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Effects of Surfactant Lipids and Surfactant Protein A on Host Defense Functions of Rat Alveolar Macrophages

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ABSTRACT

Survanta is commonly used as replacement therapy in newborn infants suffering from surfactant deficiency. We investigated the effects of Survanta and surfactant-like liposomes in the presence and absence of surfactant protein A (SP-A) on host defense functions of rat alveolar macrophages (AM). Phagocytosis of *Streptococcus pneumoniae* by AM was significantly inhibited in the presence of 100 μg/mL of Survanta. The ability of SP-A to enhance phagocytosis of *S. pneumoniae* was significantly compromised upon exposure to either Survanta or liposomes, although the overall level of phagocytosis remained higher than in the absence of SP-A. This inhibitory effect was not overcome by opsonization of the bacteria with SP-A before incubation with Survanta and AM. We also found that the ability of SP-A to mediate the association of group B *Streptococcus* with AM was compromised to a significant degree when exposed to either Survanta or liposomes in concentrations of 150 and 250 μg/mL. However, at most concentrations of Survanta or liposomes tested, the presence of SP-A resulted in significantly higher levels of bacterial association. These data show that Survanta and surfactant-like lipids suppress host defense functions of AM in the presence and absence of SP-A *in vitro*, although SP-A continues to enhance host defense functions overall. **(*Pediatr Res* 51: 220–227, 2002)**

Abbreviations

SP-A, surfactant protein A
FACS, fluorescent activated cell sorter
GBS, group B *Streptococcus*
AM, alveolar macrophage(s)
RDS, respiratory distress syndrome
DPBS, Dulbecco's PBS

RDS, a frequent complication of prematurity in neonates, is due to a deficiency in surfactant, a lipoprotein complex responsible for decreasing surface tension in alveoli (1). Over the last two decades, it has become the standard of care to provide surfactant replacement therapy for RDS since Fujiwara *et al.* (2) reported the successful treatment of human neonates with artificial surfactant. Although natural surfactant consists mainly of phospholipids, neutral lipids, and at least four proteins designated A, B, C, and D, surfactant replacement therapies in use today consist primarily of phospholipids and, in some cases, surfactant proteins B and C.

SP-A is synthesized by alveolar type II cells and Clara cells (3) and is the most abundant surfactant protein. It is not found in currently available commercial surfactant replacement therapies. Mice made SP-A deficient by homologous recombination have relatively normal pulmonary function; therefore, it appears SP-A is not essential to achieve the surface tension-lowering properties of surfactant (4). Rather, SP-A is an important mediator of the innate immune system and has been shown to influence host defense functions of AM (5–12). In addition, SP-A-deficient mice display an increased susceptibility to infection (13) and inflammation (14). Recently it has been reported that bacterial phagocytosis by AM *in vivo* can be augmented in SP-A-deficient mice by treating the mice with exogenous SP-A (5).

Phagocytic activities of AM help defend the alveoli against infection (15), and AM have been identified in the airways and alveoli of infants dying from bacterial pneumonia (16). This important population of cells begins increasing just before birth (17) and continues to accumulate rapidly over the hours and days after birth (18–20). AM have been demonstrated to avidly ingest phospholipids (17, 21), a property of AM that has been found in conjunction with a decrease in pathogen killing (22, 23). Therefore, it is not surprising that several studies have suggested that treatment with surfactant may impair AM host

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defense mechanisms (22–24). Clinically, it is difficult to distinguish infants with RDS from those suffering from congenital bacterial pneumonia or both disorders concurrently; therefore, some infants treated with surfactant replacement will have active pulmonary infection (25, 26). Recently, it has been reported that GBS-infected infants can benefit from surfactant replacement therapy to improve gas exchange; however, their mortality remains high when compared with uninfected surfactant-treated infants (27).

The role of SP-A as an integral component of the innate immune system of the lung has been well described in the literature; however, relatively little is known about how the presence of lipids may affect SP-A-mediated functions. We hypothesized that Survanta, a commonly used surfactant replacement therapy, and surfactant-like liposomes would inhibit host defense functions of AM in the presence and absence of SP-A *in vitro*. Nonetheless, we propose that the presence of SP-A may be protective against the inhibitory effects Survanta and liposomes exert on AM.

METHODS

Animals. Male pathogen-free Sprague-Dawley rats (150–250 g) were obtained from Charles River Laboratories (Raleigh, NC, U.S.A.) and Taconic Farms (Germantown, NY, U.S.A.). The Institutional Animal Care and Use Committee of Duke University approved the use of animals in this study.

Media and chemicals. DPBS without calcium chloride or magnesium chloride was obtained from GIBCO-BRL (Grand Island, NY, U.S.A.). BSA, fraction V, fatty acid poor, endotoxin-free, was from Calbiochem (La Jolla, CA, U.S.A.). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) except as noted.

Bacteria. Clinical isolates of *Streptococcus pneumoniae* and GBS were a generous gift of Dr. Roy Hopfer (Medical Microbiology Laboratory, UNC-CH Medical Center) and the University of North Carolina-Chapel Hill Medical Center. Both *Streptococcus* species were cultured on TSA-II agar plates containing 5% sheep blood (Becton-Dickinson Microbiology Systems, Cockeysville, MD, U.S.A.). Bacterial strains were titrated in suspension to correlate an absorbance at 660 nm to colony-forming units (CFU) per milliliter.

Labeling of bacteria with FITC. Bacteria were transferred 24 h after streaking from agar plates into 1 mL of DPBS. The resultant suspension was heated to 95°C for 15 min to kill the bacteria. The heat-killed bacteria were then labeled with FITC (Molecular Probes, Eugene, OR, U.S.A.) as previously described (11) with the exception that the final FITC concentration was 0.1 mg/mL. The labeled bacteria were suspended in 1 mL DPBS, and the absorbance at 660 nm was measured to estimate final bacterial concentration. Labeled bacteria were stored until use at −80°C.

Survanta. Survanta (beractant) is a natural bovine lung extract made up of phospholipids, approximately half of which are dipalmitoylphosphatidylcholine, in addition to neutral lipids, fatty acids, and surfactant proteins. The lipid portion is supplemented to replicate the surface tension-lowering properties of natural surfactant. Survanta contains 25 mg phospholipid per milliliter of 0.9% sodium chloride. Survanta's protein fraction, which is less than 1 mg/mL, is comprised of surfactant proteins B and C and does not contain SP-A according to the product information insert. Survanta was generously supplied by Ross Products Division, Abbott Laboratories, Columbus, OH, U.S.A.

Liposome preparation. All lipids were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Lipids were dried under nitrogen in a round-bottom flask and then resuspended in 0.9% sodium chloride (Mallinckrodt Baker, Inc., Paris, KY, U.S.A.) to a final concentration of 1 mg phospholipid/mL. The lipids were incubated at 37°C for 1 h with gentle shaking every 10 min. Mixing was facilitated by the addition of four glass beads to the flask just before incubation. The final liposome composition was 52% dipalmitoylphosphatidylcholine, 26% egg phosphatidylcholine, 15% dipalmitoylphosphatidylglycerol, and 7% cholesterol by weight. The liposomes were then stored in glass at 4°C.

Proteins. SP-A was purified from the bronchoalveolar lavage fluid of patients with alveolar proteinosis as previously described (28). SP-A was stored in 2–5 mM Tris-buffered water (ICN Biochemicals, Aurora, OH, U.S.A.) at −20°C. Preparations were tested for bacterial endotoxin using a *Limulus* amebocyte lysate assay (Bio-Whittaker, Walkersville, MD, U.S.A.). All preparations contained ≤0.31 pg endotoxin/μg protein. IgG from human serum (Sigma Chemical Co., St. Louis, MO, U.S.A.) was obtained as a lyophilized powder and reconstituted in saline. C1q from human serum was purchased from Advanced Research Technologies, San Diego, CA, U.S.A. Mannose-binding lectin was isolated from whole rat serum (Pel-Freeze, Rogers, AR, U.S.A.) as previously reported (29). Recombinant rat surfactant protein D (SP-D) was purified after expression in Chinese hamster ovary cells transfected with SP-D cDNA ligated into the pEE14 vector (Celltech Therapeutics, Ltd., Berkshire, UK) as previously described in detail (30).

Binding of SP-A to S. pneumoniae. FITC *S. pneumoniae* ($3.3–4.4 \times 10^8$ CFU) were suspended in 1 mL phagocytosis buffer [DPBS + 0.9 mM $CaCl_2$ (Mallinckrodt Baker, Inc., Paris, KY, U.S.A.) + 0.1% BSA] in a microfuge tube. SP-A was added to a final concentration of 25 μg/mL. The bacteria and SP-A were incubated for 1 h in the dark at room temperature on a rotator. The bacteria were then pelleted at maximal speed in a microfuge for 2 min. The bacteria were washed three times by centrifugation in phagocytosis buffer to remove excess SP-A and resuspended to a final concentration of 5×10^6 CFU/μL for immediate use.

Isolation of AM. Rats were killed by intraperitoneal injection of pentobarbital, and their tracheas were cannulated and lungs removed. Rat lungs were lavaged six times with a solution containing 140 mM sodium chloride, 5 mM KCl, 2.5 mM Na_2HPO_4, 10 mM HEPES, 0.2 mM EGTA, and 6 mM glucose, pH 7.4, at 37°C and twice with a solution containing 140 mM NaCl, 5 mM KCl, 2.5 mM Na_2HPO_4, 10 mM HEPES, 6 mM glucose, 2 mM $CaCl_2$, and 1.3 mM $MgSO_4$, pH 7.4, at 37°C. The pooled lavage was centrifuged at $228 \times g$ for 10 min at room temperature, and AM were resuspended in phago-

cytosis buffer to a final concentration of 2×10^6/mL. AM were >98% viable as determined by erythrosin-B exclusion.

Phagocytosis of S. pneumoniae by AM. Microfuge tubes were coated with 0.1% BSA at 37°C for 1 h and then rinsed twice with deionized and distilled water. Heat-killed FITC *S. pneumoniae* were added to the microfuge tubes to a final ratio of 100 bacteria per AM along with Survanta or liposomes in varying concentrations up to 100 μg/mL in the presence and absence of 25 μg/mL of SP-A. Phagocytosis buffer was used to bring the total volume of each condition to 500 μL. Survanta, liposomes, and SP-A were added to the extent that the desired concentration would be achieved in the final volume of 500 μL. The contents were vortexed for 5 s before the addition of 5×10^5 macrophages to each sample. The microfuge tubes were then incubated in a heating block at 37°C with moderate shaking for 1 h in the dark, and the assay was completed as described previously (11). Briefly, the cells were washed three times with cold DPBS and split into two sets of samples. One set was resuspended in 0.2 mg of trypan blue per milliliter in 0.02 M $NaC_2H_3O_2$, pH 5.8, to quench the signal of fluorescent bacteria on the surface of AM. The trypan blue-treated cells were washed three times with DPBS before fixation in 1% formaldehyde (Mallinckrodt Baker, Inc., Paris, KY, U.S.A.). The other set was fixed in 1% formaldehyde without treatment with trypan blue. After fixation, all samples were transferred to polystyrene round-bottom tubes (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.) and stored at 4°C in the dark until analyzed by FACS. The design of the experiments is such that the detected level of phagocytosis or cell association can vary between experiments but not within an experiment; therefore, controls were consistently repeated for each of the reported studies.

Association of GBS with AM. This assay was performed as above for phagocytosis with the following exceptions: Survanta or liposomes were added in concentrations up to 250 μg/mL, and the assay was terminated before the addition of trypan blue to a paired set of cell samples. We were unable to adequately quench the FITC signal associated with GBS with trypan blue; subsequently, FACS analysis cannot distinguish internalized bacteria from those on the surface of the AM. Therefore, for experiments employing the use of GBS, we report the results as association with AM rather than phagocytosis by AM to account for the possibility of surface-associated bacteria.

FACS analysis. FACS analysis was performed at Duke University Cancer Center. FACS employs the technique of flow cytometry to count cells and is able to distinguish cells with associated fluorescent signal from those without such signal. Approximately 10,000 cells per sample were analyzed for percent FITC-positive cells. A decrease in FITC signal by 65% or greater between the nontrypan and trypan samples was considered adequate quenching of extracellular fluorescence.

Statistical analysis. The response of the cells, either a change in the rate of phagocytosis or bacterial association with AM, was expressed as a percent of the control condition (set at 100%) for a given set of experiments. Data were analyzed using ANOVA and the Tukey test. Results reported represent the mean of at least three experiments with the addition of error bars to indicate the SEM. An asterisk indicates significant results and their corresponding p value.

RESULTS

Survanta and surfactant-like liposomes inhibit phagocytosis of S. pneumoniae by AM in a concentration-dependent manner. We sought to determine whether the presence of Survanta or surfactant-like liposomes would affect the ability of AM to phagocytose *S. pneumoniae*. At the highest concentration tested, 100 μg/mL, both Survanta and liposomes decreased phagocytosis from the control (*e.g.* the condition in which no Survanta or liposomes were present) value of 100% to 66.8 ± 6.9% ($p = 0.028$) and 56 ± 8.3%, respectively, as determined by FACS analysis (Fig. 1). Phagocytosis steadily returned toward control values in individual samples of AM exposed to decreasing concentrations of Survanta or liposomes. A representative experiment in which phagocytosis is expressed as percent FITC-positive cells is shown in Table 1.

SP-A stimulates phagocytosis of S. pneumoniae by AM. Prior studies demonstrated that 25 μg/mL human SP-A stim-

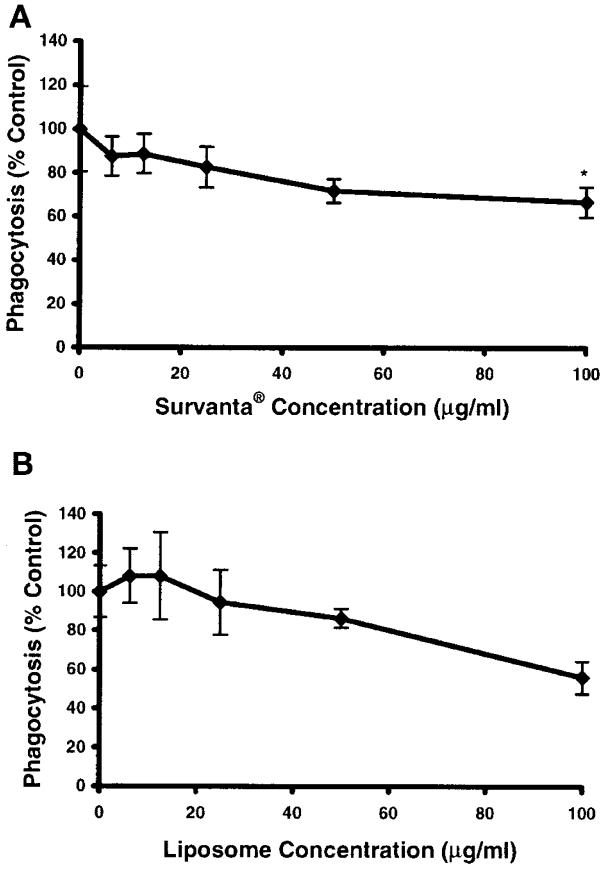

Figure 1. Survanta and surfactant-like liposomes inhibit phagocytosis of *S. pneumoniae* by AM in a concentration-dependent manner. Increasing concentrations of either (*A*) Survanta or (*B*) liposomes were incubated with AM and FITC *S. pneumoniae* in DPBS + 0.9 mM $CaCl_2$ + 0.1% BSA. Fluorescence external to the macrophages was quenched with trypan blue. Phagocytosis was measured by FACS analysis. Data are expressed as a percent of the control condition in which no Survanta or liposomes were added and represent the mean ± SEM for at least three experiments. *Significantly decreased ($p = 0.028$) from the control.

Table 1. *Surfactant-like liposomes inhibit phagocytosis of S. pneumoniae by AM in the presence and absence of SP-A*

	Liposome concentration (μg/mL)					
	0	6.25	12.5	25	50	100
No SP-A	16.5%	16.8%	13.3%	11.9%	12.7%	7.1%
+ SP-A	53.4%	51.4%	44.0%	43.1%	33.3%	27.7%

AM were incubated at 37°C for 1 h in phagocytosis buffer with FITC S. pneumoniae and varying concentrations of liposomes in the presence and absence of 25 μg/mL of SP-A. Extracellular fluorescence was quenched by the addition of trypan blue. Phagocytosis was measured by FACS analysis. Percentages shown are for FITC-positive cells of one representative experiment.

ulates phagocytosis of *S. pneumoniae* by AM (8). We tested human SP-A, human IgG, human C1q, recombinant rat SP-D, or rat mannose-binding lectin in a phagocytosis assay with *S. pneumoniae* to determine what effect, if any, they would have on the ability of AM to phagocytose bacteria. AM incubated with bacteria alone served as a control. Only SP-A was found to alter phagocytosis significantly from the baseline of 100% to 276.5 ± 90.1% ($p = 0.03$) as determined by FACS analysis (Table 2). Heat treatment of the SP-A abrogated this effect.

Survanta and surfactant-like liposomes inhibit SP-A-mediated stimulation of phagocytosis of S. pneumoniae by AM in a concentration-dependent manner. We hypothesized that Survanta and/or surfactant-like liposomes would suppress the SP-A-mediated stimulation of phagocytosis. SP-A was unable to maintain the same level of stimulation in the presence of 100 μg/mL of Survanta as well as 50 and 100 μg/mL of liposomes (Fig. 2). SP-A-mediated stimulation of phagocytosis decreased from the control value of 100% to 43.2 ± 11% ($p = 0.01$) at 100 μg/mL of Survanta and 58.2 ± 6.4% ($p = 0.001$) at 50 μg/mL of liposomes and 50.9 ± 4.8% ($p = 0.0003$) at 100 μg/mL of liposomes as determined by FACS analysis. The ability of SP-A to mediate stimulation of phagocytosis of *S. pneumoniae* approached control values as individual samples of AM were exposed to decreasing concentrations of Survanta or liposomes. A representative experiment in which phagocytosis is expressed as percent FITC-positive cells is shown in Table 1.

The ability of SP-A to stimulate phagocytosis of S. pneumoniae is preserved in the presence of lower concentrations of Survanta and surfactant-like liposomes. It was demonstrated in Figure 2 that SP-A loses its ability to maximally stimulate phagocytosis of *S. pneumoniae* by AM upon exposure to higher concentrations of Survanta and liposomes when compared with SP-A alone. We then compared the degree of phagocytosis achieved in the presence of 25 μg SP-A/mL and varying concentrations of Survanta (Table 3) or liposomes (Table 4) to the control condition, which consisted of AM and FITC *S. pneumoniae* only. We found that SP-A was able to significantly increase phagocytosis to 433 ± 91.4% ($p = 0.005$) in the absence of Survanta as well as to 411.5 ± 92.4% ($p = 0.05$) and 405.2 ± 82.5% ($p = 0.04$) in the presence of 6.25 and 12.5 μg/mL Survanta, respectively. In the absence of liposomes, SP-A increased phagocytosis by 302.8 ± 28.8% ($p = 0.002$) over control and retained stimulatory activity of 284.9 ± 15.4% ($p = 0.003$), 286 ± 25.9% ($p = 0.003$), and 266.2 ± 34.1% ($p = 0.007$) in the presence of 6.25, 12.5, and 25 μg/mL of liposomes, respectively.

Survanta inhibits phagocytosis of SP-A-opsonized S. pneumoniae by AM in a concentration-dependent manner. Previous studies demonstrated that SP-A binds to *S. pneumoniae* in a concentration- and calcium-dependent manner and acts as an opsonin to enhance phagocytosis (8). To test whether Survanta also inhibited opsonic phagocytosis, FITC *S. pneumoniae* were opsonized with 25 μg/mL SP-A before incubation with varying concentrations of Survanta and AM in phagocytosis buffer in contrast with the previously described experiments in which SP-A was present in solution during the entire incubation period. Significant inhibition remained present at both 50 and 100 μg/mL of Survanta, indicating that prevention of SP-A from binding to *S. pneumoniae* was not the only mechanism by which Survanta was acting (Fig. 3). Compared with the control value of 100%, phagocytosis was significantly decreased to 46 ± 10.4% ($p = 0.028$) and 43.4 ± 8.3% ($p = 0.018$) at 50 and 100 μg/mL of Survanta, respectively. The inhibitory effect was no longer detectable as the concentration of Survanta was lowered in individual samples.

Survanta and surfactant-like liposomes inhibit SP-A-stimulated association of GBS with AM in a concentration-dependent manner. SP-A has been demonstrated to enhance both phagocytosis and clearance of GBS by AM in the lungs of SP-A-deficient mice (5). We sought to determine what effect Survanta or surfactant-like liposomes would have on the ability of SP-A to stimulate GBS association with AM. As determined by FACS analysis, both Survanta and liposomes in concentrations of 150 and 250 μg/mL were found to significantly inhibit SP-A-stimulated association of bacteria with AM when compared with the control condition in which no Survanta or liposomes were present (Fig. 4). In the case of Survanta, the percentage of cells with associated bacteria decreased from control at 100% to 58.4 ± 10.9% ($p = 0.034$) at 150 μg/mL and 45.2 ± 12.2% ($p = 0.003$) at 250 μg/mL. Similarly, the

Table 2. *SP-A stimulates phagocytosis of S. pneumoniae by AM*

Protein	% Control
SP-A	276.5 ± 90.1*
h.i. SP-A	107.1 ± 12.5
IgG	126.6 ± 13.8
C1q	88.6 ± 13
SP-D	105.5 ± 14.7
MBL	78.4 ± 12

Phagocytosis in the absence of added protein served as the control and is set at 100%. Values are expressed as percent of control. AM were incubated at 37°C for 1 h in phagocytosis buffer with FITC S. pneumoniae and one of the following proteins: SP-A, heat inactivated SP-A, IgG, C1q, SP-D, or mannose-binding lectin (MBL). All protein concentrations were 25 μg/mL except rat SP-D, which was 1 μg/mL. Extracellular fluorescence was quenched by trypan blue. Phagocytosis was measured by FACS analysis. Results represent the mean ± SE for four experiments. * Significantly greater than control ($p = 0.03$).

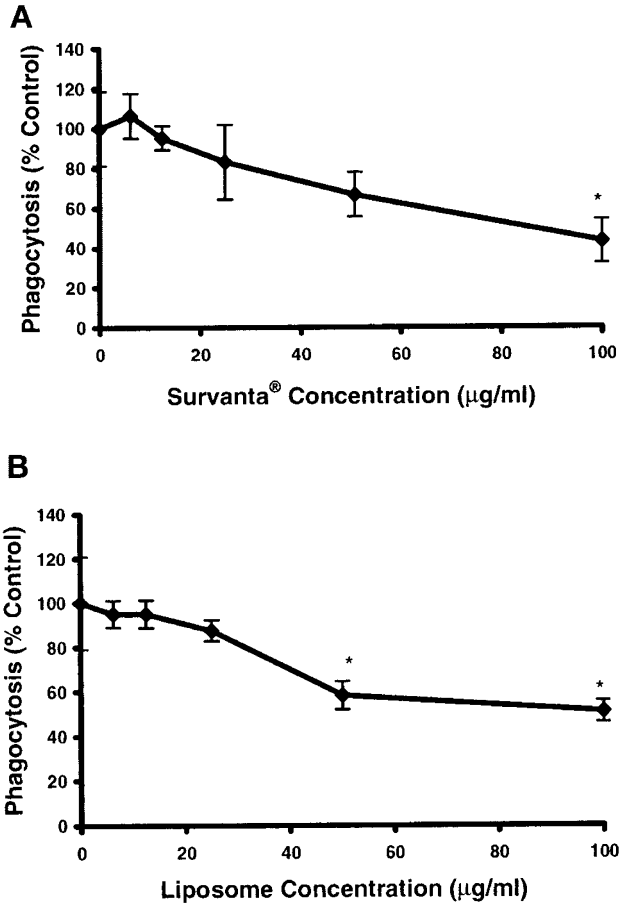

Figure 2. Survanta and surfactant-like liposomes inhibit SP-A-mediated stimulation of phagocytosis of *S. pneumoniae* by AM in a concentration-dependent manner. Increasing concentrations of (*A*) Survanta or (*B*) liposomes were incubated with AM, 25 μg/mL SP-A and FITC *S. pneumoniae* in DPBS + 0.9% $CaCl_2$ + 0.1% BSA. The analyses were performed and data expressed as described for Figure 1. *Asterisks* indicate points that are significantly different ($p \leq 0.01$) from the control (SP-A alone).

Table 3. *Effect of Survanta on SP-A-mediated stimulation of phagocytosis of S. pneumoniae by AM*

Survanta (μg/mL)	25 μg SP-A/mL
0	433 ± 91.4%*
6.25	411.5 ± 92.4%*
12.5	405.2 ± 82.5%*
25	294 ± 85.7%
50	295 ± 81.8%
100	180.2 ± 53.4%

Control (no Survanta or SP-A) phagocytosis is set at 100%; values are expressed as percent control (no SP-A). AM were incubated at 37°C for 1 h in phagocytosis buffer with FITC S. pneumoniae and varying concentrations of Survanta in the presence of 25 μg/mL of SP-A. Extracellular fluorescence was quenched by the addition of trypan blue. Phagocytosis was measured by FACS analysis. Results are mean ± SEM for at least three experiments. * Significantly greater than control ($p \leq 0.05$).

percentage of cells with associated bacteria decreased from control at 100% to 67.1 ± 10.8% ($p = 0.019$) and 67.3 ± 9.1% ($p = 0.022$) when exposed to 150 and 250 μg/mL of liposomes, respectively. In both cases, the level of bacterial association with AM returned toward control as individual samples

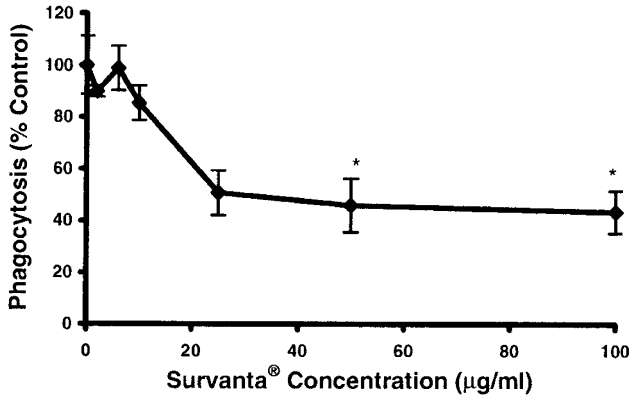

Figure 3. Survanta inhibits phagocytosis of SP-A-opsonized *S. pneumoniae* by AM in a concentration-dependent manner. FITC *S. pneumoniae* were opsonized with 25 μg/mL SP-A before incubation with AM and increasing concentrations of Survanta in DPBS + 0.9 mM $CaCl_2$ + 0.1% BSA. The analyses were performed and data expressed as described for Figure 1. *Asterisks* indicate points that are significantly different ($p < 0.03$) from the control.

of AM were exposed to decreasing concentrations of Survanta or liposomes. A representative experiment in which cell association is expressed as percent FITC-positive cells is shown in Table 5.

Association of GBS with AM increases when exposed to Survanta or surfactant-like liposomes in the presence of SP-A compared with the absence of SP-A. We demonstrate that 25 μg/mL SP-A significantly increases the association of GBS with AM. The percentage of cells with associated FITC GBS rose significantly from the control value of 100% to 203.1 ± 30.3% ($p = 0.007$) and 229.9 ± 32.8% ($p = 0.003$) in the presence of 25 μg/mL of SP-A as seen in Tables 4 and 5, respectively. At all concentrations of Survanta (Table 6) and liposomes (Table 7) tested, the presence of SP-A resulted in increased levels of association between bacteria and AM, with most reaching statistical significance ($p < 0.05$) as indicated by an asterisk.

DISCUSSION

Surfactant replacement therapies, which contain lipids and surfactant proteins B and C, have dramatically reduced mortality due to the elevated surface tension in the lungs of infants with RDS. However, little is known about the roles surfactant deficiency and surfactant replacement therapy play in the immunity of the premature lung. We hypothesized that Survanta and surfactant-like liposomes would affect the abilities of AM to associate with and phagocytose bacteria and of SP-A to mediate the functions of AM *in vitro*. SP-A, although not required for the surface tension-lowering properties of natural surfactant (4), has been found to mediate host defense functions of AM (5–12). In animal studies, a deficiency of SP-A has resulted in an increased susceptibility to infection (13) with recovery of bacterial phagocytosis after exogenous SP-A therapy (5). In RDS, there is a deficiency of pulmonary surfactant and SP-A levels have been found to be low (31–34). It is not known whether SP-A deficiency results in any clinical com-

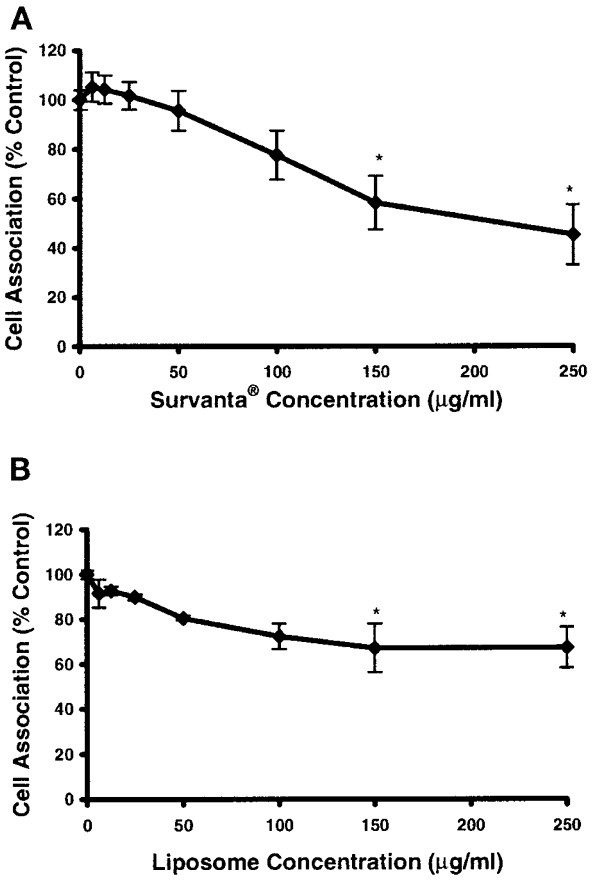

Figure 4. SP-A-mediated association of GBS with AM is inhibited by Survanta and surfactant-like liposomes in a concentration-dependent manner. Increasing concentrations of either (*A*) Survanta or (*B*) liposomes were incubated with AM, 25 μg/mL SP-A and FITC GBS in DPBS + 0.9% $CaCl_2$ + 0.1% BSA. Cell association was measured by FACS analysis. Data are expressed as a percent of the control condition in which no Survanta or liposomes were added and represent the mean ± SEM for three experiments. *Asterisks* indicate points that are significantly different ($p \leq 0.03$) from the control.

promise for infants or if its activity is modulated by the delivery of surfactant replacement.

In this study, we presented AM with *S. pneumoniae* in an *in vitro* phagocytosis assay. We found that increasing the concentration of lipids in our assay resulted in uptake of fewer bacteria by AM with statistical significance noted at 100 μg/mL of Survanta. In addition, we studied the influence of SP-A on the interactions of *S. pneumoniae* and GBS with AM in the presence of varying concentrations of Survanta and surfactant-like liposomes. We found that SP-A-mediated phagocytosis of *S. pneumoniae* by AM was decreased significantly when exposed to the higher concentrations of Survanta and surfactant-like liposomes tested. We proposed that the lipids could be interfering with the binding of SP-A to the bacteria before uptake by AM. To examine this possibility, we opsonized the bacteria with SP-A before exposure to AM and Survanta without any significant change in our results. This finding does not exclude the notion that lipids may interfere with SP-A binding to bacteria but does indicate that there must be at least another mechanism by which they are acting as well.

Table 4. *Effect of surfactant-like liposomes on SP-A-stimulated phagocytosis of S. pneumoniae by AM*

Liposomes (μg/mL)	25 μg SP-A/mL
0	302.8 ± 28.8%*
6.25	284.9 ± 15.4%*
12.5	286 ± 25.9%*
25	266.2 ± 34.1%*
50	180.1 ± 34.4%
100	156.8 ± 28.1%

Phagocytosis for the control condition (no liposomes or SP-A) is set at 100%; values shown represent percent of control. AM were incubated at 37°C for 1 h in phagocytosis buffer with FITC S. pneumoniae and varying concentrations of liposomes in the presence of 25 μg/mL of SP-A. Extracellular fluorescence was quenched by the addition of trypan blue. Phagocytosis was measured by FACS analysis. Results are mean ± SEM for three experiments. * Significantly greater than control ($p < 0.01$).

We speculate that the bacteria may experience competition as the concentration of lipids is increased due to the fact that AM are known to ingest surfactant lipids (17, 21). In the case of GBS, we found that both Survanta and surfactant-like liposomes in the higher concentrations tested decreased the ability of SP-A to mediate the association of bacteria to AM. Despite our findings that SP-A-mediated host defense mechanisms of AM are hindered in the presence of lipids *in vitro*, it is important to note that the presence of SP-A almost always resulted in overall higher levels of phagocytosis and cell association when compared with the absence of SP-A.

Surfactant exists in the alveoli as a monomolecular film at the air-liquid interface. The true composition of this film is technically impossible to sample *in situ*; however, the proteins are thought to comprise a small, albeit critical, portion. Surfactant is constantly in a state of flux, undergoing production, secretion, degradation, and recycling throughout the alveoli, a process in which AM are a key component (35). Due to the continually changing nature of surfactant, it is likely that there are regional differences throughout the lung in both its lipid-:protein ratio and lipid exposure to AM. Treatment doses of Survanta used clinically are 100 mg/kg per dose into the large airways, a portion of which reaches the alveoli to reduce surface tension. Survanta and lipid concentrations in the current study were limited to a maximum of 250 μg/mL in order to wash AM and remove excess bacteria without difficulty. SP-A was used at a concentration of 25 μg/mL, as this has been shown to enhance phagocytosis in similar studies (8, 11, 12). It is estimated that the SP-A concentration in the rat lung is in the range of 0.3–1.8 mg/mL (36); therefore, we believe that the current study utilizes lower concentrations of both the lipid and protein components of surfactant such that their ratio still falls within reason. We elected to challenge AM with many organisms in the current study. As our hypothesis stated that phagocytosis would be inhibited by the presence of Survanta and lipids, we wanted to ensure that low bacterial numbers would not influence our results. Although we cannot truly sample surfactant *in situ*, we believe our conditions reasonably represent interactions that may take place within alveoli *in vivo*.

Optimal functioning of the host defense system of the lung may be dependent on a critical balance between the protein and

Table 5. *Survanta inhibits SP-A-mediated association of GBS with AM*

Survanta concentration (μg/mL)							
0	6.25	12.5	25	50	100	150	250
86.9%	85.0%	84.2%	81.2%	73.0%	50.6%	44.4%	30.7%

AM were incubated at 37°C for 1 h in phagocytosis buffer with FITC GBS and varying concentrations of Survanta in the presence of 25 μg/mL of SP-A. Cell association was measured by FACS analysis. Percentages shown are for FITC-positive cells of one representative experiment.

Table 6. *Effect of Survanta on the association of GBS with AM in the presence and absence of SP-A*

Survanta (μg/mL)	No SP-A	25 μg SP-A/mL
0	100%	203.1 ± 30.3%*
6.25	95.3 ± 7.4%	178.8 ± 12.2%*
12.5	94 ± 9.2%	177.1 ± 12.1%*
25	95.7 ± 6.4%	172.5 ± 10.5%*
50	93 ± 7.4%	161.6 ± 9%*
100	86.2 ± 11.5%	129.7 ± 5.6%*
150	80.2 ± 10.6%	125.5 ± 9.9%*
250	76.2 ± 11.6%	93.8 ± 5.8%

Values are expressed as a percent of the association in the control condition (no Survanta or SP-A), which is set at 100%. AM were incubated at 37°C for 1 h in phagocytosis buffer with FITC GBS and varying concentrations of Survanta in the presence and absence of 25 μg/mL of SP-A. Cell association was measured by FACS analysis. Results are mean ± SEM for at least three experiments. * Significantly greater than the corresponding Survanta concentration without SP-A ($p < 0.05$).

Table 7. *Effect of surfactant-like liposomes on the association of GBS with AM in the presence and absence of SP-A*

Liposomes (μg/mL)	No SP-A	25 μg SP-A/mL
0	100%	229.9 ± 32.8%*
6.25	94.4 ± 0.9%	200.6 ± 26.7%*
12.5	102.7 ± 11%	207.4 ± 38.6%
25	103.6 ± 12.8%	201.2 ± 37.9%
50	78.4 ± 5.4%	180.9 ± 36%*
100	71.5 ± 4.8%	158.5 ± 21.9%*
150	91.2 ± 8.2%	147.6 ± 22%
250	73.3 ± 5.8%	148.9 ± 21.1%*

Values are expressed as a percent of the association in the control condition (no liposomes or SP-A), which is set at 100%. AM were incubated at 37°C for 1 h in phagocytosis buffer with FITC GBS and varying concentrations of liposomes in the presence and absence of 25 μg/mL of SP-A. Cell association was measured by FACS analysis. Results are mean ± SEM for at least three experiments. * Significantly greater than the corresponding liposome concentration without SP-A ($p < 0.05$).

lipid fractions of surfactant. The AM has been shown to ingest lipids (17, 21), which may be responsible for suppressing the oxidative burst of AM (24) as well as hindering its chemotactic responses (23) and pathogen killing (22, 23). Kremlev *et al.* (37–39) have shown that immune cell functions can be modulated by a balance between SP-A and surfactant lipids. They demonstrated that SP-A increases cell surface marker expression in a monocytic cell line (37), augments TNF-α secretion by splenocytes (38), and stimulates mitogen-induced lymphocyte proliferation (39). SP-A-induced increases were inhibited by surfactant lipids, yet maintained under certain conditions by modifying the SP-A:lipid ratio. Stamme and Wright (40) reported that SP-A enhances the binding and deacylation of lipopolysaccharide by AM, and this effect was retained in the presence of surfactant lipids. These studies, in addition to ours, provide evidence that SP-A can continue to mediate immune functions in the presence of lipids when the protein:lipid ratio is optimized.

This study provides insight into how the presence of Survanta or similar liposomes may affect functions of AM in the presence and absence of SP-A. In the newborn premature infant, it can be difficult to distinguish RDS from bacterial pneumonia (25, 26); for that reason, we have been interested in how surfactant replacement therapy might affect infants fighting an active pulmonary infection. Additionally, as a result of the evidence provided by LeVine *et al.* (13) demonstrating an increase in susceptibility to infection in SP-A-deficient mice, we wonder if surfactant-deficient premature infants would benefit from exogenous SP-A as in the LeVine *et al.* (5) follow-up study. We felt that it was first important to know whether or not SP-A could continue to mediate immune functions in the presence of a surfactant replacement therapy such as Survanta. We believe this study demonstrates that SP-A does retain this important function *in vitro*.

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