Cytotoxic Activity of the \textit{Proteus} Hemolysin HpmA

KRISTIN G. SWIHART$^\dagger$ AND RODNEY A. WELCH$^*$

Department of Medical Microbiology and Immunology, University of Wisconsin Medical School, Madison, Wisconsin 53706

Received 31 January 1990/Accepted 28 March 1990

We previously showed that \textit{hpmA} is the hemolysin determinant most commonly found among \textit{Proteus} isolates. To assess the potential contribution of HpmA to virulence, we first characterized the toxic activities of this hemolysin. Hemolytic activity was present in total cell cultures and cell-free supernatants of \textit{Proteus} clinical isolates as well as \textit{Escherichia coli} containing cloned \textit{hpm} genes. HpmA also possesses cytotoxic activity which was detected by a chromium release assay against a variety of target cell lines (Daudi, Raji, T24, U937, and Vero). Analysis of the dose response of bacterial cells against both T24 cells and erythrocytes showed that \textit{E. coli} containing cloned \textit{hpm} genes was 30-fold more cytotoxic than \textit{Proteus mirabilis} BA6163. Also, 10-fold more bacterial cells were needed to lyse T24 cells than to lyse erythrocytes. HpmA$^-$ mutants of two \textit{Proteus} strains in which the central portion of \textit{hpm} was deleted were constructed. These HpmA$^-$ mutants, which have lost the hemolytic and cytotoxic activities exhibited by their respective parent strains, demonstrate that HpmA is needed for both of these activities. In an ascending model of murine urinary tract infection, the HpmA mutant strain WPM111 behaved no differently from its parent strain, BA6163, with respect to either the level of kidney colonization or histopathological changes in the kidney. However, WPM111 had a sixfold higher 50\% lethal dose than BA6163 when injected intravenously into C3H mice.

\textit{Proteus} spp. are second only to \textit{Escherichia coli} as causes of gram-negative bacterial urinary tract infections (UTIs) (1, 39). UTIs caused by \textit{Proteus} spp. are often more serious than their \textit{E. coli}-caused counterparts because they are more often associated with pyelonephritis and renal stones (2, 10, 14). Additionally, \textit{Proteus} UTIs are less responsive than \textit{E. coli} UTIs to antibiotic therapy (2, 14).

Urease, hemolysins, immunoglobulin A protease, and pil have been proposed to contribute to \textit{Proteus} uropathogenicity (36, 38, 44). Urease is considered the major virulence factor of \textit{Proteus} spp. that cause human infections. This enzyme cleaves urea to ammonia, raising the pH of urine and leading to precipitation of struvite and apatite salts (2, 23). There is also evidence that hemolysins lead to increased virulence in \textit{Proteus} infections. When injected intravenously (i.v.) into mice, hemolytic \textit{Proteus mirabilis} strains had a lower 50\% lethal dose (LD$_{50}$) than nonhemolytic strains (26, 27). In addition, hemolytic activity has been reported to correlate with Vero cell invasion (28).

Two distinct hemolysins, HpmA and HlyA, have been found among \textit{Proteus} isolates (18, 42). We have shown that HpmA, which has calcium-independent hemolytic activity, was produced by all 63 \textit{P. mirabilis} and 23 of the 24 \textit{Proteus vulgaris} strains examined (40). The DNA sequence of \textit{hpm} has been determined, and the predicted HpmA amino acid sequence shows 63.3\% similarity to that of ShlA, the hemolysin produced by \textit{Serratia marcescens} (41). The second hemolysin, HlyA, is produced by only a few \textit{P. vulgaris} isolates (18, 37, 40). This hemolysin is similar to the \textit{E. coli} hemolysin, HlyA, with respect to its predicted amino acid sequence, operon structure, and immunological reactivity (18–20, 29). The hemolytic activity of HlyA, unlike that of HpmA, is calcium dependent (40, 46).

Little is known about the activities mediated by the \textit{Proteus} hemolysins HpmA and HlyA. However, studies of the related hemolysins, \textit{S. marcescens} ShlA and \textit{E. coli} HlyA, have been performed. ShlA is believed to lyse erythrocytes by forming a transmembrane pore (3, 34). König et al. have shown that ShlA also causes the release of leukotrienes from polymorphonuclear leukocytes (PMNs) and the release of histamine from mast cells (16), but there are no reports concerning the cytotoxic activity or target cell specificity of this hemolysin.

In addition to its hemolytic activity, \textit{E. coli} HlyA is cytotoxic to human blood granulocytes, monocytes, and lymphocytes, as well as renal tubular cells (5, 9, 15). \textit{E. coli} HlyA, like ShlA, causes the release of histamine from mast cells and the release of leukotriene from PMNs (11, 32). At lower doses, this hemolysin causes metabolic activation of PMNs, possibly through membrane perturbation. This has been proposed to cause premature activation of PMNs, and the released enzymes may damage host tissue (6).

HpmA is the most prevalent \textit{Proteus} hemolysin (40), so we wanted to assess its potential contribution to pathogenicity. Here we describe the hemolytic and cytotoxic activities of HpmA. Hemolytic activity was present in both total cell cultures and cell-free supernatants. Cytotoxic activity against several target cell types also was observed. We then constructed HpmA$^-$ mutants of two \textit{Proteus} strains; these mutants subsequently lost all the hemolytic and cytotoxic activity exhibited by their respective parent strains. When tested in vivo, one of these \textit{hpm} mutants (WPM111) had a sixfold higher LD$_{50}$ than its parent strain (BA6163) after i.v. injection of live organisms.

\textbf{MATERIALS AND METHODS}

All methods not described here are detailed in the accompanying paper (40).

\textbf{Bacterial strains, plasmids, and growth media.} The bacterial strains and plasmids used here are described in Table 1. The bacteria were grown in LB medium (21). Antibiotics were added to the medium as necessary at the following concentrations: tetracycline, 20 \textmu g/ml; ampicillin, 100 \textmu g/ml; and kanamycin, 50 \textmu g/ml. To prevent \textit{Proteus} spp. from

---

\*$^\dagger$ Corresponding author.

\*$^*$ Present address: Department of Biochemistry, University of Iowa, Iowa City, IA 52242.
swarming, 0.2 mM p-nitrophenylglycerine (Sigma Chemical Co., St. Louis, Mo.) was added to LB agar (17).

**Mutant construction.** The construction of HpmA<sup>−</sup> mutants is outlined in Fig. 1. Deletion of 1,791 bases from the center of the hpma gene was accomplished by digesting pWPM100 (which encodes the amino-terminal 140 out of 166 kilodaltons [kDa] of HpmA) with ClaI and religating at a low DNA concentration. This was an in-frame deletion, and the resultant plasmid (pWPM97) encodes a peptide containing amino acids 1 to 356 and 953 to 1220 (full-length HpmA contains 1,577 amino acids). Next, we inserted an end-filled BamHI fragment containing the kanamycin resistance (Km<sup>+</sup>) gene from pUC4K into ClaI-digested, end-filled pWPM97, resulting in pWPM99. To introduce this mutation into Proteus spp., we used pGP704, a plasmid containing the pir-dependent origin of replication from the plasmid R6K (24). pGP704 can be mobilized from E. coli to Proteus spp., when the ara functions are supplied in trans. The BglII-EcoRI fragment containing the hpmaAΔClaI::Km<sup>+</sup> allele from pWPM99 was ligated into BglII-EcoRI-digested pGP704 and transformed into SY327::pir with selection for both Km<sup>+</sup> and ampicillin resistance (Amp<sup>+</sup>). The resultant plasmid (pWPM105) was then retransformed into SM10::pir to allow conjugal transfer into Proteus spp.

Mating between Proteus spp. and E. coli was performed by growing strains to an optical density at 600 nm of 0.8. One milliliter of donor [SM10::pir(pWPM105)] was mixed with 0.5 ml of Proteus spp., placed on a filter on an LB agar plate, and incubated at 37°C for 3 h. The cells were washed off the filters and plated on LB medium containing tetracycline, kanamycin, and p-nitrophenylglycine. We were unable to use Amp<sup>+</sup> to distinguish between single crossover events, resulting in integration of the entire plasmid into the chromosome, and double crossover events, resulting in replacement of the genomic copy of hpma with the mutant copy, because of large numbers of Amp<sup>+</sup> colonies which, upon further examination, still contained pWPM105. Instead, the desired deletion mutants were detected by triplicate colony blotting with the Km<sup>+</sup> gene, the ClaI fragment deleted from hpma, and pGP704 as DNA hybridization probes. Two HpmA<sup>−</sup> mutants were isolated; WPM111 and WVP43 are the HpmA<sup>−</sup> mutants of *P. mirabilis* BA6163 and *P. vulgaris* WPV5, respectively.

**Cell culture.** Daudi cells (ATCC CCL 213; human B-cell lymphoma), Raji cells (ATCC CCL 86; human B-cell lymphoma), and U-937 cells (ATCC CRL 1593; human monocyte) were obtained from Gerald Byrne, University of Wisconsin, Madison, and maintained in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah) and 10 µg of gentamicin (Sigma) per ml. T24 cells (ATCC HTB 4; human bladder epithelium) were also obtained from Gerald Byrne and were maintained in minimal essential medium (GIBCO) with 10% fetal bovine serum and 10 µg of gentamicin per ml. Vero cells (ATCC CCL 81; African green monkey kidney) were obtained from Curtis Brandt, University of Wisconsin, Madison, and were maintained in Dulbecco minimal essential medium (GIBCO) supplemented with 5% fetal calf serum (Hyclone), 2 mM L-glutamine, 100 U of penicillin (Sigma) per ml, and 100 µg of streptomycin (Sigma) per ml. A solution containing 0.05% trypsin and 0.53 mM EDTA (GIBCO) was used to detach T24 and Vero cells for passage and chromium release assays.

**Chromium release assays.** Bacterial strains were grown to an A<sub>600</sub> of 0.9 in LB broth. The concentration of HpmA<sup>+</sup> in bacterial culture supernatants was estimated by comparing the amount of HpmA<sup>+</sup> on Coomassie blue-stained polyclamidamide gels with known concentrations of bovine serum albumin (Sigma). Target cells were washed in the appropriate culture medium described above for nucleated cells or in 0.85% saline for ovine erythrocytes. Target cells (10<sup>7</sup> nucleated cells in 1 ml of culture medium or 10<sup>10</sup> erythrocytes [RBCs] in 1 ml of 0.85% saline) were labeled by the addition of 100 µCi of sodium [[<sup>51</sup>Cr]]chromate (Amersham Corp., Arlington Heights, Ill.) and incubation at 37°C for 30 min. The cells were then washed three times in the appropriate culture medium without antibiotics or in 0.85% saline. Labeled target cells (10<sup>7</sup> nucleated cells in 100 µl of medium or

<table>
<thead>
<tr>
<th>Strain, plasmid, or bacteriophage</th>
<th>Characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH1</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1</td>
<td>D. Walker (21)</td>
</tr>
<tr>
<td></td>
<td>thi-1 thr-1 leuB26 supE44 tonA21 lacY1 recA integrated</td>
<td>J. Mekalanos (24)</td>
</tr>
<tr>
<td></td>
<td>RP4-2-T&lt;sup&gt;c&lt;/sup&gt;-Mu Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>SY327</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; araD Δ(lac-pro) argE(Am) recA56 Rif&lt;sup&gt;+&lt;/sup&gt; nalA</td>
<td>J. Mekalanos (24)</td>
</tr>
<tr>
<td>Proteus strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA6163</td>
<td><em>P. mirabilis</em> urinary tract isolate</td>
<td>H. Mobley</td>
</tr>
<tr>
<td>WPM111</td>
<td>HpmA&lt;sup&gt;−&lt;/sup&gt; mutant of BA6163</td>
<td>This study</td>
</tr>
<tr>
<td>WPV5</td>
<td><em>P. vulgaris</em> normal fecal isolate</td>
<td>This laboratory</td>
</tr>
<tr>
<td>WPM43</td>
<td>HpmA&lt;sup&gt;−&lt;/sup&gt; mutant of WPV5</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids and bacteriophage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC19</td>
<td>oriColE1 Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>21</td>
</tr>
<tr>
<td>pUC4K</td>
<td>oriColE1 Amp&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>G. Roberts (25)</td>
</tr>
<tr>
<td>pGP704</td>
<td>oriR6K mobRP4 Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>J. Mekalanos (24)</td>
</tr>
<tr>
<td>pWPM97</td>
<td>pWPM100ClaI::Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWPM100</td>
<td>pUC19::Xhol (hpmb&lt;sup&gt;+&lt;/sup&gt; hpma&lt;sup&gt;−&lt;/sup&gt; 140-kDa amino-terminal fragment)</td>
<td>41</td>
</tr>
<tr>
<td>pWPM105</td>
<td>pGP704::hpmaΔClaI::Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pWPM140</td>
<td>pUC19::Xhol-HindIII (hpmb&lt;sup&gt;−&lt;/sup&gt; hpma&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>T. Upphoff, this laboratory</td>
</tr>
<tr>
<td>pir</td>
<td>pirR6K</td>
<td>J. Mekalanos (24)</td>
</tr>
</tbody>
</table>

**TABLE 1. Bacterial strains and plasmids**
FIG. 1. Construction of pWPM105. The thick and intermediate-thickness arcs represent pUC19 and pGP704 sequences, respectively. The dotted arcs with arrowheads indicate transcription of hpm genes; those without arrowheads indicate untranscribed hpm sequences. Sizes in kilobases (kb) are indicated in the centers of the plasmids. MCS, Multiple cloning site.
$10^8$ RBCs in 100 μl of 0.85% saline) were added to wells of a flat-bottom 96-well microdilution tray. Total bacterial cell culture or cell-free supernatant (100 μl) was then added to the target cells and incubated at 37°C under 5% CO₂ for 1 h. LB medium and 1 N HCl were used to determine background and maximum levels of release of chromium, respectively, from target cells. Following incubation, target cells were pelleted by centrifugation at 208 × g for 3 min. Half of the supernatant was removed from each well and counted in a gamma counter to detect released chromium. The percent specific cytotoxicity was calculated with the following equation: percent specific cytotoxicity = [(sample cpm - background cpm)/(maximum cpm - background cpm)] × 100, where cpm indicates counts per minute.

Murine model of ascending urinary tract infection. Eight-week-old female C3H mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, Ind.). Mice were infected by urethral catheterization as previously described (13). Briefly, groups of five or six mice were anesthetized with methoxyflurane (Metofane; Pitman-Moore, Inc., Washington Crossing, N.J.), and a French no. 2 Fogarty catheter (V. Mueller, Inc., Irvine, Calif.) adapted to an Eppendorf pipette tip was inserted into the bladder through the urethra. A 50-μl volume of bacteria grown to an $A_{600}$ of 0.9 and resuspended in 0.85% saline was introduced into the bladder through the catheter. At various times after infection, mice were sacrificed and their kidneys were removed and weighed. The kidneys were then cut in half longitudinally. One half was preserved in phosphate-buffered 10% Formalin for histopathology studies. The other kidney half was homogenized in 5 ml of sterile 0.85% saline and plated at various dilutions onto LB agar plates containing p-nitrophenylglycerine. Kidneys were considered infected if bacterial colonies were detected. The limit of detection was 10 bacteria per kidney.

Determination of LD₅₀ and KID₅₀. Eight-week-old female C3H mice were obtained from Harlan Sprague Dawley. Groups of six mice each were injected i.v. with serial twofold dilutions of live bacteria grown to an $A_{600}$ of 0.9 and adjusted to the appropriate concentration in 0.85% saline. The absolute number of viable bacteria injected was confirmed with plate counts. Mice were observed twice daily for 4 days. After 4 days, surviving mice were sacrificed by carbon dioxide dislocation, and their kidneys were removed and examined as described above. LD₅₀, and 50% kidney-infective dose (KID₅₀) values were calculated (7), and statistical significance was determined by Student’s t test (33).

Histopathology studies. Histopathology studies were performed by Annette Gendron-Fitzpatrick, Department of Veterinary Science, University of Wisconsin, Madison. Kidneys preserved in phosphate-buffered 10% Formalin were sectioned, stained with hematoxylin and eosin, and examined by light microscopy.

RESULTS

HpmA hemolytic and cytotoxic activities. The potential contribution of HpmA to virulence was initially examined by studying in vitro the lytic activities mediated by this hemolysin. The hemolytic and cytotoxic activities of HpmA were measured by using $E. coli$ DH1 containing pWPM140. This plasmid encodes full-length HpmA as well as HpmB, a protein necessary for the activation and secretion of HpmA. $E. coli$ DH1 containing pUC19 was used as a control. $P. mirabilis$ BA6163 was also included for comparison. Maximum hemolytic activity was produced by $E. coli$ DH1(pWPM140) and Proteus strains during late-logarithmic-phase growth (unpublished results). Consequently, late-logarithmic-phase bacterial cell cultures and their cell-free supernatants were tested for hemolytic and cytotoxic activities. As seen in Fig. 2A, strains DH1(pWPM140) and BA6163 produced hemolytic activity, due to HpmA, which was present in both cell-free supernatants and total cell cultures. HpmA also caused equivalent lysis of human and rabbit RBCs (data not shown).

Cytotoxicity due to HpmA was initially demonstrated with T24 human bladder epithelial cells in chromium release assays (Fig. 2B). Both total cell cultures and cell-free supernatants from strain DH1(pWPM140) exhibited cytotoxic activity. Contrastingly, cytotoxic activity due to $P. mirabilis$ BA6163 was demonstrated only when total cell culture was used, even though hemolytic activity was found in both total cell cultures and cell-free supernatants (Fig. 2B). Examination of the target cell specificity of HpmA revealed that it was active against human B-cell lymphomas (Daudi and Raji), human monocytes (U-937), and African green monkey...
kidney cells (Vero), as well as T24 cells (Fig. 3). Strain DH1(pUC19) was neither hemolytic nor cytotoxic, indicating that the lytic activities observed with DH1(pWAM140) were due to HpmA (Fig. 2 and 3).

A dose-response curve depicting lysis of chromium-labeled RBCs (10⁸ per assay) or T24 cells (10⁵ per assay) versus bacterial cell number is shown in Fig. 4A. Total cell cultures of strain DH1(pWPM140) are considerably more cytotoxic than cultures of strain BA6163 against both RBCs and T24 cells, since approximately 30 times more BA6163 cells were required for cytotoxicity equivalent to that observed with DH1(pWPM140) (Fig. 4A). T24 cells were much less susceptible than RBCs to HpmA-mediated lysis, since 10⁷ DH1(pWPM140) cells were required to lyse 10⁵ T24 cells in 1 h while 10⁶ bacterial cells were able to lyse 10⁶ RBCs in that time. Similar results were obtained with P. mirabilis BA6163; more P. mirabilis cells were required to lyse T24 cells than to lyse RBCs. The fetal bovine serum present with the T24 cells but not with the RBCs accounted for a small portion of this difference, since the release of chromium from RBCs was reduced in the presence of serum to one-fifth of its level in the absence of serum (data not shown). Cell-free supernatants from strain DH1(pWPM140) yielded similar results with respect to the difference in HpmA susceptibility between T24 cells and RBCs (data not shown).

Supernatants containing approximately 250 ng of HpmA lysed 10⁴ T24 cells in 1 h, compared with the 0.2 ng of HpmA needed to lyse 10⁵ RBCs. The other nucleated cell types tested exhibited HpmA susceptibility similar to that observed with T24 cells (Fig. 3).

The kinetics of HpmA-mediated lysis over time were examined with doses of bacterial cells which exhibited approximately 70% lysis of the target cells within 1 h. Strains DH1(pWPM140) and BA6163 showed similar cytotoxicity increases over time with both RBCs and T24 cells (Fig. 4B). Culture supernatants produced much less of an increase in cytotoxicity over time (data not shown), indicating that in assays with total cell culture, more HpmA was continually being produced. No significant hemolytic or cytotoxic activity was detected against any of the target cell types tested with the control strain DH1(pUC19), even after prolonged incubation or high doses of bacterial cells (Fig. 4).

Construction of HpmA⁻ mutants. To confirm that the hemolytic and cytotoxic activities observed with Proteus spp. were due to HpmA and to examine the contribution of HpmA to virulence, allelic mutants of two Proteus strains were constructed as depicted in Fig. 1. pWPM97 contains an in-frame deletion of amino acids 356 to 953 from the aminoterminal 140-kDa truncated HpmA encoded by pWPM100, yielding a 70-kDa protein fragment. In pWPM99, insertion of the Km" determinant into pWPM97 interrupts the HpmA reading frame after the first 355 amino acids and results in a 42-kDa fragment. The HpmA fragments produced from pWPM97 and pWPM99 were secreted from E. coli DH1 (Fig. 5) but were neither hemolytic nor cytotoxic (data not shown). A portion of the altered hpmA gene present in pWPM99 was then subcloned into the suicide vector pGP704, resulting in pWPM105. pWPM105 was introduced into Proteus spp. by conjugation from the E. coli donor strain.
strain SM10::λpir, which provides in trans the factors necessary for transfer of pGPI04. Although transconjugants were obtained at a frequency of 10^{-4} to 10^{-5} per donor, most represented a single crossover event, resulting in integration of pGPM105 into the Proteus chromosome. Double crossover events, resulting in replacement of the genomic hpmA gene by the mutant hpmA gene, were detected at a frequency of 10^{-3} per transconjugant by colony hybridization. Two HpmA^- mutants, WPM111 and WPV43, were obtained by this procedure; they are the hpmA deletion mutants of P. mirabilis BA6163 and P. vulgaris WPV5, respectively. Southern analysis of total genomic DNA from WPM111 was used to confirm the identity of this mutant (data not shown).

**Phenotypic characterization and virulence of HpmA^- mutants.** Immunoblots of trichloroacetic acid-precipitated supernatants developed with anti-HpmA antiserum showed that, unlike their parent strains, the mutant strains WPV43 and WPM111 do not produce HpmA (40). The HpmA^- mutants were then compared with their parent strains with respect to hemolytic and cytotoxic activities. The HpmA^- mutants WPM111 and WPV43 were not toxic to any of the target cells tested, in contrast to their parental strains BA6163 and WPV5, which were both hemolytic and cytotoxic (Fig. 6).

To determine if HpmA contributes to pathogenicity, we initially compared strain WPM111 to its parent strain in a mouse model of ascending urinary tract infection (13). We were able to see kidney infection, with PMN infiltration and abscess formation, over a period from 1 to 5 days following introduction of Proteus spp. into the bladder (data not shown). By 1 week after introduction of Proteus spp., the kidneys were no longer infected. We used several different bacterial doses from 10^6 to 10^9 bacteria as inocula, but observed no significant difference in the kidney infection of mice given the different doses. There was no difference between the HpmA^- mutant strain WPM111 and its wild-type parent strain, BA6163, with respect to time or extent of kidney infection. Histopathological examination of hematoxylin- and eosin-stained kidney sections by light microscopy also failed to reveal any significant difference between the tissue damage caused by infection with the HpmA^- mutant and that caused by infection with the wild-type strain (data not shown).

Strain WPM111 was compared with its wild-type parent strain with respect to LD_{50} and KID_{50} following i.v. injection of live bacteria. Eight-week-old C3H mice were injected with serial twofold dilutions of bacteria. After 5 days, surviving mice were sacrificed to determine if their kidneys were infected. The LD_{50} of WPM111, the HpmA^- mutant with (mean ± standard deviation, [5.45 ± 0.45] × 10^8 bacteria), was sixfold higher than the LD_{50} of the wild type ([8.95 ± 0.03] × 10^7 bacteria). By using Student’s t test, this was shown to be a significant difference (P < 0.05). Contrastingly, only a slight, statistically insignificant difference between the KID_{50} of the mutant and the wild type was seen. The KID_{50} of the mutant strain was (2.10 ± 0.76) × 10^7 bacteria, while the KID_{50} of the wild-type strain was (1.33 ± 0.89) × 10^7 bacteria.

![FIG. 5. Production and secretion of HpmA derivatives in E. coli. Immunoblots of total cell culture or unconcentrated supernatants (10 μl) were developed with anti-HpmA antiserum. Molecular mass markers in kilodaltons are shown on the left. Lane 1, strain DH1(pWPM100) total culture; lane 2, DH1(pWPM97) total culture; lane 3, DH1(pWPM97) supernatant; lane 4, DH1(pWPM99) total culture; lane 5, DH1(pWPM99) supernatant.](http://iai.asm.org/)
**DISCUSSION**

We have shown that HpmA is the hemolysin most commonly produced by *Proteus* isolates (40), and we wanted to examine the cytotoxic activities of HpmA and its contribution to virulence in vivo. Many hemolysins, such as *E. coli* HlyA, are more appropriately named cytolsins, since they are active on many cell types in addition to RBCs. Examination of HpmA target cell specificity with cells which bacteria might encounter in UTIs shows that HpmA lyses cells of several types: human bladder epithelial cells, human B-cell lymphoma cells, human monocytes, and African green monkey kidney cells. Thus, HpmA also may be more appropriately called a cytolsin. Cytotoxic activity of the HpmA-related hemolysin, ShlA, from *S. marcescens* has not been examined. However, König et al. have shown that ShlA induces the release of leukotrienes from PMNs and the release of histamine from mast cells (16). Thus, HpmA and ShlA may have activities similar to those of *E. coli* HlyA, which is cytotoxic at high doses and at lower doses causes leukotriene and histamine release (11, 32).

HpmA-mediated hemolytic activity was observed in both total cell cultures and cell-free supernatants from *P. mirabilis* BA6163 and *E. coli* DH1(pWPM140). Contrastingly, although cytotoxic activity was detected in cell-free supernatants from strain DH1(pWPM140), cell-free supernatants from strain BA6163 exhibited no significant cytotoxic activity. The most likely explanation for these results is that cell-free supernatants from BA6163 do not contain enough HpmA to induce lysis of T24 cells. Coomassie blue-stained polyacrylamide gels of unconcentrated supernatants from BA6163 revealed no detectable HpmA, whereas an equivalent amount of supernatant from DH1(pWPM140) contains enough HpmA to be easily visualized in this gel system (40; unpublished data). Approximately 10-fold more HpmA is needed for lysis of T24 cells than for lysis of RBCs, and since undiluted supernatants from DH1(pWPM140) lysed only 50% of the T24 cells present in the assay and BA6163 is approximately 1/30 as toxic to both RBCs and T24 cells as DH1(pWPM140) is (Fig. 4A), it is not surprising that no cytotoxic activity was detected in supernatants from this strain. Supernatants from a second *Proteus* strain, WPV5, also had no detectable cytotoxic activity (data not shown).

Like HpmA, the *E. coli* hemolysin is more active against RBCs than against nucleated cells (4, 6). HpmA activity present in cell-free supernatants from strain DH1(pWPM140) lysed approximately 40 T24 cells per ng of HpmA per h and approximately 5 × 10⁵ RBCs per ng of HpmA per h. HpmA activity in supernatants decays rapidly (40), and since we have no method to quantitate active versus inactive HpmA, this number most likely reflects more HpmA than is actually required to lyse cells. Pulse-labeling experiments show that the hemolytic activity of the HpmA-related hemolysin ShlA has a half-life of 3 min at 37°C (35).

To confirm that the observed hemolytic and cytotoxic activities were due to HpmA, we constructed HpmA mutants of two *Proteus* strains. These mutants were generated by first deleting a portion of the cloned hmpA gene in *E. coli* and then introducing the altered hmpA gene into the *Proteus* strains with pGP704, which transfers conjugally from *E. coli* to *Proteus* spp., as a suicide vector (24). Using selection for resistance to tetracycline and kanamycin, we isolated mutants of two *Proteus* strains in which the genomic copy of hmpA was replaced with a mutant copy generated in vitro. Presumably, this method of mutant construction could be adapted to generate mutants of other *Proteus* genes, although modification of the suicide vector to include an antibiotic resistance gene easily scorable in *Proteus* spp. would be desirable.

While constructing these altered hmpA genes in *E. coli*, we made several observations concerning the regions of HpmA necessary for hemolytic activity and secretion. In pWP97, the in-frame deletion of amino acids 356 through 953 from the truncated HpmA resulted in loss of hemolytic and cytotoxic activities. Thus, the deleted residues may contribute directly to hemolytic activity, or, alternatively, deletion of this portion of HpmA may lead to conformational changes accounting for the loss of hemolytic activity. We have previously shown that the amino-terminal 75% of HpmA encoded by pWPM100 is sufficient for hemolytic activity (41, 42). This truncated peptide exhibits less hemolysis than full-length HpmA (K. Swihart, unpublished data). Poole et al. have likewise deleted the carboxy-terminal 25% of the related *S. marcescens* hemolysin, ShlA, while still retaining significant, although reduced, amounts of hemolytic activity. However, deletion of 57% of ShlA from the carboxy terminus did result in loss of hemolytic activity (31, 35). The results reported here for HpmA agree with those for ShlA, since the carboxy terminus is not necessary for hemolytic activity but deletion of regions from the center of HpmA results in loss of hemolytic activity.

The 70-kDa peptide produced by deletion of amino acids 356 through 953 was secreted from *E. coli* DH1 into the culture supernatant. The smaller amino-terminal 42-kDa peptide produced when the Km' determinant is inserted following amino acid 355 also was secreted from *E. coli*. Thus, only the first 355 amino acids of HpmA are required for secretion. HpmA has an amino-terminal signal sequence which has been cleaved from the secreted form (41). The results described here are similar to those of Schiebel et al., who have shown that the amino-terminal 269 amino acids of ShlA are secreted (35). They propose that ShlA crosses the cytoplasmic membrane in a signal sequence-dependent manner and then crosses the outer membrane in an ShIB-dependent manner. They further propose that sequences involved in ShIB-dependent secretion are located in the amino terminus of ShlA. The results for these related hemolysins contrast with those of other cloned genes secreted by *E. coli*; the cloned Neisseria gonorrhoeae immunoglobulin A protease, *S. marcescens* protease, and *E. coli* HlyA all require the carboxy terminus for secretion (8, 30, 45).

As expected, the *Proteus* mutants did not produce full-length HpmA. However, surprisingly, the 42-kDa peptide produced by *E. coli* containing pWP99 was not detected in the *Proteus* HpmA mutants by immunoblotting of trichloroacetic acid-precipitated supernatants. The HpmA mutants lost both hemolytic and cytotoxic activities, confirming that both activities require HpmA production. These *Proteus* strains did not produce any detectable cytotoxin other than HpmA in vitro, as evidenced by the loss of all hemolytic and cytotoxic activity against all target cell types examined.

We used a model of ascending urinary tract infection in which *Proteus* strains were introduced into the bladder through a urethral catheter to infect mouse kidneys. In this model, the HpmA mutant behaved no differently from its parent strain with respect to either the severity or time course of kidney infection or production of histopathological changes. Using a similar route of infection in rats, MacLeod et al. showed that HlyA, a hemolysin inactivated by HpmA, contributed only a fivefold difference to kidney colonization by *E. coli* (22).
When examined by i.v. injection, HpmA may play a role in virulence, either directly or indirectly, since the HpmA− mutant had a sixfold higher LD₅₀ than its wild-type parent. Peerbooms et al. have also shown that hemolytic Proteus strains have higher LD₅₀s than nonhemolytic strains after i.v. injection (26, 27). The experiments reported here extend the results of Peerbooms et al. by construction and comparison of isogenic Proteus strains. E. coli HlyA has also been shown to enhance E. coli virulence 100-fold when injected intraperitoneally into rats (43).

It is possible that HpmA acts in conjunction with other Proteus virulence factors, such as urease and pili. In animal models representing E. coli extraintestinal infections, maximum virulence is observed with strains exhibiting serum resistance and producing fimbriae as well as hemolysin (12, 22). We currently are constructing appropriate combinations of HpmA and urease mutants to test for interaction of these putative virulence factors.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-20323 from the National Institutes of Health. R.A.W. is a Pew Scholar in the Biomedical Sciences. We are grateful to Christiane Forestier, Shahaireen Pellett, and Gail Rowe for assistance in performing the animal experiments and to Annette Gendron-Fitzpatrick for performing the histopathology studies. We also thank Edward Balish, Gerald Byrne, Donna Paulnock, and their laboratories for supplying cell lines, allowing us to use their equipment, and advising us concerning the chromium release assays.

LITERATURE CITED


