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Research Article

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Detection and Partial Characterization of Antibacterial Factor(s) in Alveolar Lining Material of Rats

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ABSTRACT Intracellular killing of *Staphylococcus aureus* by alveolar macrophages is known to be enhanced by exposure to alveolar lining material. Because this material may have a role in pulmonary host defenses, we have studied its effect on pneumococci and other nonstaphylococcal organisms. Alveolar lining material from rats caused rapid killing and lysis of pneumococci. The antipneumococcal activity was localized to the surfactant-containing fraction of the fluid and was not affected by trypsin. Phospholipid extracts of the surfactant fraction or purified lamellar bodies killed pneumococci. Lysis of pneumococci by the surfactant fraction appeared to be mediated by a detergent-like activation of pneumococcal autolysin, in that bacteriolysis was prevented by substitution of ethanolamine for choline in pneumococcal cell walls, and a pneumococcal transformant that lacked autolysin was not lysed. The surfactant fraction readily killed pneumococci containing ethanolamine or the autolysin-defective transformant, and studies with tritiated methyl-D-glucose loading and release showed that killing was associated with increased bacterial cell membrane permeability. Bactericidal activity (without lysis) was observed with several nonpneumococcal gram-positive bacteria, including *Streptococcus viridans*, unspeciated respiratory streptococci, *Streptococcus pyogenes*, *Streptococcus bovis*, and Bacillus species. Purified diacylphospholipids had no antibacterial activity, however, a lysophospholipid, palmitoyl lysophosphatidylcholine, had many properties resembling the surfactant-containing fraction of lavage, including autolysin-mediated pneumococcal lysis, altered cell membrane permeability, and antibacterial activity against several gram-positive bacteria.

INTRODUCTION

It is frequently stated that inhaled bacteria are killed by alveolar macrophages (1, 2). This view is based principally on studies of aerosolized *Staphylococcus aureus* in which lung microscopy and cultures have been correlated to determine the site of bacterial killing (3–6). Other studies show that *S. aureus* are not killed readily by alveolar macrophages unless the organisms are first exposed to alveolar lining material (7, 8). LaForce et al. (7) demonstrated that it is the surfactant-containing fraction of aveolar lining material that enhances intracellular killing of *S. aureus*. The mechanism of this enhancement is unknown, and it is not known if the surfactant fraction affects nonstaphylococcal organisms. We describe here the effects of the surfactant fraction of rat alveolar lining material on pneumococci and other bacteria.

METHODS

Animals. Adult, male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were used unless indicated otherwise. They were housed in a filtered air unit. Adult male Lewis rats (Charles River Laboratories, Inc.), New Zealand white rabbits (S and S Research Animals, LaGrange, KY), albino guinea pigs (Hilltop Laboratories, Scottsdale, PA), outbred mice (Veterans Administration Hospital, Lexington, KY), and mongrel dogs (~20 kg) were used in some studies.

Lung lavage. Rats were anesthetized with pentothal intraperitoneally and exsanguinated via the abdominal aorta. Rat lungs were lavaged *in situ* as described elsewhere (9). In brief, a tracheal catheter was inserted and 5 ml of phosphate-buffered saline (PBS) (pH 7.4) was injected and withdrawn for two to three cycles. About 4 ml of fluid was recovered per rat. The fluid was centrifuged at 160 *g* for 5 min to remove leukocytes, and concentrated 20-fold by positive-pressure filtration (Amicon Diablo Ultrafilter, ≥10,000 mol wt exclusion; Amicon Corp., Scientific Sys. Div., Lexington, MA) or by centrifuging at 40,000 *g* for 20 min and resuspending the pellet in one-twentieth the volume. Guinea pigs, mice, and rabbits were sacrificed and lavaged with, respectively, 10 ml, 0.5 ml, and 20 ml of PBS. Dogs were anesthetized with intravenous sodium thiamylal and lavaged with 200 ml of PBS.

Surfactant and phospholipid preparations. The pellet obtained by centrifuging lavage fluid at 40,000 *g* for 20 min comprised the surfactant-containing fraction of lavage fluid. Phospholipid was extracted by the method of Bligh and Dyer

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(10). Lamellar bodies in surfactant were purified on a discontinuous sucrose density gradient as described by Gil and Reiss (11) and were tested for bactericidal activity on the day of preparation. Purified phospholipids and lysophospholipids were purchased (Sigma Chemical Co., St. Louis, MO).

Bacteria. Pneumococcal serotypes 1, 3, and 25 (American Type Culture Collection, Rockville, MD) were maintained in an encapsulated state by monthly passage in mice, and stored in heat-inactivated rabbit serum at 4°C (12). Dr. Alexander Tomasz (The Rockefeller University, New York) generously provided a pneumococcal "lysis-defective" transformant (R36A pneumococcus transformed with DNA from a lysis-defective mutant) containing <1% of the murein hydrolase activity of the wild strain (13). Pneumococci were grown in brain-heart infusion broth containing 7% heat-inactivated rabbit serum for 5–6 h to reach log-phase, and were washed once in PBS before use in bactericidial tests.

Radiolabeling. Pneumococcal cell walls were labeled during growth in a chemically-defined medium where the amino alcohol for cell wall synthesis was provided either as choline chloride or as ethanolamine HCl (both from Sigma Chemical Co.) (14–17). Under these conditions, the amino alcohol is incorporated exclusively into the bacterial cell wall (14) and when ethanolamine is used, it is incorporated into the identical sites normally occupied by choline (18). Pneumococci were grown overnight in synthetic medium with either 1 μCi of [^{3}H]methyl choline chloride per ml (80.0 Ci/mmol sp act, Sigma Chemical Co.) or 2.0 μCi of 1,2[^{14}C]-ethanolamine HCl per ml (4.0 mCi/mmol sp act) and enough additional unlabeled amino alcohol to permit proliferation (19). Radiolabeled bacteria (≥6,500 cpm/10^7 bacteria) were washed three times and used immediately. Release of label from cell walls was measured by incubating 10^7 radiolabeled bacteria in 0.1 ml of PBS with 0.2 ml of lavage fluid, phospholipid, or PBS for 90 min at 37°C and centrifuging at 1,600 *g* for 20 min to remove intact bacteria. Pellets and supernatants were mixed with twice their volume of Aquasol-2 (New England Nuclear, Boston, MA) for counting. Counts per minute in supernatants were expressed as percent total counts per minute, and controls (bacteria in PBS alone) were routinely subtracted from the experimental tubes.

Bacteria were labeled internally with 3-*O*-[^{3}H]methyl-D-glucose (80.0 Ci/mmol sp act, Sigma Chemical Co.) by drying 1 μCi of tritiated 3-*O*-methyl-D-glucose in a tube and adding 0.1 ml of 0.1 mM unlabeled 3-*O*-methyl-D-glucose and 0.1 ml of 10^8 washed, log-phase bacteria in PBS. After 30 min at 37°C, the bacteria (2,000–4,000 cpm per 10^8 bacteria) were washed four times in PBS at room temperature and used immediately. Release of tritiated 3-*O*-methyl-D-glucose was tested by mixing 10^8 radiolabeled washed, log-phase bacteria in 0.1 ml of PBS with 0.2 ml of lavage, phospholipid, or PBS and incubating at 37°C. The tubes were centrifuged at 10,000 *g* for 5 min and supernatants and pellets were taken up in Aquasol-2 and counted. The percent counts per minute in supernatant (minus the control) was calculated.

Bactericidal test. Washed, log-phase bacteria (10^8 in 0.1 ml of PBS) were mixed with 0.2 ml of lavage fluid, phospholipid, or PBS (as a control). After 90 min at 37°C, viable organisms were enumerated by culturing 10-fold dilutions in PBS (10^5–10^9) on 5% sheep blood agar under 5% CO_2 overnight. The percent viable bacteria was calculated.

Electron microscopy. Pneumococci were incubated with lavage or PBS for 15–30 min at 37°C. The bacteria were pelleted at 10,000 *g*, fixed, and then stained with lead citrate and uranyl acetate as described previously (20).

RESULTS

Pneumococcal killing by rat lavage. Bronchoalveolar lavage fluid from 10–12 Sprague-Dawley rats (~30 ml) was freed of leukocytes and concentrated 20-fold by filtration through an Amicon membrane (10,000-mol wt exclusion). The concentrate was incubated with washed, log-phase pneumococci at 37°C and serial cultures were performed (Fig. 1). There was a progressive decrease in the number of colony-forming units. To determine if this decrease was due to clumping of bacteria, mixtures of pneumococci and concentrated lavage were incubated for 90 min, centifuged, and gram-stained smears prepared. Only gram-negative detritus was detected, suggesting that the pneumococci had lysed (Fig. 2A). Preliminary studies showed that the antipneumococcal activity of lavage fluid fractionated with the pellet formed by centrifugation at 40,000 *g*; electron microscopy of this pellet showed membrane-like structures and lamellar bodies characteristic of surfactant (Fig. 2B). When pneumococci were incubated with the surfactant-containing pellet, cell wall defects and bacteriolysis were obvious (Fig. 2C).

Studies of the characteristics of the antipneumococcal factor(s) in rat lavage fluid are summarized in Table I. Unconcentrated lavage or the filtrate formed during concentration showed little antipneumococcal activity, whereas the retentate was active. The surfactant-containing pellet that contained most of the antipneumococcal activity showed loss of antibacterial

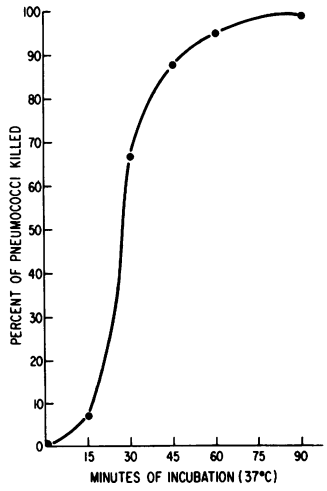

FIGURE 1 Killing of type 25 pneumococci by rat lavage fluid. Washed, log-phase bacteria (10^8 in 0.1 ml of PBS) were incubated at 37°C with 0.2 ml of 20-fold concentrated lavage, or with PBS. Aliquots from the experimental and control tubes were serially diluted in PBS and cultured on blood agar to determine percent viability. Data represent means of duplicate determinations.

TABLE I
Antipneumococcal Activity of Leukocyte-free Rat Lavage Fluid

Lavage fluid preparation	No. preparations tested	Viable pneumococci remaining*
		%
Unconcentrated	6	79±12
Concentrated 20-fold by filtration:‡		
Filtrate	5	94±6
Retentate	16	19±10
Centrifuged (40,000 *g* for 20′):		
Supernatant (concentrated 20-fold by filtration)	4	106±3
Pellet:§		
Fresh	10	12±4
Frozen (−70°C) (d)		
1	3	<1
4	3	10
7	2	95
14	2	90
Washed × 1 in PBS	3	<1
Washed × 4 in PBS	3	104
Sonicated	2	<1
Heated 60° (30′)	3	<1
Heated 100° (15′)	2	112
Incubated with:‖		
Trypsin (5%)	2	<1
0.4% serum	2	<1
4.0% serum	2	<1
8.0% serum	3	80
16.0% serum	2	90
2.0% mucin	2	91
Pneumococcal (type 3) polysaccharide (1 mg/ml)	2	<1
Purified lamellar bodies¶	2	<1
Phospholipid extract	3	<1

* Approximately 10^8 washed, log-phase type 25 pneumococci in 0.1 ml of PBS were incubated with 0.2 ml of lavage fluid or PBS (control) for 90′ at 37°C; viable bacteria were determined by preparing 10-fold dilutions and incubating aliquots on blood agar at 37°C. Data represent the mean and (for four or more determinations) SE.

‡ Positive-pressure filtration through an Amicon membrane with a 10,000-mol wt exclusion level.

§ Pellet was suspended in PBS to give a 20-fold concentration as compared with the uncentrifuged fluid.

‖ Pellet was incubated with indicated material for 30 min at 37°C and washed once in PBS before use in the bactericidal test.

¶ Separated on a discontinuous sucrose density gradient from the surfactant-containing pellet and tested on the day of preparation.

activity over 1–2 wk at −70°C or on heating at 100°C. Multiple washings of the pellet in PBS eliminated this activity. Treatment with trypsin (5% for 30 min at 37°C), sonication, or heating at 60°C did not affect the antibacterial activity, whereas it was decreased by admixture of the pellet with serum or mucin (Difco Laboratories, Detroit, MI).

Surfactant-containing pellets from 10 rats were pooled and fractionated on a discontinuous sucrose density gradient (11). The fraction rich in lamellar bodies (determined by electron microscopy) was strongly antipneumococcal, as was a phospholipid extract (10) of the crude pellet (Table I). The chloroform fraction of the phospholipid extraction contained essentially all of the activity of the original pellet (1:16 dilutions of the chloroform fraction and pellet killed, respectively, 42 and 68% of 10^8 pneumococci). The methanol fraction from the extraction contained only ~6% of the activity observed in the pellet.

Variation in antipneumococcal activity of lavage fluid. Different batches of lavage fluid varied somewhat in their antipneumococcal activity and lavage from individual Sprague-Dawley rats also varied. For example, surfactant-containing pellets from eight rats (each pellet in 0.2 ml to give 20-fold concentration) incubated for 90 min with 10^8 pneumococci gave the following percentages of viable bacteria remaining: 22, <1, <1, 36, <1, <1, 83, 44. This variation in activity could not be related to method of anesthesia or sacrifice—similar results were obtained with either intraperitoneal pentothal or with inhalation of fluothane for anesthesia, or with exsanguination or cervical dislocation for death. Also, there was no correlation between leukocyte content of lavage fluids and the antibacterial activity of resuspended pellets (Spearman's rank coefficient for 10 fluids was $r = +0.16$; $P > 0.05$).

Selectivity of bactericidal activity. Table II shows that pneumococci of different serotypes, nonpneumococcal streptococci of several species, and Bacillus species were killed by active preparations of lavage fluid. There was little apparent effect on other bacteria, but sublethal injury was not excluded.

Mechanism of pneumococcal lysis. We suspected that pneumococcal lysis caused by lavage was due to activation of murein hydrolase (*N*-acetyl muramic acid L-alanine amidase), which also causes pneumococcal lysis in old cultures and detergent- and penicillin-mediated lysis of pneumococci (13, 21). To test this hypothesis, we heated type 25 pneumococci at 60°C for 90 min to inactivate murein hydrolase (autolysin) (22) and found that heat treatment prevented lysis by concentrated rat lavage (Fig. 3A). To further test the hypothesis that lavage lysed pneumoccoci by activating autolysin, we grew type 25 pneumoccoci in defined media containing either choline (Pn-C)[1] or

[1] *Abbreviations used in this paper*: Pn-C, type 25 pneumoccoci containing choline; Pn-E, type 25 pneumoccoci containing ethanolamine.

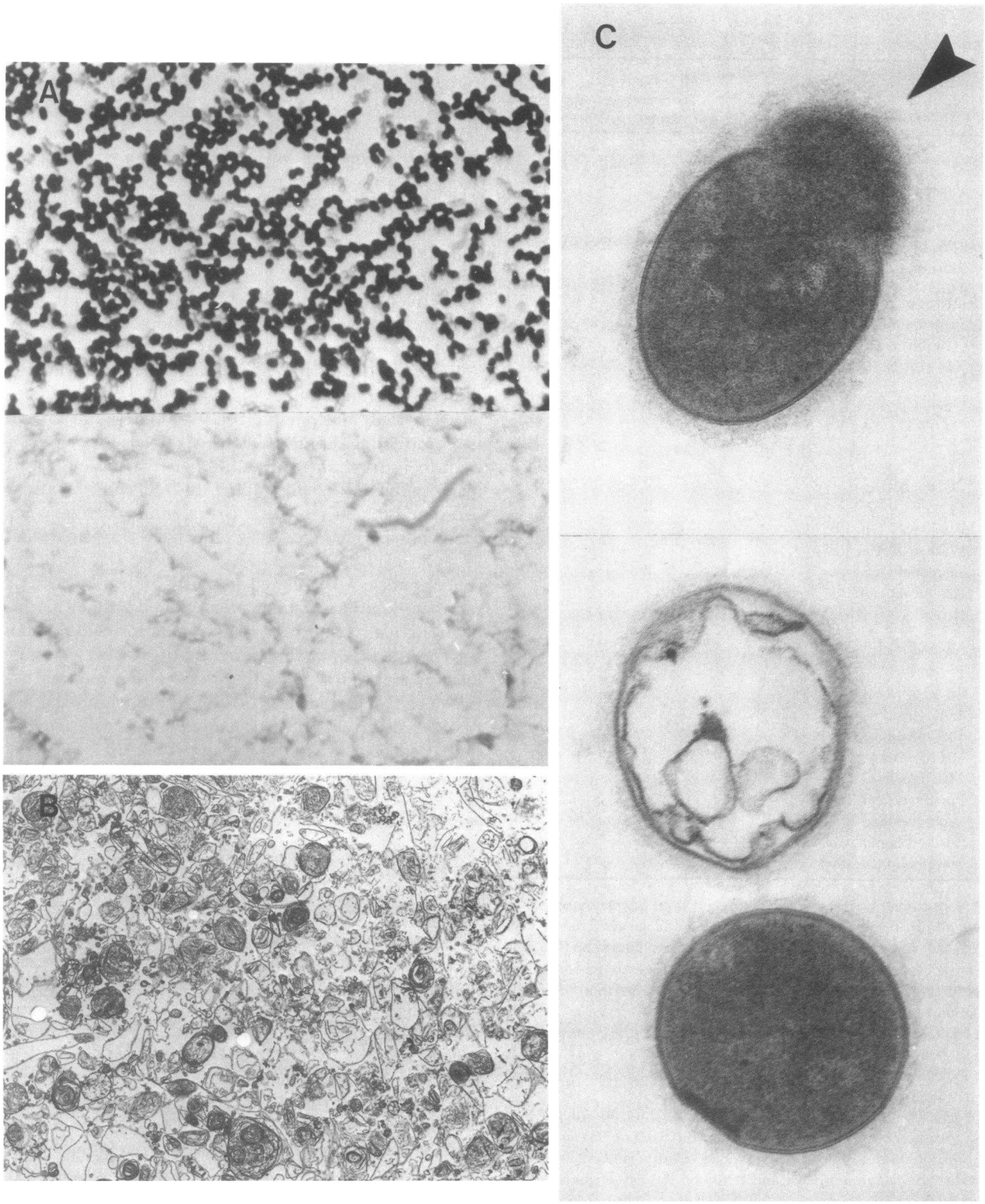

FIGURE 2 A. Photomicrographs (×1,250) of gram-stained smears of type 25 pneumococci incubated at 37°C in PBS (top half of panel) or in a 20-fold concentrate of rat lung lavage fluid (lower half). Only gram-negative detritus remained after incubation with lavage fluid. B. Electron micrograph (×3,700) of the surfactant-containing fraction of rat lavage (pellet

TABLE II

Effect of Rat Lavage Fluid on Viability of Various Bacteria

Bacteria	Viable bacteria remaining*
	%
Type 25 pneumococci	3±4‡
Type 1 pneumococci	5±5
Type 3 pneumococci	<1±0
S. viridans	<1±0
Unspeciated respiratory streptococci ("alpha-streptococci")	2±8
S. pyogenes (Lancefield group A)	<1±0
Streptococcus agalactiae (group B)	
isolate 1	105±10
isolate 2	103±7
S. fecalis (group D)	90±9
S. durans (group D)	97±8
S. bovis (group D)	7±15
Bacillus species	
isolate 1	<1±0
isolate 2	17±5
S. aureus	
isolate 1	110±10
isolate 2	101±9
Klebsiella pneumoniae	
isolate 1	91±2
isolate 2	102±8
Pseudomonas aeruginosa	116±10
Enterobacter aerogenes	89±3
Proteus vulgaris	107±7
Serratia marcescens	97±6

* 0.2 ml of 20-fold concentrated surfactant-containing pellet or PBS (control) was incubated with ~10^8 bacteria in 0.1 ml of PBS for 90 min at 37°C. Viable bacteria were determined by incubating cultures on 5% sheep blood agar overnight.

‡ Mean±SE of four or more determinations.

ethanolamine (Pn-E) as the sole source of amino alcohol for cell wall synthesis. Tomasz and co-workers (14, 17, 18) have shown that autolysin-mediated lysis of pneumococci is dependent on choline residues in the teichoic acid of the cell wall (16–18). If ethanolamine is substituted for choline, pneumococci are resistant to autolysin (13, 16, 18). As indicated in Fig. 3, Pn-C were lysed by lavage, whereas Pn-E were not (Fig. 3B and C). Further evidence that rat lavage lysed pneumococci via autolysin was obtained by testing a lysis-defective pneumococcal transformant containing <1% of the normal level of autolysin activity with lavage. Lavage did not lyse this transformant (Fig. 3D).

Quantitative studies of lavage-mediated lysis were carried out by radiolabeling pneumococci with [^{3}H]methyl choline (for type 25 Pn-C and the autolysin-defective mutant) or 1,2[^{14}C]ethanolamine (for type 25 Pn-E). The viable labeled pneumococci were incubated with lavage or PBS, and freed radiolabel was measured. Lavage caused release of radiolabel from viable type 25 Pn-C but not from type 25 Pn-E, the autolysin-defective mutant, or heat-killed type 25 Pn-C (Fig. 4).

Killing not dependent on lysis. Tritiated type 25 Pn-C, or autolysin-defective mutant and ^{14}C-labeled type 25 Pn-E were incubated with the surfactant-containing pellet of rat lavage, and viability as well as release of radiolabel were tested after 90 min at 37°C. The results (Table III) showed that release of radiolabel and killing were independent phenomena. Electron microscopy of lavage-treated autolysin-defective pneumococci showed no evidence of cell wall breakdown despite loss of viability.

The effect of lavage on nonpneumococcal bacteria was studied by examining gram-stained smears of organisms killed by incubation with a concentrated lavage. *Streptococcus viridans*, unspeciated respiratory streptococci, *Streptococcus pyogenes*, and *Streptococcus bovis* were killed by lavage without lysis. One isolate of Bacillus was almost completely lysed upon killing, whereas a second (and morphologically different) species was killed without evidence of lysis.

Effect of lavage on cell membranes. An experiment was performed to determine if bacteria that were killed by lavage without lysis had increased cell membrane permeability. Bacteria were loaded with tritiated 3-*O*-methyl-D-glucose, washed, and incubated with lavage or PBS, and the percent radiolabel released was determined. The results (Fig. 5) indicated that

obtained by centrifuging leukocyte-free lavage at 40,000 *g* for 20 min). Note membrane-like structures and lamellar bodies. The fraction was stained with lead citrate and uranyl acetate. C. Electron micrograph (×42,000) of type 25 pneumococci incubated for 30 min at 37°C with the surfactant-containing fraction of rat lung lavage fluid (concentrated 20-fold). The pneumococcus at the top of the panel shows a large cell wall defect with exudation of protoplasm (arrow). There is a bacterial "ghost" in the middle of the figure and a still intact pneumococcus at the bottom. The pneumococci were stained with lead citrate and uranyl acetate.

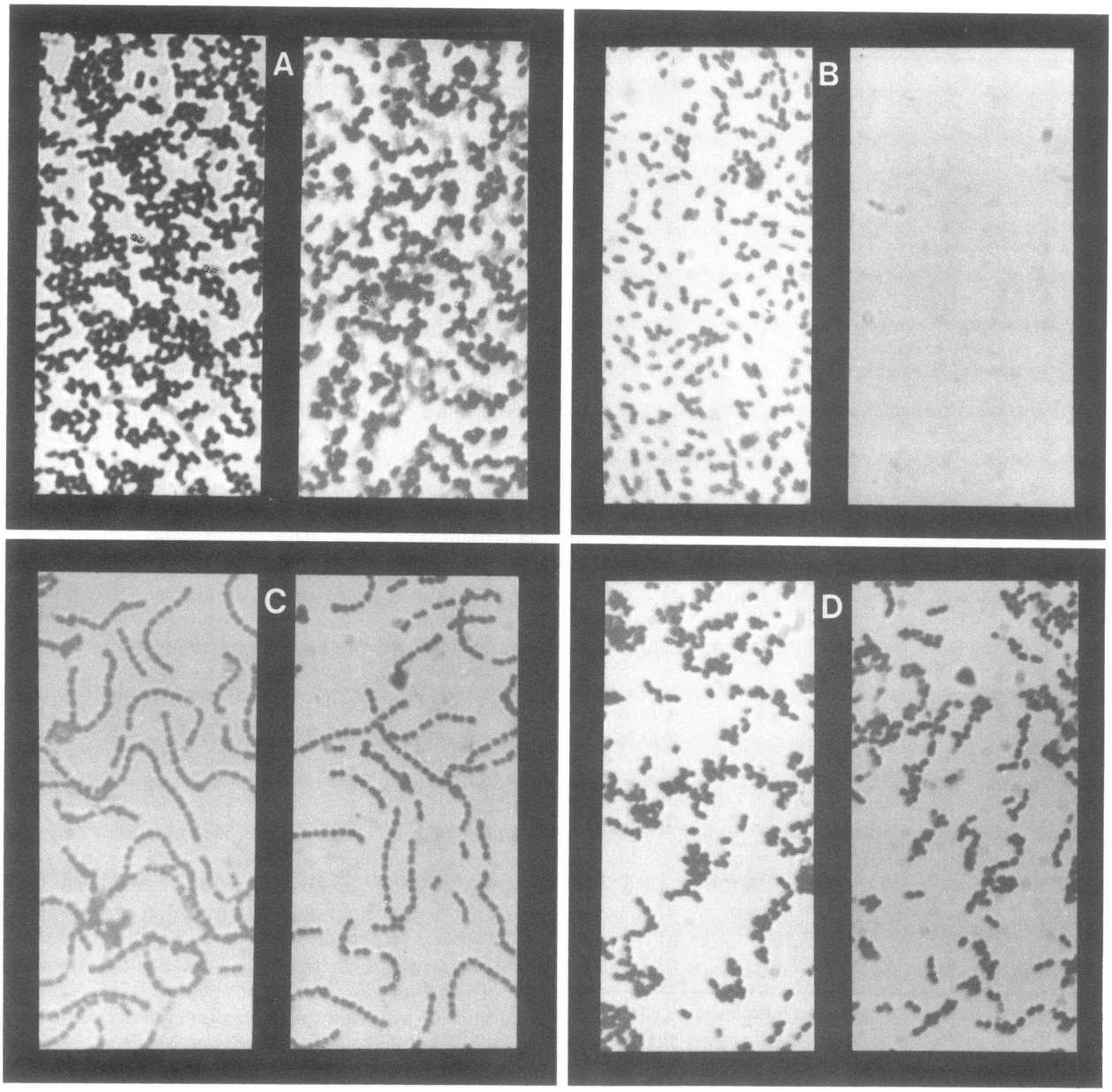

FIGURE 3 Effect of rat lung lavage fluid on pneumococci. Pneumococci were incubated with a surfactant-containing pellet of lavage (concentrated 20-fold) or PBS, for right- and left-hand panels of A–D, respectively, for 90 min at 37°C, and gram-stained smears of the sediments were photographed (×1,250). A. Heat-killed (60° for 90 min) type 25 pneumococci from broth culture. There is no apparent lysis by lavage. B. Type 25 pneumococci from defined media with choline as the only amino alcohol for cell wall synthesis. Lavage lysed the bacteria. C. Type 25 pneumococci from defined media with ethanolamine as the amino alcohol. The bacteria formed long chains, presumably due to resistance of the ethanolamine-containing cell walls to autolysin. Lavage caused no lysis. D. Pneumococcal mutant with <1% of the normal level of murein hydrolase (autolysin) activity. Lavage did not cause lysis.

lavage did alter cell membrane permeability in affected organisms.

Studies of phospholipids. Surface-active material is composed principally of diacylphospholipids including dipalmitoyl phosphatidylcholine (~50%) and numerous other phospholipids and neutral lipids (23). We incubated purified diacylphospholipids dispersed in PBS (0.2 ml) with log-phase type 25 pneumococci (10^8

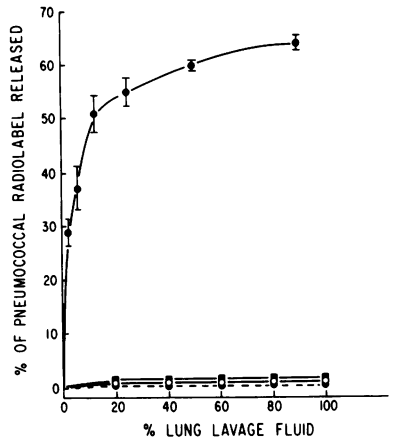

FIGURE 4 Lysis of pneumococci by rat lavage requires autolysin and cell wall choline residues. Radiolabeled pneumococci were incubated with dilutions of a 20-fold concentrate of lavage (surfactant pellet) or PBS for 90 min at 37°C, and the mixtures were contrifuged. The points indicate the percent radiolabel released from lavage-treated organisms minus PBS-treated organisms. Data represent the mean±SE of three or more determinations (SE of bottom points is deleted for clarity but was <±5%). ● —— ●, type 25 containing [^{3}H]choline; ● - - - ●, heat-killed type 25 containing [^{3}H]choline; O —— O, type 25 containing [^{14}C]ethanolamine; ■ —— ■, autolysin-defective mutant containing [^{3}H]choline.

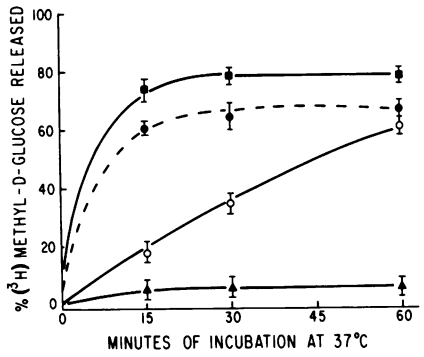

FIGURE 5 Effect of rat lavage fluid on release of tritiated 3-*O*-methyl-D-glucose from bacteria. Labeled log-phase bacteria were incubated at 37°C with a 20-fold concentrate of surfactant-containing lavage pellet, or with PBS. Results are expressed as the percent radiolabel released from lavage minus PBS-treated organisms. Data represent the mean±SE of four or more determinations. ■ —— ■, autolysin-defective mutant; ● - - - ●, *Streptococcus pyogenes*; O —— O, type 25 containing ethanolamine; ▲ —— ▲, *Streptococcus fecalis*.

in 0.1 ml PBS) for 90 min at 37°C and determined the amount of viable bacteria remaining. Phospholipids studied included dipalmitoyl phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, and phosphatidylethanolamine over a range of concentrations from 0.01 μg/ml to 1,000 μg/ml. No antipneumococcal activity was detected.

Lysophospholipids comprise several percentages of the lipids in surfactant (23) and three preparations were obtained commercially: palmitoyl lysophosphatidylcholine, myristoyl lysophosphatidylcholine, and palmitoyl lysophosphatidylethanolamine. They showed dose-related antibacterial activity against gram-positive bacteria, including pneumococci, various respiratory streptococci, group A streptococci, *S. bovis*, Bacillus species, and *S. aureus* (Table IV). The compounds appeared largely inactive against gram-negative rods. The antibacterial spectrum of lysophospholipids was thus similar to rat lavage, except palmitoyl lysophosphatidylcholine, which was particularly active against *S. aureus*.

TABLE III
Lack of Correlation of Bacterial Killing and Release of Radiolabeled Choline or Ethanolamine from Cell Walls of Pneumococci

Pneumococcus	Radiolabel released	Viable pneumococci remaining*
		%
Type 25 containing [^{3}H]choline	61±4‡	5±3
Type 25 containing [^{14}C]ethanolamine	2±1	2±6
Autolysin-defective mutant containing [^{3}H]choline	<1±1	4±4

* Viable pneumococci were incubated with a 20-fold concentrate of lavage pellet or with PBS for 90 min. Freed radiolabel is expressed as the percent radiolabel released by lavage-treated minus PBS-treated organisms.

‡ Data represent the mean±SE of four or more determinations.

Gram-stained smears of type 25 Pn-C incubated in palmitoyl lysophosphatidylcholine (150 μg/ml) showed bacteriolysis, and electron micrographs of treated organisms showed cell wall defects similar to lavage-treated pneumococci. In contrast, type 25 Pn-E and autolysin-defective pneumococci were not lysed by lysophosphatidylcholine. Gram-stained smears of the nonpneumococcal bacteria listed in Table IV showed none that were lysed by lysophosphatidylcholine except for one of the two isolates of Bacillus. Studies of radiolabeled type 25 Pn-C, Pn-E, and the autolysin-defective pneumococci confirmed these observations (Table V). Thus, lysophospholipids mimicked the effects of the surfactant-containing fraction on pneumococci and caused lysis only when the pneumococci contained autolysin and had choline residues in the cell wall.

Palmitoyl lysophosphatidylcholine altered bacterial cell membrane permeability apart from its effect on the cell wall. Studies of bacteria loaded with tritiated methyl-D-glucose showed that organisms that were

TABLE IV
*Antibacterial Activity of Lysophospholipids**

Bacteria	Palmitoyl lysophosphatidylcholine		Myristoyl lysophosphatidylcholine		Palmitoyl lysophosphatidylethanolamine	
	250 μg/ml	50 μg/ml	250 μg/ml	50 μg/ml	250 μg/ml‡	50 μg/ml
Type 25 pneumococci containing choline	<1§	27	<1	43	<1	61
Type 25 pneumococci containing ethanolamine	2	94	<1	23	27	16
Type 3 pneumococci	<1	79	<1	30	52	58
Autolysin-defective pneumococci	<1	42	14	50	33	29
S. viridans	<1	12	<1	<1	86	102
Unspeciated respiratory streptococci	<1	22	<1	58	61	79
S. pyogenes	<1	43	<1	29	66	83
Streptococcus agalactiae (isolate 1)	83	107	87	96	80	132
S. fecalis	92	91	85	105	143	115
S. durans	106	105	87	125	89	105
S. bovis	<1	25	4	109	93	113
Bacillus species (isolate 1)	7	58	<1	18	106	89
(isolate 2)	<1	40	<1	21	71	82
S. aureus (isolate 1)	16	98	60	93	64	102
(isolate 2)	35	55	73	120	108	98
(isolate 3)	16	50	71	95	113	110
(isolate 4)	25	77				
K. pneumoniae (isolate 1)	112	107	81	79	78	90
P. aeruginosa	101	95	109	95	101	78
E. aerogenes	82	115	65	73	71	121
P. vulgaris	103	112	75	101	104	99
S. marcescens	104	88	73	75	129	137
E. coli	93	95	89	96	94	152

* 10^8 log-phase, washed bacteria in 0.1 ml were incubated in 0.2 ml of the indicated concentrations of lysophospholipid or in PBS for 90 min at 37°C and viable bacteria were enumerated by culture on 5% sheep blood agar at 37°C overnight.
‡ Not completely dispersed at this concentration.
§ Percent viable bacteria remaining. Data represent the mean of two or more determinations.

killed by lysophosphatidylcholine without bacteriolysis, developed increased membrane permeability (Table VI). *Streptococcus durans*, which was resistant to killing by lysophospholipids, showed no permeability change. *Streptococcus fecalis* showed increased permeability with lysophospholipid, although its viability was not affected (Table IV); this suggests the possibility of sublethal injury. Attempts to study *S. aureus* were unsuccessful because they did not take up methyl-D-glucose.

The antipneumococcal activity of lysophosphatidylcholine could be blocked by incubation with rat lavage that had become inactive during storage at −70°C (Table VII) and, like lavage, lysophosphati-

TABLE V
Effect of Lysophospholipids on Cell Walls of Viable Pneumococci°

Bacteria	Concentration of lysophospholipid	Radiolabel released by lysophospholipid‡		
		Palmitoyl lysophosphatidylcholine	Myristoyl lysophosphatidylcholine	Palmitoyl lysophosphatidylethanolamine§
	μg/ml		%	
Type 25 pneumococci containing [^{3}H]choline	500	82	85	40
	250	83	76	28
	125	75	77	24
	62	74	70	21
	31	70	42	18
Type 25 pneumococci containing [^{14}C]ethanolamine	500	11	6	4
	250	5	3	0
	125	6	10	0
	62	6	3	1
	31	9	4	3
Autolysin-detective mutant containing [^{3}H]choline	500	1	2	—
	250	2	2	—
	125	2	4	—
	62	2	6	—
	31	0	7	—

° Washed, log-phase pneumococci (0.1 ml) were incubated in 0.2 ml of the indicated concentrations of lysophospholipid or in PBS 90 min at 37°C.

‡ Percent of radiolabel released by phospholipid-treated pneumococci minus pneumococci in PBS. Data represent the mean of two or more determinations.

§ The palmitoyl lysophosphatidylethanolamine was not completely dispersed at concentrations >125 μg/ml.

dylcholine was inactivated by admixture with mucin or fresh rat serum.

Lung lavage fluids of other species. Table VIII shows results obtained in the bactericidal test with fresh lung lavage fluids of various animal species. Animals were lavaged with PBS and the fluids concentrated 20-fold by positive-pressure filtration (10,000-mol wt exclusion level). Antibacterial activity was determined by incubating 0.2 ml of lavage with 10^8 type 25 pneumococci in 0.1 ml of PBS. Fluids from Lewis rats and dogs showed antipneumococcal activity similar to Sprague-Dawley rats. Fluids from mice showed less activity, and lavage from rabbits and guinea pigs was largely inactive.

TABLE VI
Effect of Palmitoyl Lysophosphatidylcholine (150 μg/ml) on Release of Tritiated Methyl-D-Glucose from Log-Phase Bacteria at 37°C

Bacteria	[^{3}H]methyl-D-glucose released°	
	15 min	30 min
	%	
Autolysin-defective pneumococcus	68±2‡	68±2
Type 25 pneumococci containing ethanolamine	85±2	95±3
S. viridans	—	38±1
Unspeciated respiratory streptococci	—	40±1
S. pyogenes (group A)	61±8	66±2
Bacillus species (isolate 2)	—	54±1
S. fecalis (group D)	39±8	47±0
S. durans (group D)	2±3	5±1

° Percent of radiolabel released by phospholipid treatment minus percent released in PBS.

‡ Mean±SE of three or more determinations.

DISCUSSION

The present studies indicated that the surfactant-containing fraction of rat lung lavage fluid had antibacterial activity against gram-positive bacteria and could rapidly lyse and kill pneumococci. Lysis of pneumococci was dependent on choline residues in the cell wall teichoic acid and required normal levels of murein hydrolase (autolysin) activity. These facts suggested that pneumococcal lysis was mediated by activation of pneumococcal autolysin. Tomasz and his colleagues (13–19, 21, 24) have beautifully detailed the mechanism of autolysin-induced lysis of pneumococci by detergents and penicillin. Autolysin is

TABLE VII

Effect of Inactive Rat Surfactant Preparations, Mucin, or Serum on the Antipneumococcal Activity of Palmitoyl Lysophosphatidylcholine

Reactant*	Lysophospholipid concentration	Viable pneumococci remaining: Lysophospholipid alone	Viable pneumococci remaining: Reactant plus lysophospholipid
	μg/ml	%	
Inactive rat surfactant (20-fold concentrate)	0	—	100±2‡
	50	42±9	101±6
	125	6±2	110±7
	250	<1±0	12±1
Mucin, 3%	0	—	109±4
	50	38±3	97±3
	125	12±4	86±3
	250	<1	106±7
Rat Serum, %			
0	250	<1§	—
3	250	—	51
6	250	—	47
12	250	—	66
25	250	—	75
50	250	—	88

* Reactants (in PBS) were incubated with an equal volume of the indicated concentrations of lysophospholipid for 30 min at 37°C and 0.2 ml of the mixtures was then incubated with 0.1 ml (10^8) washed, type 25 pneumococci for 90 min at 37°C.

‡ Data represent the mean±SE of four or more determinations.

§ Data represent the mean of two determinations.

strongly bound to pneumococcal Forssman antigen, which is located near the plasma membrane and is composed of teichoic acid similar to the cell wall teichoic acid, except for covalently attached lipid (25–28). Detergent or penicillin-induced lysis of pneumococci is preceded by release of Forssman antigen-containing macromolecules, suggesting dissociation of a complex of lipid, Forssman antigen, and autolysin. With detergents, this dissociation is thought to proceed through dispersion of the lipid component, after which autolysin is free to lyse the cell wall (24). Both adequate levels of autolysin and choline residues in the cell wall are necessary for lysis. In the present studies, choline- or autolysin-depleted pneumococci (and nonpneumococcal bacteria) were killed by lavage without lysis. Bacteriolysis may have facilitated pneumococcal killing, or may have been only an epiphenomenon. The mechanism of killing is uncertain but it could have resulted from permeability changes in the bacterial cell membrane and loss of cellular integrity. Studies with tritiated methyl-D-glucose established that treatment with lavage altered membrane permeability of susceptible bacteria. Virtually all of the lipid in pneumococci is in the cell membrane (24), where it may be susceptible to the same detergent-like effects as were evident during pneumococcal lysis from lavage. Examination of the rate of pneumococcal killing by lavage (Fig. 1) and the rate of development of

TABLE VIII

Effect of Lavage Fluid from Animals of Different Species on Viability of Type 25 Pneumococci

Animal	Viable pneumococci remaining (90 min at 37°C): Lewis rats	New Zealand rabbits	Albino guinea pigs	Mongrel dogs	Mice*
			%		
1	<1	84	102	52	71
2	80	79	99	35	26
3	<1	108	103	16	67
4	<1	10	96	17	70
5	<1	93	65		8
6	95	80	85		
7	4		115		
8	23		102		
9	14				
10	71				
Mean±SE	29±12	76±14	96±5	29±8	48±13

* Lavage from five mice were pooled for each determination.

the membrane permeability changes (Fig. 5), suggests that permeability changes may have preceded bacterial death.

The properties of the bactericidal factor(s) of rat lavage were partially characterized in the present studies. Bactericidal activity was demonstrated with purified lamellar bodies; these bodies contain some protein but are predominantly phospholipid (29). Trypsin did not affect the antibacterial activity of crude surfactant preparations and phospholipid extracts were very active, suggesting that the antibacterial factor(s) were lipids rather than proteins. Candidate substances for the antibacterial activity of rat surfactant include diacylphospholipids (surfactant is >50% dipalmitoyl phosphatidylcholine) (23, 29) and lysophospholipids. We found no antibactericidal activity in vitro with diacylphospholipids, including dipalmitoyl phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, or phosphatidylethanolamine. In contrast, lysophospholipids, which comprise several percent of the total lipid in the lung (23, 30, 31), are detergents with known antimicrobial activity (32, 33). Mardh and Taylor-Robinson (32) showed that lysophosphatide (structure unspecified) killed *Clostridium perfringens*, anaerobic streptococci, pneumococci and *Neisseria meningitidis*, *S. fecalis*, and *Streptobacillus moniliformis*, at concentrations varying from 1 to 250 μg/ml. Gram-negative rods, *Staphylococcus epidermidis*, *Candida albicans*, and several protozoa were resistant to >500 μg/ml. A lytic effect of phosphatide (structure unspecified) has also been demonstrated with L-variants of several bacteria and mycoplasmas (33) and with certain lipid-containing viruses (e.g., influenza) (32). We confirmed and extended observations on the antibacterial effects of lysophospholipids. Palmitoyl lysophosphatidylcholine, which has been identified in surfactant (23), had characteristics similar to the antibacterial factor(s) in rat lavage. It caused autolysin-mediated lysis of pneumococci and altered bacterial membrane permeability. The bacterial strains affected by lavage and lysophosphatidylcholine were similar and several of the same Streptococcus species and Bacillus species were killed. Further, lysophosphatidylcholine was inactivated by mucin or serum, analogous to lavage. These observations do not establish that lysophospholipids are the antibacterial factor(s) in rat lavage, but the role of these compounds in pulmonary defenses obviously warrants further evaluation. We studied two lysophosphatides other than dipalmitoyl lysophophatidylcholine and some differences in antibacterial activity were noted. In canine surfactant there are at least 18 different lysophosphatidylcholines (34), and the antibacterial activity of these molecules might be expected to vary with their exact composition. Various fatty acids have been shown (35–37) to have antimicrobial activity and also might be considered as candidate substances for antibacterial activity in lavage. Full characterization of the antibacterial factor(s) awaits their isolation.

The significance of antibacterial factor(s) in lavage depends on whether they aid bacterial clearance in vivo. Previous studies of the clearance of inhaled *S. aureus* support the view that alveolar killing occurs soley in the macrophages (1–6). However, *S. aureus* is killed more readily by alveolar macrophages after exposure to alveolar lining material (7–8). Neither we nor previous investigators (7–8) have detected staphylococcal killing by lavage but we did not exclude the possibility that lavage caused sublethal injury to *S. aureus*. Sublethal injury to bacteria with antibiotics can greatly facilitate phagocytosis (38), and/or intracellular killing (39, 40), and a similar process might operate in vivo with factors in alveolar lining material.

The respective roles of alveolar macrophage and extracellular factors in early clearance of inhaled nonstaphylococcal bacteria are not shown. Recently, LaForce and Boose (41) detected a peptide in rabbit lavage that facilitates deoxycholate killing of *Escherichia coli* in vitro. This peptide is not in the surfactant pellet, is heat-stable (at 95°C), and can be destroyed with trypsin. Thus, it is distinct from the factor(s) described in the present paper. If the peptide can kill gram-negative bacteria in conjunction with deoxycholate (a detergent) in vitro, it may have antibacterial activity in vivo in combination with detergents like lysophospholipids.

There is some evidence that inhaled pneumococci are not killed solely by alveolar leukocytes, in that they are rapidly killed in nonimmune decomplemented rats, and clearance does not correlate with opsonic requirements in vitro (42). Clearance of aerosolized pneumoccoci is almost as rapid in nonimmune decomplemented guinea pigs as in rats, however, and we have not detected antipneumococcal activity in guinea pig lavage. The interspecies and intraspecies variations in antipneumococcal activity of lavage are not explained. They may simply represent inactivation of antibacterial factors during collection by contaminating substances such as mucus. Studies of surfactant in various species (43) and different members of the same species (44) do not show major differences in the overall composition of surfactant lipids or in total lysophosphatide content, but qualitative differences in the lipids (such as the particular fatty acid residues in the lysphosphatides) may affect their antibacterial properties.

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