INFECTION AND IMMUNITY, June 1991, p. 2036–2042
0019-9567/91/062036-07$02.00/0

Vol. 59, No. 6

Cytotoxicity of the HpmA Hemolysin and Urease of *Proteus mirabilis* and *Proteus vulgaris* against Cultured Human Renal Proximal Tubular Epithelial Cells

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Received 11 January 1991/Accepted 23 March 1991

Proteus mirabilis*, a common agent of nosocomially acquired and catheter-associated bacteriuria, can cause acute pyelonephritis. In ascending infections, bacteria colonize the bladder and ascend the ureters to the proximal tubules of the kidney. We postulate that *Proteus* species uses the HpmA hemolysin and urease to elicit tissue damage that allows entry of these bacteria into the kidney. To study this interaction, strains of *Proteus mirabilis* and *P. vulgaris* and their isogenic hemolysin-negative (*hpmA*) or isogenic urease-negative (*ureC*) constructs were overlaid onto cultures of human renal proximal tubular epithelial cells (HRPTEC) isolated from kidneys obtained by immediate autopsy. Cytotoxicity was measured by release of soluble lactate dehydrogenase (LDH). Two strains of *P. mirabilis* inoculated at 10^6 CFU caused a release of 80% of total LDH after 6 h, whereas pyelonephritogenic hemolytic *Escherichia coli* CFT073 released only 25% at 6 h ($P < 0.012$). Ten *P. mirabilis* isolates and five *P. vulgaris* isolates were all hemolytic and cytotoxic and produced urease which was induced by urea. The HpmA hemolysin is apparently responsible for the majority of cytotoxicity in vitro since the hemolysin-negative (*hpmA*) mutants of *P. mirabilis* and *P. vulgaris* were significantly less cytotoxic than wild-type strains. *P. mirabilis* WPM111 (hemolysin negative) was used to test the effect of urease-catalyzed urea hydrolysis on HRPTEC viability. In the presence of 50 mM urea, WPM111 caused the release of 42% of LDH versus 1% at 6 h in the absence of substrate ($P = 0.003$). We conclude that the HpmA hemolysin of *Proteus* species acts as a potent cytotoxin against HRPTEC. In addition, urease apparently contributes to this process when substrate urea is available.

The percentage of elderly people in the U.S. population is increasing rapidly. Many of these people will reside in nursing homes. As part of their health care in this setting, at least half will require urinary catheterization for management of incontinence (11, 24). However, long-term urinary catheterization inevitably leads to polymicrobial bacteriuria (38). Nosocomially acquired, *Proteus mirabilis* is cultured very frequently from the urine of patients with long-term indwelling urinary catheters (22, 37). As one of the most common uropathogens isolated in this patient population, it can cause serious kidney infections characterized as acute pyelonephritis (4, 6, 8, 9, 29, 37) and is known for its ability to initiate renal stone formation (21). After *Escherichia coli*, *P. mirabilis* is the second leading cause of bacteremia in elderly people (31). Although much less common, *Proteus vulgaris* is also cultured from the urine of long-term catheterized patients (38).

Several virulence determinants have been postulated for *P. mirabilis* including urease (1, 12), hemolysin (28), fimbriae (19), and flagellum-mediated motility (25). Of these, urease has been demonstrated by us to be a critical virulence factor (12). The concentration of urea in urine can reach 0.5 M (10), which is saturating substrate concentration ($>10 \times K_m$) for this enzyme (13). Excess levels of ammonia produced by ureolysis cause alkalinization and supersaturation of polyvalent cation and anions. At pH values above 8, these ions precipitate, usually in the form of struvite, i.e., $MgNH_3PO_4$, and carbonate-apatite, i.e., $Ca_{10}(PO_4CO_3OH)_6(OH)$, resulting in the formation of bladder and kidney stones (10). *P. mirabilis* is the most commonly isolated uropathogen from patients with struvite and carbonate-apatite stones (21). Ammonia also seems to have a cytotoxic effect on kidney cells in culture. Braude and Siemienski (1) found that the rise in pH associated with the hydrolysis of urea was toxic for monkey kidney cells in vitro. Additionally, alkalinization of kidney tissue may protect the organism from host immunity by inactivating the complement cascade (17).

Most isolates of *P. mirabilis* and *P. vulgaris* also produce a high titer of secreted hemolysin (HpmA) (30) that is genetically distinct from that encoded by the *hly* locus of *E. coli* (33, 39). Peerbooms et al. (26, 27) reported that hemolysin-producing *Proteus* strains are cytotoxic for Vero cells and that strains with high hemolysin titers had a lower 50% lethal dose for mice when injected intravenously than strains with low hemolysin titers. These investigators also correlated high hemolytic titer with the ability to invade Vero cells (28). Although these data strongly suggest that hemolysin is a cytotoxin, it has been difficult to draw sound conclusions for two reasons. (i) Most, if not all, *P. mirabilis* strains have some hemolytic activity (27, 29), and therefore a true negative control strain was not evaluated in most of these reports. (ii) Intravenous injection of organisms does not mimic the natural course of ascending urinary tract infection in which bacteria move up the urethra to the bladder and ascend the ureters to the kidney epithelium. In this report, we circumvent these pitfalls by using isogenic hemolysin mutants of *P. mirabilis* and *P. vulgaris* which have charac-

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terized deletions in the hemolysin gene and completely lack hemolytic activity.

Because *P. mirabilis* localizes preferentially in the kidney (5), it is important to study the interaction of bacterium and the appropriate renal target cell. In vitro cell culture techniques have been used in this study to examine interactions of *Proteus* strains with human renal proximal tubular epithelial cells (HRPTEC), which may represent the target cell for the development of acute pyelonephritis. We hypothesize that both the HpmA hemolysin and urease in the presence of substrate urea are potent cytotoxins for cultured HRPTEC. Our findings support this thesis.

MATERIALS AND METHODS

Bacterial strains. *P. mirabilis* HI4320 (MR/P and MR/K fimbriated, hemolysin$^+$, urease$^+$) and *P. mirabilis* BA6163 (MR/P fimbriated, hemolysin$^+$, urease$^+$) were isolated from women with urinary catheter-associated bacteriuria (38). *P. mirabilis* BA6163 was used for construction of strain WPM111, a deletion mutation of *hpmA*, as previously described by Swihart and Welch (34) by using the suicide vector pGP704 (18). *P. mirabilis* HI4320 was used for introduction of a deletion mutation in urease structural gene *ureC* by homologous recombination (12) also using pGP704 (18). *P. vulgaris* isolates and other *P. mirabilis* isolates were cultured at concentrations of $\geq 10^5$ CFU/ml from the urine of elderly (≥65 years old) patients with indwelling urinary catheters in place for ≥30 days (38) or from the urine of patients with the clinical symptoms of acute pyelonephritis (19). *P. vulgaris* WPV5 and its isogenic *hpmA* hemolysin mutant WPV43 have been described previously (34).

Urease activity. Bacterial cultures were inoculated into modified Luria broth (containing, per liter, 10 g of tryptone [Difco], 5 g of yeast extract, 8.5 g of NaCl, and 100 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.2; adjusted to 300 mosM by dilution) and incubated without shaking at 37°C. When included, urea was added to 50 mM, a concentration shown to give maximal induction of urease (21). At 3, 4.5, 6, and 12 h, cultures were sampled (90, 60, 20, and 20 ml, respectively) and harvested by centrifugation (10,000 × *g*, 10 min, 4°C). Cell pellets were resuspended in 1.7 ml of 20 mM sodium phosphate (pH 6.8) and ruptured in a French pressure cell (5-ml cell, 3/8-in [ca. 0.9-cm] piston) at 20,000 lb/in^2. Lysates (1 ml) were centrifuged in a microcentrifuge for 1 min, and supernatants were assayed directly for urease activity by using the phenol red spectrophotometric assay as previously described (13). Protein concentration was determined by the method of Lowry et al. (16) by using bovine serum albumin as a standard. Activities are reported as micromoles of NH_3 liberated per minute per milligram of protein.

Hemolytic titers. Bacterial suspensions were sampled directly from the tissue culture wells of the lactate dehydrogenase (LDH) assay (see below) and diluted serially twofold. Samples (100 μl) were mixed with a 1% suspension of sheep erythrocytes (100 μl) and incubated at 37°C and read after 1 h (19). Hemolytic titer is defined as the reciprocal of the last dilution to give complete hemolysis of erythrocytes.

Hemagglutination. Hemagglutination patterns characteristic of MR/K-fimbriated and MR/P-fimbriated *Proteus* strains were determined as described previously (19).

Isolation and culture of HRPTEC. HRPTEC were isolated from recently autopsied kidneys, cultured, and characterized as previously described (35). 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, reseeded at a density of 1×10^5 to 2×10^5 cells per ml of medium onto tissue culture dishes (Miles Laboratory, Elkhart, Ind.), and used for experiments when monolayers were confluent.

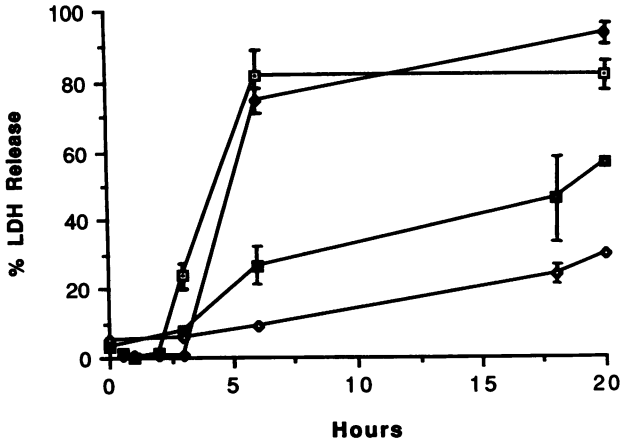

FIG. 1. LDH release by cultured HRPTEC exposed to strains of *P. mirabilis* or *E. coli* CFT073 and its hemolysin-negative derivative. Confluent monolayers of HRPTEC were overlaid with bacterial suspensions (10^6 CFU). At intervals, samples were taken from tissue culture wells, and soluble LDH was determined and expressed as a percentage of enzyme released by treatment of the cell culture with Nonidet P-40. Symbols: ⊡, *P. mirabilis* HI4320 (peak hemolytic titer of 1:512); ◆, *P. mirabilis* CFT403 (peak hemolytic titer of 1:2); ■, *E. coli* CFT073 (peak hemolytic titer of 1:2); ◆, *E. coli* CFT073 *hlyD*::Tn*phoA* (nonhemolytic). Data are means of duplicate determinations from a representative of three experiments. Standard deviations are shown when they exceed 2% LDH release.

Bacterial inocula. *Proteus* strains were inoculated into Luria broth and grown for 18 h with aeration at 37°C. These cultures (0.25 ml) were used to inoculate 50-ml portions of modified Luria broth with or without 10 mM urea which were then incubated for 3 h at 37°C with aeration. Cultures were diluted to 10^6 CFU/ml in modified Luria broth with and without 50 mM urea. This suspension was used simultaneously to inoculate tissue culture, to assay hemolytic titer, and to determine urease activity. These cultures were incubated statically at 37°C.

LDH determination. To assay for bacteria-induced cytotoxicity, the release of LDH from epithelial cell culture 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 (20, 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} by use of a Gilford Response spectrophotometer. Typical assays ran for 1 min. For estimation of total activity, monolayers were disrupted by the addition of

TABLE 1. Hemolysin activity, urease activity, and cytotoxicity of *Proteus* strains for cultured HRPTEC[a]

Species and strain	CFU/ml (10^9)[b]		Hemolytic titer[c]		Urease activity[d]				Cytotoxicity[e]			
					4.5 h		12 h		4.5 h		12 h	
	4.5 h	12 h	4.5 h	12 h	Unind[f]	Ind[g]	Unind	Ind	Unind	Ind	Unind	Ind
P. mirabilis												
CFT295	1.1	2.9	512	256	2.9	24.7	0.1	2.4	97	106	105	104
HU2450	4.0	ND[h]	128	256	0.4	25.5	0.2	7.3	97	132	87	123
CFT106	2.0	7.1	128	128	1.3	14.8	0.7	2.8	88	106	102	108
SA1387	2.4	3.8	128	16	0.1	23.5	0.2	7.3	109	110	104	102
MI159	1.5	7.4	64	32	0.2	20.1	0.2	4.4	91	98	98	101
EI1131	1.1	1.1	16	—[i]	0.2	25.7	0.1	9.0	96	130	105	115
CFT37	2.2	4.0	16	32	0.8	24.0	0.6	6.8	98	113	89	99
MA2489	1.0	3.1	16	8	0.1	24.1	0.1	6.7	88	104	97	109
DR3282	2.3	6.0	8	8	0.1	29.6	0.2	12.6	93	102	98	101
CFT403	1.5	4.8	4	—	3.1	39.4	2.8	11.7	47	62	79	84
P. vulgaris												
BR481	0.2	2.6	64	128	1.3	13.3	0.1	1.6	100	112	104	143
BR2026	0.5	3.1	64	64	0.9	14.5	0.1	1.7	98	95	103	97
BR2358	0.4	<.1	64	64	1.1	16.3	0.2	1.8	83	96	95	90
BR740	0.5	1.7	32	64	1.6	12.8	0.1	1.5	92	96	100	74
GO1312	0.5	2.5	32	64	1.5	12.4	0.2	2.0	98	120	100	112

[a] All data derived from single experiments with duplicate determinations for each data entry.
[b] Quantitative cultures from wells of tissue culture plates, uninduced with urea.
[c] Reciprocal titer represents the last twofold dilution of bacterial suspension sampled from tissue culture plates that produced complete lysis of 3% sheep erythrocyte suspension in 1 h. Data were derived from cultures not induced with urea. Titers taken with urea-induced cultures were not significantly different from those taken with uninduced cultures.
[d] Values are expressed as micromoles of NH_3 per minute per milligram of protein.
[e] Release of lactate dehydrogenase (percentate of total) from cultured HRPTEC.
[f] Unind, cultures grown in the absence of urea.
[g] Ind, cultures grown and assays done in the presence of 50 mM urea.
[h] ND, not determined.
[i] —, no measurable hemolytic titer.

Nonidet P-40 (Sigma) 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 total activity. Assays were run in duplicate for each bacterial inoculation. Mean values were compared by the *t* test.

RESULTS

Cytotoxicity of bacterial strains for cultured HRPTEC. We have previously shown that the *E. coli* hemolysin was cytotoxic for cultured HRPTEC (20). As a comparison, we measured the cytotoxicity of *P. mirabilis* strains by using the release of LDH from the cell cultures of HRPTEC as an index of cytotoxicity. We selected two strains of *P. mirabilis*, HI4320 and CFT403, which had peak hemolytic titers of 1:512 and 1:2, respectively. Bacterial cultures of each strain overlaid on cell cultures demonstrated a rapid release of LDH after 2 (HI4320) and 3 h (CFT403) (Fig. 1). Approximately 80% of total LDH had been released by both strains at 6 h, in sharp contrast to hemolytic *E. coli* CFT073, which demonstrated approximately 25% release at 6 h ($P < 0.012$) and less than 60% at 20 h ($P < 0.017$). *P. mirabilis* strains displayed little additional LDH release between 6 and 20 h. A hemolysin-negative mutant of *E. coli* CFT073 *hlyD*::Tn*phoA* (20), demonstrated significantly less cytotoxicity than the *E. coli* parent strain ($P = 0.0018$ at 20 h).

Hemolysin, urease, and cytotoxic activities of *P. mirabilis* and *P. vulgaris* isolates. To test cytotoxicity as a general phenomenon for *Proteus* isolates, we measured LDH release from HRPTEC cultures at 4.5 and 12 h when overlaid with each of 10 *P. mirabilis* and each of 5 *P. vulgaris* isolates. Hemolytic titers were measured directly from the cell culture system at the times of assay. In addition, urease activity was measured at the same time points from static cultures, induced or not with 50 mM urea, grown in parallel (i.e., from the same inoculum) with tissue culture (Table 1). All *Proteus* isolates were found to be cytotoxic for HRPTEC at both 4.5 and 12 h. All strains were hemolytic as well. All strains with a hemolytic titer of ≥1:8 displayed strong cytotoxicity at both 4.5 and 12 h. *P. mirabilis* CFT403, which had a titer of 1:4, showed markedly reduced cytotoxicity at both 4.5 and 12 h. At 4.5 or 12 h, there was no significant difference ($P > 0.1$) between the rates of LDH release, as a group, for *P. mirabilis* strains and those of *P. vulgaris* strains.

All strains were urease positive and inducible with urea. *P. mirabilis* strains were induced an average of 27-fold at 4.5 h and 14-fold at 12 h to levels of 25.1 and 7.1 μmol of NH_3 per min per mg of protein, respectively. *P. vulgaris* strains displayed significantly ($P = 0.002$, 4.5 h, induced) lower urease activities and were induced an average of 11-fold at 4.5 h and 12-fold at 12 h to levels of 13.9 and 1.7 μmol of NH_3 per min per mg of protein, respectively. The cytotoxicity of urea-induced cultures was generally higher than, but not significantly different ($P > 0.1$) from, that of uninduced cultures. Medium containing urea alone in the absence of bacteria resulted in little or no measurable cytotoxicity.

Cytotoxicity of hemolysin-negative mutants. To test whether the *Proteus* HpmA hemolysin was specifically responsible for cytotoxicity, as has been demonstrated for the HlyA hemolysin of *E. coli* (2, 3, 7, 15), we tested isogenic strains of *P. mirabilis* and *P. vulgaris* with specific deletions in the chromosomal *hpmA* locus (34) (Fig. 2). Again, wild-type *P. mirabilis* BA6163 elicited rapid LDH release (>90% LDH release at 6 h), whereas the isogenic *hpmA* derivative, WPM111, showed no LDH release at 6 h ($P = 0.003$) and less than 15% LDH release at 12 h ($P = 0.0009$) (Fig. 2A). This

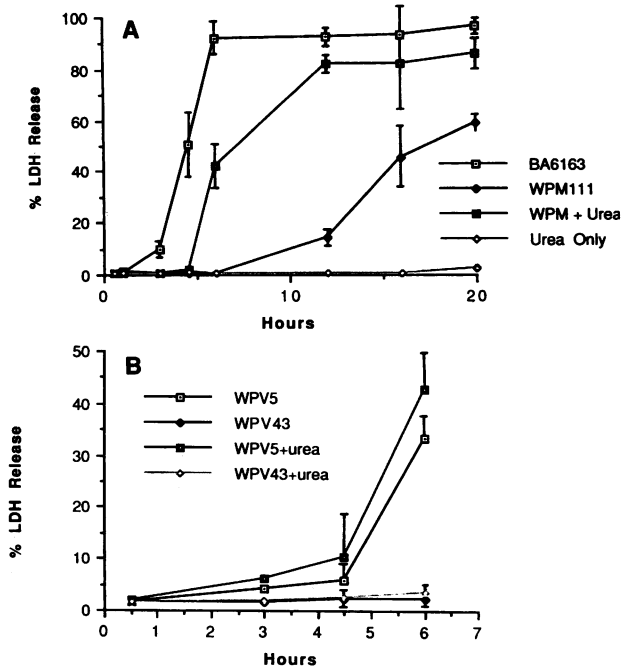

FIG. 2. Cytotoxicity of *P. mirabilis*, *P. vulgaris*, and their hemolysin-negative derivatives. Cell cultures were overlaid with bacterial suspensions and LDH release was measured as described in the legend to Fig. 1. When included, urea was added to 50 mM. (A) *P. mirabilis* BA6163 (hemolysin$^+$, urease$^+$); *P. mirabilis* WPM111 (hemolysin$^-$, urease$^+$). WPM + Urea, strain WPM111 assayed in the presence of urea. (B) *P. vulgaris* WPV5 (hemolysin$^+$, urease$^+$); *P. vulgaris* WPV43 (hemolysin$^+$, urease$^+$). Data are means ± standard deviation of the three separate experiments each with duplicate determinations. Standard deviations that exceed 2% LDH release are shown.

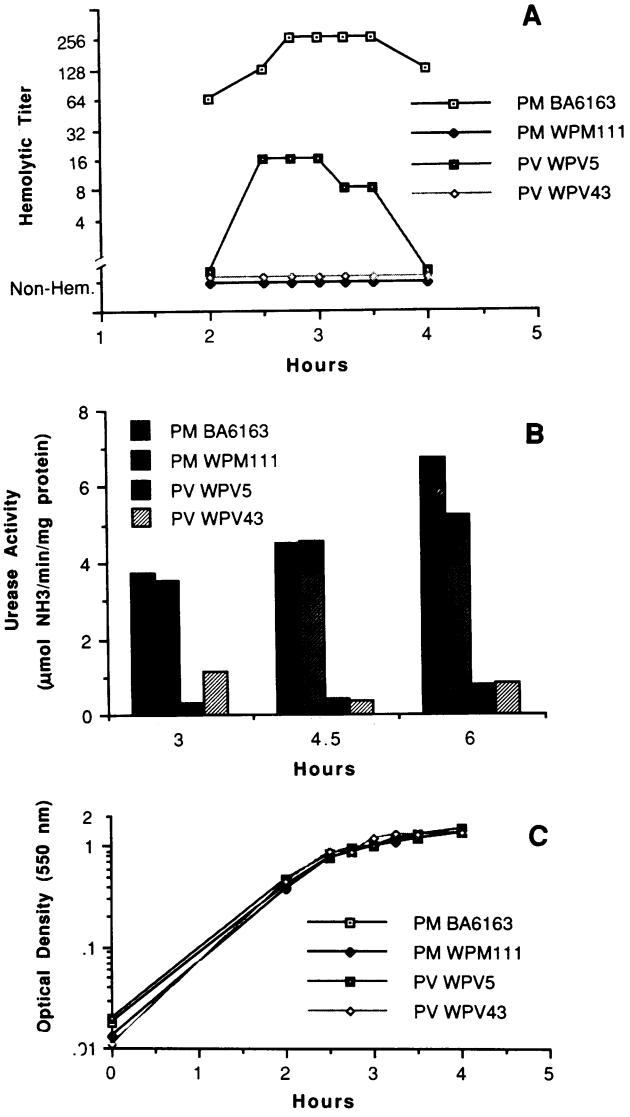

FIG. 3. Hemolytic activity, urease activity, and growth of *P. mirabilis* (PM) and *P. vulgaris* (PV) overlaid onto HRPTEC cultures. (A) Reciprocal hemolytic titer of bacterial culture growing on HRPTEC from the experiments described in the legend to Fig. 2. (B) Urease activity of soluble protein derived from French press lysates of statically grown *P. mirabilis* and *P. vulgaris* and their *hpmA* mutants. (C) Viable counts of *Proteus* strains and their hemolysin-negative mutants growing on HRPTEC cultures. Hemolytic activity and urease activities are means of three separate experiments. Growth curves are representative of three determinations.

demonstrated that the HpmA hemolysin was the principal cytotoxin responsible for the killing of HRPTEC. It is interesting to note that the hemolysin-negative *P. mirabilis* was nearly as cytotoxic as the hemolysin-positive *E. coli* depicted in Fig. 1, suggesting that other *Proteus* factors may play a minor role in cytolysis.

Very similar conclusions can be drawn for *P. vulgaris* WPV5 and its isogenic *hpmA* hemolysin mutant WPV43 (Fig. 2B). LDH release for *P. vulgaris* WPV5 was not as rapid as for *P. mirabilis*, but it reached 34% by 6 h as compared to 3% for the *hmpA*-negative mutant, WPV43, at 6 h (P = 0.0002). For *P. vulgaris*, LDH release increased sharply between 4.5 and 6 h.

For the experiments described in Fig. 2, hemolytic titers of *P. mirabilis* BA6163 peaked at 1:256, 2.75 h after inoculation onto HRPTEC (Fig. 3A). Peak hemolysis was followed by marked cytotoxicity at 6 h (Fig. 2A). The HpmA-negative mutant, WPM111, demonstrated no hemolysis at any time point; this correlated with lower levels of LDH release when compared with the wild type. Hemolytic titers of *P. vulgaris* WPV5 peaked at 1:16, 2.5 h after inoculation. The HpmA-negative mutant WPV43 was always nonhemolytic. At all time points, urease activities of *P. mirabilis* BA6163 and its *hpmA* mutant were not significantly different ($P > 0.1$) but were an average of 7.5-fold higher than those of *P. vulgaris* WPV5 and WPV43, the hemolysin mutant (Fig. 3B). Differences in hemolytic titer and cytotoxicity were not due to differences in cell density since quantitative cultures revealed similar numbers of bacteria per milliliter for all strains at all time points (Fig. 3C).

Cytotoxicity of urease-mediated urea hydrolysis. Availability of hemolysin-negative mutants allowed the testing of the effect of the ureases of *P. mirabilis* and *P. vulgaris* in the absence of the cytotoxic hemolysin. When *P. mirabilis* WPM111 (hemolysin negative) was overlaid on cell culture in the presence of 50 mM urea, a marked increase in cytotoxicity was observed in comparison to the cytotoxicity of cultures inoculated in the absence of urea (Fig. 2A) at 6 h (P = 0.025), 12 h (P = 0.019), and 20 h (P = 0.05). Urea in

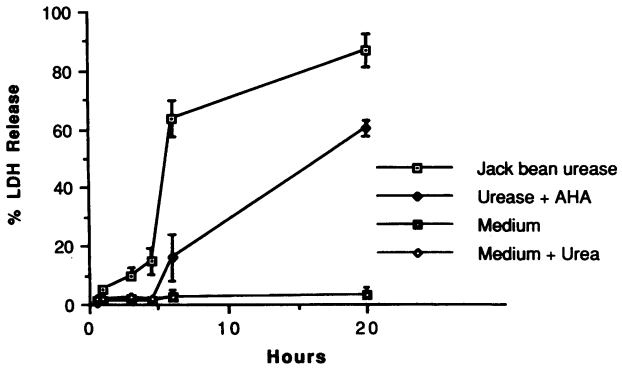

FIG. 4. Cytotoxicity of jack bean urease for cultured HRPTEC. Jack bean urease (10 μg/ml) with and without acetohydroxamic acid (AHA) (500 μg/ml), a specific inhibitor of urease, was added to culture medium overlaying HRPTEC. Urea was added to 50 mM, and cytotoxicity was assessed by LDH release. Data are means ± standard deviations of duplicate determinations from one of three representative experiments. LDH release for jack bean urease was significantly higher than for jack bean urease plus acetohydroxamic acid at 4.5 h ($P = 0.025$), 6 h ($P = 0.016$), and 20 h ($P = 0.017$).

the medium in the absence of bacterial cells had no toxic effect on cell cultures.

To demonstrate that an active urease itself (i.e., in the absence of substrate urea) was not responsible for cytotoxicity, we tested *P. mirabilis* HI4320 and its urease-negative derivative in the cell culture system. No difference in cytotoxicity ($P > 0.1$) was observed between the isogenic pair (data not shown), indicating that the urease protein in the absence of urea substrate was not directly involved in LDH release but rather that the HpmA hemolysin was causing the cytolysis. It was not a surprising finding that the urease protein itself does not contribute additionally to cytotoxicity unless urea is hydrolyzed, liberating ammonia.

Urease-catalyzed urea hydrolysis was sufficient to mediate cytotoxicity in the absence of bacterial cells. We tested the effect of adding purified jack bean urease to the cell cultures (Fig. 4) in amounts comparable to those of induced *P. mirabilis* strains (activity equaled 25 μmol of NH_3 per min per mg of protein). At 6 h, 63% of LDH had been released. When the action of jack bean urease was inhibited by acetohydroxamic acid, a competitive inhibitor of the enzyme, only 17% of LDH was released. LDH release by jack bean urease was significantly higher ($P \leq 0.025$) than that by jack bean urease plus acetohydroxamic acid at 4.5, 6, and 20 h.

DISCUSSION

The HpmA hemolysins of *P. mirabilis* and *P. vulgaris* are potent cytotoxins in vitro for cultured HRPTEC. Isogenic strains of these uropathogens, which contained specific deletions within the structural gene (*hpmA*) for the hemolysin, were used to unambiguously demonstrate highly significant differences in LDH release from these cultured human cells. The cytotoxic activities of *Proteus* strains far exceeded cytotoxic activity of a hemolytic pyelonephritogenic strain of *E. coli*. There was a trend towards cytotoxicity being proportional to the hemolytic titer against sheep erythrocytes. However, this effect was seen only with a few strains, and the cytotoxicity of strains with low hemolysin titers appeared to be delayed only for a short period of time. These data suggest that any level of hemolysin production may be sufficient to cause irreversible cytolytic events in HRPTEC.

The vast majority of *Proteus* isolates have been shown to possess only *hpmA* hemolysin determinants which encode a 166-kDa calcium-independent cytotoxin that can be found both associated with the bacterial cell and released into the culture medium (34). Bacterial cells as well as cell filtrate were found previously by Swihart and Welch (34) to be cytotoxic for a number of other cell lines including Daudi, Raji, T24, U937, and Vero.

The construction of hemolysin-negative mutants has also allowed us to examine the specific contribution of bacterial urease to cytotoxicity. Having eliminated the hemolysin-mediated cytotoxicity (34), we have determined the effect of adding substrate urea to these cell cultures. Hydrolysis of urea, present in high concentrations in the urinary tract, by the urease of *P. mirabilis* results in rapid cell death of cultured renal epithelial cells. Urease activity rose steadily throughout exponential and stationary phases. Presumably, cell killing resulted from increases in the concentration of ammonium hydroxide formed upon substrate hydrolysis. We have reported similar cytotoxicity for a human gastric adenocarcinoma cell line exposed to urea and the strongly urease-positive bacterium *Helicobacter pylori* (32). In the *H. pylori* study, percent cell killing correlated quantitatively with ammonia concentration in the medium. Although ammonia concentrations were not directly measured in the present study, we suspect that the presence of excess ammonia initiated the cytolytic events that were observed in the absence of hemolysin and the presence of urease and urea.

In the current study we have used cultured HRPTEC for cytotoxicity studies. The majority of human urinary tract infections develop by the ascending route by colonization of the introitus, passage from the urethra to the bladder, and ascension of the ureters to the kidney. *Proteus* species appear to have a predeliction for kidney tissue since these organisms have been found, in bladder washout studies, to localize within the kidney rather than the bladder (5). Entry into the kidney parenchyma is guarded by the renal proximal tubular epithelial cells, which form tubules that are one cell thick. It follows, therefore, that these cells represent a critical barrier to the development of acute pyelonephritis. We feel that these cultured cells are particularly relevant for the study of pathogenesis of urinary tract infection because they are of human origin, are not transformed cells, represent the target tissue (i.e., the kidney), and presumably express relevant receptor molecules for interaction with bacterial fimbriae or nonfimbrial adhesins.

Although *P. mirabilis* and *P. vulgaris* possess very similar genes for both HpmA hemolysin (33) and urease (14, 23), *P. mirabilis* appears to express both proteins at higher levels than do *P. vulgaris* strains. The release of LDH by parent strains (Fig. 2) demonstrates a more rapid release by the *P. mirabilis* strain than by *P. vulgaris*. At 4.5 h, however (Table 1), there is no significant difference in accumulated cytotoxicity. As a group, *P. mirabilis* strains produced about twice the urease activity, when induced with urea, as did *P. vulgaris* (Table 1).

It is interesting that the role of hemolysin and urease in pathogenesis of urinary tract infection and pyelonephritis may not be directly reflected by these in vitro assays. *P. mirabilis* WPM111, the *hpmA*-negative mutant, and its parent strain BA6163 were used to challenge CBA mice trans-

urethrally (34). There was no significant difference between these two strains in either colonization of the kidneys or histological damage. This was an unexpected finding because of the potent cytotoxicity observed, in this study, for the HpmA hemolysin in vitro.

On the other hand, urease, which appears to have a less dramatic effect in vitro, proved to be critical in vivo for the development of acute pyelonephritis. A urease-negative construction of *P. mirabilis* HI4320 and the parent strain were used to challenge CBA mice transurethrally (12). The urease-positive strain colonized the kidneys in 200-fold higher levels of CFU per gram of tissue than the urease-negative mutant. It stands to reason that the hemolysin may play a more subtle role in pathogenesis that is yet to be determined, whereas urease appears to be a critical virulence determinant for *Proteus* species.

ACKNOWLEDGMENTS

This work was supported in part by grants AI-23328, AG-04393, and AI-20323 from the National Institutes of Health.

We thank Anna Trifillis and Mary Ann Kahng for providing cell cultures.

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