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Article **Pulmonology**

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See the related Commentary beginning on page 1453.

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The pulmonary collectins, surfactant proteins A (SP-A) and D (SP-D), have been reported to bind lipopolysaccharide (LPS), opsonize microorganisms, and enhance the clearance of lung pathogens. In this study, we examined the effect of SP-A and SP-D on the growth and viability of Gram-negative bacteria. The pulmonary clearance of *Escherichia coli K12* was reduced in SP-A–null mice and was increased in SP-D–overexpressing mice, compared with strain-matched wild-type controls. Purified SP-A and SP-D inhibited bacterial synthetic functions of several, but not all, strains of *E. coli*, *Klebsiella pneumoniae*, and *Enterobacter aerogenes*. In general, rough *E. coli* strains were more susceptible than smooth strains, and collectin-mediated growth inhibition was partially blocked by coincubation with rough LPS vesicles. Although both SP-A and SP-D agglutinated *E. coli K12* in a calcium-dependent manner, microbial growth inhibition was independent of bacterial aggregation. At least part of the antimicrobial activity of SP-A and SP-D was localized to their C-terminal domains using truncated recombinant proteins. Incubation of *E. coli K12* with SP-A or SP-D increased bacterial permeability. Deletion of the *E. coli* OmpA gene from a collectin-resistant smooth *E. coli* strain enhanced SP-A and SP-D–mediated growth inhibition. These data indicate that SP-A and SP-D are antimicrobial proteins that directly inhibit the proliferation of Gram-negative bacteria in a macrophage- and aggregation-independent manner by increasing the permeability of the microbial cell membrane.

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Introduction

The process of respiration deposits aerosolized particulate matter, including infectious microbes, in the distal airspaces of the lung. Rapid bacterial clearance in naive hosts is primarily based on recognition of pathogen-associated molecular patterns, such as dense bacterial carbohydrate arrays, by host pattern recognition receptors, such as the mannose receptor on the surface of macrophages (1, 2). There is recent evidence that two oligomeric proteins that are secreted into the lining fluid of the airways and alveoli, surfactant proteins A (SP-A) and D (SP-D), serve important roles in pulmonary innate immune defense (3). This set of functions was initially suggested by their structural similarities with mannose-binding protein A (4), including N-terminal collagen-like regions and C-terminal C-type lectin carbohydrate recognition domains (CRDs), which identified them as pattern recognition receptors and members of the collectin family of preimmune opsonins (5). The pulmonary collectins have been shown to bind bacterial, fungal, viral, and mycobacterial organisms, to enhance the in vitro phagocytosis and intracellular killing of a variety of pulmonary pathogens, and to modulate the function of macrophages (for review see ref. 3). SP-A and SP-D have recently been reported to play physiologically relevant roles in the clearance of Gram-negative organisms from the lungs of experimental animals through macrophage-dependent pathways (6, 7). Collectively, these data suggest that SP-A and SP-D function as broad-spectrum opsonins for microbial pathogens and as immunomodulators that regulate the inflammatory response in the alveolar space (8).

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Nonstandard abbreviations used: surfactant protein (SP); mouse SP (mSP); recombinant mouse SP (rmSP); lipopolysaccharide (LPS); rat SP (rSP); recombinant rat SP (rrSP); human SP (hSP); carbohydrate recognition domain (CRD); Luria broth (LB); bronchoalveolar lavage (BAL); outer membrane protein A (OmpA); absorbance units (AU); actinomycin D (Act D); a recombinant C-terminal fragment of SP-A (ΔSP-A); a recombinant C-terminal fragment of SP-D (ΔSP-D).

Several laboratories have examined the molecular basis of the interaction between SP-A or SP-D and pathogen-associated molecular patterns. Both collectins bind to lipopolysaccharide (LPS), a cell wall component of all Gram-negative bacteria composed of an amphipathic lipid A moiety, core oligosaccharides, and a variable O-antigen polysaccharide domain (9, 10). LPS interactions are mediated by calcium-dependent binding of the C-terminal domains of the proteins to the lipid A domain for SP-A, and the core oligosaccharides and O-antigen domains for SP-D (11). SP-A binds preferentially to rough LPS strains containing truncated or absent O-antigen domains. SP-A has also been reported to interact with peptide ligands on the surface of microorganisms, including a P2 outer membrane protein of *Haemophilus influenzae* (12). Binding of both collectins to *Escherichia coli* and other Gram-negative bacteria results in agglutination, which may enhance phagocytosis by the alveolar macrophage (13), but little is known about other effects of collectin/bacterial interactions on microbial physiology.

The purpose of this study was to examine the macrophage-independent effects of SP-A and SP-D on the growth and viability of the Gram-negative bacterium *E. coli*.

Methods

SP-A and SP-D infection models. SP-A$^{-/-}$ mice (a gift of J. Whitsett and T. Korfhagen) were developed from ES cells after disruption of the mouse SP-A (mSP-A) gene by homologous recombination and maintained by breeding with Swiss Black mice, as previously described (14). The targeted SP-A gene was then bred into the C3H/HeN background through nine generations. All comparisons made in the SP-A–null background were between C3H/HeN SP-A$^{-/-}$ mice and C3H/HeN SP-A$^{+/+}$ (wild-type) controls. For SP-D in vivo studies, Swiss Black mice with elevated pulmonary SP-D levels and wild-type Swiss Black control mice were used. Lung-specific overexpression of rat SP-D (rSP-D) in Clara cells and alveolar type II cells was accomplished using the human SP-C (hSP-C) promoter, as previously described (15). For infection experiments, mice were anesthetized by inhalation of isoflurane, endotracheally intubated with a 22-gauge feeding needle (Roboz Surgical Instrument Co., Rockville, Maryland, USA), inoculated with 5×10^6 *E. coli* organisms (100 μl), and allowed to awaken. Six hours later, mice were anesthetized with pentobarbital, and euthanized by transection of the dorsal aorta. Using sterile technique, the lungs were removed, and pulverized with a Dounce homogenizer. Lung homogenates were diluted in saline, spread onto Luria broth (LB) ampicillin plates, and incubated overnight at 37°C. Colonies were counted, and data were expressed as CFUs per lung. Animals were housed in positively ventilated microisolator cages with automatic recirculating water, located in a room with laminar, high efficiency particle accumulation–filtered air. The animals received autoclaved food, water, and bedding. Mice used in experimental procedures were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee at University of Cincinnati School of Medicine.

Production of mutant recombinant proteins. The cDNA for mSP-D was generated by RT-PCR of mouse lung RNA. Truncated mutant forms of SP-A and SP-D containing only the C-terminal domains (A81–F228 of rSP-A and D203–F355 of mSP-D) were developed by overlapping extension PCR (16) using rSP-A or mSP-D cDNA templates and expression in insect cells (17, 18). Recombinant SP-A and SP-D were purified from the culture media by calcium-dependent adsorption to mannose-Sepharose 6B or maltose-Sepharose 6B columns, respectively (19). SP-A was dialyzed against 25 mM HEPES alone (pH 7.4), and SP-D was dialyzed against 10 mM HEPES (pH 7.4) and 150 mM NaCl. Then they were stored at –20°C.

Purification of native SP-A and SP-D. Surfactant was isolated by bronchoalveolar lavage (BAL) of Sprague-Dawley rats that had been treated 1 month earlier with intratracheal silica to enhance the collectin yield, as previously described (20–22). Rat SP-D was purified from the BAL of silica-pretreated rats by calcium-dependent adsorption of the 12,000-*g* supernatant to maltose-Sepharose columns. Human SP-A was isolated from the lung washings of patients with the lung disease alveolar proteinosis by a modification of the method of Suwabe et al. (23). Briefly, hSP-A was purified from the cell-free surfactant pellet of BAL by serial sedimentation and resuspension (five times) in buffer containing 10 mM Tris, 150 mM NaCl, and 1 mM $CaCl_2$, release by incubation with 10 mM Tris, 150 mM NaCl, and 2 mM EDTA, and adsorption of the recalcified supernatant to mannose-Sepharose affinity columns. After elution with 2 mM EDTA, gel exclusion chromatography with Superose 6 (Amersham Biosciences, Piscataway, New Jersey, USA) was used to remove contaminating SP-D. The hSP-A was dialyzed against buffer containing 50 mM NaCl and 25 mM HEPES and stored at –20°C. The EDTA content of all native and recombinant collectin preparations after dialysis was assessed by a modification of the spectrophotometric assay of Kratochvil and White, using β-phenanthrolene–disulfonic acid as the indicator (24).

Protein assays. Routine protein concentrations were determined with a bicinchoninic acid protein assay kit (BCA; Pierce Chemical Co., Rockford, Illinois, USA) using BSA as a standard. Protein samples were separated by 8–16% SDS-PAGE and stained with Coomassie blue or silver (25). For immunoblot analysis, protein species were transferred to nitrocellulose membranes and reacted with rabbit polyclonal anti–hSP-A antibody (26) or anti–hSP-D antibody (a gift of E.C. Crouch, Washington University, St. Louis, Missouri, USA). Blots were developed by HRP-dependent oxidation of a chemiluminescent substrate (ECL; Amersham Biosciences) and visualized using autoradiography.

LPS preparations. LPS vesicles were generated by probe sonication of stock solutions of *E. coli* J5 LPS (Sigma-Aldrich, St. Louis, Missouri, USA; 10 mg/ml in H_2O) at room temperature for 7 minutes (550 Sonic Dismembrator; Fisher Scientific Co., Pittsburgh, Pennsylvania, USA). LPS was isolated from Gram-negative bacterial isolates by acetone/phenol extraction (27). LPS samples were size-fractionated on 4–12% Bis-Tris PAGE gels (NuPAGE Novex Gels; Invitrogen, San Diego, California, USA) and stained with silver.

Bacterial strains. *E. coli K12* transfected with the plasmid PVL 1393 (Invitrogen, Carlsbad, California, USA) to confer ampicillin resistance were used for all in vitro and in vivo experiments, except where otherwise specified (28). Laboratory strains of *E. coli* tested included four rough strains: J5 (American Type Culture Collection, Manassas, Virginia, USA), HB101a (a gift of M. Welsh, University of Iowa, Iowa City, Iowa, USA), HB101b (a gift of M. Karp, University of Turku, Turku, Finland), and LCD25 (a *K12* acetate auxotroph; List Biologicals, Campbell, California, USA); and two smooth strains: 0111:K58 (B4) and 055:K59 (B5; both from American Type Culture Collection). *E. coli* clinical isolates (Ec1–Ec4) were purchased from the Pennsylvania State University Gastroenteric Disease Center, Wiley Laboratory (University Park, Pennsylvania, USA), and *Enterobacter aerogenes* and *Klebsiella pneumoniae* clinical isolates were gifts of J. Rhodes (University of Cincinnati, Cincinnati, Ohio, USA). Isogenic mutants based on smooth parental *E. coli K1* strain E98 were used to test the role of outer membrane protein A (OmpA) in resistance to collectin-mediated growth inhibition (29). These isogenic mutants included: (a) ΔOmpA, which is OmpA-null E98; (b) ΔOmpAv, which is OmpA-null E98 containing empty vector; and (c) ΔOmpA+, which is OmpaA-null E98 corrected by overexpression of OmpA. Isogenic *E. coli K12* bacteria (a gift of M. Apicella, University of Iowa) containing the full *K12* LPS core region and genes for enzymes that add back portions of the O-antigen were used to map domains of *E. coli* that may be necessary for collectin interactions. These bacteria included *E. coli* pGEM-3zf9(+), which contains the pGEM plasmid only; and *E. coli* pGEMLOS7 and *E. coli* pGEMLOS4, which express glycosyltransferases that add two sugars and five sugars, respectively, to the *K12* LPS core region (30). *E. coli* MLK217 (htrB mutant), a *K12* variant that is defective in an acyltransferase responsible for acyl side chain modification of the 3-OH position of lipid A (31), was used to test the importance of lipid A structural integrity in collectin-mediated bacterial killing.

Assays of bacterial density and aggregation. *E. coli* were grown in LB (Difco Laboratories, Detroit, Michigan, USA) in the presence of 50 μg/ml ampicillin overnight. A 10-μl aliquot of the stationary-phase bacteria was added to 2 ml of LB, 50 μg/ml ampicillin, and various concentrations of SP-A or SP-D. The tubes were shaken at 300 rpm for up to 7 hours, and bacterial density was monitored by measurement of light scattering at a wavelength of 400 nm in a spectrophotometer at 1-hour intervals. Bacterial aggregation was also qualitatively assessed by viewing of aliquots of bacterial cultures under the light microscope.

Assays of bacterial growth. Inhibition of bacterial growth was assessed using a radial diffusion method (32). Molten SeaKem LE agarose (0.8–1.0%) (FMC BioProducts, Rockland, Maine, USA) in buffer containing 10 mM sodium phosphate and 1.0% LB medium was mixed with 2×10^6 *E. coli* at a temperature of 40°C and allowed to harden by cooling. Agar composed of tryptone (40 g/l), yeast extract (20 g/l), and agarose (1%) was layered on top. Albumin (Sigma-Aldrich; 1 mg/ml), lysozyme (Sigma-Aldrich; 0.1 and 1.0 mg/ml), or pulmonary collectins (0.1 and 1.0 mg/ml) were added to 5-μl wells bored in the agar. After overnight incubation at 37°C, the plates were visually inspected for clearing around the wells.

Assessment of macromolecular synthesis. Bacteria were grown overnight in LB, spun, and resuspended at 1:200 dilution. After shaking at 37°C for 3 hours to mid–log phase, the organisms were washed in HBSS and resuspended in labeling media at an OD_{400nm} of approximately 0.2 absorbance units (AU). For assessment of RNA synthesis, bacteria were incubated for various time periods in HBSS containing 4 μCi/ml ^{3}H-uridine (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). For assessment of protein synthesis, bacteria were suspended in HBSS containing 40 mM Tris, 0.1% casamino acids, 0.9% NaCl, and 0.4 μCi/ml ^{14}C-L amino acid mixture (PerkinElmer Lifesciences Inc., Boston, Massachusetts, USA). In some experiments, *E. coli* J5 LPS vesicles were also added. After incubation in labeling media at 37°C for various periods of time in the presence or absence of SP-A or SP-D, the reactions were stopped by addition of ice-cold 10% trichloroacetic acid. The precipitated cell products were captured on filter membranes and counted in a scintillation counter. Control experiments in which *E. coli K12*–aggregating antibodies E*K12* and PM were added were also performed (33).

Assays of bacterial permeability. We assessed the effect of the pulmonary collectins on *E. coli* outer membrane integrity by determining susceptibility to the impermeant antibiotic actinomycin D (Act D; Sigma-Aldrich). *E. coli K12* RNA synthesis was measured in the presence or absence of collectins and Act D (10 μg/ml) by ^{3}H-uridine labeling as outlined above (34). Protein release from *E. coli* exposed to the collectins was measured using the thiol-specific fluorophore ThioGlo 1 (CALBIOCHEM, San Diego, California, USA) (35). *E. coli* were exposed to rSP-A or rSP-D in HBSS at various concentrations for 15 minutes at 37°C, and then sedimented by centrifugation. The supernatant was incubated with 10 μM ThioGlo1 in the presence and absence of the protein-denaturing agent SDS, and thiol-containing proteins were detected using a fluorescent plate reader with excitation and

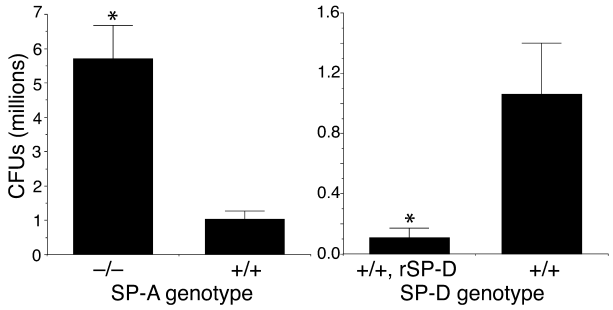


Figure 1
Modulation of *E. coli* clearance from the lungs of pulmonary collectin mouse models. Bacterial CFUs in lung homogenates were quantified 6 hours after intratracheal inoculation with 5×10^6 *E. coli* in age- and inbred strain–matched SP-A$^{-/-}$ and SP-A$^{+/+}$ C3H/HeN mice, or Swiss Black SP-D–overexpressing mice (+/+, rSP-D) and littermate controls (+/+). Data are mean ± SEM; $n = 10$ per group; $^*P < 0.05$.

emission maxima of 405 nm and 535 nm, respectively. Thiol-containing proteins were quantified by subtraction of fluorescence due to released glutathione (i.e., signal obtained in the absence of SDS) and protein-only controls (i.e., signal obtained in the absence of *E. coli*) from total fluorescence. Bacterial membrane integrity was also assessed by differential staining with the permeant fluorescent probe STYO 9 and the impermeant fluorescent probe propidium iodide (*Bac*Light; Molecular Probes Inc.). Fluorescence was measured at excitation and emission maxima of 485 nm and 535 nm, respectively, for STYO 9, and 485 nm and 620 nm, respectively, for propidium iodide (36). In all permeability assays, the permeabilizing antimicrobial peptide mellitin (Sigma-Aldrich) was used as a positive control.

Statistical analysis. All data shown are mean ± SEM. Comparisons between groups were made using the unpaired Student's *t* test, and $P < 0.05$ was considered to indicate statistical significance.

Results

Altered pulmonary clearance of E. coli in collectin mouse models. The role of pulmonary collectins in the pulmonary clearance of *E. coli K12* was tested in genetically engineered SP-A–null (SP-A$^{-/-}$) mice and mice that overexpress SP-D (SP-D$^{+/+,rSP\text{-}D}$). The SP-A–null allele developed by gene targeting in Swiss Black mice (14) was bred into the C3H/HeN strain to provide a genetically homogeneous background. *E. coli K12* CFUs in lung homogenates were compared 6 hours after endotracheal inoculation with 5×10^6 *E. coli K12*, and the results are shown in Figure 1. There was a fivefold decrease in the clearance of *E. coli K12* from SP-A$^{-/-}$ mice compared with SP-A$^{+/+}$ mice ($5.68 \times 10^6 \pm 0.97 \times 10^6$ and $1.00 \times 10^6 \pm 0.25 \times 10^6$ CFUs, $n = 10$ mice per group, $P < 0.05$) and a tenfold increase in the clearance of *E. coli K12* from SP-D$^{+/+,rSP\text{-}D}$ mice compared with littermate controls ($0.11 \times 10^6 \pm 0.06 \times 10^6$ and $1.06 \times 10^6 \pm 0.34 \times 10^6$, $n = 10$ mice per group, $P < 0.05$). These data suggest that SP-A and SP-D contribute to pulmonary host defense against *E. coli*. Prior studies with other Gram-negative organisms suggest that the mechanisms of collectin-mediated pulmonary clearance may include aggregation, opsonization, and enhanced intracellular killing (3). The experiments below were performed to explore the hypothesis that SP-A and SP-D directly affect the growth and viability of *E. coli*.

Proteins associated with the surfactant pellet inhibit the growth of E. coli. The effect of human surfactant-associated proteins on the biosynthetic capacity of *E. coli K12* was tested using an RNA synthesis assay. Alveolar lavage fluid from a patient with alveolar proteinosis was centrifuged at low speed to remove cellular components, and at high speed to sediment the surfactant pellet. Proteins that were associated with surfactant in a calcium-dependent manner were eluted with EDTA, dialyzed into calcium-containing buffer, and purified by mannose-Sepharose affinity chromatography. The peak fraction from the carbohydrate affinity column

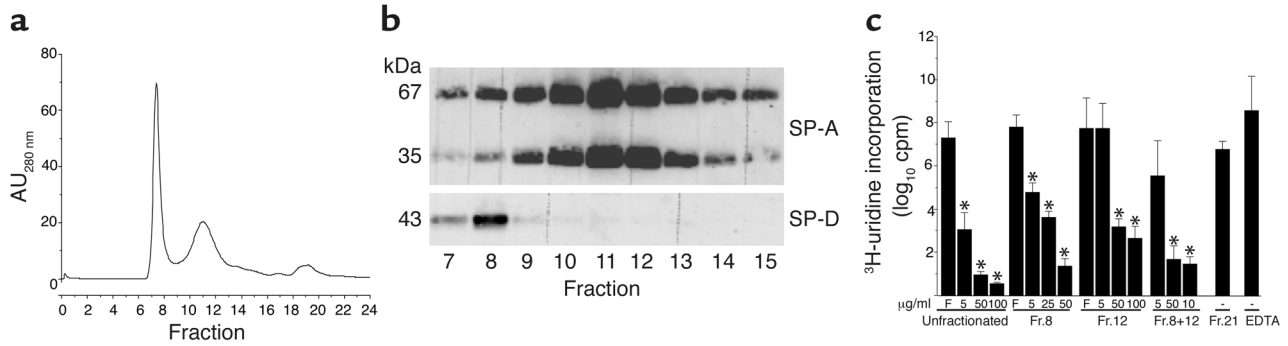


Figure 2
Antimicrobial activity of calcium-bound surfactant-associated proteins. Human surfactant was repeatedly washed by sedimentation in the presence of calcium, eluted with EDTA, purified by mannose-Sepharose affinity chromatography, and size-fractionated by fast protein liquid chromatography on a Superose 6 column. (**a**) Elution of proteins from the column was monitored by UV absorbance at a wavelength of 280 nm. (**b**) The SP-A and SP-D content of individual fractions was determined by Western analysis. (**c**) The antimicrobial activity of the original sample and selected fractions, including 8, 12, and the tenfold concentrate of fraction 21, was assessed by measurement of ^{3}H-uridine incorporation in *E. coli K12*. Controls shown included protein-free ultrafiltrates (molecular weight cutoff of 10,000) of the most concentrated sample used in each set (designated F), and 2 mM EDTA, the highest possible concentration of EDTA in any sample. Data are mean ± SEM; $n = 3$; $^*P < 0.01$.

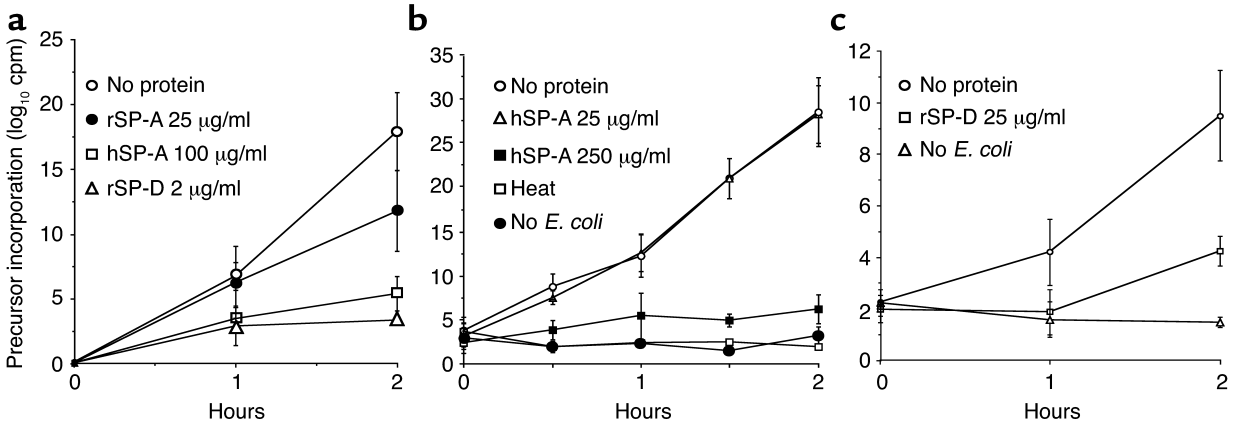


Figure 3
Time course of inhibition of *E. coli K12* macromolecular synthesis by rat and human pulmonary collectins. *E. coli K12* were incubated in the presence of ^{3}H-uridine (**a**) or ^{14}C-amino acids (**b** and **c**) and pulmonary collectins including hSP-A, rSP-A, or rSP-D for up to 2 hours. At the indicated time points, bacterial RNA or proteins were precipitated, collected on filters, and counted using a scintillation counter. Controls including no bacteria, no added protein, and heat-killed bacteria (heat) are shown. Data are mean ± SEM; $n = 3$.

was size-separated by gel exclusion chromatography (Figure 2a), and the 1-ml eluted fractions were tested for collectin content by Western analysis (Figure 2b), and for antimicrobial activity by ^{3}H-uridine incorporation into *E. coli K12* (Figure 2c). There were two major absorbance peaks (A_{280nm}), one centered at fractions 7–8, which contained both SP-A and SP-D, and one centered at fractions 11–12, which contained only SP-A (Figure 2b). Both fraction 8 and fraction 12, as well as fractions 8 and 12 combined, inhibited RNA synthesis in *E. coli K12* in a dose-dependent manner similar in magnitude to that of growth inhibition by the unfractionated proteins applied to the column (Figure 2c). RNA synthesis in *E. coli K12* was not inhibited by the protein-free ultrafiltrate (molecular weight cutoff of 10,000) of the most concentrated collectin fractions tested in each set. There was also no activity in fractions flanking the major peaks, including fraction 5 (not shown) and fraction 21.

The inhibition of RNA synthesis by purified SP-A and SP-D was assessed over a 2-hour period, and the results are shown in Figure 3. Both hSP-A at 100 μg/ml and rSP-D at 2 μg/ml markedly suppressed ^{3}H-uridine incorporation at 1 hour, and there was little additional labeling at 2 hours (Figure 3a). In contrast, rSP-A at 25 μg/ml did not significantly inhibit growth at 1 hour or at 2 hours, but there appeared to be a trend toward inhibition at the later time point (P = NS) (Figure 3a).

The pulmonary collectins also inhibited protein synthesis (Figure 3, b and c). In the absence of added surfactant proteins, the incorporation of ^{14}C-amino acids into *E. coli K12* rose linearly over a 2-hour incubation

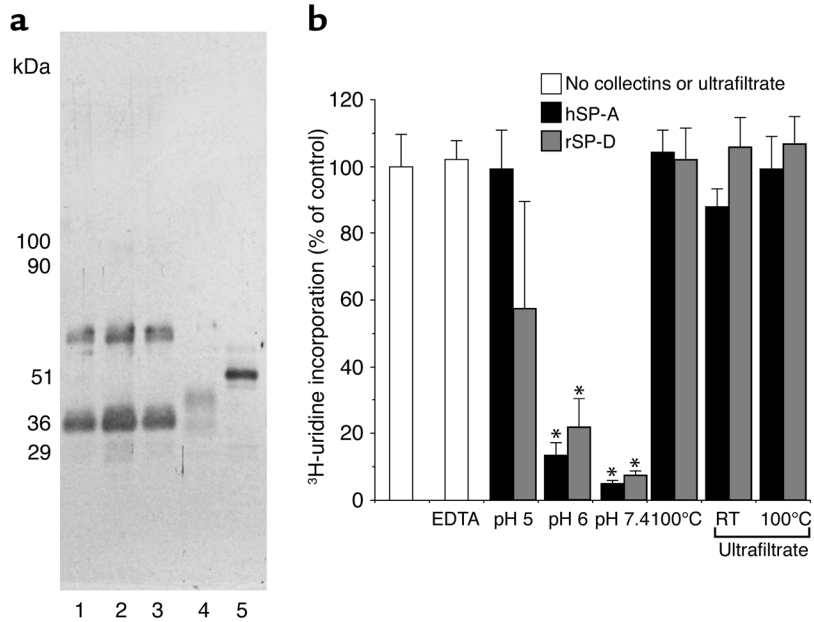


Figure 4
Specificity of collectin-mediated growth inhibition of *E. coli K12*. (**a**) The purity of collectins used in the study, including the EDTA eluate of the surfactant pellet (lane 1), fast protein liquid chromatography fractions 8 (lane 2) and 12 (lane 3), rSP-A (lane 4), and rSP-D (lane 5), was assessed by silver staining after 8–16% SDS-PAGE electrophoresis. (**b**) ^{3}H-uridine incorporation by *E. coli K12* was assessed alone and in the presence of 2 mM EDTA; in the presence of hSP-A (100 μg/ml) or rSP-D (10 μg/ml) at pH 5.0, 6.0, and 7.4; in the presence of boiled hSP-A (100 μg/ml) or rSP-D (10 μg/ml); and in the presence of the ultrafiltrate (molecular weight cutoff of 10,000) of hSP-A (100 μg/ml) or rSP-D (10 μg/ml) before boiling (room temperature; RT) and after boiling (100°C). Data are mean ± SEM; $n = 3$; *$P < 0.01$.

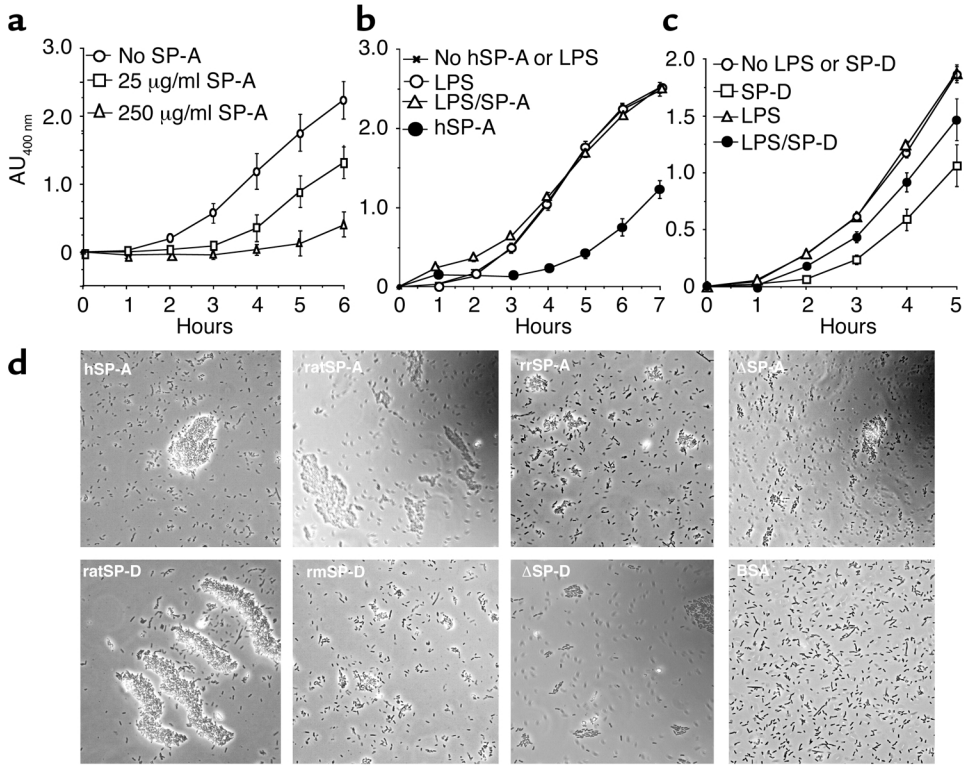


Figure 5
Attenuation of *E. coli K12*–induced light scattering by SP-A and SP-D. (**a**) *E. coli K12* were mixed with 25 μg/ml (open squares) or 250 μg/ml (open triangles) hSP-A or no protein (open circles) and incubated in LB at 37°C in a shaking water bath. Bacterial density was monitored by absorbance at 400 nm. (**b**) *E. coli* grown as above were incubated with hSP-A (filled circles), 300 μg/ml J5 LPS (open circles), 300 μg/ml J5 LPS plus 100 μg/ml hSP-A (open triangles), or no additives (x's). (**c**) *E. coli* grown as above were incubated with 100 μg/ml rmSP-D (open squares), 300 μg/ml J5 LPS (open triangles), or 300 μg/ml J5 LPS plus 100 μg/ml rmSP-D (filled circles). Data are mean ± SEM; $n = 3$. (**d**) Bacterial aggregation was assessed under the light microscope after incubation of *E. coli K12* with hSP-A, rSP-A, rrSP-A, a C-terminal fragment of SP-A containing residues A81–F228 (ΔSP-A), rSP-D, rmSP-D, a C-terminal fragment of SP-D containing residues D203–F355 (ΔSP-D), or BSA, and viewed with the light microscope.

period. Protein synthesis was almost completely inhibited by 250 μg/ml hSP-A throughout the entire labeling period, while 25 μg/ml hSP-A had no effect (Figure 3b). Rat SP-D at 25 μg/ml suppressed ^{14}C-amino acid incorporation at 1 hour and at 2 hours, but some additional labeling occurred in the second hour (Figure 3c). These data indicate that the pulmonary collectins rapidly inhibit bacterial RNA and protein synthesis, and that SP-D is a more potent inhibitor of macromolecular synthesis in *E. coli K12* than SP-A.

Several experiments were performed to assess the optimal conditions and specificity of collectin-mediated growth inhibition. Inspection of silver-stained SDS-PAGE gels revealed the typical reduced molecular species for hSP-A at 35 kDa and 66 kDa, for rSP-A at 26, 32, and 38 kDa, and for rSP-D at 40 kDa, and an overall purity for all proteins of greater than 95% (Figure 4a). As shown in Figure 4b, SP-A inhibited *E. coli K12* growth at pH 6.0 ($P < 0.01$) or pH 7.4 ($P < 0.01$), but not at pH 5.0. Growth inhibition by SP-D was also significant at pH 6.0 ($P < 0.01$) and pH 7.4 ($P < 0.01$) but was less consistent at pH 5.0 (P = NS). Boiling of the collectins (100°C) destroyed their antimicrobial activity. EDTA contamination was not responsible for growth inhibition, since 2 mM EDTA (the maximum possible level of contamination) did not inhibit *E. coli K12* growth, and the measured EDTA concentrations of the protein reagents ranged from only 0 to 22 μM (average 7.0 ± 3.2 μM) for hSP-A, from 3 to 20 μM (average 13.5 ± 2.5 μM) for rSP-A, and from 0 to 16 μM for rSP-D (average 5.4 ± 2.3 μM). In addition, the ultrafiltrates of the highest collectin concentrations tested had no antimicrobial activity.

Pulmonary collectin/bacterial interactions. We assessed interactions of SP-A and SP-D with *E. coli K12* by measuring their effects on light scattering of cultured organisms over time. *E. coli K12* were grown to the stationary phase overnight, diluted 1:200 into LB with ampicillin, and agitated at 37°C for various time periods alone or in the presence of collectins. Light scattering was monitored in a spectrophotometer at a wavelength of 400 nm (Figure 5). In the absence of SP-A, the *E. coli* grew logarithmically and the OD of the culture increased steadily to a peak OD_{400nm} of about 2.0 AU at 6 hours (Figure 5a). The increase in OD was partially inhibited by 25 μg/ml hSP-A (maximum OD = 1.25 AU), and markedly inhibited by 250 μg/ml hSP-A (maximum OD = 0.25 AU). To explore potential

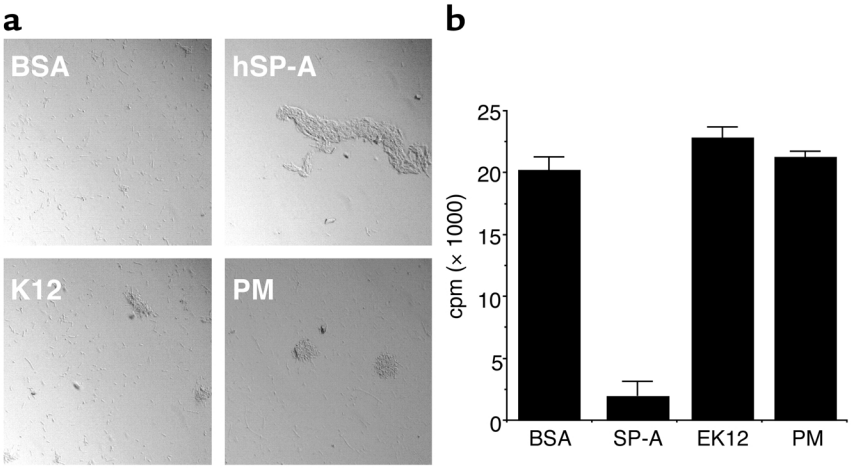


Figure 6
Antibody-induced aggregation does not affect RNA synthesis in *E. coli K12*. (**a**) *E. coli K12* were incubated in the presence of BSA, hSP-A (100 μg/ml), or *E. coli*–aggregating EK12 (100 μg/ml) or PM antibody (10 μg/ml) for 1 hour at 37°C and then visualized under the light microscope. (**b**) *E. coli* were incubated for 1.5 hours with ^{3}H-uridine prior to assessment of RNA synthesis as above. Data are mean ± SEM; $n = 3$.

ligands for SP-A and SP-D on the bacterial surface, *E. coli*/collectin interactions were also assessed in the presence of LPS. The inclusion of excess *E. coli* J5 LPS vesicles (300 μg/ml) completely blocked the hSP-A–mediated (100 μg/ml) attenuation in light scattering (Figure 5b) but had no effect on OD in the absence of collectins. Recombinant mouse SP-D (rmSP-D) (100 μg/ml) also reduced bacterial density from 1.81 to 1.06 AU, by a mechanism that was partially reversed with excess LPS vesicles (1.46 AU) (Figure 5c). The collectins had no effect on light scattering induced by growing *Staphylococcus aureus* (not shown). Light microscopy revealed bacterial aggregation after incubation of *E. coli K12* with hSP-A, rSP-A, rrSP-A, rSP-D, or rmSP-D (Figure 5d). It revealed more limited aggregation by truncated mutant forms of SP-A (ΔSP-A) and SP-D (ΔSP-D) that contained only the C-terminal domains (A81–F228 of rSP-A or D203–F355 of mSP-D), but no aggregation by BSA controls (Figure 5d). These data indicate that the collectins reduce *E. coli K12* density in culture, by a mechanism that includes LPS-dependent aggregation.

The role of aggregation in microbial growth inhibition by the collectins. Bacterial agglutination by the collectins complicates the assessment of their antimicrobial activity in several ways. One concern is that aggregation may induce bacteria to downregulate microbial synthetic functions indirectly (37). To address this issue, *E. coli K12* were incubated with aggregating antibodies E*K12* or PM directed against surface components of the organism, and metabolic precursor incorporation was measured (Figure 6).

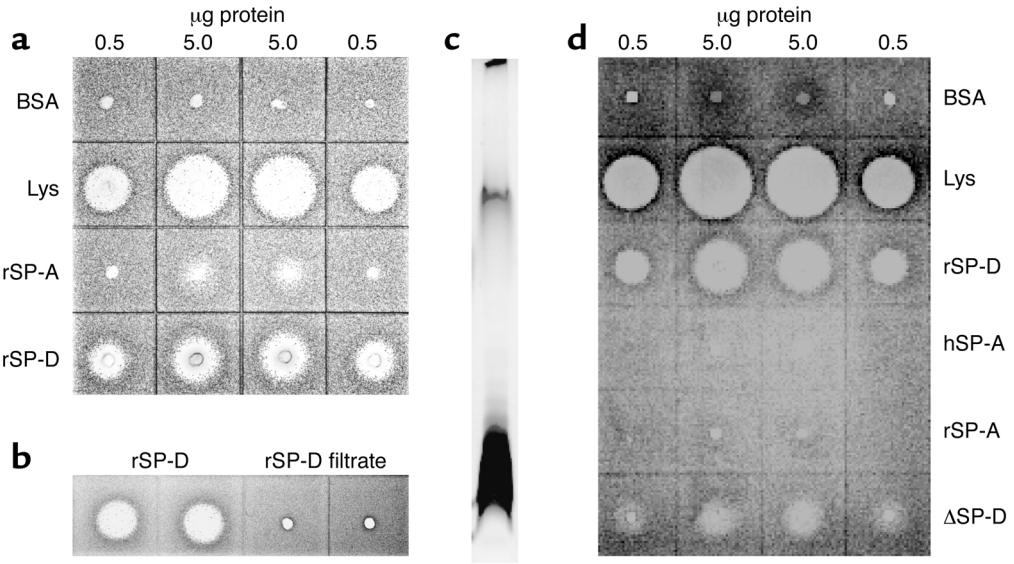


Figure 7
Inhibition of bacterial growth by SP-A and SP-D is independent of bacterial aggregation. Molten agarose was mixed with *E. coli K12* (**a** and **b**) or a clinical *E. coli* isolate from a septic patient (**d**), plated in Petri dishes, and allowed to cool. Wells were bored in the agar, and proteins (0.5 or 5.0 μg/well) were added for overnight incubation. (**a**) BSA, lysozyme (Lys), rSP-A, or rSP-D was incubated with *E. coli K12*. (**b**) *E. coli K12* was incubated with 5 μg rSP-D (first two wells) or the protein-free filtrate (molecular weight cutoff of 10,000) from the 1-mg/ml rSP-D reagent (last two wells). (**c**) Silver-stained SDS-PAGE gel of clinical *E. coli* isolate showing slow- and fast-migrating species, consistent with smooth LPS phenotype. (**d**) Clinical *E. coli* isolate incubated with the same proteins as *E. coli K12*, as well as hSP-A and ΔSP-D, a D203–F355 C-terminal fragment of recombinant mouse SP-D containing only the C-terminal domains.

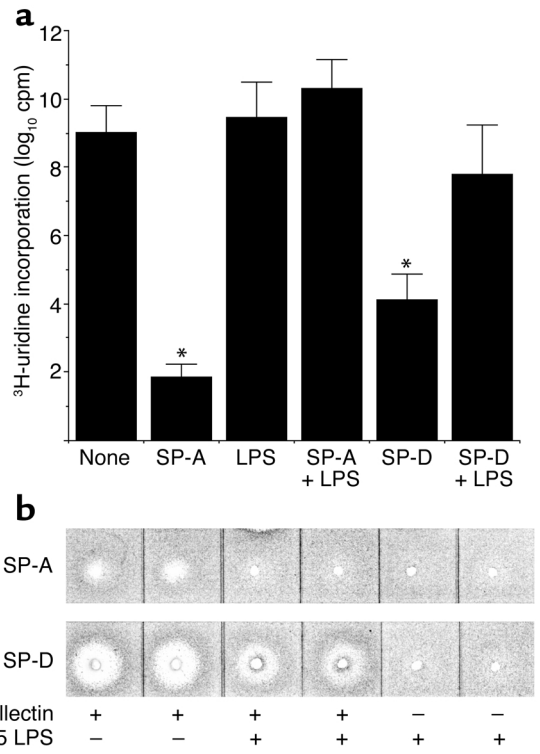


Figure 8

Growth inhibition by rSP-A and rSP-D is blocked by J5 LPS. (**a**) *E. coli K12* were grown in the presence of hSP-A (50 or 100 μg/ml) or rSP-D (5 μg/ml) and *E. coli* J5 LPS vesicles (300 μg/ml) for 90 minutes at 37°C. ^{3}H-uridine incorporation was measured as above. Data are mean ± SEM; n = 3–5 per group; $^*P < 0.01$ compared with *E. coli* alone. (**b**) *E. coli*–impregnated agar was prepared as above, and 0.5 μg of rSP-A or rSP-D was added to the well in the presence or absence of *E. coli* J5 LPS vesicles (100 μg/ml). Control wells with J5 LPS alone are also shown.

Both E*K12* and PM resulted in bacterial aggregation, albeit to a lesser extent than hSP-A. However, E*K12*, PM, BSA, and a nonspecific antibody (not shown) all failed to inhibit RNA synthesis, while 100 μg/ml hSP-A produced marked inhibition of ^{3}H-uridine incorporation.

Additional experiments were performed to determine the direct effects of the pulmonary collectins on microbial growth in the absence of bacterial aggregation, using a radial diffusion method, and the results are shown in Figure 7. Wells were bored into agar impregnated with *E. coli K12*. Proteins concentrated to 0.1 or 1 mg/ml were introduced into the wells in 5-μl aliquots, and the plates were incubated overnight at 37°C. Lysozyme at 0.5 or 5 μg/well produced dose-dependent clearing around the wells, but 5 μg albumin per well had no effect. Both rSP-A and rSP-D inhibited the growth of *E. coli*, although rSP-D consistently produced greater zonal clearing than rSP-A (Figure 7a). The protein-free filtrate (molecular weight cutoff of 10,000) from the highest rSP-A (not shown) and rSP-D (Figure 7b) concentrations used had no effect on *E. coli* growth. Similarly, 2 mM EDTA did not produce zonal clearing (not shown). The effect of collectins on the growth of an *E. coli* strain obtained from the blood of a septic patient was assessed. LPS isolated from the organism migrated as both low– and high–molecular weight species on a silver-stained SDS-PAGE analysis, consistent with a smooth LPS phenotype (Figure 7c). Both rSP-D and ΔSP-D but not hSP-A or rSP-A inhibited the growth of the clinical *E. coli* isolate (Figure 7d). A truncated form of rSP-D containing only the C-terminal domains, including the neck region and CRD (amino acids D203–F355 of rSP-D; ref. 38), produced zonal clearing in the plates at 0.5 μg/well and more easily visible inhibition of bacterial growth at 5 μg/well. These data indicate that SP-A and SP-D inhibit *E. coli* growth in an aggregation-independent manner, that SP-D has antimicrobial activity against both laboratory and clinical *E. coli* strains, and that at least part of the growth inhibition by SP-D is mediated by the C-terminal domains of the protein.

Role of LPS in collectin-mediated growth inhibition. We examined the importance of collectin/LPS interactions in the antimicrobial activity of SP-A and SP-D by assessing RNA synthesis in *E. coli K12* in the presence of excess LPS vesicles, in smooth and rough *E. coli* laboratory strains, and in *E. coli K12* bacteria with mutations that alter cell surface LPS. *E. coli* J5 LPS vesicles (300 μg/ml) were coincubated with *E. coli* and rSP-A (100 μg/ml) or rSP-D (25 μg/ml) in the presence of ^{3}H-uridine. LPS blocked the inhibitory effects of both collectins on RNA synthesis but had no effect on growth when added alone (Figure 8a). LPS also blocked growth inhibition by both surfactant proteins in the radial diffusion assay, albeit to a greater extent for SP-A than for SP-D (Figure 8b). The effect of SP-A and SP-D on ^{3}H-uridine incorporation in several *E. coli* laboratory strains was tested (Figure 9a) and correlated with LPS subtype based on migration on SDS-PAGE gels (Figure 9b). Partial restoration of the O-antigen of *E. coli K12* was found to have no effect on the antimicrobial effects of the collectins, using bacteria transfected with one of three plasmids: the empty plasmid pGEM-3zF9(+); pGEMLOS7, which directs expression of a glycosyltransferase that adds two sugars (galactose and *N*-acetylglucosamine) to the *K12* core region; or pGEMLOS4, which directs expression of a glycosyltransferase that adds five sugars (galactose or *N*-acetylglucosamine-galactose-*N*-acetylglucosamine-galactose-*N*-acetylglucosamine) to the *K12* core region. Similarly, both SP-A and SP-D inhibited the growth of the deep rough mutant *E. coli* MLK217, which expresses a modified lipid A moiety lacking the 3-OH acyl side chain, to the same extent that they inhibited the growth of the *E. coli K12* parental strain. Both collectins inhibited RNA synthesis in rough *E. coli* strains J5 ($P < 0.01$ for both), HB101b ($P < 0.01$ for SP-A, $P < 0.05$ for SP-D), and LCD25 ($P < 0.01$ for SP-A, $P < 0.05$ for SP-D), but only SP-A significantly inhibited HB101a ($P = 0.01$). It is interesting to note that HB101 LPS has a more complex SDS-PAGE profile than *E. coli K12* or J5 strains, suggesting that more extensive carbohydrate modification may play a role in resistance to SP-D. The finding that neither SP-A nor SP-D significantly inhibited RNA synthesis in the smooth LPS strains 0111:B4 or 055:B5 is also consistent with a role for

O-antigen domains in shielding the organism from the effects of the collectins. Collectively, these data suggest that LPS plays a role in collectin-mediated microbial-growth inhibition, but the precise molecular targets involved are unknown.

The effect of SP-A and SP-D on ^{3}H-uridine incorporation in several Gram-negative clinical isolates was tested and correlated with LPS subtype based on migration on SDS-PAGE gels (Figure 9). RNA synthesis was inhibited by SP-A but not SP-D in *E. coli* isolates Ec1 ($P < 0.05$) and Ec2 ($P < 0.01$); neither SP-A nor SP-D inhibited RNA synthesis in Ec3. These three isolates had smooth LPS subtypes based on their migration on SDS-PAGE gels. Neither SP-A nor SP-D exhibited antimicrobial activity against the rough isolate Ec4. Both SP-A and SP-D inhibited ^{3}H-uridine incorporation by two different *E. aerogenes* species, and two *K. pneumoniae* isolates were sensitive to inhibition by SP-A (SP-D was not tested). Both the *Klebsiella* and the *Enterobacter* species had relatively simple LPS profiles on SDS-PAGE analysis. These data indicate that the collectins exhibit antimicrobial activity for some but not all Gram-negative clinical species.

Deletion of OmpA increases the susceptibility of *E. coli* to growth inhibition by the pulmonary collectins. OmpA plays important roles in the membrane integrity of *E. coli* (39). The effects of the pulmonary collectins on the growth of a set of isogenic *E. coli K1* OmpA mutant bacteria were tested in a radial diffusion assay (Figure 10). The parental *E. coli K1* clinical isolate was a smooth variant (Figure 10a) and was relatively resistant to growth inhibition by rSP-A, hSP-A, and recombinant SP-A and SP-D forms, but sensitive to rSP-D (Figure 10b). Deletion of OmpA (ΔOmpA) resulted in sensitivity to growth inhibition by all native and recombinant collectins, including a truncated rrSP-A containing only the C-terminal domains of the molecule (ΔSP-A) (18). Complementation of the ΔOmpA bacteria with a vector directing expression of OmpA (ΔOmpA+), but not with an empty vector alone (ΔOmpAv), partially restored resistance of the bacterium to collectin-mediated growth inhibition. These data indicate that OmpA protects *E. coli* from growth inhibition by the pulmonary collectins, that deletion of OmpA can convert a collectin-resistant smooth LPS strain to a susceptible smooth LPS strain, and that the C-terminal domain of SP-A exhibits antimicrobial activity.

The pulmonary collectins increase bacterial permeability. The effect of the pulmonary collectins on the integrity of the *E. coli* outer membrane was assessed by measurement of protein efflux, Act D sensitivity, and vital DNA dye exclusion. The ThioGlo reagents are a group of maleimide naphthopyranones that rapidly react with accessible cysteines to yield a fluorescent product with a detection limit as low as 10 nM protein thiol (35). Since rSP-A and rSP-D do not contain free cysteines, this method can be used to measure release of thiol-containing proteins from collectin-treated organisms. *E. coli* exposed to rSP-A or rSP-D at 50–100 μg/ml for 15 minutes showed a significant increase in the content of thiol-containing proteins in the extracellular space

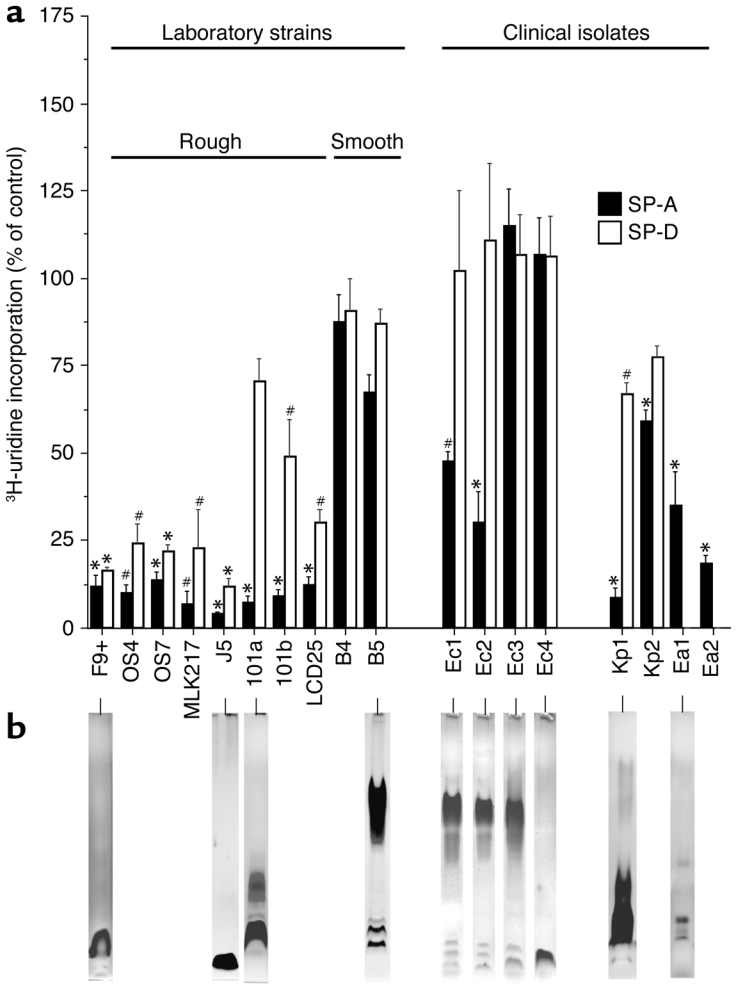


Figure 9
Growth inhibition of smooth and rough laboratory strains of *E. coli* and other Gram-negative isolates by pulmonary collectins. The effect of hSP-A (100 μg/ml) and rSP-D (10 μg/ml) on ^{3}H-uridine uptake by laboratory strains and clinical isolates was assessed (**a**) and correlated with the LPS profile on SDS-PAGE analysis (**b**). The following rough *E. coli* strains are shown: an *E. coli K12* strain containing an empty pGEM vector (F9+), and isogenic strains with pGEM-driven expression of glycosyltransferases that add two (OS4) and five (OS7) sugars to the core oligosaccharide; a *K12* mutant with a defective lipid A missing an acyl chain (MLK217); *E. coli* J5; two luminescent *E. coli* HB101 strains (101a and 101b); and LCD25, an *E. coli K12* acetate auxotroph. The following smooth *E. coli* strains are shown: 0111:K58 (B4) and 055:K59 (B5). Clinical isolates shown include four *E. coli* isolates, three smooth variants (Ec1, Ec2, and Ec3) and one rough variant (Ec4); two *K. pneumoniae* strains (Kp1 and Kp2); and two *E. aerogenes* strains (Ea1 and Ea2). Data shown are mean ± SEM; $n = 3$; $^*P < 0.01$, $^\#P < 0.05$.

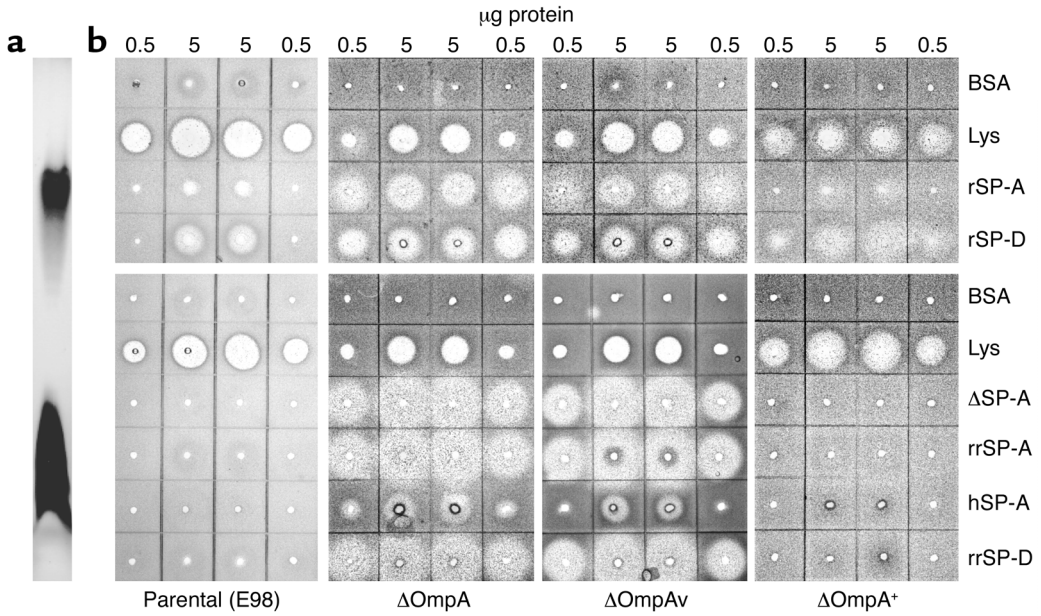


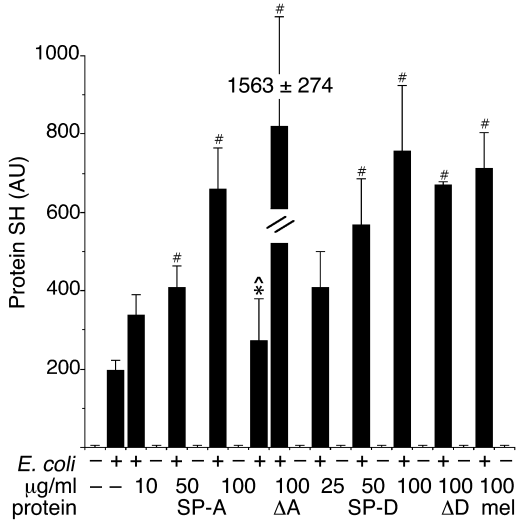
Figure 10
Deletion of OmpA enhances the susceptibility of smooth *E. coli* variant E98 to growth inhibition by SP-A and SP-D. (**a**) Silver-stained SDS-PAGE of *E. coli* strain E98. (**b**) A clinical isolate of E98 (parental), an isogenic OmpA-negative (ΔOmpA) mutant, and ΔOmpA bacteria containing either an empty vector (ΔOmpAv) or a plasmid directing overexpression of OmpA in the OmpA-negative background (ΔOmpA+) were embedded in agar and incubated overnight with albumin, lysozyme, rSP-A, rSP-D, rrSP-A, a C-terminal fragment of rrSP-A (ΔSP-A), hSP-A, and rrSP-D at 0.5 μg/well or 5.0 μg/well, as indicated.

compared with unexposed *E. coli K12*, but boiled SP-A had no effect (Figure 11). Truncated recombinant SP-A (ΔSP-A) and SP-D (ΔSP-D) fragments containing only the C-terminal domains also enhanced protein release. SP-A and SP-D were roughly as potent as mellitin, a peptide which is known to increase the permeability of Gram negative organisms in inducing the release of protein from *E. coli*. Levels of glutathione and freely accessible thiols (SDS-independent fluorescence) in the supernatants of treated organisms were also monitored but were not significantly different between the groups (not shown). Although this assay cannot distinguish between protein secretion and protein leakage, the rapid onset of the effect (15 minutes) suggests that collectins increase membrane permeability. Further, the data demonstrated that C-terminal domains of SP-A and SP-D, which contain the CRD, are sufficient to increase protein release from *E. coli K12*.

Figure 11
Incubation of *E. coli K12* with the pulmonary collectins results in release of protein into the extracellular space. *E. coli* were incubated with rSP-A, boiled rSP-A (^), a C-terminal fragment of SP-A (A81–F228, denoted ΔA), rSP-D, a C-terminal fragment of SP-D (D203–F355, denoted ΔD), or the permeabilizing antimicrobial peptide mellitin (mel) in HBSS for 15 minutes at 37°C and then sedimented by centrifugation. Thiol-containing proteins released into the extracellular space were measured using the fluorescent dye ThioGlo1. Controls in which bacteria or bacteria plus collectins were omitted are also shown. Data are mean ± SEM; n = 4–5. Statistical significance of differences in SH level in the presence and absence of collectins and controls is shown; $^{\#}P < 0.05$, $^{*}P < 0.01$.

Act D is an antibiotic that intercalates into DNA and inhibits RNA synthesis, but that does not ordinarily cross intact cell membranes. Coincubation of *E. coli K12* with 1–10 μg/ml hSP-A enhanced growth inhibition by Act D (10 μg/ml), which had no effect when added alone (Figure 12). Conversely, exposure of *E. coli* to low concentrations of SP-A and SP-D resulted in an increase in dose-dependent growth inhibition by Act D (Figure 12, b and c). These data suggest that SP-A enhances access of Act D to intracellular compartments.

Finally, vital dye staining also indicated that SP-A and SP-D increased membrane permeability. STYO 9 is green



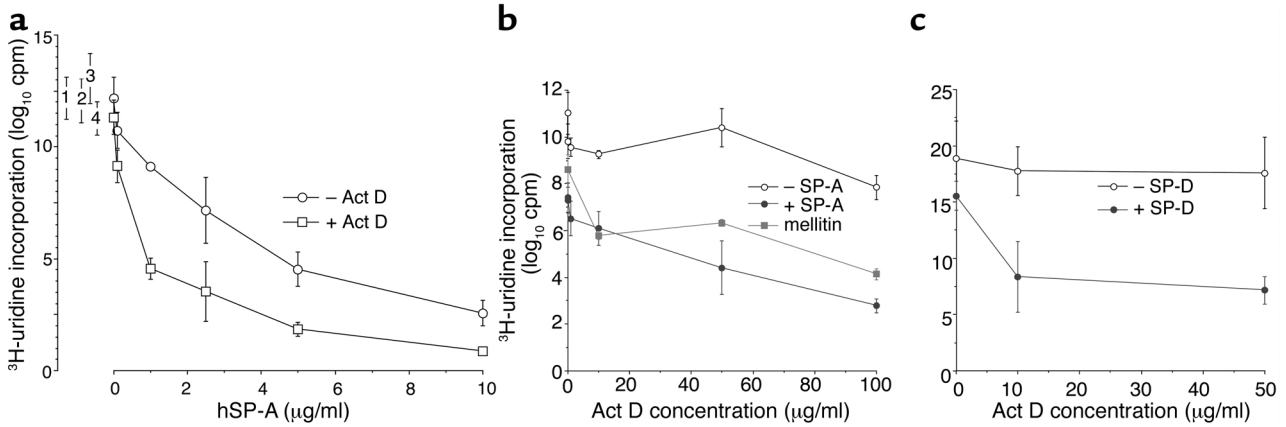


Figure 12
The pulmonary collectins enhance membrane permeability to Act D. (**a**) *E. coli K12* were incubated with 1–10 μg/ml hSP-A, in the presence and absence of 10 μg/ml Act D, and incorporation of ^{3}H-uridine was measured. RNA synthesis was also assessed in the presence of: 1, Act D (10 μg/ml) alone; 2, protein-free ultrafiltrate from 10 μg/ml rSP-A; 3, 0.05% DMSO vehicle; 4, ultrafiltrate plus Act D (10 μg/ml). (**b** and **c**) ^{3}H-uridine incorporation by *E. coli K12* incubated with a fixed SP-A, SP-D, or mellitin concentration and varying Act D concentrations. Data are mean ± SEM; n = 4–7.

fluorescent nucleic acid stain that penetrates all bacteria, while propidium iodide is a red fluorescent nucleic acid stain that enters only bacteria with damaged cell membranes. The ratio of red to green fluorescence emitted by bacteria stained with an appropriate mixture of these stains reflects cellular permeability and viability. The pulmonary collectins, at concentrations as low as 25 μg/ml SP-A and 5 μg/ml SP-D, enhanced *E. coli* nuclear staining with an impermeant fluorescent dye within 30 minutes, consistent with a rapid increase in membrane permeability and decreased bacterial viability (Figure 13).

Discussion

These data indicate that SP-A and SP-D inhibit the growth and synthetic function of some clinical and laboratory strains of *E. coli* and other Gram-negative bacteria. This direct antimicrobial function is distinct from the macrophage-dependent host defense properties of the pulmonary collectins, including opsonization and enhanced intracellular killing, that have been described previously. At least a portion of the antimicrobial activity of both collectins maps to their C-terminal domains and can be partially inhibited with excess LPS, suggesting an interaction between the CRD and glycoconjugates of the microbial surface. Laboratory strains with less extensive LPS modification were generally more susceptible to collectin-mediated growth inhibition than the smooth strains. The finding that deletion of OmpA enhanced the susceptibility of a smooth *E. coli* strain to SP-A and SP-D also highlights the importance of membrane function in collectin-mediated growth inhibition. Exposure of *E. coli* to SP-A or SP-D enhanced nuclear staining with propidium iodide, increased permeability to Act D, and increased release of thiol-containing proteins into the

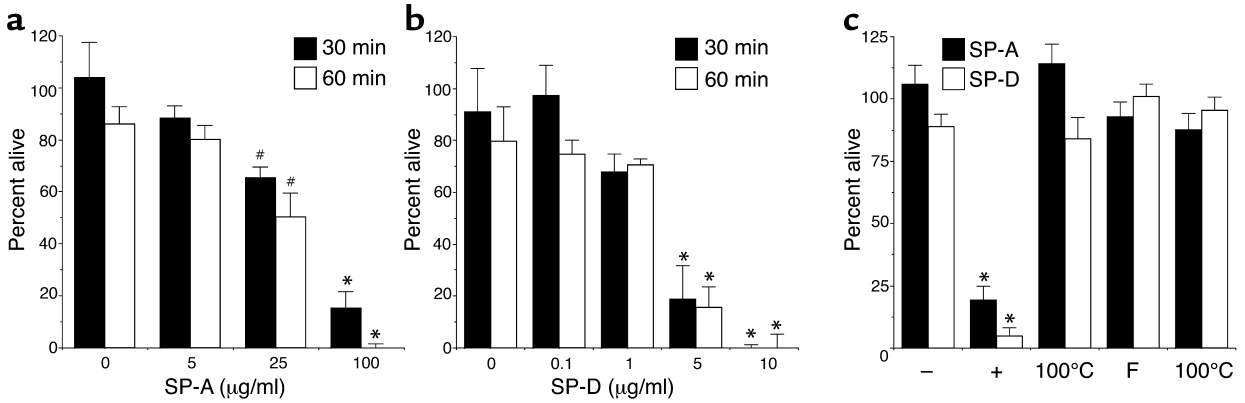


Figure 13
Collectin-induced reduction in viability of *E. coli*. (**a** and **b**) *E. coli K12* were incubated with rSP-A (**a**) or rSP-D (**b**) at various concentrations for 30 minutes at 37°C and then with green fluorescent nucleic acid stains STYO 9 and propidium iodide to assess viability. Data are mean ± SEM; n = 4–5. Statistical significance of differences in viability in the presence and absence of collectins is shown ($^{\#}P < 0.05$, $^{*}P < 0.01$). (**c**) Control experiments comparing viability of *E. coli K12* grown in the absence (–) and presence (+) of collectins (100 μg/ml rSP-A, 10 μg/ml rSP-D), boiled collectins (100°C), or the ultrafiltrate of collectins (F) and boiled collectins (F100°C); $^{*}P$<0.01.

extracellular milieu, indicating that the mechanism of growth inhibition involves damage to the cell membrane. Protein release was also induced by the C-terminal collectin fragments, suggesting that at least part of the membrane-permeabilizing activity is located in the neck and CRD domains of the protein. We conclude that SP-A and SP-D directly inhibit microbial growth by increasing membrane permeability through a C-terminal domain–dependent mechanism and speculate that they may play a role in macrophage-independent control of Gram-negative bacterial proliferation in the airspace.

SP-A and SP-D are members of the C-type lectin family, an ancient group of carbohydrate-binding proteins with diverse biologic functions including host defense (8), blood-component coagulation (40), membrane adhesion (41), clearance of serum glycoproteins and oxidized lipoprotein particles (42), and inhibition of serum ice-crystal formation (43). The collectins are a small subfamily of the C-type lectins, including only SP-A, SP-D, and mannose-binding lectin (MBL) in humans, that are distinguished by their oligomeric structural organization and biologic roles as pattern recognition opsonins and immunomodulatory proteins (44). All three human collectins contain N-terminal triple-helical collagen-like regions and coiled-coil neck domains involved in trimerization. MBL and SP-A assemble into 18 subunit oligomers composed of six disulfide-linked trimers that are aligned in a flower bouquet–like pattern, while SP-D forms a dodecameric cruciform structure composed of four trimers radiating from a disulfide-linked hub (45). The affinity and specificity of the collectins for bacterial surfaces are based on their unique carbohydrate-binding motifs and the spatial orientation of their multiple CRDs, which are arrayed at the C-terminal end of each trimerized subunit at angles and intervals that are optimal for interactions with cell surface ligands on microbes (46). The effect of collectin binding on bacterial physiology has not been previously studied, to our knowledge.

Reduced bacterial agglutination, phagocytosis, and intracellular killing all likely contribute to the defect in pulmonary clearance in SP-A$^{-/-}$ mice in this study and others (6, 7). However, compared with specific antibody and complement, collectins are actually quite weak opsonins, usually producing only modest enhancement of phagocytosis (up to 2- to 2.5-fold) (3, 47). In addition, the pulmonary collectins are also expressed in compartments that do not generally contain macrophages (e.g., SP-D in sweat glands) (48–50). These observations suggested to us that collectins may possess macrophage-independent antimicrobial activity.

Our data indicate that pulmonary surfactant fractions that contain SP-A and SP-D inhibit the biosynthetic capacity of bacteria. Other low–molecular weight antimicrobial proteins in lavage fluid, including lysozyme, defensins, and cathelicidins (51), would not be expected to copurify with surfactant proteins through the multiple steps employed, including calcium-dependent cosedimentation with the surfactant pellet, mannose-Sepharose affinity chromatography, and size separation by gel filtration. Furthermore, SDS-PAGE analysis indicated that SP-A and SP-D isolated using the procedures outlined in Methods were more than 95% pure. The antimicrobial activity of the collectins was destroyed by boiling and diminished under low pH conditions, and no activity was detected in the filtrate (molecular weight cutoff of 10,000) of any of the protein reagents used. Pulmonary surfactant proteins derived from diverse sources and purified by different methods, including native collectins purified from the alveolar lavage of rats and humans, and affinity-purified recombinant pulmonary collectins from rat and mouse, all exhibited direct antimicrobial activity. Collectively, these results indicate that inhibition of bacterial proliferation is an intrinsic property of SP-A and SP-D.

Both SP-A and SP-D are known to induce the agglutination of Gram-negative bacteria, which can have important effects on microbial physiology or access to nutrients. It is unlikely that downregulation of synthetic function induced by agglutination of the organism significantly contributed to growth inhibition in the *E. coli* solution-phase assays in this study, since aggregation mediated by antibacterial antibodies did not affect metabolic-precursor incorporation (37). In addition, growth inhibition also occurred upon exposure of agar-embedded *E. coli* to the pulmonary collectins, and the C-terminal fragments of SP-A and SP-D, which aggregated bacteria poorly, retained their antimicrobial properties in permeability and radial diffusion assays. Bacteria divide and migrate within the gel, however, and the possibility that SP-A and SP-D induce microaggregation or alter microbial migration cannot be completely excluded. Nonetheless, collectively these data are consistent with the hypothesis that at least part of the antimicrobial effect of SP-A and SP-D is independent of aggregation.

The pulmonary collectins inhibited the growth of most rough laboratory *E. coli* strains tested, but not the smooth laboratory strains. The clinical isolates that were susceptible to SP-A included two of four *E. coli* strains, two of two *K. pneumoniae* strains, and two of two *E. aerogenes* strains, while the only organisms sensitive to SP-D were one of two *Klebsiella* species and a smooth *E. coli* strain isolated from the blood of a septic patient (Figure 7). We speculate that the clinical isolates have molecular adaptations that increase their resistance to collectin-mediated growth inhibition, and that killing in vivo may require combinatorial interactions with other antimicrobial defenses in the airspace.

The results presented indicate that SP-A and SP-D kill bacteria by interactions with the cell membrane. Free LPS vesicles blocked collectin-mediated aggregation and growth inhibition, most likely by competing with the bacterial surface for collectin binding. Most rough *E. coli* laboratory strains were susceptible to collectin-mediated growth inhibition, while smooth laboratory strains were not, suggesting that the O-antigen may protect the organism, perhaps by sterically inhibiting interactions with core oligosaccharide or lipid A

targets. However, addition of up to five sugars to the *E. coli K12* core region did not affect susceptibility to the collectins, and one of the *E. coli* clinical strains that was resistant to both collectins was clearly a rough variant. Although lipid A is thought to be the predominant SP-A ligand of LPS, deletion of the 3-OH acyl chain had no effect on growth inhibition by either collectin.

Several lines of evidence suggested that the collectins increase the permeability of the bacterial cell membrane. Incubation of *E. coli* with both collectins increased access of the nucleic acid staining dye propidium iodide and the antibiotic Act D to intracellular targets. The membrane defect occurs rapidly, since collectins induced protein release from bacteria within 15 minutes and increased permeability to nuclear stains within 30 minutes. Genetically altered bacteria provide additional evidence that the collectins inhibit cell growth by affecting membrane function. OmpA is a highly conserved bacterial protein that plays an important role in the structure of the outer membrane (29). Degradation of OmpA by neutrophil elastase compromises membrane integrity and causes cell death (39). Deletion of OmpA in smooth-type *E. coli K1* bacteria in this study resulted in enhanced susceptibility to SP-A– and SP-D–induced growth inhibition, perhaps by increasing access of the collectins to membrane targets. These data indicate that the resistance to collectin-mediated growth inhibition in a smooth *E. coli* can be overcome by deletion of OmpA, and they are consistent with the hypothesis that collectins exert their effects at the level of the cell membrane. Microbial membrane disruption is a common antimicrobial theme among several host defense proteins in the alveolar lining fluid, including enzymatic cell wall–degrading molecules such as lysozyme (52) and low–molecular weight cationic peptides such as the defensins (53). The molecular mechanism of the latter is thought to involve ionic interactions between positively charged residues of the protein and negative charges on the bacterial cell surface. The structural basis of the membrane-destabilizing effects of SP-A and SP-D are not known, but the pH dependence of the collectin antimicrobial activity suggests that ionic interactions may be important. The C-terminal domains of collectins appear to be involved, since both ΔSP-A and ΔSP-D increased protein release from *E. coli* and produced growth inhibition in the radial diffusion assays at high (i.e., likely supraphysiologic) concentrations within the agar. The crystal structure of SP-A has not been reported, but the cleft between the three CRDs of SP-D presents a large positively charged surface that could potentially bind and disrupt bacterial membranes (54). C-terminal fragments of SP-A and SP-D have also been reported to enhance neutrophil chemotaxis (55), clearance of respiratory syncytial virus (56), and *Aspergillus fumigatus* infection (57), and to reduce allergic pulmonary hypersensitivity to *A. fumigatus* (58).

Inhaled organisms that reach the most distal airspaces are deposited in the alveolar lining layer, a unique two-phase compartment that is bounded on the lumenal margin by a surfactant phospholipid membrane(s) and on the epithelial margin by an aqueous layer. Alveolar macrophages, which are considered primary defenders of the alveolus, patrol the hypophase but may not have access to organisms that are shielded by surfactant membranes at the air-liquid interface. SP-A preferentially associates with membrane interfaces and is greatly enriched in large surfactant aggregates such as tubular myelin, which form the reservoir for the surfactant film. The alveolar localization of SP-D is less clear and is assumed to be the aqueous compartment, although the protein is known to associate with phosphatidylinositol and with surfactant aggregates (8). The concentrations of pulmonary collectins that inhibited bacterial growth and viability in this study were well within the estimated range of physiologic alveolar lining fluid levels for the pulmonary collectins, estimated to be 300–1,800 μg/ml for SP-A and 36–216 μg/ml for SP-D (3). The one possible exception is the use of SP-D concentrations of up to 1 mg/ml in the radial diffusion assays. However, SP-D is a large, extended molecule that diffuses poorly in agar, and the actual collectin concentrations in the agar around the wells are likely to be much lower than 1 mg/ml. The intimate association of SP-A and SP-D with surfactant may both retain them in the alveolar compartment and position them in the first line of defense against inhaled microbes that are deposited on the surfactant film (8).

Our data are consistent with novel roles for SP-A and SP-D as antimicrobial proteins. Cooperative interactions of SP-A and SP-D with other antimicrobial proteins in the epithelial lining fluid may result in synergistic inhibition of bacterial proliferation (59). The reduced collectin levels found in the BAL fluid in idiopathic pulmonary fibrosis, Gram-negative pneumonia, and cystic fibrosis may play a role in the pathogenesis of acute or recurrent infections in those diseases (45, 60). Future challenges include elucidation of the bacterial mechanisms that determine resistance or sensitivity to SP-A and SP-D, and definition of the relative physiologic importance of the antibiotic properties of the collectins in host defense at the air-lung interface.

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