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Isolation of an ovine pulmonary surfactant-associated anionic peptide bactericidal for *Pasteurella haemolytica*

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ABSTRACT **Ovine pulmonary surfactant is bactericidal for *Pasteurella haemolytica* when surfactant and bacteria mixtures are incubated with normal ovine serum. To isolate this component, surfactant (1 mg/ml) was centrifuged at 100,000 × g_{av}, and the supernatant was fractionated by HPLC. Fractions were eluted with acetonitrile (10–100%)/0.1% trifluoracetic acid and tested for bactericidal activity. Amino acid and sequence analysis of three bactericidal fractions showed that fraction 2 contained H-GDDDDDD-OH, fraction 3 contained H-DDDDDDD-OH, and fraction 6 contained H-GADDDDD-OH. Peptides in 0.14 M NaCl/10 μM $ZnCl_2$ (zinc saline solution) induced killing of *P. haemolytica* and other bacteria comparable to defensins and β-defensins [minimal bactericidal concentration $(MBC)_{50}$ range, 0.01–0.06 mM] but not in 0.14 M NaCl/10 mM sodium phosphate buffer, pH 7.2/0.5 mM $CaCl_2$/0.15 mM $MgCl_2$ (MBC_{50} range, 2.8–11.5 mM). Bactericidal activity resided in the core aspartate hexapeptide homopolymeric region, and MBC_{50} values of aspartate dipeptide-to-heptapeptide homopolymers were inversely proportional to the number of aspartate residues in the peptide. *P. haemolytica* incubated with H-DDDDDD-OH in zinc saline solution was killed within 30 min. Ultrastructurally, cells contained flocculated intracellular constituents. In contrast to cationic defensins and β-defensins, surfactant-associated anionic peptides are smaller in size, opposite in charge, and are bactericidal in zinc saline solution. They are members of another class of peptide antibiotics containing aspartate, which when present in pulmonary secretions may help clear bacteria as a part of the innate pulmonary defense system.**

Epithelial cells, resident and inflammatory cells, and serous and mucous secretions in the mammalian respiratory tract contain antimicrobial peptides for innate protection against bacterial infections. In ruminants these peptides, called β-defensins, are intracellular and consist of 29–34 amino acids with six conserved cysteine residues for intramolecular disulfide bonding (1). β-Defensins are usually arginine-rich, cationic proteins with broad antimicrobial, antiviral, and cytotoxic activity (1). Some can be chemotactic, opsonic, or may modulate hormonal responses (1). Active peptides originate from posttranscriptionally modified charge-neutralized preprodefensins (2).

One peptide, tracheal antimicrobial peptide, is found in columnar cells of the pseudostratified epithelium throughout the conducting airways of ruminants (3). Like other β-defensins, tracheal antimicrobial peptide is 4084 Da in size, cationic, and has *in vitro* antimicrobial activity against Gram-negative bacteria, Gram-positive bacteria, and yeasts (4).

Defensins and β-defensins are also part of the phagolysomal killing mechanisms of mononuclear and polymorphonuclear leukocytes on the mucosa of the respiratory tract (1). These include alveolar macrophage cationic peptides 1 and 2 from rabbits (5, 6), neutrophil peptides HNP 1–4 from humans (7), neutrophil peptide GPNP from guinea pigs (8), and neutrophil peptides NP 1–5 from rabbits (9).

Usually defensins and β-defensins are not found in mucous and serous respiratory secretions. However, antimicrobial activity exists in bronchoalveolar lavage and amniotic fluids. This is due to a number of ill-defined peptides that are variable in size from 630 to 3400 Da (10, 11), contain hydrophilic and anionic amino acids (10, 12), and require zinc as a cofactor for antimicrobial activity (10–12).

Ovine pulmonary surfactant is also bactericidal when bacteria and surfactant mixtures are incubated with normal serum (13). Bactericidal activity associates with a filterable (<10,000 Da), heat-stable component partially destroyed by digestion with leucine aminopeptidase.

In this paper we describe the isolation and properties of three small ovine pulmonary surfactant-associated anionic peptides (SAAP) containing homopolymeric regions of aspartate. This work provides definitive evidence that such peptides, with zinc, are bactericidal for both Gram-positive and Gram-negative bacteria. These peptides are very different from the well-known cationic defensins, magainins, and cecropins and are members of another class of peptide antibiotics that when present in pulmonary secretions may help clear bacteria as part of the innate pulmonary defense system.

MATERIALS AND METHODS

Bacteria. *P. haemolytica* serotype A1 strain 82-25, *Klebsiella pneumoniae* ATCC 10031, *Escherichia coli* ATCC 12795, *Staphylococcus aureus* ATCC 29213, and *Streptococcus faecalis* ATCC 29212 were used. *P. haemolytica* A1 is among those serotypes causing enzootic pneumonia in sheep. The other organisms were used to assess the bactericidal spectrum of SAAP. Organisms were grown in tryptose broth at 37°C for 3 hr, pelleted by centrifugation at 5900 × g_{AV} for 15 min at 4°C, and resuspended in either of two solutions; test buffer 0.14 M NaCl/10 mM sodium phosphate buffer, pH 7.2/0.5 mM $CaCl_2$/0.15 mM $MgCl_2$ (13) or zinc saline solution 0.14 M NaCl/10 μM $ZnCl_2$. Bacterial suspensions were adjusted in a spectrophotometer and diluted as described (13).

Preparation and Fractionation of Surfactant. Pulmonary surfactant was prepared as described (13), washed twice in distilled water, and lyophilized. Surfactant from one sheep (no. 4) was bactericidal without normal serum, that from another (sheep no. 814) was bactericidal with normal ovine serum, and surfactant from two (sheep nos. 217 and 3149) were not bactericidal for *P. haemolytica* in a described assay (13).

For fractionation, surfactant (sheep 814) was reconstituted to 1.0 mg (dry weight)/ml with distilled water and centrifuged

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Abbreviations: MBC, minimal bactericidal concentration; SAAP, surfactant-associated anionic peptide(s).

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at 100,000 × g_{AV} for 1 hr at 4°C. A series of 50-μl samples (totaling 300 μl) of the 100,000 × g_{AV} surfactant supernatant were separated (in six runs) on a C_{18} column (μBondapak, 15-μm particle size, 3.9 × 300 mm, Waters, using a Beckman 126 pump and 168 diode-array detector, and a Waters WISP sample processor). Timed fractions over 30 min were eluted with a gradient of acetonitrile (10–100%)/0.1% trifluoracetic acid with a flow rate of 1 ml/min. Fractions were collected (Foxy fraction collector, ISCO), pooled with similar fractions of previous runs, and evaporated to dryness under vacuum. Residues were dissolved in petroleum ether or acetonitrile and evaporated to dryness with nitrogen or dissolved in milli-Q water and lyophilized. Residues were reconstituted to 300 μl with test buffer and diafiltrated over a 500-Da ultrafiltration membrane (YC05; Amicon). Fractions were then tested as described below for peptide-induced killing of *P. haemolytica* by normal ovine serum.

Fraction 3, eluted with 55–79% acetonitrile, induced killing of *P. haemolytica* by normal ovine serum. This fraction was dissolved in water/acetonitrile, 60:40 and separated by HPLC as described above. Timed fractions were eluted in a 40–70% acetonitrile gradient over 30 min with a flow rate of 1 ml/min. Fractions were collected, evaporated to dryness, dissolved in acetonitrile, and again evaporated to dryness with nitrogen. Residues were reconstituted to 300 μl of original volume with test buffer and diafiltrated over a 500-Da ultrafiltration membrane. Three fractions (fraction 2 eluted with 44–46% acetonitrile, fraction 3 eluted with 48–50% acetonitrile, and fraction 6 eluted with 56–58% acetonitrile) were bactericidal for *P. haemolytica* with normal ovine serum.

Amino Acid Analysis and Peptide Sequence. Fractions 2, 3, and 6 were analyzed for amino acid content (Applied Biosystems amino acid analyzer model 420/130) after gas-phase hydrolysis at 150°C. The data was collected and calculated using an Applied Biosystem 920A data analysis module. Sequence analysis was determined by the Edman degradation method on a liquid-pulse sequencer (Applied Biosystems model 477A/120). Sequence was determined by the Protein Facility (Iowa State University, Ames).

Peptide Synthesis. Peptides were supplied by Chiron. They were synthesized on a grafted polymer surface in a Multipin peptide synthesis format with *N*-α-9-fluorenylmethoxycarbonyl-(Fmoc)-protected amino acids. Side-chain deprotection/cleavage was done by acidolysis.

Peptides were purified on a Vydac (Hesperia, CA) (protein) C_4 column (0.46 cm × 25 cm, 4-μm particle size, 300 Å core size). Fractions, in 0.1% (vol/vol) orthophosphoric acid in water were eluted over 15 min with a 0–100% gradient of 0.1% (vol/vol) orthophosphoric acid in 60% (vol/vol) acetonitrile in water. Mass spectrometer analysis was done with a Perkin–Elmer Sciex API III spectrometer with an ionspray ion source and ion-counting detection. Peptides were 95–99% pure.

One- to six-residue amino acid homopolymers of aspartate and poly(DL-aspartate) (6800 Da; Sigma) were also used; they were resuspended in distilled water and diluted in test buffer or zinc saline solution.

Bactericidal Assay. The direct bactericidal activity of all four surfactants on *P. haemolytica* was determined as described (13) using test buffer; other solutions were as follows: 0.14 M NaCl; 0.14 M NaCl/10 μM EDTA; 0.14 M NaCl/10 μM EDTA containing 20 μM $MgCl_2$, 20 μM $CaCl_2$, or 20 μM $ZnCl_2$; 0.14 M NaCl/10 mM sodium phosphate buffer (PBS); PBS/10 μM EDTA; and PBS/10 μM EDTA containing 20 μM $MgCl_2$, 20 μM $CaCl_2$, or 20 μM $ZnCl_2$.

The direct bactericidal activity of HPLC fractions, synthesized peptides, or commercial amino acids and peptides was determined similarly on *P. haemolytica* or the other bacterial species and diluted to obtain a minimal bactericidal concentration $(MBC)_{50}$. Tests were done in both test buffer and zinc saline solution. To detect other factors that might be involved in surfactant-induced killing by serum, a number of test solutions and controls were used. These included (*i*) test buffer or zinc saline solution, (*ii*) dilutions of peptide, (*iii*) nonbactericidal surfactant (sheep 3149), (*iv*) dilutions of peptide/nonbactericidal surfactant, (*v*) serum, (*vi*) dilutions of peptide/serum, (*vii*) nonbactericidal surfactant/serum, and (*viii*) dilutions of peptide/nonbactericidal surfactant/serum.

The rate of killing was determined by incubating *P. haemolytica* with 0.5 mM H-DDDDDD-OH in zinc saline solution and sampling at 10-min intervals.

In all tests, the number of viable bacteria was determined by culturing 150 μl from each well in triplicate (50 μl per spot) on trypticase soy agar/5% defibrinated sheep blood and incubating the plates overnight at 37°C. Each replicate contained a set of three counts. Percentage killing was calculated as [1 − (colony-forming units of test mixtures in ii, iv, vi, or viii colony-forming unit buffer or control mixtures in i, iii, v, or vii, respectively)] × 100. MBC_{50} values were calculated from linear regression of the *X* intercept value using percentage killed to derive the initial slope between the highest concentration of peptide showing no growth and the lowest concentration of peptide showing growth. The Statistical Analysis System (SAS; version 6.07) was used to assess analysis of variance and nonlinear least-squares for each peptide.

Electron Microscopy. Zinc saline solution, containing 2.2 × 10^8 colony-forming units of *P. haemolytica* per ml, was split into two 10-ml amounts. Pyrogen-free water (1 ml) was added to one tube, and 0.5 mM H-DDDDDD-OH in pyrogen-free water (1 ml) was added to the other tube. After 30 min at 37°C, *P. haemolytica* incubated without peptide contained 2.3 × 10^8 colony-forming units per ml, and *P. haemolytica* incubated with 0.5 mM H-DDDDDD-OH contained 5.3 × 10^3 colony-forming units per ml. A sample of cells were negatively stained with 2.0% phosphotungstic acid, pH 7.0, and the remainder of cells in both suspensions was processed, cut, and stained as described (13). Cell morphology was assessed in a Philips EM-410 electron microscope. X-ray spectral microanalysis, to detect cell-associated zinc, was determined from carbon-coated grids in a carbon holder of a JEOL model 1200EX scanning and transmission electron microscope with a Kevex, Delta IV detector. Spectra were created at ≈350 counts per sec for 60 sec with the specimen tilted at 25°.

RESULTS

Effect of Cations on Surfactant Bactericidal Activity. Not all surfactant preparations exhibit the same patterns of bactericidal activity, and these patterns can be influenced by buffer composition (Table 1). For example, the bactericidal

Table 1. Effect of EDTA and zinc on ovine pulmonary surfactant killing of *P. haemolytica* serotype A1

Solution containing surfactant*	Cytotoxicity of surfactant,† %			
	814	3149	4	217
Test buffer/serum‡	99.4§	0.0	100.0	0.0
NaCl	29.5	16.2	99.1	0.0
NaCl/EDTA	0.0	11.7	96.8	0.0
NaCl/EDTA/$ZnCl_2$	18.3	49.8	98.6	40.5
PBS¶	81.0	0.0	100.0	0.0
PBS/EDTA	88.0	0.0	100.0	0.0
PBS/EDTA/$ZnCl_2$	77.7	0.0	100.0	0.0

*Concentrations of 0.14 M NaCl, 10 μM EDTA, and 20 μM $ZnCl_2$ were used.
†Surfactant concentration was 1.0 mg/ml.
‡Bactericidal test previously described by Brogden (13) used 0.14 M NaCl/10 mM sodium phosphate buffer, pH 7.2/0.5 mM $CaCl_2$/0.15 mM $MgCl_2$.
§Percentage cytotoxicity of seven replications.
¶Solution of 0.14 M NaCl/10 mM sodium phosphate buffer, pH 7.2.

activity of surfactant from sheep 4 and 814 (with high bactericidal activity) was not greatly influenced by phosphate, zinc, or EDTA in the test mixtures. Generally, bactericidal activity was lower when surfactant solutions contained 0.14 M NaCl and EDTA; bactericidal activity was then restored when $ZnCl_2$ was added. Bactericidal activity was high in PBS, PBS/EDTA, and PBS/EDTA/$ZnCl_2$ solutions. Saline or PBS/$MgCl_2$ or $CaCl_2$ had no effect on bactericidal activity (data not shown).

Bactericidal activity of surfactant from sheep 3149 (with low inherent bactericidal activity) decreased slightly in NaCl/EDTA and was restored in NaCl/EDTA/$ZnCl_2$ solutions (Table 1). Bactericidal activity of surfactant from sheep 217 (without bactericidal activity) was enhanced in NaCl/EDTA/$ZnCl_2$ (Table 1). In both cases, there was no bactericidal activity in PBS with or without EDTA and $ZnCl_2$. Saline or PBS/$MgCl_2$ or $CaCl_2$ had no effect on bactericidal activity (data not shown).

Isolation of Peptide. Fraction 3, eluted with 55–79% acetonitrile from HPLC separation of the 100,000 × g_{AV} surfactant supernatant (Fig. 1), induced killing of *P. haemolytica* by normal ovine serum similar to that seen with surfactant. A combination of mass spectrometry and assay of bactericidal activity showed that the latter was associated with a 746-kDa component. This component was collected and separated in a shallow 40–70% acetonitrile gradient (Fig. 2). Three fractions (fraction 2 eluted with 44–46% acetonitrile, fraction 3 eluted with 48–50% acetonitrile, and fraction 6 eluted with 56–58% acetonitrile) were bactericidal.

Analysis and Sequence of Peptide. Fractions 2, 3, and 6 contained only three amino acids (Table 2). Fraction 2 contained asparagine/aspartate and glycine at a ratio of 6:1; fraction 3 contained only asparagine/aspartate; and fraction 6 contained asparagine/aspartate, glycine, and alanine at a ratio of 5:1:1. Sequence analysis revealed three different heptapeptides; H-GDDDDDD-OH (fraction 2), H-DDDDDDD-OH (fraction 3), and H-GADDDDD-OH (fraction 6).

MBC_{50} of Synthetic Peptides. Synthesized peptides in zinc saline solution induced killing of *P. haemolytica* similar to surfactant by normal serum (MBC_{50} range, 0.01–0.06 mM), but peptides in test buffer did not (MBC_{50} range, 2.8–11.5 mM) (Table 3). Addition of surfactant and serum to dilutions of peptide in zinc saline solution did not increase bactericidal activity, but dilutions of peptide in test buffer did. Synthesized peptide H-DDDDDDD-OH (fraction 3) in zinc saline solution induced killing of other Gram-negative and Gram-positive

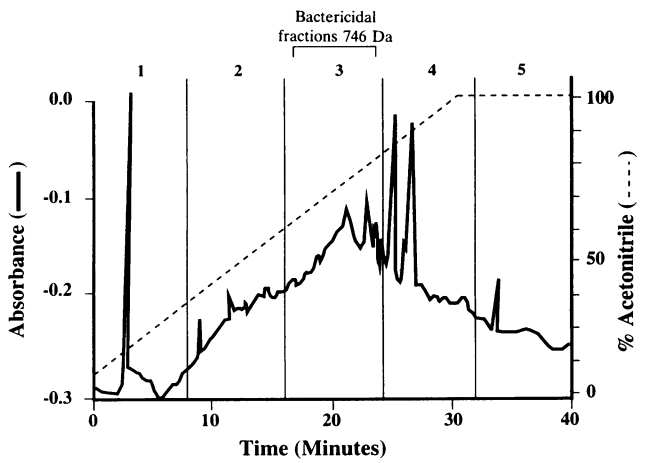

FIG. 1. HPLC chromatogram (254- to 280-nm absorbance spectra) of 100,000 × g_{AV} supernatant of ovine pulmonary surfactant (1 mg/ml) suspension. Fractions were eluted with a gradient (10–100% over 30 min) of acetonitrile in 0.1% trifluoracetic acid. Fraction 3, eluted by 55–79% acetonitrile, had bactericidal activity.

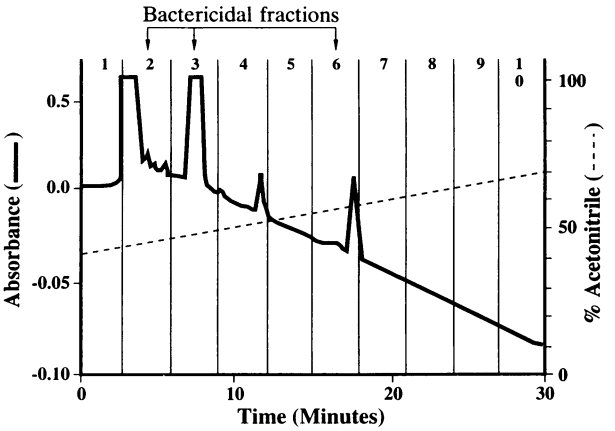

FIG. 2. HPLC chromatogram (213- to 217-nm absorbance spectra) of fraction 3 after it was collected, dissolved in water/acetonitrile (60:40) and separated with a gradient (40–70% over 30 min) of acetonitrile in 0.1% trifluoracetic acid. Fractions 2, 3, and 6 had bactericidal activity.

bacteria (MBC_{50} range, 0.62–1.39 mM), but peptide in test buffer did not (MBC_{50} > 7.31 mM) (Table 4).

One- to six-residue amino acid homopolymers of aspartate and poly(DL-aspartate) (6800 Da) in zinc saline solution were bactericidal directly for *P. haemolytica*. MBC_{50} decreased with the number of aspartate residues in the homopolymer and followed a nonlinear exponential curve (Fig. 3). Poly(DL-aspartate) was not as bactericidal as smaller aspartate peptide fragments (MBC_{50}, 1.77 mM). The same homopolymers of aspartate in test buffer were considerably less bactericidal (MBC_{50} range, 7.34–26.23 mM).

P. haemolytica was killed within 30 min when incubated with H-DDDDDD-OH in zinc saline solution and sampled at 10-min intervals.

Electron Microscopy. After 30-min incubation in zinc saline solution, *P. haemolytica* was morphologically normal. Cells were covered with a tight outer envelope and intact cytoplasmic membrane and contained dense cytoplasm. At 30-min incubation with 0.5 mM H-DDDDDD-OH in zinc saline solution, negative-stained cells appeared normal. Channels could not be seen in the outer membrane. However, thin-sectioned cells showed evidence of intracellular damage. Cellular contents began to flocculate. Flocculation became progressively worse until the cellular contents cleared from the cell cytoplasmic space. Spectral scans did not detect any increase in zinc associated with either the bacterial envelope or cytoplasmic contents in injured cells.

DISCUSSION

Ovine pulmonary surfactant preparations are bactericidal directly for *P. haemolytica*, indirectly bactericidal when *P. haemolytica* (and other Gram-negative bacteria) and surfactant mixtures are incubated with normal serum (13), or not bactericidal at all (Table 1). The reason for this variability is

Table 2. Amino acid analysis of bactericidal fractions isolated after the second HPLC separation

Amino acid	Fraction 2*	Fraction 3*	Fraction 6†
Asx	5.83‡	6.42	5.25
Gly	0.68		1.00
Ala			1.01

*Mean of two runs on same hydrolysis.
†Mean of three runs on same hydrolysis.
‡Relative ratio by amino acid analysis.

Table 3. Killing of *P. haemolytica* serotype A1 by ovine pulmonary SAAP, surfactant, and serum mixtures

Incubation mixture	Buffer*†	MBC_{50} of SAAP, mM: Fraction 2 H-GDDDDDD-OH (765.7 Da)	Fraction 3 H-DDDDDDD-OH (823.8 Da)	Fraction 6 H-GADDDDD-OH (721.6 Da)
Peptide	Test buffer	11.49 A	8.52 A	10.10 A
	Zn-saline	0.04 c	0.02 b	0.02 c
Peptide/surfactant	Test buffer	6.44 C	4.43 B	5.18 B
	Zn-saline	0.05 b	0.01 c	0.04 b
Peptide/serum	Test buffer	10.38 B	4.95 B	5.59 B
	Zn-saline	0.05 b	0.02 a	0.04 b
Peptide/surfactant/serum	Test buffer	5.93 C	2.79 C	4.75 C
	Zn-saline	0.06 a	0.03 a	0.04 a

Means with the same capital or small letter in each column are not significantly different ($P < 0.05$).
*Test buffer is 0.14 M NaCl/10 mM sodium phosphate buffer, pH 7.2/0.5 mM $CaCl_2$/0.15 mM $MgCl_2$.
†Zn-saline buffer is 0.14 M NaCl/10 μM $ZnCl_2$.

not clearly understood but thought to be related to innate pulmonary defense against microbial infection. It is possible SAAP may be produced directly by pulmonary tissue or be a cleaved fragment secondary to other metabolic cascade events in the lung. Regardless, the concentration and composition of SAAP in surfactant from different sheep and the presence of physiologic zinc would likely be responsible for the variability seen in Table 1. Interaction of SAAP with other surfactant components (e.g., surfactant proteins or phospholipids) is likely and may also influence bactericidal activity.

From sheep 814 surfactant, three small, anionic peptides were isolated and sequenced. Synthesized peptides were moderately bactericidal in test buffer (Tables 3 and 4) fro *P. haemolytica* and other Gram-positive and Gram-negative bacteria. Because bactericidal activity of bronchoalveolar lavage and amniotic fluid was reported to be inhibited by solutions containing phosphate and enhanced by solutions containing zinc (10-12), surfactant was suspended in 0.14 M NaCl/10 μM $ZnCl_2$. Bactericidal activity of surfactant (Table 1), synthesized SAAP (Tables 3 and 4), and commercial aspartate peptides were all enhanced.

These peptides differ significantly from defensins and β-defensins found in epithelial, resident, and inflammatory cells of the respiratory tract. For example, SAAP are found in respiratory secretions (13) and require zinc as a cofactor for antimicrobial activity. They are also opposite in charge (anionic vs. cationic) and considerably smaller in size (722–824 Da vs. 3400–4084 Da) than defensins and β-defensins. Like defensins and β-defensins, SAAP are active against a number of Gram-negative and Gram-positive bacteria at comparable concentrations (e.g., 0.1–1.0 mM) (4, 14). SAAP are members of another class of peptide antibiotics containing homopolymeric regions of aspartate, which if present in pulmonary secretions may help clear bacteria as a part of the innate pulmonary defense system.

Table 4. Killing of bacteria by ovine pulmonary SAAP

Organism	Buffer*†	MBC_{50} of SAAP, mM, Fraction 3 H-DDDDDDD-OH (823.8 Da)
K. pneumoniae ATCC 10031	Test buffer	>7.31
	Zn-saline	0.62 (0.26)‡
E. coli ATCC 12795	Test buffer	>7.31
	Zn-saline	0.64 (0.16)‡
S. aureus ATCC 29213	Test buffer	>7.31
	Zn-saline	1.21 (0.37)‡
S. faecalis ATCC 29212	Test buffer	>7.31
	Zn-saline	1.39 (0.30)‡

*Test buffer is 0.14 M NaCl/10 mM sodium phosphate buffer, pH 7.2/0.5 mM $CaCl_2$/0.15 mM $MgCl_2$.
†Zn-saline buffer is 0.14 M NaCl/10 μM $ZnCl_2$.
‡Means (SEs) of six replications.

Bacterial killing appears to be due to a combination of pulmonary surfactant peptides and zinc in the serum. Ovine serum contains 15 μM zinc (15). The zinc is bound loosely to serum proteins, particularly albumin (16), perhaps explaining why albumin can substitute for normal serum in the assay (13). However, the exact role of zinc in the bactericidal mechanism is not known. Clearly, phosphate inhibits bactericidal activity of SAAP, and zinc enhances bactericidal activity (Tables 1 and 3). But not all bactericidal surfactants increase in activity and not all nonbactericidal surfactants become bactericidal in solutions containing zinc. Bactericidal activity (i.e., MBC_{50}) is inversely related to the number of aspartate residues in the peptide.

The mechanism of bacterial killing is not like that for defensins and β-defensins, which bind to the bacteria surface-forming channels in the membrane and increase membrane permeability in a charge- or voltage-dependent manner (17). SAAP–zinc complexes could not be detected on the bacterial surface by x-ray spectral microanalysis, and membrane chan-

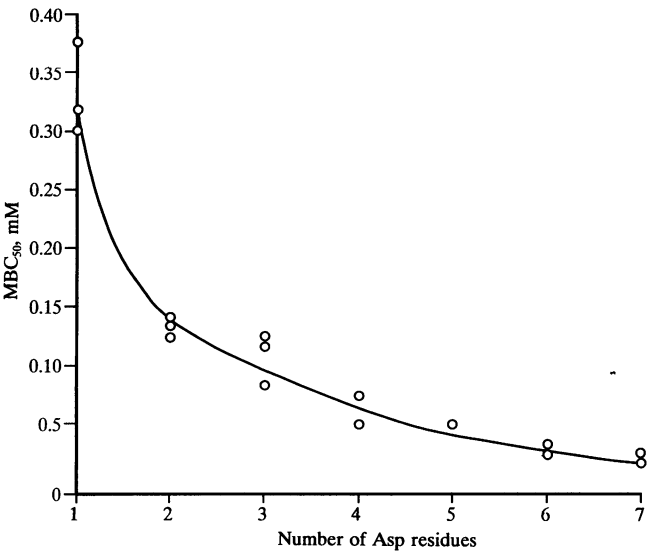

FIG. 3. Killing of *P. haemolytica* serotype A1 after incubation in zinc saline solution containing one- to six-residue amino acid homopolymers of aspartate. MBC_{50} decreases with the number of aspartate residues in the homopolymer and follows a nonlinear exponential curve with the formula, where figures in parentheses are SE values:

$$MBC_{50} = 0.579 \exp(-0.602\ \mathrm{Asp}).$$
$$(0.048) \quad (0.052)$$

nels could not be seen directly by electron microscopy in either negative-stained or thin-sectioned cells. Rather, the mechanism of cell death may be related to the pH of surfactant containing anionic peptide or involve a metabolic event by which the anionic peptide and zinc are taken up by the bacterial cell. Aspartate forms stable complexes with zinc (18), and synthetic aspartate and aspartate polymers were bactericidal in zinc saline solution. A similar mechanism may be involved in the action of a low-molecular-weight peptide from human amniotic fluid (10). The peptide of 630 Da contains three glutamic acid, two glycine, and one lysine residues and required zinc for bactericidal activity. Glutamic acid forms stable complexes with zinc similar to aspartic acid (18).

The origin of SAAP is distinct from tracheal antimicrobial peptide (3, 4) and alveolar macrophage microbicidal cationic protein (5). SAAP composition suggests that it may be part of a charge-neutralizing sequence incorporated into a number of cationic proteins found in the lung—for example, a major basic protein found in the granules of eosinophils (19). Major basic protein is a 13.8-kDa basic protein due to a predominance of arginine and lysine residues. It may participate in airway hyperresponsiveness of asthmatic patients (19) and help to clear helminth infestations (20). This protein is synthesized as a larger precursor with an amino-terminal signal sequence for targeting to the endoplasmic reticulum, a charge-neutralizing anionic propiece, and the mature defensin at the carboxyl terminus (2). There are several negatively charged glutamate and aspartate residues in the propiece, which is near a positively charged lysine cleavage site (2). The aspartate-containing peptide found in ovine pulmonary secretions may originate from eosinophil major basic protein released to combat parasitic infestation. Lungworm infestations, in the respiratory tract of sheep, are characterized by accumulations of eosinophils (21).

Alternately, the peptide may also be part of a charge-neutralizing fragment of a surfactant-associated proprotein. SAAP copurifies with surfactant through a series of differential and gradient centrifugation. After frozen storage, it can be then separated from surfactant by ultracentrifugation at $100{,}000 \times g_{AV}$. SAAP may be an individually expressed product or an enzymatic posttranslational modification byproduct of larger surfactant-associated proprotein. The surfactant-associated proteins SP-A, SP-B, SP-C, and SP-D, produced by type II epithelial cells, are all known to be posttranslationally modified by peptidases at the alveolar epithelial surface (22-24). SP-B and SP-C are produced with heterogeneous NH_2 terminals that are trimmed with amino peptidases to functional proteins (22). It is possible that SAAP is a byproduct of enzymatic trimming; this would explain the presence of such a small peptide in the purified surfactant working solution after the extensive differential and density-gradient centrifugation procedure.

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