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Ovine Pulmonary Surfactant Induces Killing of *Pasteurella haemolytica*, *Escherichia coli*, and *Klebsiella pneumoniae* by Normal Serum

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Pulmonary surfactant has been shown to play an increasingly important role in bacterial clearance at the alveolar surface in the lung. This study describes a bactericidal mechanism in which ovine pulmonary surfactant induces killing of *Pasteurella haemolytica* by normal serum. To demonstrate killing, six bacterial species were incubated first with pulmonary surfactant for 60 min at 37°C and then with serum for an additional 60 min at 37°C. *P. haemolytica* type A1 strains 82-25 and L101, a *P. haemolytica* type 2 strain, *Escherichia coli*, and *Klebsiella pneumoniae* were susceptible and *Pasteurella multocida*, *Serratia marcescens*, and *Pseudomonas aeruginosa* were not susceptible to killing by ovine pulmonary surfactant and normal serum. No bacteria incubated with bovine pulmonary surfactant were killed by normal serum. Although the species origin of pulmonary surfactant was selective, the species origin of serum was not. *P. haemolytica* incubated with ovine pulmonary surfactant was killed by fetal calf serum, gnotobiotic calf serum, pooled normal sheep serum, pooled normal rabbit serum, and pooled guinea pig serum. Ultrastructurally, killed *P. haemolytica* suspensions contained dead cells and cells distorted with vacuoles between the cytoplasmic membrane and the cytoplasm. The mechanism of killing did not correlate with concentrations of complement or lysozyme or titers of residual antibody in either the pulmonary surfactant or the serum, and killing was reduced by preincubation of surfactant with *P. haemolytica* lipopolysaccharide. Preliminary characterization of both surfactant and serum implicate a low-molecular-weight proteinaceous component in the surfactant and serum albumin in the serum. This mechanism may help clear certain gram-negative bacteria from the lungs of sheep as a part of the pulmonary innate defense system.

Pasteurella haemolytica is a gram-negative bacterium that selectively inhabits the upper respiratory tract of ruminants (4, 15, 16, 20, 29) and, during periods of stress, heavily colonizes the nasal mucosa (15). At this time, large numbers of organisms may be inhaled. In spite of this, the organism does not easily become established in the lung without predisposing factors. A cell-mediated clearance mechanism is questionable because *P. haemolytica* produces a potent leukotoxin cytotoxic for alveolar macrophages and neutrophils (27, 37). Therefore, an alternate clearance mechanism involving pulmonary secretions may be at work.

P. haemolytica is killed after incubation with antiserum as a result of a complement-mediated killing mechanism (2, 26, 33). However, it is not killed after incubation with normal serum (2). *P. haemolytica* can also be killed by bronchoalveolar lavage fluid from calves with low titers of antibody (26). In this paper, I report a bactericidal mechanism in which pulmonary surfactant renders *P. haemolytica* susceptible to killing by normal serum. This mechanism appears to be similar to that proposed by others (10, 11, 23–26) in that surfactant induces sublethal bacterial cellular injury. However, it differs considerably from those studies in the mechanism of action, which requires the presence of normal serum. This mechanism may help substantiate the results of previous studies that report the injury, but not death, of gram-negative bacteria incubated with pulmonary surfactant (11).

MATERIALS AND METHODS

Bacterial strains and cultivation. *P. haemolytica* type A1 strains 82-25 and L101, a *P. haemolytica* type 2 strain, *Pasteurella multocida* type 3A strain P-1059, *Klebsiella pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* FD type 1 (ATCC 27312), *Serratia marcescens* (ATCC 14756), and *Escherichia coli* O26:B6 (ATCC 12795) were used. *P. haemolytica* 82-25 is an isolate from ovine pneumonia, and strain L101 is an isolate from bovine pneumonia.

For the bactericidal test, strains were grown in tryptose broth at 37°C with constant stirring for 3 h. After incubation, cultures were centrifuged at 5,900 × g_{av} (average centrifugal force) for 15 min at 4°C and the bacterial pellet was resuspended in test buffer consisting of phosphate-buffered saline (PBS) containing 0.14 M NaCl, 10 mM sodium phosphate (pH 7.2), 0.5 mM $CaCl_2$, and 0.15 mM $MgCl_2$. The suspensions were adjusted in the spectrophotometer (80% transmittance, 600 nm; Coleman model 35; Bacharach Instrument Co.) to contain 1.0×10^8 CFU/ml and diluted 10^5-fold to 10^3 CFU/ml in test buffer.

Bacterial extracts. Capsular polysaccharide from *P. haemolytica* P-1075 (1) was obtained from Chris Adlam (Langford, Inc., Guelph, Canada) and reconstituted to a concentration of 1.0 mg/ml. Lipopolysaccharide (LPS) from *P. haemolytica* 82-25 (type A1) was prepared by phenol-chloroform-petroleum ether extraction as previously described (14) and reconstituted to a concentration of 1.0 mg/ml.

Preparation, treatment, and fractionation of surfactant. Two 2-year-old Columbia ewes weighing 60 to 80 kg and one 4-month-old calf weighing 160 kg were anesthetized and exsanguinated. Four rats were also anesthetized and exsanguinated. The lungs were excised and lavaged to total lung capacity with PBS, pH 7.2, containing 100 μg of gentamicin per ml. The lavage fluid was centrifuged at 200 × g_{av} for 30

min at 4°C to remove the alveolar macrophages. Ovine and bovine lavage fluid was then centrifuged at 9,150 × g_{av} for 30 min at 4°C to pellet the surfactant (6, 7). The surfactant pellet was suspended in PBS, layered over 0.7 M sucrose in PBS, pH 7.2, and centrifuged at 8,000 × g_{av} for 30 min at 4°C. The surfactant band was collected, centrifuged at 27,000 × g_{av} for 30 min at 4°C, washed twice in distilled water, and lyophilized. Rat surfactant was used directly. Each surfactant was adjusted to 1 mg (dry weight) per ml in test buffer before use. Titers of complement (8, 28, 38), lysozyme (35) concentrations, and titers of antibody to *P. haemolytica* 82-25 LPS (31) were determined in ovine surfactant and no complement, lysozyme, or specific antibody was detected.

To characterize the nature of the inducing factor, ovine surfactant was heated at 56 or 100°C for 30 min. Separate samples of surfactant (0.5 ml) were digested individually with 125 μg of proteinase K (Amresco, Solon, Ohio) at 56°C for 30 min, 1.0 mg of trypsin, 1.0 mg of phospholipase C (Sigma Chemical Co., St. Louis, Mo.) or 250 μg of phospholipase D from *Corynebacterium pseudotuberculosis* (courtesy of Glenn Songer, University of Arizona, Tucson) for 30 min at 37°C. Proteinase K, trypsin, and the phospholipases were then inactivated by heating the mixture at 100°C for 10 min.

Surfactant was also centrifuged at 100,000 × g_{av}, and the supernatant was removed. The surfactant pellet was washed three times with test buffer and again pelleted at 100,000 × g_{av}. The final pellet was resuspended to the original surfactant volume. The supernatant was filtered through a 10,000-Da-cutoff filter (Centricon 10; Amicon, Beverly, Mass.). The filtrate (0.625 ml) was then digested with 2.5 mg (25 U) of leucine aminopeptidase (Sigma Chemical Co.) for 1 h at 37°C and boiled for 10 min to inactivate the enzyme.

Surfactant was fractionated so each group of components could be checked for the inducing factor. For this, an allotted portion of surfactant was mixed with chloroform and methanol and separated into a chloroform, lipid-containing phase and a methanol-water phase as described by Bligh and Dyer (3). The chloroform phase was collected and dried under a stream of nitrogen. The methanol-water phase was also collected, frozen, and lyophilized. The total lipid extract from the chloroform phase contained approximately 70.0% of the total weight of surfactant, and the total aqueous extract contained the remaining 30.0%. Suspensions of total lipid extract (0.7 mg/ml) and total aqueous extract (0.3 mg/ml) were then made by vortex mixing them in test buffer followed by brief sonification for 10 s.

Phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and sphingomyelin were separated from total lipid extract by thin-layer chromatography (39) and visualized with iodine. Spots were scraped from the plate, and each phospholipid was eluted from the silica with chloroform and dried under nitrogen. A suspension of phosphatidylcholine (0.5 mg/ml) and suspensions (0.05 mg/ml each) of phosphatidylglycerol, phosphatidylethanolamine, and sphingomyelin were then made in proportion to their presence in the total lipid extract (5) by vortex mixing them in test buffer followed by brief sonification for 10 s.

Surfactant-associated proteins were precipitated from surfactant with 1-butanol as described by Haagsman et al. (17). The precipitated protein was dried under nitrogen and washed twice in 20 mM–octyl-β-D-glucopyranoside–100 mM NaCl–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4) and dialyzed against the same buffer for 48 h. The insoluble material was removed by centrifugation at 100,000 × g_{av} for 60 min. The supernatant was removed, dialyzed against water, and lyophilized. The proteins were resuspended to a concentration of 0.3 mg/ml in test buffer.

Active surfactant extracts and the 100,000 × g_{av} supernatant were analyzed for medium-length fatty acids (C14 to C20). Briefly, a portion of surfactant or supernatant was resuspended in 2.5 ml of 2 N HCl and heated at 100°C for 16 h. Chloroform (2.5 ml) was then added, and it was removed to another tube containing 1.5 ml of methanol and HCl (11 parts methanol to 1 part concentrated HCl) and heated at 55°C for 30 min. The mixture was washed three times with 2.5 ml of distilled water; the water layer was removed each time. The chloroform was evaporated to dryness under a stream of nitrogen. The methylated fatty acids were resuspended in 1 ml of hexane and analyzed with a gas-liquid chromatograph with flame ionization detection (model 5890A; Hewlett-Packard, Avondale, Pa.).

Preparation, treatment, and fractionation of serum. Fetal calf serum, gnotobiotic calf serum, pooled normal sheep serum, pooled normal rabbit serum, pooled guinea pig serum, complement 4-deficient guinea pig serum, and pooled rat serum were used and kept frozen at −80°C throughout the study. Titers of complement (8, 28, 38), lysozyme concentrations (35), and titers of antibody to *P. haemolytica* 82-25 LPS (31) were measured in all serums. Complement was only detected in the fresh guinea pig serum shown in Table 5 (titer, 1:126). Lysozyme was detected in rabbit (118 μg/ml) and guinea pig (45 μg/ml) sera. Antibody was detected in sheep (titer, 1:64 to 1:128), rabbit (titer, 1:8), and guinea pig (titer, 1:32) sera. All other sera were negative for these constituents.

To characterize the nature of the terminal killing component, serum was heated at 56°C for 30 min to inactivate complement. Serum was fractionated so each group of components could be checked for the factor. For this, serum was delipidated with butanol and di-isopropyl ether as described by Cham and Knowles (9). The lipid phase was collected and dried under nitrogen. Serum lipid was reconstituted in test buffer and mixed by brief sonification for 10 s. Ovine serum albumin (fraction V; Sigma Chemical Co.) was resuspended to 35 mg per ml of test buffer.

Bactericidal test. The bactericidal test was performed with Immulon 1 microtiter plates (Dynatech, Alexandria, Va.) as follows. To start, 150 μl of test buffer was put into the buffer control wells (row A), 100 μl of test buffer and 50 μl of surfactant (1 mg/ml) were put into the surfactant control wells (row B), 100 μl of test buffer was put into the serum control wells (row C), and 50 μl of test buffer and 50 μl of surfactant (1 mg/ml) were put into the surfactant-plus-serum wells (row D). Then, 50 μl of bacterial cells (10^3 CFU/ml) was added to all wells in rows A, B, C, and D. The plate was shaken on a microtiter plate shaker (Lab-line Instruments, Inc., Melrose Park, Ill.) and incubated at 37°C for 60 min. After incubation, 50 μl of serum was added to the wells in rows C and D. The final volume in all wells was 200 μl. The plate was again shaken and incubated at 37°C for 60 min. The number of viable bacteria was determined by culturing 150 μl from each well in triplicate (50 μl per spot) on Trypticase soy agar containing 5% defibrinated sheep blood and incubating the plates overnight at 37°C. Each replicate contained a set of three counts.

In one test, ovine surfactant was incubated with capsular polysaccharide or LPS for 60 min prior to the bactericidal test to determine whether *P. haemolytica* surface components could block ovine surfactant-induced killing by ovine serum. In other tests, treated surfactant, treated serum,

TABLE 1. Killing of *P. haemolytica* 82-25 (type A1) in ovine pulmonary surfactant and ovine serum mixtures

Incubation mixture	CFU[a]	% Killed[b]
Test buffer[c]	52.0 ± 2.0	0.0
Ovine surfactant	35.4 ± 1.3	31.9
Normal ovine serum	54.0 ± 0.9	0.0
Surfactant + serum	0.6 ± 0.1	98.8

[a] Means ± standard errors of 15 replications.
[b] % Killed = [1 − (CFU incubation mixture/CFU test buffer)] × 100.
[c] Test buffer, 10 mM PBS, pH 7.2, with 0.5 mM $CaCl_2$ and 0.15 mM $MgCl_2$.

individual surfactant components, or individual serum components were substituted. Percent killing was calculated as [1 − (CFU incubation mixture/CFU test buffer)] × 100.

Electron microscopy. A bactericidal test with ovine surfactant, *P. haemolytica* 82-25, and ovine serum was performed. After incubation, 100 μl of 5% glutaraldehyde in 0.1 M cacodylate buffer was added and the plate was incubated on ice for 30 min. After fixation, the wells in each of rows A, B, C, and D were pooled into four samples and centrifuged for 5 min at 5,900 × g_{av} at 4°C. The supernatant was removed, and 0.5 ml of warm agarose (in 0.05 M cacodylate buffer) was added to the tubes and placed on ice. The agarose pieces were then washed twice in 0.05 M cacodylate buffer and stained with 1.0% OsO_4 for 60 min. The agarose pieces were again washed in 0.05 M cacodylate buffer and dehydrated by a series of graded ethanol solutions (6). The dehydrated samples were cleared in propylene oxide, infiltrated, and embedded in Epon. Thin sections were cut and stained with lead citrate and uranyl acetate.

Protein A-colloidal gold staining was also performed on sections to characterize the precipitated protein surrounding the bacterial cells incubated in serum. Sections, on a nickel grid, were incubated in a saturated solution of sodium metaperiodate for 30 min at room temperature. The sections were washed three times for 5 min each in 10 mM Tris-buffered saline, pH 7.4, with 0.05% Tween 20 and 1.0% bovine serum albumin (BSA). The sections were then incubated in blocking buffer (10 mM Tris-buffered saline with 0.05% Tween 20 and 5% BSA) for 30 min at room temperature. Dilute protein A-colloidal gold (1:10 in 10 mM Tris-buffered saline with 0.05% Tween 20 and 1.0% BSA) was then added to the sections and incubated for 60 min at room temperature. The sections were then washed in 10 mM Tris-buffered saline with 0.05% Tween 20 and 1.0% BSA, stained with uranyl acetate and lead citrate, and examined.

RESULTS

Bactericidal test. The CFU and percent killing of *P. haemolytica* incubated in test buffer, ovine surfactant, ovine serum, and ovine surfactant with ovine serum are shown in Table 1. Organisms were not killed after incubation in either test buffer or normal ovine serum. However, the numbers of organisms incubated in surfactant were approximately 70% of the numbers in test buffer, and this difference was statistically significant (*t* test; $P < 0.05$). The numbers of organisms incubated in ovine surfactant with ovine serum fell to virtually 0. The rate of acquiring sensitivity to ovine serum was rapid and occurred within 60 min when *P. haemolytica* was incubated in ovine surfactant and sampled at 10-min intervals and then incubated for 60 min in ovine serum (Fig. 1) or when *P. haemolytica* was incubated in ovine surfactant for 60 min and then incubated in ovine serum and sampled at 10-min intervals (Fig. 2). Similarly, *P. haemolytica* incubated with ovine surfactant was also killed by fetal calf serum, gnotobiotic calf serum, rabbit serum, and guinea pig serum.

Killing was specific and only occurred when *P. haemolytica* was incubated first with ovine surfactant and then with serum. *P. haemolytica* was not killed if the components were reversed in order or if the components were mixed together (data not shown). Also, this killing mechanism was selective. *P. haemolytica* type A1 strains 82-25 and L101, a *P. hae-*

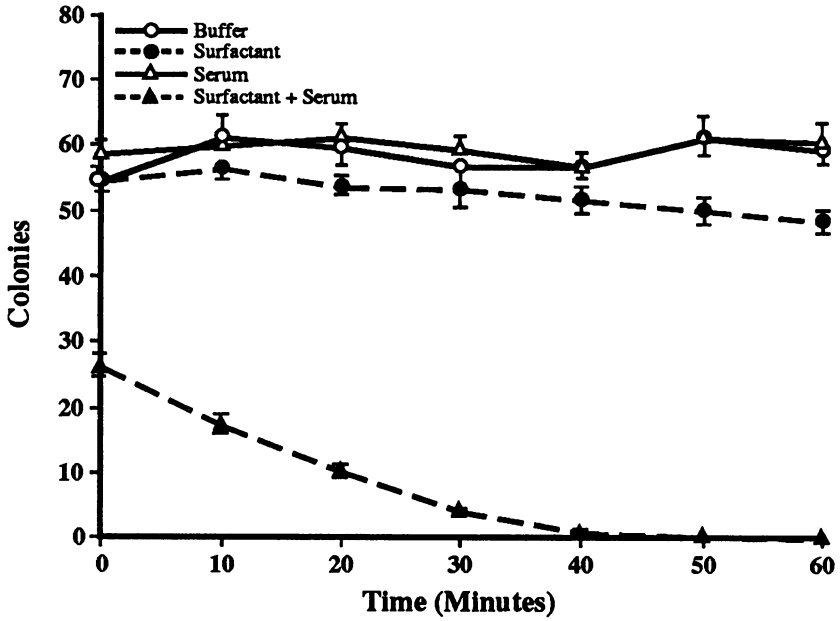

FIG. 1. Killing of *P. haemolytica* 82-25 (type A1) after incubation in ovine surfactant and sampling at 10-min intervals followed by incubation for 60 min in normal ovine serum. Data represent mean CFU ± standard errors of eight replications.

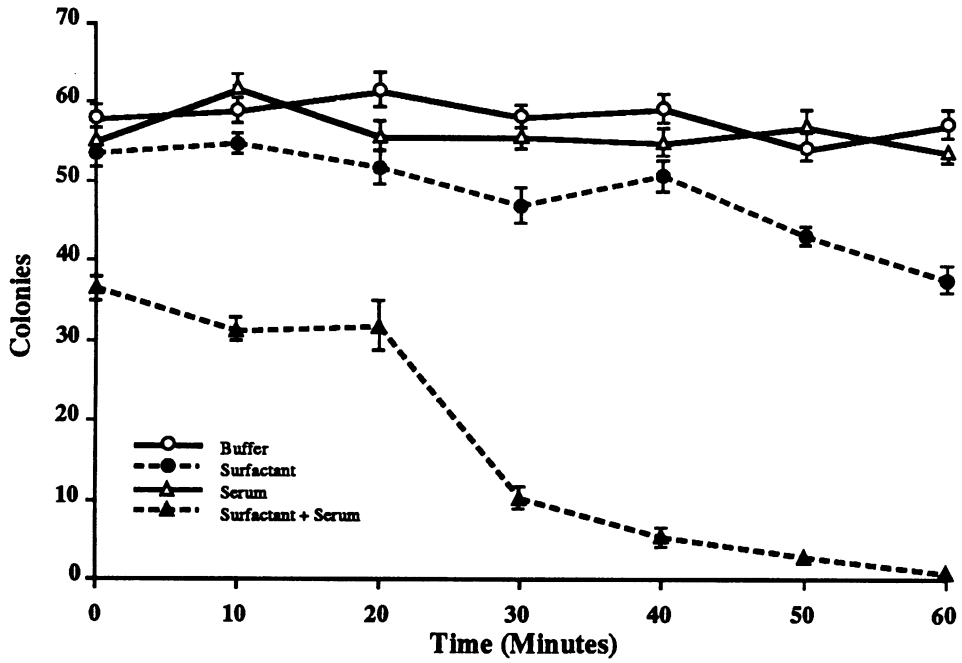

FIG. 2. Killing of *P. haemolytica* 82-25 (type A1) after incubation in ovine surfactant for 60 min followed by incubation in normal ovine serum and sampling at 10-min intervals. Data represent mean CFU ± standard errors of eight replications.

molytica type 2 strain, *E. coli*, and *K. pneumoniae* were susceptible and *P. multocida*, *S. marcescens*, and *P. aeruginosa* were not susceptible to killing by this mechanism (Table 2). No organisms incubated with bovine surfactant were killed by ovine, bovine, or rabbit serum. *P. haemolytica* type A1 strain 82-25 was not killed when incubated with rat surfactant and rat serum (data not shown). Ovine surfactant-induced killing by normal serum did not correlate with the presence or absence of lysozyme in serum.

Effect of treatments on surfactant. The CFU and percent killing of *P. haemolytica* incubated in ovine surfactant exposed to heat, proteolytic enzymes, phospholipases, or after centrifugation are shown in Table 3. Surfactant-induced killing by normal serum was substantially reduced if *P. haemolytica* LPS was incubated with surfactant for 60 min prior to the bactericidal test (*t* test; $P < 0.05$). Surfactant-induced killing by normal serum was not reduced when surfactant was preincubated with *P. haemolytica* capsular polysaccharide, heated at 56 and 100°C, or digested with proteinase K, trypsin, phospholipase C, or phospholipase D.

After centrifugation at 100,000 × g_{av}, the supernatant fluid induced killing by normal serum, but the resuspended surfactant pellet did not (Table 3). Filtration of the supernatant through a 10,000-Da cutoff filter (Centricon 10) followed by digestion with leucine aminopeptidase substantially reduced killing by normal serum (*t* test; $P < 0.05$). Fatty acids were not found by gas chromatography in the supernatant from the 100,000 × g_{av} centrifugation.

Effect of surfactant components. After extraction by the method of Bligh and Dyer (3), both the reconstituted total aqueous extract and total lipid extract induced killing of *P. haemolytica* by normal serum (Table 4). Interestingly, the total-lipid extract control without serum was cytotoxic for *P. haemolytica*, but the individual phospholipid controls without serum were not (data not shown). The isolated phospholipids induced some killing of the organism by serum (15.2 to 36.5%) that differed significantly from that by both test buffer and surfactant with serum controls (*t* test; $P < 0.05$). The inability of individual phospholipids to induce substantial killing of *P. haemolytica* by serum confirms the results of phospholipase digestion in Table 3.

Effect of treatments on serum. The killing of *P. haemolytica* was not affected by heating sera at 56°C for 30 min. *P. haemolytica* was not killed after incubation in normal guinea pig serum, heated guinea pig serum, complement C4-deficient guinea pig serum, or heated guinea pig serum containing 2 mM EDTA. Yet, *P. haemolytica* was killed by all of these sera when it was preincubated with ovine surfactant (Table 5).

The killing of *P. haemolytica* was not dependent upon antibodies in the surfactant or serum. *P. haemolytica* incubated with ovine surfactant was killed by both normal ovine serum (titer, 1:128) and normal ovine serum absorbed three

TABLE 2. Killing of bacteria in ovine pulmonary surfactant with ovine, bovine, and rabbit serum mixtures

Bacterium (strain or type)	% Killed[a] in serum		
	Ovine	Bovine	Rabbit
P. haemolytica (82-25)	98.3	98.3	99.0
P. haemolytica (L101)	89.5	90.0	94.0
P. haemolytica (type 2)	84.0	79.3	83.5
E. coli	71.3	93.8	98.0
K. pneumoniae	80.0	90.1	86.5
P. multocida	0.0	0.0	0.0
S. marcescens	0.0	0.0	0.0
P. aeruginosa	0.0	0.0	0.0

[a] % Killed = [1 − (CFU incubation mixture/CFU test buffer)] × 100. Values are means of four replications.

TABLE 3. Effect of physical and enzymatic treatment of ovine pulmonary surfactant on killing of *P. haemolytica* 82-25 (type A1) by ovine serum

Treatment	CFU[a] (no. of replications)	% Killed[b]
Test buffer[c]	40.2 ± 1.7 (31)	0.0
Surfactant	0.1 ± 0.1 (22)	99.8
Preabsorption of surfactant		
Capsular polysaccharide	4.2 ± 0.9 (4)	89.6
LPS	28.3 ± 3.2 (4)	29.6
Heat (min)		
56°C (30)	0.7 ± 0.4 (2)	98.3
100°C (30)	0.0 ± 0.0 (4)	100.0
Enzyme		
Proteinase K	0.8 ± 0.4 (4)	98.0
Trypsin	0.1 ± 0.1 (4)	99.8
Phospholipase C	0.8 ± 0.3 (4)	98.0
Phospholipase D	4.4 ± 0.9 (4)	89.1
Centrifugation at 100,000 × g_{av}		
Supernatant	1.2 ± 0.5 (2)	97.0
Filtrate (10,000 Da)	0.1 ± 0.1 (4)	99.8
Leucine amino peptidase	21.8 ± 2.0 (4)	45.8
Pellet	64.0 ± 1.3 (2)	0.0

[a] Means ± standard errors.
[b] % Killed = [1 − (CFU incubation mixture/CFU test buffer)] × 100.
[c] Test buffer, 10 mM PBS, pH 7.2, with 0.5 mM $CaCl_2$ and 0.15 mM $MgCl_2$.

times with glutaraldehyde-fixed cells (titer, 0). Likewise, *P. haemolytica* was also killed by fetal calf serum and gnotobiotic calf serum with no titers of antibody and rabbit serum and guinea pig serum with low titers of antibody to strain 82-25.

Effect of serum components. Ovine surfactant-sensitized *P. haemolytica* 82-25 was killed by both delipidated serum extract (100% of the cells killed) and reconstituted serum lipid extract in PBS (100% of the cells killed). However, the latter observation may not be involved in the surfactant-induced killing mechanism because serum lipid extract controls without surfactant were cytotoxic for strain 82-25 (100% of the cells killed).

Fraction V ovine albumin, at the relative concentration found in serum (35 mg/ml), also killed surfactant-sensitized *P. haemolytica* 82-25 (87.8% of the cells killed).

TABLE 4. Killing of *P. haemolytica* 82-25 (type A1) incubated in fractions of ovine pulmonary surfactant and ovine serum

Incubation mixture	CFU[a] (no. of replications)	% Killed[b]
Test buffer[c]	40.7 ± 1.4 (32)	0.0
Surfactant	0.1 ± 0.1 (22)	99.8
Total lipid extract	1.3 ± 0.3 (24)	96.9
Phosphatidylcholine	31.1 ± 1.5 (14)	23.6
Phosphatidylglycerol	27.4 ± 1.1 (20)	32.7
Phosphatidylethanolamine	34.5 ± 1.1 (20)	15.2
Sphingomyelin	25.9 ± 1.4 (20)	36.5
Total aqueous extract	2.3 ± 0.5 (20)	94.3
Surfactant proteins	30.9 ± 1.1 (12)	24.0

[a] Means ± standard errors.
[b] % Killed = [1 − (CFU incubation mixture/CFU test buffer)] × 100.
[c] Test buffer, 10 mM PBS, pH 7.2, with 0.5 mM $CaCl_2$ and 0.15 mM $MgCl_2$.

TABLE 5. Killing of *P. haemolytica* 82-25 (type A1) in ovine pulmonary surfactant with treated guinea pig serum mixtures

Incubation mixture	CFU[a] (no. of replications)	% Killed[b]
Test buffer[c]	63.3 ± 1.3 (16)	0.0
Fresh serum[d]	9.6 ± 0.6 (4)	84.8
Heated serum[e]	8.5 ± 0.8 (4)	86.6
C4-deficient serum	19.4 ± 1.6 (4)	69.4
Heated serum with EDTA	3.0 ± 0.4 (4)	95.3

[a] Means ± standard errors.
[b] % Killed = [1 − (CFU incubation mixture/CFU test buffer)] × 100.
[c] Test buffer, 10 mM PBS, pH 7.2, with 0.5 mM $CaCl_2$ and 0.15 mM $MgCl_2$.
[d] Complement titer, 1:126.
[e] Heated at 56°C for 30 min.

Electron microscopy. *P. haemolytica* 82-25 incubated in PBS had a normal morphology. Cells were covered with a tight outer envelope and intact cytoplasmic membrane and contained dense cytoplasm (Fig. 3a). Most cells incubated with surfactant had a normal morphology as seen in the buffer controls, but some distortion was evident in a few cells (Fig. 3b). These cells had intact cytoplasmic membranes, but the outer envelope was distended and the interspace was dilated. Cells incubated in serum had a normal morphology (Fig. 3c). However, a halo of serum protein was visible around most cells. This halo of protein could not be labeled with protein A-colloidal gold (cells not shown). Cells incubated first with surfactant and then with serum were in various stages of lysis (Fig. 3d). Many of these cells were distorted with vacuoles between the cytoplasmic membrane and the cytoplasm. In some cells, the outer envelope was distended and the interspace was dilated. Some cells were characterized by a break in the outer envelope, with a loss of cellular content.

DISCUSSION

P. haemolytica, *E. coli*, and *K. pneumoniae* were killed by a mechanism first initiated by pulmonary surfactant and then terminated by normal serum. The killing of *P. haemolytica* did not correlate with the presence of complement, antibodies, or lysozyme in either the surfactant or the serum. The exact nature of the inducing factor in surfactant is not known. However, preliminary characterizations of both surfactant and serum implicate a low-molecular-weight proteinaceous component in the surfactant and serum albumin in the serum.

Damage of bacteria by surfactant has been reported with a number of bacterial species (10, 11, 23–26). One mechanism is mediated by free fatty acids found in the neutral lipids of pulmonary surfactant (10, 11). The free fatty acids were bactericidal against pneumococci of different serotypes, nonpneumococcal streptococci of several species, and gram-positive bacteria but not gram-negative bacteria (11). Another mechanism was mediated by a low-molecular-mass polypeptide (3,400 Da) with zinc as a cofactor (23, 24). This polypeptide was not lethal directly but facilitated the killing of gram-negative bacteria by deoxycholate (23). Cell injury was associated with increased cell membrane permeability (24).

Not only is the damage induced to *P. haemolytica* by ovine surfactant remarkably similar to the damage induced to *E. coli* by mouse, rabbit, and human surfactant (14, 23, 24), but so are a number of characteristics of the components

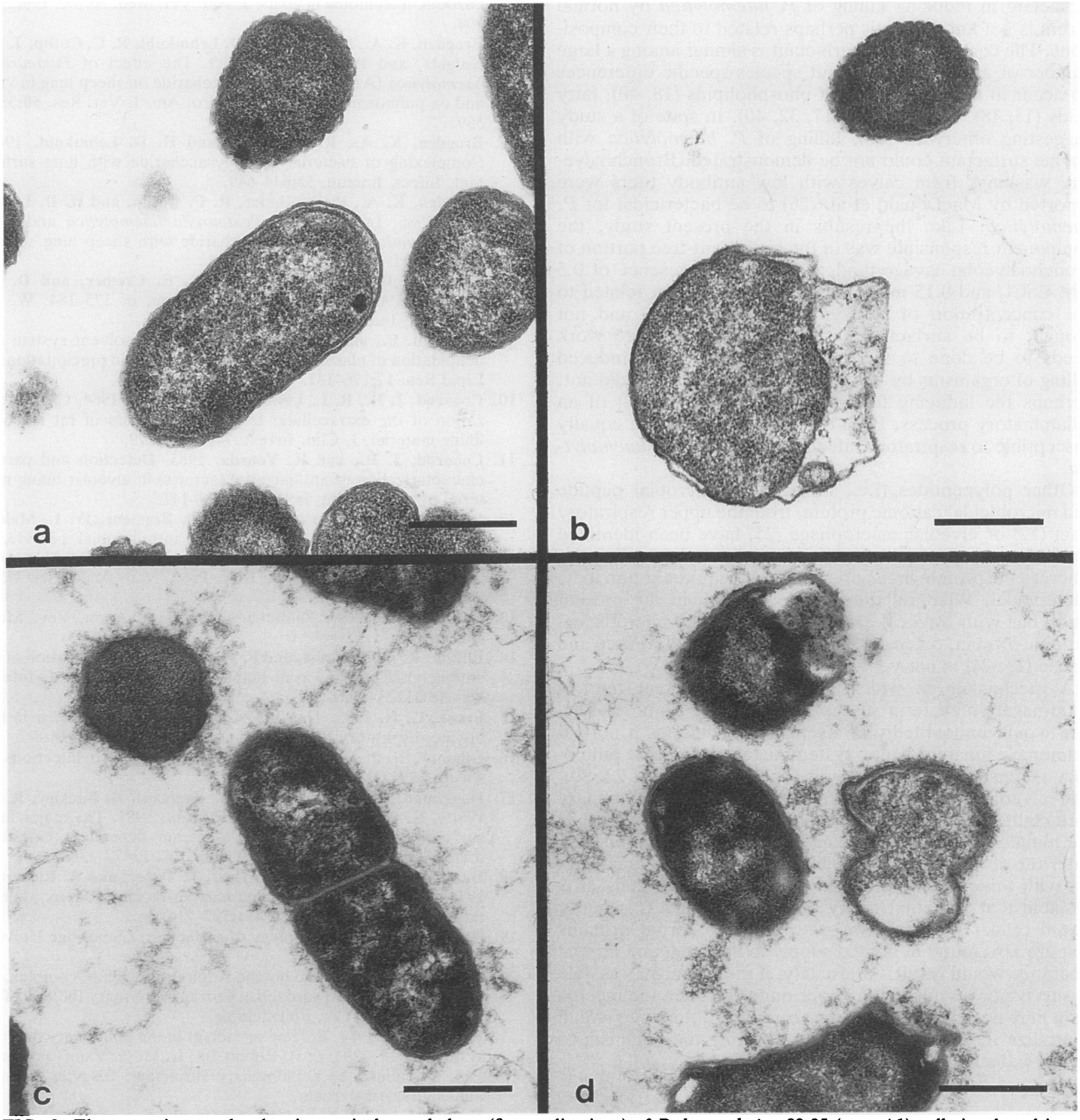

FIG. 3. Electron micrographs showing typical morphology (five replications) of *P. haemolytica* 82-25 (type A1) cells incubated in test buffer (a), ovine surfactant (b), normal ovine serum (c), and ovine surfactant with ovine serum (d). Bars = 0.5 μm.

involved. These include (i) activity against *E. coli*, (ii) location of the component in the surfactant-free portion of bronchoalveolar lavage fluid (23), (iii) heat stability at 100°C, and (iv) induction by a small (<10,000 Da) component selectively sensitive to proteolytic enzymes. The polypeptide of LaForce and Boose (23) was destroyed with trypsin, but the inducing factor in whole surfactant described here was not destroyed by either trypsin or proteinase K. However, when the inducing factor was separated from the 100,000 × g_{av} particulate and filtered through a 10,000-Da-cutoff filter, it was partially destroyed by digestion with leucine aminopeptidase. The reasons for this are not yet clear. However, incubation of *E. coli* with ovine surfactant under the conditions in this study did not affect its ability to grow on MacConkey agar (data not shown). LaForce and Boose thought that if the peptide could kill gram-negative bacteria in conjunction with deoxycholate (or detergent) in vitro, it may have antibacterial activity in vivo in combination with detergents such as lysophospholipids (14, 23, 24). The final antibacterial activity instead is likely due to albumin in normal serum.

The reason for the differences between ovine and bovine

surfactant in inducing killing of *P. haemolytica* by normal serum is not known but is perhaps related to their compositions. The composition of surfactant is similar among a large number of animals (18, 36) but species-specific differences do occur in the proportions of phospholipids (18, 40), fatty acids (13, 18), and proteins (17, 32, 40). In spite of a study suggesting otherwise (26), killing of *P. haemolytica* with bovine surfactant could not be demonstrated. Bronchoalveolar washings from calves with low antibody titers were reported by MacDonald et al. (26) to be bactericidal for *P. haemolytica*. Like the results in the present study, the component responsible was in the surfactant-free portion of bronchoalveolar lavage fluid, active in the presence of 0.5 mM $CaCl_2$ and 0.15 mM $MgCl_2$, heat stable, not related to the concentration of lysozyme or complement, and not thought to be surfactant directly. However, more work needs to be done to learn why ovine surfactant induced killing of organisms by serum but bovine surfactant did not. Perhaps the inducing factor is secreted as a result of an inflammatory process. Both of these ruminants are equally susceptible to respiratory infections caused by *P. haemolytica*.

Other polypeptides (i.e., tracheal antimicrobial peptide and microbicidal cationic protein) from the upper respiratory tract (12) or alveolar macrophage (25) have been identified and have antimicrobial activity. All are bactericidal directly, whereas the protein in the present study requires serum to be bactericidal. What relationship the protein in the present study has with tracheal antimicrobial peptide, microbicidal cationic protein, defensins, or that reported by LaForce and Boose (23, 24) is not yet known.

A mechanism in which pulmonary surfactant renders gram-negative bacteria susceptible to killing by normal serum would undoubtedly be useful to the lung as a normal pulmonary innate defense system augmenting other pulmonary defense mechanisms involving surfactant (21, 22, 30). The alveoli of the lung are covered with a pulmonary surfactant layer on the epithelial surface (19). This layer is the initial surface that invading bacteria contact when they enter the alveoli. As bacteria become established and interact with this layer (34) as well as with epithelial cells, it is possible that sublethal injury may then result. This injury would render the organism susceptible to serum proteins that are also found in limited supply in the lung, and normal clearance would result. Alternately, if the organism was able to survive or be inhaled in larger numbers, then the mechanism may not work. Organisms secreting LPS in vivo would neutralize the killing mechanism, allowing the organism to thrive, inducing pneumonia.

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