Surfactant dysfunction and lung injury due to the *E. coli* virulence factor hemolysin in a rat pneumonia model

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Surfactant dysfunction and lung injury due to the *E. coli* virulence factor hemolysin in a rat pneumonia model. *Am J Physiol Lung Cell Mol Physiol* 292: L632–L643, 2007; doi:10.1152/ajplung.00326.2006.—This study tests the hypothesis that the virulence factor hemolysin (Hly) expressed by extraintestinal pathogenic *Escherichia coli* contributes to surfactant dysfunction and lung injury in a rat model of gram-negative pneumonia. Rats were instilled intratracheally with CP9 (wild type, Hly-positive), CP9*hlyA* (Hly-minus), CP9/pEK50 (supraphysiological Hly), or purified LPS. At 6 h postinfection, rats given CP9 had a decreased percentage content of large surfactant aggregates in cell-free bronchoalveolar lavage (BAL), decreased large aggregate surface activity, decreased PaO2/FIO2 ratio, increased BAL albumin/protein levels, and increased histological evidence of lung injury compared with rats given CP9*hlyA* or LPS. In addition, rats given CP9/pEK50 or CP9 had decreased large aggregate surface activity, decreased PaO2/FIO2 ratios, and increased BAL albumin/protein levels at 2 h postinfection compared with rats given CP9*hlyA*. The severity of permeability lung injury based on albumin/protein levels in BAL at 2 h was ordered as CP9/pEK50 > CP9 > CP9*hlyA* > normal saline controls. Total lung titers of bacteria were increased at 6 h in rats given CP9 vs. CP9*hlyA*, but bacterial titers were not significantly different at 2 h, indicating that increased surfactant dysfunction and lung injury were associated with Hly as opposed to bacterial numbers per se. Further studies in vitro showed that CP9 could directly lyse transformed pulmonary epithelial cells (H441 cells) but that indirect lysis of H441 cells secondary to Hly-induced neutrophil lysis did not occur. Together, these data demonstrate that Hly is an important direct mediator of surfactant dysfunction and lung injury in gram-negative pneumonia.

Lung surfactant dysfunction; *Escherichia coli*; bacterial virulence

PNEUMONIA IS THE SECOND MOST frequent hospital-acquired infection, with ~300,000 cases occurring per year in the United States (8, 17, 32, 33, 55). More than 60% of nosocomial pneumonias are caused by gram-negative enteric bacilli, with *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Enterobacter sp.*, *Acinetobacter*, and *Stenotrophomonas* being most commonly isolated (8–10, 32). Gram-negative bacteria can induce severe lung disease and respiratory failure, leading to associated crude mortalities ranging from 24–76% and estimated attributable mortalities ranging from 20–50% (2, 8, 32, 55). These mortality figures translate into ~36,000–80,000 deaths annually in the United States (2, 8, 32, 55), with relatively little improvement in mortality reported over the last 10–15 years. The estimated cost of nosocomial pneumonias caused by gram-negative bacilli in this country is greater than 1 billion dollars per year (43, 55, 57).

Although the goal of host defense is to eradicate invading pathogens, an overexuberant or prolonged proinflammatory phase may result in host-mediated lung injury (16, 34, 51, 60). Therefore, the ideal host response is to maximize bacterial clearance and minimize host-mediated collateral damage to host tissue. Gram-negative bacilli commonly contain virulence factors that resist bacterial clearance and also can contribute to pulmonary damage (6, 15, 48, 58). Hemolysin (Hly) is the most common toxin present in strains of *E. coli* associated with extraintestinal infection (25). Hly is a pore-forming extracellular toxin from the RTX (repeats-in-toxin) family that has been shown to mediate death via apoptosis or necrosis depending on concentration in a wide range of cell types including macrophages, epithelial cells, and neutrophils (6, 56). Hly is present in a number of pathogens responsible for gram-negative pneumonia, including *E. coli*, *Proteus sp.*, *Klebsiella sp.*, *Serratia marcescens*, and *P. aeruginosa* (6). Animal models have investigated the role of Hly in the pathogenesis of urinary tract infection (35, 39), but the mechanisms by which this bacterial factor contributes to the pathogenesis of pneumonia at the cellular level in vivo are unclear. This paper examines the importance of Hly in increasing the severity of surfactant dysfunction and lung injury in a rat model of gram-negative pneumonia.

The role of Hly in acute pulmonary injury and surfactant dysfunction is studied using the live *E. coli* strain CP9, a Hly-positive wild-type extraintestinal pathogenic *E. coli* (ExPEC), and two isogenic derivatives either deficient in Hly (CP9*hlyA*) or overexpressing Hly (CP9/pEK50). The primary hypothesis tested is that the presence of Hly in ExPEC increases the severity of surfactant dysfunction and lung injury following intratracheal inoculation of rats with these microorganisms. Our prior work has shown that Hly contributes to lung injury in rats given ExPEC and induces neutrophil necrosis/lysis both in vitro and in vivo (47). The present paper...
extends this work to focus on effects of Hly on surfactant dysfunction involving large aggregate content and surface activity in rats with E. coli pneumonia. Lung surfactant is known to be abnormal in activity or composition in several types of acute pulmonary injury (see Refs. 37 and 54 for review), but little information is available on the specific influence of Hly on surfactant aggregate content and activity as studied here. Additional complementary in vitro experiments address whether Hly directly damages the integrity of cultured H441 cells or indirectly injures these cells via Hly-mediated neutrophil lysis. These latter studies are included as a first step in assessing whether lung epithelial cell damage resulting directly or indirectly from Hly could be one potential contributor to aggregate-related surfactant dysfunction in E. coli pneumonia in vivo.

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 described in detail previously (24, 42). CP9 possesses many of the characteristics of typical ExPEC strains (44) and has been shown to be highly virulent in a urinary tract infection model (41), an intraperito-
neal infection model (45), and a pneumonia model (40, 46). CP9hlyA is a TnphoA1-generated derivative of CP9 in which the structural gene for hemolysin (hlyA) is disrupted. CP9hlyA has previously been confirmed to be non-hemolytic after growth on blood agar plates and by an absence of red blood lysis in a quantitative hemolysis assay (49). Stability of the genotype/phenotype of CP9hlyA was confirmed by assessing hemolysin production. CP9pEK50 is a derivative that produces supraphysiological levels of Hly by virtue of possessing the hlyA gene. CP9pEK50 was generated derivative of CP9 in which the structural gene for hemolysin (hlyA) is disrupted. CP9hlyA has previously been confirmed to be non-hemolytic after growth on blood agar plates and by an absence of red blood lysis in a quantitative hemolysis assay (49). Stability of the genotype/phenotype of CP9hlyA was confirmed by assessing hemolysin production. CP9pEK50 is a derivative that produces supraphysiological levels of Hly by virtue of possessing the hlyA gene.

**E. coli titer measurements.** Total lung titers of E. coli at 2 and 6 h were determined by enumerating bacteria in both BAL and post-BAL lung tissue. Lungs were excised intact, weighed, suspended in normal saline to a total weight of 10 g (assumed to equate to 10 ml), and homogenized on ice (3 bursts of 3-s duration each) using a Polytron PT-2000 homogenizer (Brinkman Instruments, Westbury, NY). Serial 10-fold dilutions of BAL and post-BAL lung homogenates were performed in PBS and cultured to assess bacterial cfu in duplicate on LB plates at 37°C. Titers of E. coli (cfu/ml) were determined in tissue and BAL, multiplied by the appropriate volume (10 ml for the lung homogenate or the recovered volume of BAL), and summed to yield the total lung titer in cfu/lung.

**Histological evaluations of lung injury.** Histological evaluations of lung injury severity were performed on lung tissue obtained from subgroups of rats not subjected to BAL. Lungs were removed en bloc following tracheal cannulation and flushing of the vasculature with 20 ml of HBSS injected into the right ventricle. The lungs were fixed with 10% neutral buffered formalin at an inflation pressure of 20 cmH2O for 24 h, and gross sections (3 from the left lung and 1 from each of the right lung lobes) were paraffin-embedded. Thin sections (4 μm) were cut and stained with hematoxylin and eosiin using standard methods (26). Slides were evaluated for the severity of lung tissue injury by an experienced pathologist (Dr. James Woytash, Dept. of Pathology, SUNY at Buffalo) who was blinded to animal group assignments.

**Total protein and phospholipid content, albumin concentration, and surfactant aggregate content of cell-free BAL.** Total phospholipid in cell-free BAL was measured by the phosphomolybdate assay of Ames (1), and total protein was determined by the method of Lowry et al. (29) modified by the addition of 15% SDS to allow accurate quantitation in the presence of lipid. Albumin concentrations in cell-free BAL were measured by ELISA using a polyclonal rabbit anti-mouse albumin antibody (a gift from Dr. Daniel Remick, Univ. of Michigan, Ann Arbor, MI) and horseradish peroxidase-labeled goat anti-rabbit immunoglobulin (Pharmingen, San Diego, CA) (11). Rat albumin (Sigma, St. Louis, MO) was used as a standard. Additional studies examined the content and activity of large surfactant aggregates isolated from cell-free BAL by centrifugation at 12,500 rpm (3 from the left lung and 1 from each of the right lung lobes) were paraffin-embedded. Thin sections (4 μm) were cut and stained with hematoxylin and eosiin using standard methods (26). Slides were evaluated for the severity of lung tissue injury by an experienced pathologist (Dr. James Woytash, Dept. of Pathology, SUNY at Buffalo) who was blinded to animal group assignments.

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**Pulsating bubble measurements of surfactant activity.** The surface activity of large surfactant aggregates was assessed during cycling at a physiological rate of 20 cycles/min at 37 ± 0.5°C on a pulsating bubble surfactometer (General Transco, Largo, FL; formerly Electro-nectics, Buffalo, NY) (14). A small air bubble, communicating with ambient air, was formed in a 40-μl aliquot of surfactant in a plastic sample chamber mounted on the pulsator unit of the surfactometer. The bubble was oscillated between maximum and minimum radii of 0.55 and 0.4 mm while the pressure drop across the air-water interface...
was measured with a precision pressure transducer. Surface tension at minimum bubble radius (minimum surface tension) was calculated as a function of time of pulsation from the measured pressure drop at end-compression and the Laplace equation for a spherical interface (14, 19). Surfactant samples were examined at a uniform phospholipid concentration of 1 mg/ml in 150 mM NaCl + 2 mM CaCl$_2$, pH 7.0.

**In vitro assessments of the direct effects of Hly on H441 cells.** H441 cells, a human pulmonary adenocarcinoma epithelial cell line (American Type Culture Collection, Rockville, MD), were propagated in RPMI 1640 medium supplemented with 2 mM l-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate (American Type Culture Collection, Manassas, VA), and 10% heat-inactivated fetal calf serum (GIBCO/Invitrogen, Grand Island, NY). The medium also contained 100 units of penicillin and 100 µg of streptomycin per ml (GIBCO/Invitrogen). Cells were grown to confluence in 75-cm$^2$ tissue culture flask (Corning, Corning, NY). H441 cells were detached with a 0.25% trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO) and resuspended in 20% plasma-PBS (pH 7.4). H441 cell suspensions were placed in a 24-well tissue culture plate (5 × 10$^5$ cells/250 µl media per well; Nunc, Rochester, NY) and incubated at 37°C for 1 h with 1.0 × 10$^5$ cfu (in 250 µl PBS) of either CP9 (Hly-positive), CP9hlyA (Hly-minus), or PBS alone. Following incubation, the tissue culture plate was centrifuged at 110 g at 4°C for 6 min (Sorvall Legend RT centrifuge), and the supernatant was removed and retained on ice at 4°C. Utrapure formaldehyde (2% in a volume of 500 µl) was added to the wells, and the plate was incubated for 20 min at 4°C. The cells were removed from the wells, followed by the addition of another 500 µl of 2% formaldehyde to the wells and incubation at 4°C for 10 min to ensure maximal recovery of cells. Aliquots of cells from a given well were subsequently pooled, washed once with 3 ml of PBS, and resuspended in 500 µl of PBS, and 86 µl of PBS was added containing 1 × 10$^5$ sulforhodamine-impregnated polystyrene beads (6-µm diameter beads; Polysciences, Warrington, PA). H441 cell counts were determined by assessing the number of cells in each final suspension using a flow cytometer (Becton Dickinson Biosciences Immunocytometry Systems, San Jose, CA). Data acquisition was terminated after 1,000 bead events were detected per sample to ensure that an equal volume of cell suspension was analyzed for each sample (each sample contained the same bead concentration). A gate in the forward-scatter vs. side-scatter plot discriminated H441 cells and beads from bacteria and “debris,” and a subsequent backgating of the side-scatter vs. FL2 channel of the cytometer discriminated H441 cells from the beads so that epithelial events could be determined. Results were presented as the percent of H441 cells remaining after exposure to the various *E. coli* strains compared with H441 cells exposed to PBS alone.

**In vitro assessments of Hly-induced neutrophil lysis on H441 cells.** To determine whether Hly-mediated lysis of neutrophils could indirectly lyse epithelial cells, tissue culture plate well inserts (#136935, Corning, Corning, NY) and incubated at 37°C for 1 h, and cells in the bottom wells were then collected and counted by flow cytometry as described above under in vitro assessments of the direct effects of Hly on H441 cells.

**Statistical analyses.** Graphic displays and descriptive statistics for all parameters measured at each time point for each group were expressed as means ± SE. Pairwise comparisons were made using Student’s t-test with α = 0.05 and adjusted for multiple comparisons such that $P < 0.05$ (number of comparisons) was considered significantly different.

**RESULTS**

Hly mediates a significant increase in lung injury at 6 h in rats. To determine the effects of Hly on lung injury in vivo, rats initially underwent challenge with CP9 (wild-type, Hly-positive), its isogenic derivative CP9hlyA (Hly-minus), or normal saline. A fourth group of rats received purified LPS for the purposes of comparison since this bacterial component has been used as a surrogate for bacteria in previously described studies on gram-negative pneumonia (3, 4, 36). Rats instilled intratracheally with CP9, CP9hlyA, or LPS had differing degrees of lung injury at 6 h postinoculation. Arterial oxygenation ($PaO_2/FIO_2$ ratio) was reduced to the greatest extent at 6 h in rats that received CP9 (67 ± 9 mmHg) compared with CP9hlyA (243 ± 54 mmHg) or LPS (237 ± 70 mmHg) ($P < 0.05$, Fig. 1). $PaO_2/FIO_2$ ratios in rats given CP9hlyA or LPS were, however, lower than those found for control rats instilled with normal saline (411 ± 41 mmHg) ($P < 0.05$, Fig. 1). Arterial oxygenation in rats given CP9 met the criteria for clinical acute respiratory distress syndrome (ARDS) at 6 h, whereas rats given CP9hlyA or LPS had $PaO_2/FIO_2$ ratios consistent with clinical acute lung injury (ALI) based on the definitions of the American-European Consensus Committee (5).

Rats receiving CP9 also had more severe lung injury based on increased leakage of albumin into the alveolar spaces compared with rats given CP9hlyA or LPS (Fig. 2A). Albumin concentrations by ELISA in cell-free BAL at 6 h were more than twice as large for rats given CP9 (2,604 ± 252 µg/ml) compared with rats given CP9hlyA (1,189 ± 130 µg/ml) ($P < 0.0005$, Fig. 2A). Rats given LPS or saline had much lower BAL albumin concentrations (216 ± 13 µg/ml for LPS and 97 ± 19 µg/ml for saline, $P = 0.0005$ compared with Fig. 2A). Rats given CP9 also had increased total BAL protein based on colorimetric assay compared with rats given...
Compared with those receiving LPS or saline \((P < 0.0005\) for saline and \(P < 0.023–0.0001\) for LPS, Fig. 3).

The decreased arterial oxygenation and higher levels of albumin/protein in BAL in CP9-infected rats in Figs. 1–3 correlated with histological evidence of lung injury at 6 h postinfection (Fig. 4). Lung injury and cellular influx were not apparent in lung sections of rats instilled with normal saline (Fig. 4A). Lung sections of animals that received LPS (Fig. 4B), CP9 (Fig. 4C), or CP9\(^{hlyA}\) (Fig. 4D) had similar degrees of cellular influx (mainly neutrophils) and perivascular edema around blood vessels associated with bronchi and bronchioles. However, the rank order of lung injury as demonstrated by disruption of the alveolar-capillary wall architecture, extravasated red blood cells, and intra-alveolar deposition of fibrin and cellular debris was CP9 > CP9\(^{hlyA}\) > LPS.

**Hly mediates significant lung surfactant abnormalities in rats at 6 h.** Surfactant dysfunction was assessed by measurements of large aggregate percent content and surface activity in lavage from rats instilled intratracheally with CP9, CP9\(^{hlyA}\), or LPS (Figs. 5 and 6). Amounts of total phospholipid in cell-free BAL at 6 h were increased for rats given CP9 \((0.66 \pm 0.04 \text{ mg})\) or CP9\(^{hlyA}\) \((0.50 \pm 0.04 \text{ mg})\) compared with LPS \((0.41 \pm 0.03 \text{ mg})\) or saline \((0.37 \pm 0.02 \text{ mg})\) \((P < 0.05\) or less). However, a much lower percentage of total phospholipid was contained in the large surfactant aggregate fraction in BAL from infected animals. The relative severity of surfactant dysfunction in terms of large aggregate percent content was ordered as CP9 \((\text{worst}) > CP9^{hlyA} > LPS\) (Fig. 5). Rats given CP9 had only 28 ± 2% of total BAL phospholipid in the large aggregate fraction compared with 43 ± 1% for rats given CP9\(^{hlyA}\), 46 ± 1% for rats given LPS, and 61 ± 3% for control rats given saline (all values are \(P < 0.0005\) or less compared with CP9, Fig. 5). The surface activity of large surfactant aggregates obtained at 6 h from rats given CP9, CP9\(^{hlyA}\), or LPS was also significantly reduced compared with aggregates from control rats given normal saline (Fig. 6). Impairments in the surface tension lowering ability of large aggregates had the same relative order of severity of CP9 \((\text{worst}) > CP9^{hlyA} > LPS > \text{saline}\) found for large aggregate content (Fig. 5). Surface tension lowering curves for each of the four groups were significantly different from each other \((P < 0.0001\) or less for all group comparisons at a given time of bubble pulsation) (Fig. 6). After 20 min of bubble pulsation, resuspended large aggregate surfactant obtained at 6 h postinfection from rats given CP9 reached minimum surface tension values of 20.9 ± 0.7 mN/m compared with <1 mN/m for large aggregates from control rats given saline. At the same 20-min time of pulsation, minimum surface tension values for large aggregates from rats given CP9\(^{hlyA}\) were also significantly elevated at 13.6 ± 1.1 mN/m, whereas large aggregates from rats given intratracheal LPS had minimum surface tensions of 4.4 ± 1.4 mN/m that were only slightly elevated over controls.

**Hly increases the survival of E. coli in the rat pneumonia model at 6 h.** To assess the effects of Hly on bacterial survival at 6 h postchallenge, titers of CP9 and CP9\(^{hlyA}\) were measured. After challenge, wild-type CP9 \(E. \text{coli}\) were able to grow within the lungs, increasing in titer from 1.44 ± 0.02 × 10^7 \text{ cfu} to 9.1 ± 3.1 × 10^7 \text{ cfu} at 6 h postinoculation (total lung titer). In contrast, the isogenic Hly-deficient derivative CP9\(^{hlyA}\) underwent clearance, decreasing in titer from 1.18 ± 0.12 × 10^7 \text{ cfu} to 5.6 ± 1.6 × 10^6 \text{ cfu} by 6 h.
postinfection (Fig. 7). These data show that Hly contributes to the survival of *E. coli* within the lungs at 6 h but also suggest that some of the differences in surfactant and lung injury found above at this time between CP9 and CP9hlyA might have been influenced by the higher titer of CP9. To address this potential confounding variable, additional studies were carried out at 2 h postchallenge when the total lung titers of CP9 and CP9hlyA were not significantly different (1.76 ± 0.23 × 10⁸ cfu for CP9 and 1.37 ± 0.30 × 10⁷ cfu for CP9hlyA, Fig. 7). In addition, studies at 2 h also utilized the Hly overexpressing *E. coli* strain CP9/pEK50 at a slightly lower titer (6.1 ± 0.4 × 10⁸). The results of experiments at 2 h are reported below.

Severity of lung injury and surfactant dysfunction at 2 h in rats given CP9, CP9hlyA, CP9/pEK50, or saline. Experiments at 2 h postinfection utilized CP9 (wild-type, Hly-positive), CP9hlyA (Hly-minus), CP9/pEK50 (supraphysiological levels of Hly), and normal saline as a control. Rats infected with CP9/pEK50 or CP9 had decreased PaO₂/FIO₂ ratios and increased BAL albumin/protein concentrations at 2 h postbacterial challenge compared with rats given CP9hlyA (Fig. 8, A–C). Rats given CP9/pEK50 or CP9 had similar PaO₂/FIO₂ ratios of 163 ± 33 mmHg and 189 ± 25 mmHg, respectively, both of which met the oxygenation criteria for clinical ARDS (5). These PaO₂/FIO₂ ratios were significantly lower than found for rats given CP9hlyA (346 ± 51 mmHg, *P* < 0.01) or saline (384 ± 15 mmHg, *P* < 0.0005 (Fig. 8A). Albumin and total protein contents in cell-free BAL at 2 h postinfection were ordered as CP9/pEK50 (worst) > CP9 > CP9hlyA > saline controls. The concentration of albumin by ELISA for rats receiving CP9/pEK50 (1,890 ± 200 μg/ml) was greater than that of rats given CP9 (1,110 ± 104 μg/ml) (*P* < 0.01, Fig. 8B). Albumin concentrations for rats receiving CP9hlyA (584 ± 99 μg/ml) were significantly lower than for rats given either CP9/pEK50 (*P* < 0.0005) or CP9 (*P* < 0.01) and significantly larger than for saline controls (102 ± 34 μg/ml) (*P* < 0.0005) (Fig. 8B). Total protein levels in BAL by colorimetric assay followed a pattern identical to albumin (absolute levels of total protein and albumin were again not directly comparable because of assay differences). Total BAL protein was larger for rats given CP9/pEK50 (1,914 ± 115 μg/ml) compared with CP9 (1,533 ± 75 μg/ml) (*P* < 0.01, Fig. 8C). Total BAL protein for rats given CP9hlyA (679 ± 73 μg/ml) was significantly lower compared with CP9/pEK50 (*P* < 0.00005) or CP9 (*P* < 0.00005) and significantly higher than control rats given saline (112 ± 13 μg/ml) (*P* < 0.00005, Fig. 8C). Stained lung tissue sections at 2 h indicated a relative histological severity of injury ordered as CP9/pEK50 > CP9 > CP9hlyA, with alveolar-capillary wall destruction at this early time point present only in animals challenged with the supraphysiological, Hly-producing strain CP9/pEK50 (data not shown).

Consistent with the severe lung injury in rats given either CP9/pEK50 or CP9, both groups had significant detriments
in the surface activity of large surfactant aggregates on the pulsating bubble apparatus (Fig. 9). Minimum surface tension values after 20 min of bubble pulsation were 16 ± 1 mN/m (CP9/pEK50), 15 ± 1 mN/m (CP9), 10 ± 1 mN/m (CP9hlyA), and 1 ± 0 mN/m (saline) (Fig. 9). Surface tension lowering curves for large aggregates from rats given CP9/pEK50 or CP9 were not statistically different from one another at bubble pulsation times of ≥ 5 min. However, surface tension lowering curves for large aggregates from rats given CP9hlyA or saline were statistically different from each other as well as from both CP9/pEK50 and CP9 at all.
time points of bubble pulsation ($P < 0.0005$ or less at a fixed time of pulsation).

In vitro studies showing that Hly lyses H441 cells directly, but not indirectly via Hly-mediated neutrophil lysis. It has recently been reported from our laboratory that Hly directly damages human neutrophils in vitro and can cause neutrophil necrosis in rats in vivo (47). This prior work suggests that Hly might mediate pulmonary damage either directly or indirectly via the release of histotoxic components from neutrophils. To test these possibilities in terms of potential lung epithelial cell lysis, a set of studies was done using the transformed H441 pulmonary epithelial cell line. First, the direct effects of Hly on H441 cells were studied by exposing these cells in culture to bacterial strains differing in their production of Hly and assessing lysis following 60 min of incubation at 37°C. The magnitude of H441 lysis was significantly greater when these cells were exposed to CP9 compared with the isogenic Hly-minus derivative CP9hlyA (Fig. 10).

A final set of experiments addressed whether Hly-mediated neutrophil lysis could indirectly (secondarily) cause the lysis of H441 cells. To accomplish this, a trans-well assay system was utilized as detailed in MATERIALS AND METHODS. In brief, the top well of the system contained either CP9 alone, neutrophils...
alone (PMNs), or CP9 and neutrophils (this latter condition generated neutrophil lysis that was a required condition of the assay). The bottom well contained either H441 epithelial cells (Fig. 11, A or B, PMNs in the top well) or neutrophils (Fig. 11B, PMNs in the bottom well). When CP9 alone or neutrophils were in the top well, or CP9 and neutrophils were in the top well, there was no significant difference in the percent change in H441 cells in the bottom well (means ± SE for n = 6 animals).

Fig. 10. E. coli Hly directly lyases H441 epithelial cells. Live CP9 (wild-type, Hly-positive) or CP9hlyA (Hly-negative) E. coli were directly interacted with H441 epithelial cells at 37°C for 60 min. Following incubation, cells were fixed with formaldehyde and counted via flow cytometry (see MATERIALS AND METHODS). Data are shown as the percent of H441 cells remaining after exposure to the two E. coli strains (means ± SE, n = 4). *P < 0.05 compared with CP9, paired t-test.

Fig. 11. Indirect effect of Hly-mediated lysis of neutrophils on the lysis of H441 epithelial cells. A: results are shown for H441 epithelial cells in the bottom well, and either CP9 alone, neutrophils alone (PMNs), or both in the top well. Histotoxic factors in the top well were capable of diffusing into the bottom well (see B). After incubation for 60 min at 37°C, H441 cells in the bottom well were fixed with formaldehyde and counted by flow cytometry. There was no significant difference in the percent change in H441 cells in the bottom well when CP9 alone, neutrophils alone, or both were in the top well (means ± SE for n = 3). B: results in this panel confirm that the trans-well system was working properly. For each experiment, it was confirmed that CP9 lysed neutrophils in the top well [lysis of 87 ± 7% of PMNs; column labeled “PMNs (top well)“]. It was also confirmed that histotoxic molecules were able to diffuse into the bottom well. The column labeled “PMNs (bottom well)” represents the case when CP9 and neutrophils were in the top well and neutrophils (not H441 cells) were in the bottom well. The release of histotoxic molecules from lysis of neutrophils in the top well resulted in lysis of neutrophils in the bottom well (56 ± 6% of neutrophils in the bottom well).
phils alone were in the top well, no lysis of H441 cells in the bottom well occurred (Fig. 11A). In addition, no lysis of H441 cells in the bottom well was observed when CP9 and neutrophils were present together in the top well (conditions that resulted in neutrophil lysis) (Fig. 11A). Thus, under the conditions of this in vitro assay, Hly-mediated lysis of neutrophils did not cause indirect lysis of H441 cells. To confirm that histotoxic components of lysed neutrophils were able to diffuse from the top well into the bottom well, purified human neutrophils were placed in some of the bottom wells. In contrast to what was observed with H441 cells, neutrophils in the bottom well were significantly lysed when both neutrophils and CP9 were in the top well (Fig. 11B, PMNs in the bottom well). In addition, the lysis of neutrophils in the top well when CP9 bacteria were also present was confirmed for each experiment (Fig. 11B, PMNs in the top well).

**DISCUSSION**

This paper has examined the effects of the *E. coli* virulence factor Hly on surfactant dysfunction and acute pulmonary injury in rats in vivo using a wild-type extraintestinal pathogenic strain of *E. coli* (CP9) and isogenic derivatives that varied in their production of Hly, i.e., CP9hlyA (Hly-minus) or CP9/pEK50 (supraphysiological Hly). The presence of Hly in CP9 was shown to increase surfactant dysfunction based on reductions in large aggregate percent content and surface activity (Figs. 5, 6, and 9) as well as to worsen the severity of lung injury based on Pao2/Fio2 ratio, albumin/protein levels in BAL, and histological analysis of stained tissue sections compared with rats given CP9hlyA at 2 and 6 h postinfection (Figs. 1–4, and 8). In addition, rats given the Hly overproducing CP9/pEK50 mutant had the most severe permeability lung injury (highest levels of albumin/protein in BAL) of any group studied at 2 h postinfection (Fig. 8, B and C). Although rats challenged with CP9hlyA had less prominent surfactant deficits and lung injury severity than animals given CP9 or CP9/pEK50, these animals still had reduced aggregate surface activity, increased BAL protein, and impaired arterial oxygenation compared with saline controls (Figs. 1–6, 8, and 9). This indicates that factors in addition to Hly also contribute to pulmonary pathology in *E. coli* pneumonia. Surfactant abnormalities and lung injury were relatively small in rats challenged with LPS as opposed to live CP9 or CP9hlyA bacteria, showing that this purified toxin is a poor surrogate for tracheally instilled live microorganisms in studying lung injury in pneumonia models.

In addition to investigating the influence of Hly on surfactant dysfunction and lung injury, experiments also assessed the effects of this virulence factor on bacterial clearance. The presence of Hly in CP9 led to higher total lung titers of this bacterium at 6 h, but not at 2 h, after intratracheal inoculation compared with rats given the Hly-minus mutant CP9hlyA (Fig. 7). This difference in bacterial titers was a potential confounding factor in establishing the contributions of Hly to surfactant dysfunction and lung injury at the 6-h time point. However, the fact that the same pattern of decreased large aggregate surface activity and increased lung injury severity for CP9 vs. CP9hlyA was found at 2 h postinfection (when the titers of the organisms were the same) clearly demonstrates that surfactant and pulmonary abnormalities were associated with the presence of Hly instead of with larger numbers of CP9 bacteria per se. This interpretation is also consistent with results showing that infection with the Hly overproducing mutant CP9/pEK50 generated even greater permeability lung injury than CP9 at 2 h postinfection (Fig. 8, B and C). In addition, in vitro studies showed that CP9 was able to directly induce the lysis of H441 transformed pulmonary epithelial cells (Fig. 10). Together, these data demonstrate that Hly is an important virulence factor in *E. coli* pneumonia and that its presence directly contributes to surfactant dysfunction based on decreased large aggregate content and surface activity and also to increased lung permeability injury.

The magnitude of the surface activity deficits measured for large surfactant aggregates in our study likely underestimates the actual functional deficits in surface tension lowering that occurred in the alveoli of *E. coli*-infected animals in vivo. This is because our experiments examined the intrinsic surface activity of large surfactant aggregates resuspended at a uniform concentration in 0.15 M NaCl + 2 mM CaCl2 for bubble surfactometer assessments to facilitate activity comparisons between groups. However, the original BAL fluid recovered from injured animals contained substantial amounts of albumin (Figs. 2 and 8), which along with other plasma proteins is known to inhibit lung surfactant surface activity (e.g., Refs. 22, 23, 37). It is highly probable that surface activity deficits would have been more severe than reported in Figs. 6 and 9 if all of the original inhibitory BAL protein had remained present during bubble measurements. This effect would be expected to be most pronounced in BAL from rats infected with CP9 or CP9/pEK50, which originally contained the greatest amounts of protein (Figs. 2 and 8). The decreases found in the intrinsic surface activity of large aggregates in the present work likely were caused in part by plasma proteins that became associated with surfactant aggregates in BAL and were thus present during bubble measurements. Davidson et al. (12) have recently shown that plasma proteins can become incorporated into large surfactant aggregates during gastric aspiration lung injury in rats, resulting in a decrease in the intrinsic surface activity of aggregates as found here in *E. coli* pneumonia (Figs. 6 and 9). In addition, lipids and/or apoproteins in lung surfactant aggregates from animals with inflammatory lung injury can become chemically degraded or altered in compositional ratio (12).

A second aspect of surfactant dysfunction in our experiments was a decreased percent content of large surfactant aggregates in cell-free BAL from rats receiving CP9 compared with CP9hlyA (Hly-minus) or LPS (Fig. 5). Large surfactant aggregates obtained by centrifugation from cell-free BAL from normal animals have greater surface activity and apoprotein content compared with smaller aggregates (Refs. 37 and 59 for review). Large surfactant aggregates in lavage have been reported to be depleted in percent content during several forms of acute pulmonary injury (7, 12, 18, 20, 28, 53). A variety of centrifugation methods and conditions are utilized to obtain large surfactant aggregates from cell-free BAL, and our experiments focused on an aggregate fraction obtained by simple centrifugation at 12,500 g. This centrifugation condition has been used previously to obtain highly active large surfactant aggregates for the preparation of calf lung surfactant extract, which is the basis of the clinical exogenous surfactant Infasurf...
(Ref. 37 for review). Similar centrifugation conditions have also been used in studying large surfactant aggregates in animal models of acute lung injury (e.g., Refs. 7, 12, 20, 21). In the present investigation, injured vs. control animals had substantial reductions in the percent content of this normally active large surfactant aggregate fraction relative to total BAL phospholipid at 6 h postinfection (Fig. 5). Reductions in the percent content of large surfactant aggregates in rats with *E. coli* pneumonia could result from an increased conversion to small aggregates in the alveolar hypophase, or from injury to alveolar type II pneumocytes that alters surfactant reuptake, recycling, or metabolism. In addition, decreases in the percent content of large aggregates could also reflect the entry of non-surfactant phospholipid material into the alveolar lumen from the injured pulmonary interstitium. Consistent with our prior work (40), rats infected with CP9 bacteria were found to have increased amounts of total phospholipid (as opposed to large aggregate phospholipid) in BAL. The present study did not investigate the relative importance of different mechanisms contributing to the reduced percent content of large surfactant aggregates in CP9-injured animals, and this needs to be addressed in more detail in future work.

One significant design aspect of the present experiments involved the use of live extraintestinal *E. coli* (i.e., CP9 and isogenic derivatives lacking or overexpressing Hly). The use of CP9 guaranteed a physiologically relevant level of expression for Hly in vivo as well as a physiological manner of host presentation compared with purified bacterial products. The use of live organisms also allowed the potential for direct bacterial-host cell interactions that could modify pulmonary responses by pathways not present with the use of purified bacterial components. As noted earlier, the importance of using live *E. coli* organisms was apparent when the effects of LPS were compared with live *E. coli*. Surfactant dysfunction and lung injury were significantly less severe in animals instilled with LPS (3.6 mg/kg body wt) compared with live CP9 and CP9*hlyA* bacteria (Figs. 1–6).

Another technical issue in this study was the use of H441 cells to assess whether the effects of Hly on cell injury were directly or indirectly mediated by Hly-induced neutrophil lysis. Mechanisms by which Hly might mediate pulmonary damage potentially include causing direct injury or lysis of lung cells or, alternatively, inducing such effects indirectly via Hly-mediated release of histotoxic components from neutrophils (47). As noted earlier, Hly in CP9 had a direct effect in increasing the lysis of H441 epithelial cells compared with the Hly-negative strain CP9*hlyA* (Fig. 10). However, some lysis of H441 cells still occurred for the latter Hly-negative strain compared with saline controls, indicating that bacterial virulence factors other than Hly also promote epithelial cell lysis. It is also noteworthy that while Hly in CP9 did increase H441 cell lysis in vitro (Fig. 10), relatively high bacterial titers of $1 \times 10^9$ cfu/ml were required. Unpublished data from our laboratory indicate that 2 logs less of CP9 are able to nearly completely lyse freshly purified rabbit alveolar epithelial cells in vitro. The high titers of CP9 needed to lyse H441 cells in the present experiments indicate that this transformed pulmonary cell line may have increased resistance to bacteria-induced lysis compared with normal epithelial cells. Thus our inability to demonstrate that Hly-induced neutrophil lysis could secondarily cause the lysis of H441 cells in vitro (Fig. 11) does not exclude the possibility that such an effect could occur with pulmonary epithelial cells in vivo. Further studies will be required to investigate this issue as well as the mechanistic contributions of specific pulmonary inflammatory/immune responses to surfactant dysfunction and cellular injury in the rat *E. coli* pneumonia model.

In summary, the results of this study demonstrate that substantial surfactant dysfunction is present in the pathophysiology of *E. coli*-induced lung injury and is worsened in the presence of the bacterial virulence factor Hly. Pulmonary surfactant plays essential roles in reducing the work of breathing, normalizing lung mechanics and gas exchange, and protecting against edema (37, 38). The finding that lung surfactant abnormalities contribute functionally to *E. coli*-induced pneumonitis suggests that exogenous surfactant replacement therapy may prove beneficial in treating this condition. A number of studies have demonstrated that exogenous surfactant supplementation interventions can improve lung function and/or outcome in animal models of ALI/ARDS associated with pulmonary bacterial infection or endotoxin administration (e.g., Refs. 13, 30, 31, 50, 52). Studies on the efficacy of natural and synthetic exogenous lung surfactant formulations in improving lung function and outcome in gram-negative pneumonia in the rat model examined in this paper are currently in progress in our laboratory.

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