E. coli virulence factor hemolysin induces neutrophil apoptosis and necrosis/lysis in vitro and necrosis/lysis and lung injury in a rat pneumonia model

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Departments of 1Medicine and 2Microbiology, 3The Witebsky Center for Microbial Pathogenesis, 4Veterans Administration Western New York Healthcare System, Departments of 5Anesthesiology, 6Pathology, and 7Pediatrics, 8Center of Excellence in Bioinformatics and Life Sciences, University at Buffalo, Buffalo, New York

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Russo, Thomas A., Bruce A. Davidson, Stacy A. Genagon, Natalie M. Warholic, Ulrike MacDonald, Patrick D. Pawlicki, Janet M. Beanan, Ruth Olson, Bruce A. Holm, and Paul R Knight III. E. coli virulence factor hemolysin induces neutrophil apoptosis and necrosis/lysis in vitro and necrosis/lysis and lung injury in a rat pneumonia model. Am J Physiol Lung Cell Mol Physiol 289: L207–L216, 2005. First published April 1, 2005; doi:10.1152/ajplung.00482.2004.—Enteric gram-negative bacilli, such as Escherichia coli are the most common cause of nosocomial pneumonia. In this study a wild-type extraintestinal pathogenic strain of E. coli (ExPEC) (CP9) and isogenic derivatives deficient in hemolysin (Hly) and cytotox necrotizing factor (CNF) were assessed in vitro and in a rat model of gram-negative pneumonia to test the hypothesis that these virulence factors induce neutrophil apoptosis and/or necrosis/lysis. As ascertained by in vitro caspase-3/7 and LDH activities and neutrophil morphology, Hly mediated neutrophil apoptosis at lower E. coli titers (1 × 10⁵–6 cfu) and necrosis/lysis at higher titers (≥1 × 10⁷ cfu). Data suggest that CNF promotes apoptosis but not necrosis or lysis. We also demonstrate that annexin V/7-amino-actinomycin D staining was an unreliable assessment of apoptosis using live E. coli. The use of caspase-3/7 and LDH activities and neutrophil morphology supported the notion that necrosis, not apoptosis, was the primary mechanism by which neutrophils were affected in our in vivo gram-negative pneumonia model using live E. coli. In addition, in vivo studies demonstrated that Hly mediates lung injury. Neutrophil necrosis was not observed when animals were challenged with purified lipopolysaccharide, demonstrating the importance of using live bacteria. These findings establish that Hly contributes to ExPEC virulence by mediating neutrophil toxicity, with necrosis/lysis being the dominant effect of Hly on neutrophils in vivo and by lung injury. Whether Hly-mediated lung injury is due to neutrophil necrosis, a direct effect of Hly, or both is unclear.

Escherichia coli; gram-negative bacilli; cytotoxic necrotizing factor-1

NOSOCOMIAL PNEUMONIA ACCOUNTS for 15% of hospital-acquired infections (2). Gram-negative enteric bacilli such as Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Enterobacter sp., Acinetobacter, and Stenotrophomonas are the most common cause of hospital-acquired pneumonia, being implicated in 55–85% of cases (8–10, 24). Severe disease usually occurs when these agents cause pneumonia, with associated crude mortality and estimated attributable mortality ranging from 24 to 76% and 20 to 50%, respectively, which translates into 36,000–80,000 deaths annually (2, 8, 24, 52). Over the last 10–15 years, there has been little improvement in the outcome from this infection. The estimated annual cost of nosocomial pneumonias alone due to gram-negative bacilli in this country is greater than one billion dollars (55).

A goal of our laboratory is to identify strategies to decrease the morbidity and mortality caused by gram-negative pneumonia. To accomplish this, we have been studying neutrophil-pathogen interactions in a rat model of bacterial pneumonia, using E. coli as a model pathogen (37, 38, 43). The importance of neutrophils in protecting against infection in the lung is as great as any other site in the body (46). Increased susceptibility and severity of pneumonia occur in the setting of neutropenia and with genetically inherited (e.g., chronic granulomatous disease) or drug-induced (e.g., steroids) disorders of neutrophil function (25). In animal models, enhanced recruitment and activation of neutrophils by TNF-α and CXC chemokines result in enhanced bacterial clearance and survival in gram-negative pneumonia (14, 28, 48, 49, 51). Likewise, inhibition of CXC receptor CXCR2 results in decreased pulmonary neutrophil recruitment, bacterial clearance, and survival (28, 49).

To combat this critical antimicrobial host defense system, extracellular bacterial pathogens possess virulence factors that not only protect against the bactericidal activity of neutrophils (7) but also have the capability to impede neutrophil pulmonary response. We have previously demonstrated that capsule and the O-antigen moieties of lipopolysaccharide (LPS) inhibit the recruitment of neutrophils into the pulmonary compartment after bacterial challenge (37). This is due, in part, to capsule and O-antigen decreasing pulmonary neutrophil chemotaxis mediated by both the formyl peptide and nonformyl peptide receptors (38).

Inducing neutrophil apoptosis or necrosis is another mechanism by which extracellular bacterial pathogens may subvert the host’s pulmonary defense. Most neutrophils that migrate into the lungs will die there via apoptosis (11, 16). However, the time frame in which this occurs can be modified by both host and nonhost factors. As a general rule, proinflammatory mediators delay apoptosis, and, in contrast, downregulators (e.g., IL-10) and the process of phagocytosis accelerate apoptosis (22, 44). These findings can be interpreted as being beneficial to the host. If proinflammatory mediators increase functional neutrophil longevity, then their phagocytic potential

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will be maximized. Once phagocytosis is complete or down-regulation of the inflammatory process has begun, neutrophils undergo elimination via apoptosis. The ability of E. coli to modulate this homeostatic process may enhance its pathogenic potential within the lung. The E. coli toxins alpha-hemolysin (Hly) and cytotoxic necrotizing factor (CNF)-1 are candidates for affecting neutrophil apoptosis or necrosis. Hly is a member of the repeats-in-toxin family of pore-forming toxins. E. coli Hly is toxic to a wide range of cells by inserting into the plasma membrane to generate a transmembrane pore. CNF is a member of a family of bacterial toxins that target the Rho family of small GTP-binding proteins in mammalian cells (6). Previous studies have demonstrated that these virulence factors can directly induce apoptosis or necrosis, pending concentration, in a variety of cell types (5, 12, 13, 15, 18, 30, 54).

In this study a wild-type extraintestinal pathogenic strain of E. coli (CP9) and isogenic derivatives deficient in Hly and CNF were assessed in vitro and in a rat model of gram-negative pneumonia to test the hypothesis that these virulence factors mediate neutrophil apoptosis and/or necrosis/lysis.

MATERIALS AND METHODS

Bacterial strains and media. The model pathogen CP9 is an E. coli blood isolate cultured from a patient with sepsis and has been previously described in detail (19, 39). CP9 possesses many of the characteristics of typical ExPEC strains (40) and is highly virulent in a urinray tract infection model (36), an intraperitoneal infection model (42), and a pneumonia model (35, 43). CP9hlyA is a Tapho1A'-generated derivative of CP9 in which the structural gene for Hly (hlyA) is disrupted. CP9hlyA was confirmed to be nonhemolytic by a lack of hemolysis after growth on blood agar plates and by an absence of red blood lysis in a quantitative hemolysis assay (47). CP9hlyA pEK50 has restoration of its Hly phenotype via complementation with the hlyA-containing plasmid pEK50 (21). CP9/pEK50, by virtue of possessing the wild-type Hly operon and the multicopy plasmid pEK50, produces supraphysiological amounts of Hly. CP9cnf1 is an isogenic derivative of CP9, in which the gene for CNF has been partially deleted as described previously (a gift from Dr. Hank Lockman) (34). Stability of the genotype/phenotype of CP9hlyA was confirmed by Hly production and of CP9cnf1 by PCR amplification of cnf. All strains were maintained at −80°C in 50% Luria-Bertani (LB) broth and 50% glycerol. For in vitro assays bacterial strains were grown in LB broth (5 g yeast extract, 10 g tryptone, 10 g NaCl/l). Incubations were performed at 37°C unless otherwise described.

Pulmonary infection model. Animal studies were reviewed and approved by the University at Buffalo and Veterans Administration Institutional Animal Care Committee. An established rat (Long-Evans) model for studying pulmonary damage was used as reported (35, 43). In brief, Long-Evans rats (250–300 g) were anesthetized with 3% halothane in 100% oxygen until unconscious and then subjected to RNase (RNase A + T1) digestion (Pharmingen). Protected mRNA species were phenol-chloroform-isooamy alcohol extracted and ethanol precipitated. The dried precipitate was dissolved in loading buffer and run on a 0.4-mm 5% acrylamide gel. The dried gel was loaded into a phosphorimaging cassette and exposed for 2–15 h, and the image was captured and analyzed using a Bio-Rad Molecular Imaging System (Hercules, CA). We identified specific mRNA species by comparing to migration distances of the unprobed controls, and the band densities were normalized to the housekeeping gene transcript L32. The mRNA species Fas antigen, bcl-x, Fas ligand, caspase 1–3, bax, and bcl-2 were quantitated. All reagents and equipment are carefully maintained as RNase-free (33). Neutrophil purification. “Naive” neutrophils were purified from blood by healthy human volunteers using Polymorph prep (Axis-Shield; Poc, Oslo, Norway) per manufacturer instructions. After purification, neutrophil preparations consisted of ~96–100% neutrophils and ~0–4% lymphocytes.

Assessment of neutrophil morphology. Human neutrophils, purified from blood, were used to assess cellular morphology after in vitro exposure to bacteria or normal saline. Rat neutrophils, recovered by BAL, were used to assess morphology after pulmonary instillation of normal saline, LPS, or bacteria. After 1 h of exposure at 37°C in vitro and 6 h in vivo, a 50-μl aliquot was diluted 1:200 in Isoton II solution (Beckman Coulter), and the leukocyte concentration was determined using a Multisizer 3 Coulter Counter (Beckman Coulter). We prepared a cytospin by diluting cells to a final concentration of 5 × 10⁷ leukocytes, using a Cytospin 3 cytocentrifuge (Shandon, Pittsburgh, PA), staining with Diff-Quik reagents (Baxter, Miami, FL), and examining under light microscopy (Nikon microscope ECLIPSE 80i; Nikon Instruments, Melville, NY). Photomicrographs were taken with a SPOT camera and SPOT INSIGHT software (version 4.0.4; Diagnostic Instruments, Sterling Heights, MI).

Caspase-3/7 assay. Caspase-3/7 activity was measured by conversion of the nonfluorescent caspase substrate Z-DEVD-R110 to the fluorescent rhodamine 110 as per the instructions (Apo-ONE homogenization kit, Promega, Madison, WI). In vitro activity was measured in triplicate in a white, flat-bottom, 96-well Microtiter (Thermo Labsystems, Franklin, MA) after 5 × 10⁵ purified neutrophils were exposed to various E. coli strains at various titers or no bacteria (negative control) for 1 h at 37°C. In vivo activity was measured from cells obtained by BAL 6 h after pulmonary instillation of normal saline, LPS (670 μg), or E. coli [~1 × 10⁷ colony-forming units (cfu)]. Fluorescence was measured continuously by a SPECRama (Molecular Devices, Sunnyvale, CA) and data acquisition utilized SOFTmax PRO (version 3.1, Molecular Devices). Caspase activity was expressed as the Vmax.
maximum slope on the linear portion of the curve) for a given bacterial or LPS concentration. Due to the fact that caspase-3/7 activity was compared between bacterial and nonbacterial groups (e.g., CP9 and LPS) and that caspase-3/7 activity may not have correlated directly with bacterial titers, caspase-3/7 activity was not normalized. Nonetheless, differences in cfus between E. coli groups were small. For in vitro studies, the mean differences in cfu between CP9 and its mutant derivatives CP9hlyA, CP9cnf1, and CP9hlyA/pEK50 were 0.26, 5.7, and 7.5%, respectively. For in vivo studies, the mean difference in cfu between CP9 and CP9hlyA was 1.4%. Results are the mean of two independent experiments.

**LDH assay.** LDH activity was measured via a coupled enzymatic assay, which results in the conversion of a tetrazolium salt into a red formazan product, which was detected colorimetrically (CytoTox96, Promega). In vitro activity was measured in triplicate in a 96-well microtissue culture plate (Becton Dickinson, Franklin Lakes, NJ) after 5 × 10⁶ purified neutrophils were exposed to various E. coli strains at various titers for 1 h at 37°C. Neutrophils in plasma not exposed to bacteria, plasma alone, or plasma plus lysis buffer established baseline LDH activity. Neutrophils plus lysis buffer or LDH served as positive controls. In vivo activity was measured from BAL obtained 6 h after pulmonary instillation of normal saline, LPS (645 µg), or E. coli (CP9, CP9hlyA, ~1 × 10⁷ cfu). Absorbance at 490 nm was measured continuously by a SPECTRAMax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) and data acquisition utilized SOFTmax PRO (version 3.1, Molecular Devices). LDH activity was expressed as the Vmax. Due to the fact that LDH activity was compared between bacterial and nonbacterial groups (e.g., CP9 and LPS) and that LDH activity may not have correlated directly with bacterial titers, LDH activity was not normalized. Nonetheless, differences in bacterial titers between E. coli groups were small. For in vitro studies, the mean differences in cfu between CP9 and its mutant derivatives CP9hlyA, CP9cnf1, and CP9hlyA/pEK50 were 0.58, 4.19, and 3.6%, respectively. For in vivo studies, the mean difference in cfu between CP9 and CP9hlyA was 1.4%. In vitro results were the mean of two independent experiments and in vivo results were the mean of 11 or 12 animals per group (four experiments with two or three animals per group).

**Neutrophil lysis assay.** Neutrophil lysis was measured by a flow cytometry-based assay. Purified neutrophils (5 × 10⁵) were exposed to various E. coli strains at various titers or no bacteria (negative control). After incubation for 1 h at 37°C, 1 × 10⁶ sulfrohodamine-impregnated polystyrene beads (6 µm; Polysciences, Warrington, PA) (FL-1) were added to the neutrophil suspension. Neutrophil lysis was determined by assessing the number of R-phycoerythrin (R-PE)-CD15-labeled neutrophils (FL-2) in the suspension using a FACSCalibur flow cytometer (Becton Dickinson Biosciences Immunocytometry Systems, San Jose, CA). Data acquisition for each sample was terminated after 2,000 bead events had been detected. This ensured that an equal volume of cell suspension was analyzed for each sample since each sample contained the same bead concentration. A gate in the forward-scatter vs. side-scatter plot discriminated neutrophils and beads from “debris” and a subsequent back-gating of the side-scatter vs. FL-2 discriminated the neutrophils from the beads and enabled neutrophil events to be counted.

**Flow cytometry assessment of neutrophil apoptosis and necrosis/lysis in vitro.** Purified neutrophils (5.0 × 10⁵) were resuspended in 100 µl of buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) and incubated with various strains of E. coli at various titers for 1 h at 37°C. Five microliters of annexin V-FITC (BD Biosciences, San Diego, CA) were added to the suspension and incubated at room temperature for 10 min, followed by the addition of 5 µl of 50 µg/ml 7-amino-actinomycin D (7-AAD, BD Biosciences) and incubation at room temperature for 10 min. The neutrophil population was identified by R-PE-conjugated CD15 (BD Biosciences) (FL-2). Annexin V-positive/7-AAD-negative cells were designated as early apoptotic, and annexin V- and 7-AAD-positive cells were designated as late apoptotic.
and to a lesser degree Hly diminished the abundance of Fas and bcl-x mRNA (CP9 compared with CP9\textit{cnf1} and CP9\textit{hlyA} in Fig. 1). \textit{bcl}-x is an oncogene that inhibits apoptosis (4) and has been shown to protect against apoptosis in both neutrophils and macrophages (31, 45, 53). Therefore, the repression of \textit{bcl}-x by CNF1 and Hly is consistent with a proapoptotic role for these virulence traits. Although controversial, repression of Fas antigen by CNF1 and Hly is most consistent with an antiapoptotic effect (1). Therefore, our next study focused on the role of Hly on modulating apoptosis. Furthermore, since mRNA abundance studies utilized whole lung mRNA derived from multiple cells, an apoptotic effect of live CP9, CP9\textit{cnf1}, and CP9\textit{hlyA} on purified neutrophils in vitro was subsequently assessed.

\textbf{Effect of Hly on neutrophil morphology in vitro.} We assessed the effect of Hly on neutrophil morphology in vitro by exposing purified neutrophils to 1 \times 10^4–7 cfu of CP9 (wt) and CP9\textit{hlyA} (Hly-negative) for 1 h and subsequent examination of Diff-Quik-stained neutrophils on a cytospin slide (Fig. 2). Neutrophils exposed to 10^4 cfu of CP9 demonstrated the same normal cellular morphology as neutrophils not exposed to bacteria (Fig. 2, A and B). Neutrophils exposed to 10^5 cfu of CP9 demonstrated early apoptotic changes, manifested by a ruffled membrane and clearly apoptotic cells with pyknotic nuclei (Fig. 2C). Neutrophils exposed to 10^6 cfu of CP9 demonstrated occasional apoptotic changes; however, the majority were undergoing various stages of necrosis (Fig. 2D). Greater than 90% of neutrophils exposed to 10^7 cfu of CP9 demonstrated advanced necrosis (Fig. 2E). In contrast, neutrophils exposed to 10^4 and 10^5 cfu of CP9\textit{hlyA} demonstrated the same normal cellular morphology as neutrophils not exposed to bacteria (Fig. 2, F and G, respectively). Neutrophils exposed to 10^6 cfu of CP9\textit{hlyA} demonstrated some cells with apoptotic changes (Fig. 2H). Neutrophils exposed to 10^7 cfu of CP9\textit{hlyA} demonstrated a mixed morphology with the majority consisting of normal cells, but occasional apoptotic cells and cells in early stages of necrosis were also observed (Fig. 2I). These findings demonstrate that the effect of Hly on neutrophils is dependent on the bacterial concentration. At 10^4 cfu, no effect was observed, at 10^5 apoptotic changes were observed, and at 10^6 and 10^7 necrosis became the dominant feature. These data also demonstrate that although Hly was required for these changes, other bacterial factors are also most likely contributory as evidenced by the abnormal neutrophil morphology observed in some cells after exposure to 10^7 cfu of CP9\textit{hlyA} (Fig. 2f). To further assess and quantitate the effects of Hly and CNF on neutrophil apoptosis and necrosis/lysis at various concentrations, alternative assays were utilized.
Effect of Hly and CNF on neutrophil 3/7 caspase activity in vitro. The effect of Hly and CNF on neutrophil 3/7 caspase activity, an alternative indicator of apoptosis, was assessed in vitro (Fig. 3). Purified neutrophils (5 × 10^6) were exposed to various titers of CP9 (wt), CP9cnf1 (CNF-negative), CP9hlyA (Hly-negative), CP9hlyApEK50 (Hly-positive via complementation) at 37°C for 1 h. Caspase-3/7 activity was measured by a fluorescence assay (see MATERIALS AND METHODS) and expressed as the Vmax (maximum slope on the linear portion of the curve) for a given bacterial concentration. *P < 0.0001 compared with all bacterial titers. #P < 0.0001 compared with CP9hlyA and CP9cnf1 at the same titer, respectively.

Effect of Hly on neutrophil LDH activity in vitro. Effect of Hly on neutrophil necrosis/lysis, a flow cytometry-based assay was developed that measured neutrophil lysis (see MATERIALS AND METHODS for details). Purified neutrophils were exposed to 1 × 10^7 cfu of CP9 (wt), CP9hlyA (Hly-negative), CP9cnf1 (CNF-negative), and CP9hlyApEK50 for 1 h at 37°C. Subsequently, after data acquisition for each sample was terminated following detection of 2,000 sulforhodamine-impregnated polystyrene beads (6 μm) events, neutrophil-gated events were measured (Fig. 5). Compared with neutrophils not exposed to bacteria and neutrophils exposed to CP9hlyA, a significant decrease (P < 0.0001, unpaired t-test) in neutrophil events was detected after neutrophils were exposed to E. coli strains that express Hly (CP9, CP9cnf1, CP9hlyApEK50). These data further demonstrate that at titers of ≥10^7 cfu of CP9 strains that produce Hly at normal expression levels neutrophils primarily undergo necrosis/lysis.

Annexin V/7-AAD staining was an unreliable assessment of apoptosis using live E. coli. Annexin V and 7-AAD staining, combined with analysis via flow cytometry, were initially used to examine how Hly and CNF modified neutrophil apoptosis in vitro. However, these data demonstrated that annexin V/7-AAD staining was an unreliable assessment of apoptosis under these experimental conditions using live E. coli. Neutrophils identified as early apoptotic after exposure to 10^7 cfu of CP9 were in fact necrotic as was subsequently established by cellular morphology, caspase-3/7, and LDH activity (data not shown).
Exposure to maximal in vitro LDH activity was detected after neutrophils were challenged with LPS (Fig. 7B). After challenge with CP9 (Hly-positive), a fraction of neutrophils demonstrated cellular changes consistent with necrosis (Fig. 7, C and D). After challenge with CP9hlyA (Hly-negative), occasional neutrophils demonstrated cellular changes consistent with an early stage of necrosis, albeit not as pronounced as what was observed after challenge with CP9 (wt) (Fig. 7, E and F). Many, but not all, of the necrotic neutrophils had phagocytosed bacteria (Fig. 7, C–F), thereby establishing their identity. Apoptotic changes were not observed in neutrophils after challenge with either CP9 or CP9hlyA. Together, these findings support that necrosis, not apoptosis, is the primary mechanism by which Hly affects neutrophils in our in vivo gram-negative pneumonia model.

Effect of Hly on lung injury in vivo. The finding that Hly mediated neutrophil necrosis within the pulmonary compartment prompted an assessment of lung injury. Long-Evans rat lungs were instilled intratracheally with normal saline, purified LPS (670 μg), or ~1 × 10⁷ cfu of the E. coli strains CP9hly (produces no Hly), CP9 (produces physiological levels of Hly), or CP9/pEK50 (produces supraphysiological levels of Hly). Lung injury was assessed by measurement of oxygenation (PaO₂/FIO₂) and leakage of albumin into the pulmonary compartment (BAL albumin). Compared with the control animals (normal saline), BAL albumin was increased in animals after bacterial challenge. Furthermore, BAL albumin was significa-
cantly greater in animals challenged with the Hly-producing strains CP9 and CP9/pEK50 compared with the Hly-deficient strain CP9*hly (Table 1). Likewise, oxygenation was significantly decreased in animals challenged with CP9 and CP9/pEK50 compared with CP9*hly. The diminished oxygenation and increased albumin leakage in the presence of hemolysin were not due to an increased clearance of CP9*hly compared with CP9 and CP9/pEK50 or differences in total BAL neutrophils (Table 1). Together, these findings demonstrate that Hly either directly or indirectly mediates lung injury.

DISCUSSION

ExPEC are a group of successful, highly evolved pathogens capable of causing serious infections in both healthy and immunocompromised hosts (41). Because ExPEC are extracellular pathogens, the ability to evade professional phagocytes, such as neutrophils, would greatly enhance their pathogenic capability. In this paper, we tested the hypothesis that the ExPEC toxins Hly and CNF1 modulate neutrophil apoptosis and necrosis/lysis. Our data demonstrate that in vitro Hly induces apoptosis at bacterial titers \(10^7\) cfu, but at higher titers, Hly causes necrosis/lysis (Figs. 2–5). When the bioactivity of Hly was assessed in an in vivo rat pneumonia model, Hly primarily caused neutrophil necrosis/lysis (Figs. 6 and 7). Likewise, lung injury was increased in animals challenged with \textit{E. coli} strains that produced Hly compared with challenge with an isogenic Hly-deficient derivative (Table 1). Whether Hly-mediated...
lungs is consistent with both CNF and Hly modulating apoptosis in vivo (26). In addition, results from our experiments suggest that ExPEC possesses additional bacterial virulence factors that cause neutrophil necrosis, even in the absence of Hly, neutrophils were observed to undergo necrotic changes (Fig. 2f). Furthermore, results from in vivo lung injury experiments demonstrated that lung injury still occurs in the absence of Hly (Table 1). Whether this injury is mediated by unidentified bacterial factors or host factors remains undetermined.

An important aspect of the experimental design of these studies was the use of a clinically relevant extraintestinal pathogenic strain of *E. coli* (CP9) (not a commensal or intestinal pathogenic strain of *E. coli*) that possesses the full repertoire of virulence factors needed to be a successful extraintestinal pathogen and isogenic derivatives deficient in Hly repertoire of virulence factors needed to be a successful extraintestinal pathogen and isogenic derivatives deficient in Hly.

### Table 1. Effect of hemolysin on lung injury in vivo

<table>
<thead>
<tr>
<th>Lung instillate</th>
<th>Total Lung Bacterial Titer, Log cfu</th>
<th>Pao/Fio₂, mmHg</th>
<th>BAL Albumin, µg/ml</th>
<th>Total BAL Neutrophils</th>
</tr>
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<tbody>
<tr>
<td>Normal saline</td>
<td>377 ± 14</td>
<td>102 ± 34</td>
<td>4.4 ± 10² ± 2.8 × 10⁴</td>
<td></td>
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<tr>
<td>CP9/hly (HLY-negative)</td>
<td>386 ± 21†</td>
<td>584 ± 99‡</td>
<td>8.4 × 10⁵ ± 2.5 × 10⁵</td>
<td></td>
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<tr>
<td>CP9 (wild-type, Hly-positive)</td>
<td>186 ± 24</td>
<td>1,110 ± 104</td>
<td>1.3 × 10⁶ ± 5.9 × 10⁵</td>
<td></td>
</tr>
<tr>
<td>CP9/pEK50 (increased Hly production)</td>
<td>160 ± 33</td>
<td>1,889 ± 201</td>
<td>9.7 × 10⁵ ± 2.9 × 10⁵</td>
<td></td>
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</table>

All values are expressed as means ± SE; *n = 6 for each group. Hly, hemolysin; BAL, bronchoalveolar lavage; cfu, colony-forming units. *P = 0.005 and 0.01, CP9/pEK50 compared with CP9 and CP9/hly, respectively (2-tailed, unpaired r-test); †P = 0.0002 and 0.0003, CP9/hly compared with CP9 and CP9/pEK50, respectively (2-tailed, unpaired r-test); ‡P = 0.004 and 0.0002, CP9/hly compared with CP9 and CP9/pEK50, respectively (2-tailed, unpaired r-test).

The findings from these experiments have important implications. They demonstrate that one of the mechanisms by which Hly contributes to ExPEC virulence is through increasing neutrophil death. Our data demonstrated that in vitro the effects of Hly are dose dependent, inducing apoptosis at bacterial titers <10⁵ cfu, but at higher titers, causing necrosis/lysis. Our in vivo data suggest that, in at least the rat pneumonia model, which appears to be a reasonable approximation of the clinical scenario in patients, necrosis/lysis is the dominant effect of Hly on neutrophils. First, the challenge inoculum for in vivo experiments was ~1 × 10⁷ cfu. This is the minimal inoculum needed to reproducibly cause pneumonia (37) and is similar to that used in other pulmonary infection models (27, 32, 50). At the time of harvest, mean BAL *E. coli* titers were 1.5 × 10⁶ ± 4.1 × 10⁵ cfu/ml and total lung titers were 1.1 × 10⁸ ± 4.9 × 10⁷ cfu. In humans a quantitative BAL culture of ≥10⁵–10⁷/ml supports the diagnosis of ventilator-associated pneumonia (29, 56). In in vitro studies, neutrophils started to demonstrate necrotic changes with a bacterial titer 2 × 10⁵/ml (Fig. 4); however, given the surface area of the lung, it is unclear how the measured in vivo titers equate to cfu per milliliter. In addition, we appreciate that it may be difficult to visualize apoptotic neutrophils ex vivo due to their rapid clearance by pulmonary macrophages. However, BAL cellular caspase-3/7 activity (>95% of BAL cells were neutrophils) was significantly less than the activity observed in in vitro studies that utilized the same number of cells (Figs. 3 and 6A). It is also possible that neutrophils are protected from apoptosis by the ensuing inflammatory milieu within the lung (3, 17). Together, these data support necrosis/lysis as the primary action of Hly on neutrophils in vivo. Whether Hly-mediated apoptosis occurs in other cells, at other sites of infection, or at lower titers in vivo awaits further study.

The biologic ramifications of Hly’s mediating neutrophil necrosis/lysis are less clear, but the pathogenesis of pneumonia can be affected on at least two levels. First, Hly’s mediating neutrophil necrosis/lysis would be predicted to affect phagocytosis and bactericidal activity. In support of that supposition are data from a previous study from our laboratory, which demonstrated that neutrophils contributed to the clearance of *E. coli* in the rat pneumonia model (43). In that study, neutrophil-depleted and neutrophil-replete animals were compared. Second, the fate of pulmonary neutrophils is not only important for host defense, but also for lung injury. Under normal physiological conditions, pulmonary neutrophils undergo apoptosis and clearance by macrophages (11, 16). However, a significant number of neutrophils undergoing necrosis/lysis may contribute to pulmonary injury via release of histotoxic granule contents (23). In addition, necrosis, as opposed to apoptosis, is
proinflammatory. Data from this study demonstrate that Hly mediated lung injury (Table 1). However, whether this damage is direct or indirect and whether neutrophil necrosis is contributory are unclear. We have previously established that pulmonary damage correlated with bacterial titer but not pulmonary myeloperoxidase (43). This finding suggests, but does not establish, that acute lung injury in E. coli pneumonia is mediated by either bacterial or nonneutrophil host factors. A more complete understanding of the consequences of neutrophil necrosis due to Hly may lead to a decrease in acute lung injury and improved outcomes from gram-negative pneumonia.

GRANTS

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