyelitis in four mice and moderate pyelitis in two mice, and one mouse developed moderate pyelonephritis (radial bands composed of moderate numbers of lymphocytes and macrophage inflammatory cells, accompanied by low numbers of neutrophils, extending through the cortex and medulla). There were no kidney lesions in 12 of the 18 mice (63%).

Urolithiasis. Two days after challenge, no animal (0 of 38) was found to have a bladder stone. One week after challenge, gross examination revealed bladder stones in 12 of 39 (31%) mice challenged with 10^9 CFU of the parent strain; no bladder stones were observed in 41 mice challenged with 10^9 CFU of the mutant strain ($P = 0.0013$). At 2 weeks after challenge, 8 of 20 (40%) mice challenged with 10^9 CFU of the parent strain developed bladder stones; no bladder stones were observed in 20 mice challenged with 10^9 CFU of the mutant strain ($P = 0.002$). Stone analysis revealed a composition of phosphate, magnesium, and a small amount of potassium, consistent with the composition of struvite (magnesium ammonium phosphate).

DISCUSSION

Bacteriologic culture and evaluation of renal pathology showed a significant reduction in the virulence of the urease-negative mutant compared with that of the urease-positive parent strain. The ID_{50} for transurethral challenge of the isogenic mutant was 1,000-fold higher than that of the parent strain. The urease-positive *P. mirabilis* parent strain HI4320 colonized the kidneys of mice at high levels. The urease-

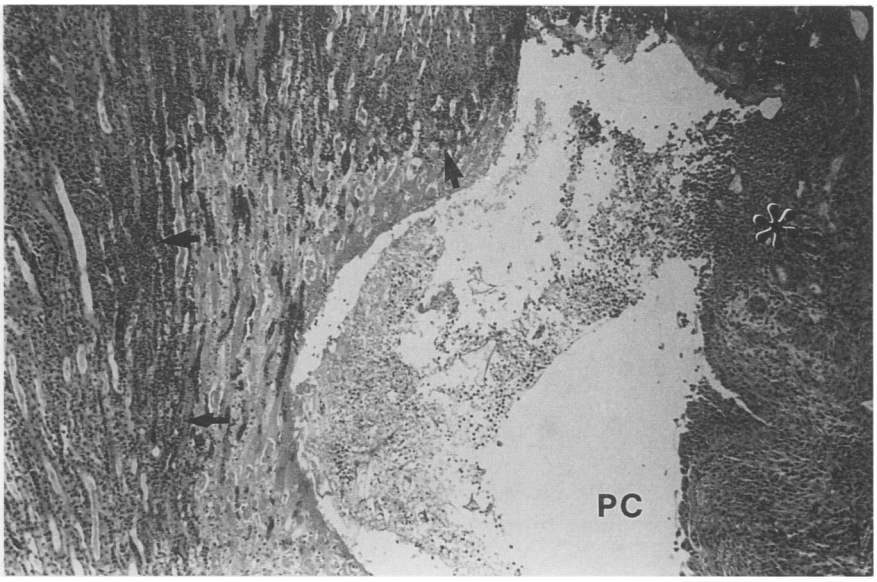

FIG. 3. Severe necrotizing pyelonephritis (renal pathology score, 5+) at 1 week postchallenge with the *P. mirabilis* parent strain HI4320. The uroepithelium is ulcerated (asterisk). The lumen of the renal pelvic cavity (PC) is distended with exudate. There is acute necrosis of tubules adjacent to the renal pelvis. The surrounding parenchyma is infiltrated with moderate to large numbers of inflammatory cells (arrows).

negative mutant, on the other hand, was unable to establish significant infection in the kidney in most mice and was cleared from the bladder by 1 week.

The destructive renal lesion caused by the parent strain was characterized by extensive acute necrosis of the tubules in the papilla, medulla, and renal cortex, with a margin of intense neutrophil infiltration at the junction of the necrotic area with viable tubules. The severe necrotizing pyelonephritic lesion was observed in mice sacrificed at 1 week postchallenge. Similar lesions were observed in rats examined at 3 days and 1 week after transurethral challenge with *P. mirabilis* (28) and in rats (4, 5) and mice (15, 16) after hematogenous infection. The wedge-shaped necrotizing lesion was reported to be characteristic of *P. mirabilis* as opposed to other uropathogenic bacteria (5).

In this study, we observed seven deaths among 20 (35%) mice over a 2-week period after transurethral administration of 10^9 CFU. All of these mice showed extensive and severe necrotizing pyelonephritis by pathologic examination, suggesting that renal infection was the cause of death. These numbers are lower than those reported by Moayeri et al. (18), who noted 16% (9 of 57) deaths in BALB/c mice intravesicularly administered 2×10^8 CFU of *P. mirabilis*. Differences observed could be attributed to the bacterial

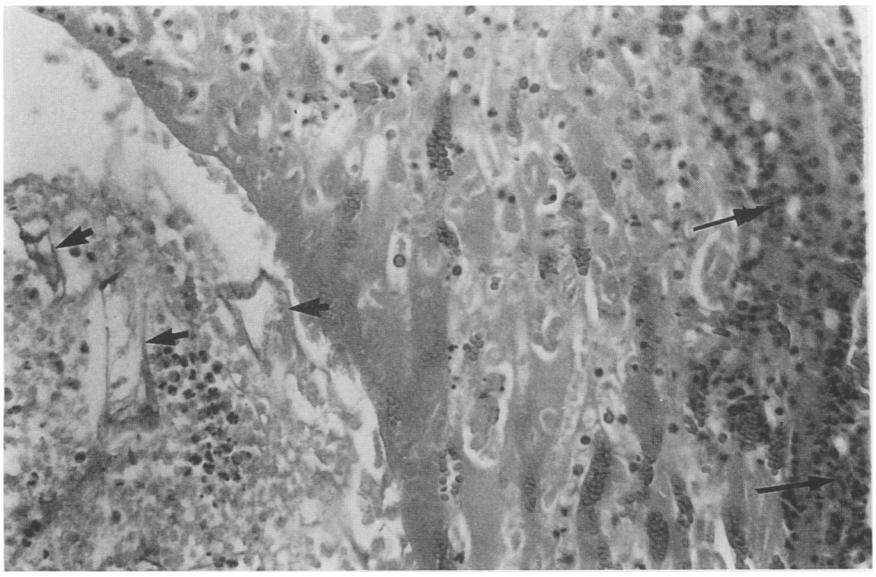

FIG. 4. High-power photomicrograph of the kidney represented in Fig. 3 showing the pelvis and adjacent renal parenchyma. There is acute necrosis of renal tubules, with infiltration of moderate numbers of inflammatory cells (large arrows) at the perimeter. The lumen of the pelvis contains exudate, neutrophils, and struvite stones (small arrows).

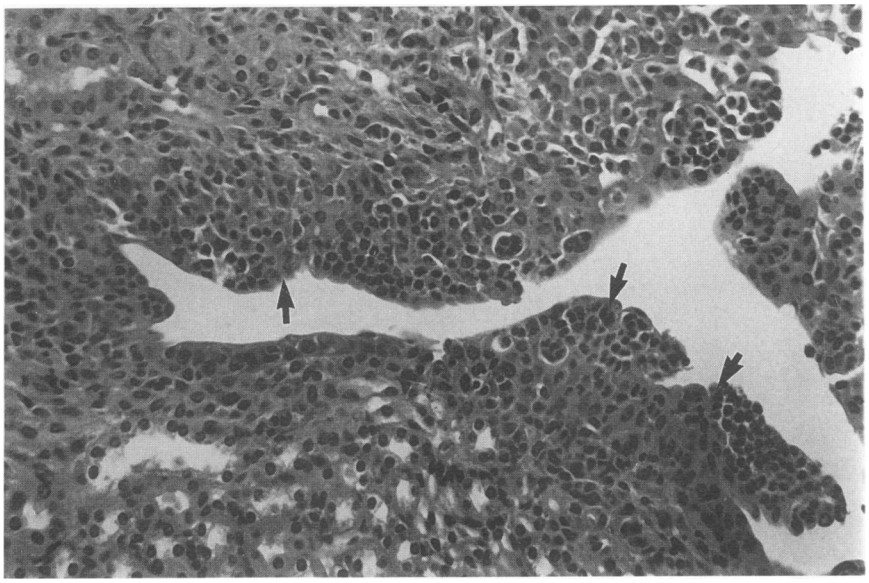

FIG. 5. Severe pyelitis (renal pathology score, 3+) at 1 week postchallenge in a mouse challenged with 10^9 CFU of the *P. mirabilis* parent strain. The uroepithelium is thickened and irregular (arrows). There is diffuse and moderate infiltration of neutrophils and lymphocytes adjacent to the pelvis and within the uroepithelium.

strain (isolate from a human with catheter-associated bacteriuria in this study versus an isolate from a renal stone from a patient with a history of acute pyelonephritis), the challenge dose, or a difference in the susceptibility to infection of CBA mice and BALB/c mice.

Another notable difference was the absence of observed infection stones in the BALB/c study (18). While Moayeri et al. found no stones in the kidneys of animals, we recovered eight stones from the bladders of 20 animals at 2 weeks after challenge. The stones were identified as struvite ($MgNH_4PO_4$) on the basis of high levels of magnesium and phosphate. This is the characteristic composition of infection stones in humans with *Proteus* urinary tract infections. The differences in the reports of the presence of stones could be attributed simply to a failure to inspect the bladder lumen in the previous study (18) but also to a fivefold-higher inoculum in the present study or a higher urease activity of *P. mirabilis* HI4320 (this study) than of *P. mirabilis* K7 (18). These activities, however, have not been compared.

The urease-negative mutant retained a low level of pathogenicity for the uroepithelium lining the pelvic cavity, possibly through expression of other virulence determinants. At

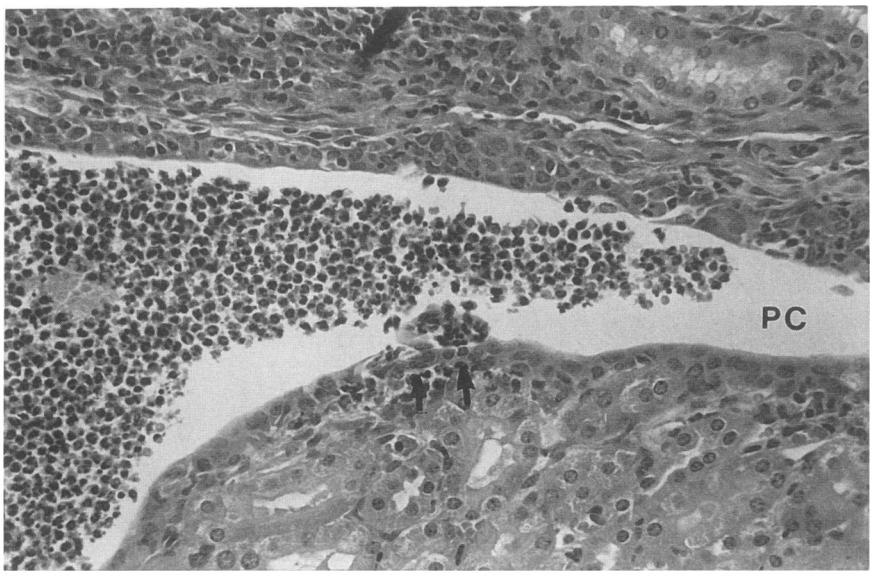

FIG. 6. Moderate pyelitis (renal pathology score, 2+) in the kidney of a mouse 1 week after challenge with 10^9 CFU of the *P. mirabilis* urease-negative mutant. There is mild focal necrosis of epithelial cells lining the pelvis (arrows). Moderate numbers of neutrophils and necrotic epithelial cells are present in the lumen of the pelvic cavity (PC). There are moderate numbers of mononuclear inflammatory cells in the renal papilla.

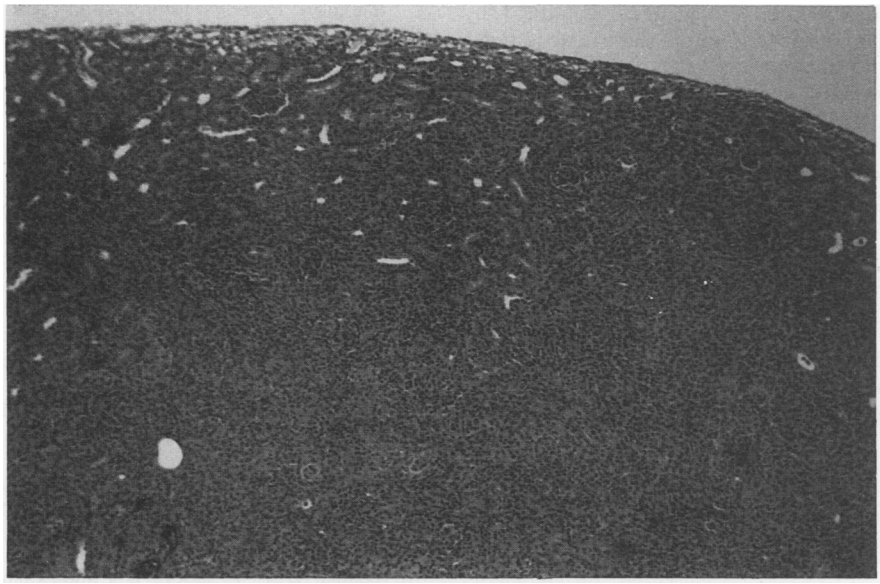

FIG. 7. Mouse kidney with a renal pathology score of 5+ at 2 weeks postchallenge with 10^9 CFU of the *P. mirabilis* parent strain. The area of the kidney cortex in which tubules have been destroyed and replaced by fibrosis and moderate mononuclear inflammatory cell infiltration is shown.

1 week after challenge with 10^9 CFU of the urease-negative mutant, 75% of the mice demonstrated mild to moderate pyelitis. The damage was transient, and 63% of the mice showed no damage at 2 weeks postchallenge.

A urease-negative mutant produced by ethyl methanesulfonate chemical mutagenesis (not isogenic) was reported to retain the ability to cause renal damage in mice challenged intravenously (15). However, lesions were described subjectively as being smaller in diameter, with cortical necrosis absent in the mice challenged with the mutant strain (15, 16). This could indicate that the embolic lesions caused by the parent and mutant strains had differences in the pathologic mechanisms.

The mechanisms by which urease could contribute to virulence include (i) availability of urea as a nutrient, (ii) obstruction of the ureters by struvite stones, and (iii) ammonia-induced cytotoxicity of the renal epithelium.

The absence of necrotizing pyelonephritis in mice challenged with the urease-negative strain could be explained by the lack of adequate growth of the mutant strain in the urinary tract of the mouse. Growth limitation for mutants that lack urease is consistent with the low numbers of bacteria recovered from the urinary tract after challenge. That is, urease may simply provide *P. mirabilis* with a means for acquiring nitrogen for protein and DNA synthesis. Urea is plentiful in urine (0.4 to 0.5 M [8]) and enters the bacterium by diffusion across the outer and inner membranes. It is hydrolyzed by the cytosolic urease to ammonia and carbon dioxide. Liberated ammonia can be coupled to polypeptide synthesis. Therefore, the function of urease may be to provide usable nitrogen essential for growth of *P. mirabilis* in urine, and the lack of urease may cause reduced bacterial multiplication. There is no reason to believe, however, that nitrogen is limiting in urine. It is unclear, however, whether non-urea nitrogen is limiting for *P. mirabilis* in urine.

A strong association was noted between urease production and stone formation. Urolithiasis was observed only in animals infected with the parent strain, confirming that the mechanism of urinary stone formation, which is a feature of *P. mirabilis* infections (5), is a result of urease-catalyzed hydrolysis of urea. Previous studies have demonstrated that urease is critical for crystallization of struvite stones (8) deposited around the bacterium (29). The process of urolithiasis appeared to require more than 2 days to produce visible stones since none of the infected animals was found with a stone after 2 days, whereas 31% (12 of 39) of the animals harbored stones after 1 week. Obstruction of urine flow by these crystal depositions may potentiate growth of *P. mirabilis* in the kidney. Reflux of urine, in this case, may inhibit clearance of the organism.

The experiments with mice did not enable us to determine whether urease has an indirect role in causing renal parenchymal tissue necrosis by affecting colonization or whether urease is directly responsible for the tissue damage via hydrolysis of urea. The ammonia released from urease-catalyzed urea hydrolysis is sufficient to elevate the pH from 7 to 9 and equilibrates with water to form ammonium hydroxide. We have previously shown that damage to cultured human renal proximal tubular epithelial cells was induced by a hemolysin-negative strain of *P. mirabilis* in the presence, but not the absence, of 50 mM urea (20). Also, the urease-negative mutant did not cause cytolysis. These observations indicate that in cell culture systems, cytotoxicity correlates with urease-catalyzed hydrolysis of urea, liberating ammonia. It is possible that exposure of kidney epithelium to these nonphysiological conditions is a mechanism of significant necrosis to the kidney. This conclusion is consistent with the results of experiments demonstrating that treatment with hydroxyurea, a urease inhibitor, reduced bacteriuria, kidney infection, and severity of kidney lesions induced by hematogenous *P. mirabilis* (17).

The results of this study demonstrate that the urease of *P. mirabilis* is a critical virulence determinant in the development of experimental pyelonephritis. Previous studies document that treatment of rats and mice with urease inhibitors causes reduced bacteriuria and pyelonephritis after transurethral infection with *P. mirabilis* (1, 24). The introduction of a urease gene into a urease-negative mutant of *Staphylococcus*

saprophyticus restored virulence, resulting in cystitis (7). The enzyme is well adapted for function in the urinary tract. The genes encoding the enzyme are induced by substrate urea and are, thus, probably always being expressed during an active infection. There does not appear to be a feedback mechanism (such as high concentrations of ammonia) for shutting off synthesis of the enzyme. Therefore, there could be a relentless production of the enzyme in response to the constant elimination of urea as a nitrogenous waste by the host. This continuous supply of substrate favors conditions under which urine pH remains elevated and provides the niche enjoyed by uropathogenic *P. mirabilis*. Further studies are needed to determine the mechanisms by which urease participates in the pathophysiology of pyelonephritis caused by *P. mirabilis* and, in particular, to evaluate its role in the pathogenesis of tubule necrosis.

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