Surfactant Protein D Enhances Phagocytosis and Killing of Unencapsulated Phase Variants of Klebsiella pneumoniae

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Pulmonary surfactant protein D (SP-D) is a collagenous C-type lectin (collectin) that is secreted into the alveoli and distal airways of the lung. We have studied the interactions of SP-D and alveolar macrophages with Klebsiella pneumoniae, a common cause of nosocomial pneumonia. SP-D does not agglutinate encapsulated K. pneumoniae but selectively agglutinates spontaneous, unencapsulated phase variants, such as Klebsiella strain K50-3OF, through interactions with their lipopolysaccharides (LPS). These effects are calcium dependent and inhibited with maltoose but not lactose, consistent with involvement of the SP-D carbohydrate recognition domain. Precoating of K50-3OF with SP-D enhances the phagocytosis and killing of these organisms by rat alveolar macrophages in cell culture and stimulates the production of nitric oxide by the NR-8383 rat alveolar macrophage cell line. SP-D similarly enhances the NO response to K50-3OF LPS adsorbed to Latex beads under conditions where soluble LPS or SP-D, or soluble complexes of SP-D and LPS, do not stimulate NO production. Our studies demonstrate that interactions of SP-D with exposed arrays of Klebsiella LPS on a particulate surface can enhance the host defense activities of alveolar macrophages and suggest that activation of macrophages by SP-D requires binding to microorganisms or other particulate ligands. Because unencapsulated phase variants are likely to be responsible for the initial stages of tissue invasion and infection, we speculate that SP-D-mediated agglutination and/or opsonization of K. pneumoniae is an important defense mechanism for this respiratory pathogen in otherwise healthy individuals.

A critical physiological function of pulmonary surfactant is to decrease surface tension at the air-blood interface. However, surfactant and other components of the airspace lining material also contribute to pulmonary host defense. In particular, there is increasing evidence that surfactant-associated proteins A and D (SP-A and SP-D, respectively) contribute to the defense against inhaled microorganisms (12, 52, 62, 64). These proteins, which are synthesized by alveolar and nonciliated bronchiolar epithelial cells, are members of a family of collagenous, C-type lectins called collectins. The collectins are assembled as multimers of trimeric subunits, each consisting of a short amino-terminal cross-linking domain, a triple helical collagen domain, and a trimeric, mannose-type carbohydrate recognition domain (CRD). The most compelling evidence for an important role of the lung collectins in host defense has come from recent studies of SP-A deficiency in transgenic models. For example, SP-A−/− mice show increased sensitivity to airway challenge by group B streptococci, Pseudomonas aeruginosa, and Staphylococcus aureus (30, 32, 33). Significantly, the host defense defects are corrected by the instillation of organisms in the presence of purified SP-A. SP-D−/− mice show abnormalities in surfactant homeostasis (8, 26) and in their response to challenge with certain viral pathogens (31). However, bacterial challenge experiments have not yet been reported.

Several different mechanisms could contribute to the host defense activities of the lung collectins. At least two of these mechanisms involve interactions of collectins with glycoconjugates on microbial surfaces. For example, the collectins can mediate bridging interactions between microbial cell wall glycoconjugates and collectin receptors on phagocytic cells with resulting enhancement of internalization and killing (12, 43, 52). Another lectin-dependent mechanism involves microbial agglutination, a process that can lead to enhanced phagocytosis and which could influence mechanical or mucociliary clearance. In addition, collectins can modulate the activity of other receptors required for efficient phagocytosis. For example, SP-A has been shown to increase the surface expression of macrophage Fc (59) and mannose receptors (22).

SP-D shows high-affinity binding to certain gram-negative lipopolysaccharides (LPS) and is an efficient agglutinin of bacteria that express these surface structures (28). Both monocytes/macrophages and neutrophils express glycoconjugates that can interact with SP-D (12). In addition, at least one potential receptor for SP-D on alveolar macrophages has been identified (20). Thus, there is the possibility that SP-D can mediate bridging interactions between microorganisms and the phagocyte surface, thereby leading to enhanced microbial binding and/or internalization.

Despite these activities, the hypothesis that SP-D can enhance the internalization and killing of microorganisms remains largely unproven (53). SP-D enhances the internalization of specific strains of influenza A virus by neutrophils, but these effects are secondary to viral aggregation, which enhances the interactions of the viral hemagglutinin with its sialylated receptors on the leukocyte surface (12, 17). Precoating
of Pneumocystis carinii with SP-D enhances binding to alveolar macrophage, but internalization is not increased (36, 45), and SP-D binding does not enhance the internalization of Escherichia coli J5 by alveolar macrophages (50). On the other hand, SP-D enhances the internalization and killing of certain mucoid strains of P. aeruginosa, and an uncharacterized SP-D receptor was implicated in this process (54). Although Madan et al. found that SP-D enhances the internalization and killing of Aspergillus fumigatus by alveolar macrophages (37), other investigators found no evidence of enhanced internalization of the fungus by the phagocytic cells (1).

In order to better understand the role of SP-D in host defense against pulmonary pathogens, we examined the interactions of SP-D with Klebsiella pneumoniae. This choice of a model system was guided by several considerations. First, K. pneumoniae is an important pulmonary pathogen and a common cause of gram-negative nosocomial pneumonia (51). Second, the structures of surface polysaccharides of many K. pneumoniae serotypes have been extensively characterized (24, 46). Third, there is considerable information regarding the interactions of specific strains of this organism with other C-type lectins. These include SP-A and the macrophage mannose receptor. Both proteins can enhance the internalization and killing by alveolar phagocytes of Klebsiella serovars that express capsular polysaccharides containing a di-mannose repeating unit (3, 22). Lastly, in preliminary experiments we observed the preferential SP-D-dependent agglutination of unencapsulated K. pneumoniae serovars (41).

An important aspect of Klebsiella biology is the propensity of these bacteria to undergo phase variation in the expression of capsular polysaccharides. Although the phenomenon of phase variation in capsular expression has been long appreciated for E. coli and many other bacterial organisms (45), it has been only recently documented for K. pneumoniae (38). Phase variation represents an apparently random, bidirectional, on-off control of bacterial genes that can take place at relatively high frequency, i.e., approximately 10,000 times more frequently than classical mutational events (44). Thus, changes in environmental conditions can rapidly select for spontaneous variants with the most suitable phenotype. Because the capsule is the outermost structure of the cell wall and can vary markedly in thickness, phase variation in the expression of capsular polysaccharides has the potential to profoundly influence host-microbe interactions. For example, capsules could potentially preclude colonization or infection by limiting the accessibility of bacterial adhesins to their receptors on epithelial cells, mask binding sites on the bacterial cell wall required for efficient phagocytosis killing, or present their own binding sites for innate or immune opsonins.

In the present studies, we show that SP-D specifically interacts with the LPS of unencapsulated phase variants of K. pneumoniae and that the binding of SP-D enhances bacterial internalization and killing. In addition, SP-D increases the production of nitric oxide metabolites by macrophages in response to the bacteria.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The unencapsulated derivatives of strain K21a and the K50 strain were obtained as described elsewhere (38, 55). A spontaneous unencapsulated mutant, 50-30F, from the parent K50 strain was obtained by selecting nonmucoid colonies as described previously (38). For these experiments, bacteria were grown on Trypomycye soy agar (Difco) for 24 h at 37°C and harvested by scraping the confluent growth. The bacteria were resuspended at the desired density in either phosphate-buffered saline (0.1M NaCl, 0.02 M PO4 [pH 7.2]), HEPES-buffered RPMI (pH 7.5), or F-12 Nutrient Mixture as indicated. The last two of these media were supple-
mented with 0.25% (wt/vol) NaHCO3, 1% (wt/vol) glucose, 15% (vol/vol) heat-inactivated newborn bovine serum (Beith Sera, Bet-Haemek, Israel), 100 μg of streptomycin per ml, and 100 U of penicillin G per ml. CFU counts on agar plates showed that a density of 1.00 optical density at 700 nm (OD700) unit is equivalent to approximately 2 × 10^6 and 5 × 10^6 CFU/ml for encapsulated and unencapsulated variants, respectively. Stocks with a 50-fold higher cell density were stored at −70°C with 20% (vol/vol) glycerol. On the day of assay, bacteria were washed three times to remove the glycerol, diluted to the desired density, and incubated on ice pending use for various assays.

**Biological and chemical reagents.** Lipoteichoic acid (LTA) was extracted and purified from S. pyogenes M type 3, and preparation of anti-LTA antisera were described previously (57). Antisera to K. pneumoniae LPS and anti-Streptococcus pyogenes LTA were prepared in rabbits (10, 48). All media and buffers were assayed for the presence of bacterial endotoxin by the gel-clot technique using the Limulus amebocyte lysate (Pyrogenet, M.A., Bioproducts Inc., Walkersville, Md.) and used only when the amount of endotoxin were less than 75 pg/ml. Human recombinant SP-D (RsSP-D) dodecamers were prepared as described previously (11). For most of the present studies, contamination with soluble endotoxin was not an issue because SP-D was used to coat bacteria or beads that were washed with endotoxin-free buffer prior to their exposure to macrophages. Nevertheless, the level of endotoxin contamination was routinely quantified using a sensitive, end-point chromogenic microplate assay (Chromogenix, Milan, Italy) with E. coli O111:B4 endotoxin as the standard. The endotoxin content of the purified recombinant proteins was 0.3 to 5 ng/ml or < 50 pg of endotoxin per μg for our stock solutions. For individual assays of SP-D activity, the stock was further diluted more than 10-fold in endotoxin-free medium, giving final endotoxin concentrations of <500 pg/ml, much less than those associated with detectable macrophage activation in our system. With the dilutions of stock protein used in the chromogenic assay, EDTA did not interfere with the determination of endotoxin concentration; in addition, there was no effect of SP-D at concentrations up to 10 μg/well on the detection of purified LPS (data not shown).

**Assay of glucuronic acid.** The amount of capsular glucuronic acid per mass unit of Klebsiella (10^9 CFU/ml) was determined by extracting the exopolysaccharide with Zwittergent 3-14. This was followed by quantitative measurement of uronic acid by the method of Blumenkranz and Asboe-Hansen (6, 13, 55).

**Purification and characterization of LPS.** The LPS from Klebsiella strains K50-30F and K21a from E. coli Y1088 were purified by the hot phenol-water method (48, 63). The extracted LPS was examined by the whole-cell lysate method of Hitchcock (19). Briefly, bacteria were suspended in 100 μl of lysis buffer containing 2% sodium dodecyl sulfate (SDS), 4% 2-mercaptoethanol, 10% glycerol, 1 M Tris (pH 6.8), and bromophenol blue. Lysates were heated at 100°C for 10 min and subjected to proteinase K digestion (1.25 mg/ml) at 60°C for 60 min. The purified preparations were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (29) without urea on 5 to 10% slab gels and visualized by silver staining (60).

**Lectin blotting of LPS.** LPS blotting was performed by limited modification of the method of Burnette et al. (9). Following SDS-PAGE, the LPS was blotted onto nitrocellulose sheets (70 V, 0.4 to 0.9 A, 1.5 h, 4°C). The sheets were cut into 3-mm-wide strips and incubated overnight at room temperature in 50 mM Tris-buffered saline (TBS)–2% bovine serum albumin (BSA)–10 mM CaCl2 (pH 7.4) containing 10 μl of human SP-D (66 μg/ml). The strips were washed four times with TBS and incubated for 1 h at room temperature with rabbit anti-human SP-D (diluted 1:1,000) in TBS–2% BSA (pH 7.4). Excess antibody was removed by washing with TBS, and the strips were then incubated for 1 h with biotinylated goat anti-rabbit immunoglobulin G antibodies (Dianova, Hamburg, Germany) (1:1,500) in TBS–2% BSA. After washing in TBS, avidin-biotin complex (SP-D; Dianova) was added, and the strips were incubated for 1 h prior to washing with TBS. Bound SP-D was visualized using 4-chloro-1-naphthol as substrate.

**Assays for SP-D-induced bacterial agglutination.** A glass slide agglutination assay was used for the rapid qualitative assessment of agglutinating activity. In this assay 0.02-ml aliquots of bacterial suspension (5 × 10^9 CFU/ml) in HBS were placed on a glass slide and mixed with 0.02 ml of various concentrations of RsSP-D in HBS supplemented with 10 mM CaCl2, buffer alone, or buffer containing the desired concentration of potential inhibitor. After 1 min of gentle agitation, agglutination was assessed visually and graded from 1 to 4. Control incubations were performed in calcium-free buffer.
Agglutination was quantified using a spectrophotometric assay that monitors the rate of bacterial sedimentation. Bacteria were suspended at a density of 0.7 OD$_{600}$ in HBS buffer at a final concentration of 10 mM CaCl$_2$ and stored on ice prior to the assay. Aliquots of bacterial suspension (0.8 ml) were then transferred to 1.5-ml polypropylene tubes (Fisherbrand catalog no. 14-885-942). Following the addition of 0.2 ml of SP-D (1.5 μg of RS-SP-D per ml in the presence or absence of selected inhibitors) or 0.2 ml of the corresponding buffer, cuvettes were sealed and the mixtures were gently rotated end over end for 45 min at room temperature. The sedimentation of bacteria or bacterial aggregates was then monitored by recording the change in light transmission at 700 nm over a period of 15 min. The 50% inhibition of SP-D-induced aggregation was determined by plotting percent inhibition as a function of agent concentration, and the percent inhibition was derived from the change in light transmission at 15 min. The density changes in reaction mixtures containing bacteria alone or bacteria and SP-D were considered 100 and 0% inhibition, respectively. During this incubation period there was no significant change in the light transmission of reaction mixtures containing bacteria alone (unpublished data). There is also no time-dependent change in transmission when microgram amounts of SP-D are incubated with calcium in the absence of bacteria (unpublished observations).

**Assay for agglutination of LPS-coated latex particles.** Adsorption of LPS and LTA onto latex beads was based on a previously described method of adsorbing glycosconjugates (15). Equal volumes of latex beads (1.1-μm diameter; Sigma catalog no. LB-11) were mixed with LPS or LTA (200 μg/ml) in HBS. The mixture was incubated for 4 h at room temperature and centrifuged at 7,000 rpm for 1 min in a Herrme 233x microcentrifuge. The pellet was resuspended in HBS, and the procedure was repeated twice to remove nonsorbed LPS or LTA. The latex bead pellets were resuspended to the original volume in HBS containing 0.2% Tween. For the test, the latex suspension was diluted with HBS containing 2% Tween (vol/vol), and 10 mM CaCl$_2$ (in the absence or presence of 50 mM maltose) to give an OD$_{600}$ of 0.7. Aliquots of the bead suspension (0.8 ml) were distributed to disposable plastic cuvettes, and 0.2 ml of SP-D was added at the desired concentration. The cuvettes were sealed with Parafilm, and the mixtures were rotated end over end at room temperature for 45 min. The OD$_{600}$ was recorded after 25 min.

**Coating of Klebsiella or latex beads with SP-D.** Bacterial suspensions (5 × 10$^9$ CFU/ml) were prepared in PBS or in PBS supplemented with 20 mM CaCl$_2$ or 20 mM EDTA. Equal volumes of the bacterial suspensions and PBS or PBS containing up to 10 μg of SP-D per ml were incubated for 60 min at room temperature. The bacteria were then washed three times with endotoxin-free PBS by centrifugation at 10,000 rpm in a Hermle 233x microcentrifuge to remove unbound SP-D. The pellets of SP-D-coated or uncoated bacteria were resuspended to the original density in PBS and maintained at 4°C pending use in the phagocytosis experiments. Similar conditions were used for the coating of LPS-latex beads prepared as described above.

Sonicated lysates were prepared by treating a sample of the bacterial suspension in RPMI-1640 medium on ice for 5 min using an ultrasonic disintegrator (Misonix Inc., Farmington, N.Y.) at 20 kc/s. The sonicated bacterial suspension was centrifuged (10,000 rpm for 10 min), and the supernatant was filtered through 0.2-μm-pore-size filters (Sartorius AG, Goettingen, Germany). Under these conditions we recovered approximately 167 pg of soluble LPS per 10$^9$ K50-3OF, which corresponds to approximately 56% of the total bacterial LPS as measured by the Limulus assay using purified K50 LPS as the standard.

**Isolation of rat alveolar macrophages.** Rat alveolar macrophages (AM) were obtained by bronchoalveolar lavage (27, 49). The cell suspension was centrifuged at 150 × g for 10 min, and the cell pellet was washed with HBS.

Light microscopic assay of bacterial association with AM. Rat AM were resuspended in HBS (10$^7$ cells/ml) and transferred in 0.2-ml aliquots to six-well plates containing glass coverslips (22 by 22 mm). After 30 min at room temperature, the coverslips were washed three times with HBS using an oscillatory shaker (1 min, 150 rpm). The monolayer of adherent macrophages was overlaid with 1 ml of HBS to which 0.02 ml of the SP-D-coated and uncoated bacterial suspension was added. The cultures were rotated at 150 rpm at 37°C, and the monolayers were then washed free of nonbound bacteria as described above. The number of viable cells for each condition was determined and stained by light microscopy in order to enumerate the average number of associated bacteria per macrophage. Specifically, we counted 300 to 500 cells with one or more associated bacteria (42).

**ELISA of bacterial internalization.** Bacterial internalization was quantified using minor modifications of an enzyme-linked immunosorbent assay (ELISA) optimized for Klebsiella (2). Briefly, macrophage monolayers in 96-well plates were incubated with 100-μl suspensions of uncoated or SP-D-coated Klebsiella for 30 min at 37 or 4°C. After washing to remove the unbound bacteria, triplicate monolayers from each group were fixed with methanol for 10 min (zero time). Parallel sets of triplicate monolayers were incubated in the presence of SP-D-coated bacteria at 37 or 4°C. At the indicated time, the monolayers were fixed to stop the ingestion process. Bound, extracellular bacteria were quantified by ELISA using a rabbit antiserum to Klebsiella prepared using heat-killed bacteria.

**Growth assay of bacterial killing.** Monolayers of AM were prepared on coverslips as described above. To determine the number of live bacteria bound at zero time a parallel set of triplicate monolayers was lysed with 0.2 ml of 0.2% SDS. Following 1 min of agitation, the lysates were temporarily stored in the cold. Two additional triplicate sets of monolayers were incubated for 90 min at 37 and 4°C, prior to lysis with SDS. The CFU counts in the lysates were then estimated as described elsewhere (58). Briefly, 0.1 ml of the lysates was added to 3 ml of Lorian broth (Difco) and incubated at 4°C at 37°C, and the optical density was recorded. The optical density is proportional to the initial number of live bacteria associated with the macrophages after washing off the nonbound bacteria (58). Thus, a reduction in optical density as compared to the time zero samples represents a reduction of the number of live bacteria during the indicated period of incubation. In preliminary experiments we verified that SP-D alone does not inhibit bacterial growth under the conditions of assay.

**Tetrazolium dye reduction assay of bacterial killing.** The tetrazolium dye reduction assay was performed as described elsewhere (47). Briefly, 0.1-ml aliquots of a suspension of 10$^8$ rat alveolar macrophages in HBS were transferred to 96-well tissue culture plates (Nunc, Roskilde, Denmark). The macrophages were incubated at room temperature for 30 min to allow the cells to attach. The monolayers were washed free of nonadherent macrophages and exposed to a 0.1-ml suspension of K50-3OF (5 × 10$^{10}$ CFU/ml), uncoated or precoated with SP-D as described above. The monolayers were incubated at 37°C for 30 min, washed free of nonbound bacteria, and overlaid with 0.1 ml of HBS buffer. From one set of triplicate monolayers the buffer was removed, and 0.1 ml of Lorian broth supplemented with 0.02% (vol/vol) Tween was added and the cultures were shaken for 1 min to lyse the macrophages. One plate was transferred to the cold, and additional plates were incubated for 90 min at 4°C and at 37°C for 45 or 90 min. At the end of the incubation period, the medium was removed from the monolayers. Lorian broth supplemented with Tween was added, and the mixtures were shaken and placed in the cold. All monolayers were transferred simultaneously to 37°C for 3 h to allow growth of bacteria that remained associated with the macrophage monolayers before lysis. At the end of this incubation, 0.02 ml of tetrazolium dye stock solution was added to all wells as originally described. After shaking and incubating for 15 min at 37°C, the absorbance of the purple reduction product was quantified by OD$_{540}$ using a plate reader. The intensity of the color is proportional to the growth of the bacteria present in the cell and to the initial number of bacteria associated with the macrophages after washing off nonbound bacteria. Thus, a reduction of color intensity as compared to time zero samples represents a reduction in the number of live bacteria during the indicated period of incubation.

**NO production by stimulated rat AM.** For these experiments we utilized the rat alveolar macrophage cell line NR-8383. Briefly, 3.5 × 10$^9$ macrophages in 0.15 ml of F-12 medium were transferred to 96-well culture plates (Nunc) and incubated for 24 h at 37°C in a humidified 5% CO$_2$ atmosphere. Fifty microliters of 1 ml of 10$^9$ CFU/ml K50-3OF or K50-3OF with 0.1 μM sodium nitroprusside (regulated to a final concentration of 10$^{-5}$ M sodium nitroprusside) were added. After 24 h of incubation the plates were centrifuged for 10 min at 2,000 rpm and the supernatant was harvested and stored at −70°C. NO production was measured as the concentration of the nitrite in the supernatant according to the Greiss reaction (5, 16). Briefly, 0.05 ml of the supernatant was transferred to 96-well plates and incubated for 10 min at room temperature with 0.05 ml of the Greiss reagent. The absorbance at 560 nm was determined, and the nitrite concentration was calculated using a standard curve from 1 to 100 μM sodium nitrite. Because the absolute amount of NO production varied between experiments, data from separate experiments were normalized to the NO response obtained with 0.1 μg of LPS per ml, a potent stimulus for NO production.

For some experiments macrophages were exposed to latex beads coated with purified K50-3OF LPS. As a control, we also examined the effects of uncoated latex beads, purified SP-D, K50-3OF LPS, or the combination of purified LPS and SP-D at the indicated concentrations. The LPS was purified and beads were coated as described above.

**RESULTS**

In our preliminary studies we employed a slide agglutination test to screen eight Klebsiella strains carrying K2, K3, K7, K21a, K26, K32, K36, K50, K55, K62, K61, K67, and K70 types of capsular polysaccharides and found that none were aggluti-
nated by SP-D at concentrations up to 10 μg/ml (45). In contrast, unencapsulated derivatives of K21a and K50 serotypes were agglutinated by SP-D at 0.5 and 4 μg/ml, respectively (Table 1). The SP-D-induced agglutination of the unencapsulated strains was calcium dependent and inhibited by 50 mM maltose, suggesting that the carbohydrate recognition domain of the collectin is involved in the agglutination reactions. Because the encapsulated parental strains bind to the macrophage mannose receptor and are efficiently internalized and killed via this lectin-dependent mechanism in the absence of SP-D (3), and because SP-D does not further increase binding of encapsulated Klebsiella with alveolar macrophages (see below), our studies focused on the unencapsulated phase variants, particularly, K50-3OF.

SP-D binds to the LPS of unencapsulated Klebsiella phase variants. Previous studies have shown that the major target molecule for SP-D on E. coli Y1088 is the LPS (28). In addition, it has been shown that SP-D can bind to at least one form of purified K. pneumoniae LPS in solid-phase binding assays (35). However, several different structures of LPS are expressed by K. pneumoniae, and, like most gram-negative bacteria, these organisms express a variety of other cell surface glycoconjugates that might interact with SP-D (46). Accordingly, studies were performed to determine whether SP-D induced agglutination of unencapsulated Klebsiella involved interactions of SP-D with LPS. Agglutination of K50-3OF, as monitored by spectrophotometry, was calcium-dependent and inhibited by maltose (Fig. 1). The 50% inhibitory concentration of maltose was 11 ± 2 mM (mean ± standard deviation), in the range of that previously reported for E. coli (28). Significantly, 40 mM lactose, a structurally related disaccharide that does not efficiently bind to SP-D, did not inhibit the agglutination of either K50-3OF or E. coli Y1088 strains.

In addition, purified LPS isolated by hot phenol extraction effectively inhibited the agglutination of K50-3OF (Fig. 2). The 50% inhibitory concentration of LPS isolated from K50-3OF or from E. coli Y1088 was 4.0 ± 0.7 and 3.1 ± 0.5 μg/ml, respectively. While these values were not significantly different (P > 0.1), the 50% inhibitory concentration of LPS from Klebsiella sp. strain K21a was significantly greater (P < 0.01) being 20 ± 1.5 μg/ml for the same strain. The 50% inhibitory concentration of K21a LPS was also significantly higher (P < 0.05) than that of E. coli Y1088 LPS (62 ± 3 17 ± 2 μg/ml) for SP-D-induced agglutination of E. coli Y1088 strain.

To confirm the specificity for the LPS and the ability of LPS to mediate agglutination, latex beads were coated with purified cell wall components and assayed for SP-D-mediated agglutination. LPS isolated from the K50-3OF strain and with LTA from S. pyogenes exhibited strong agglutination with 1:10 dilutions of anti-Klebsiella LPS and anti-LTA antisera, respectively, by the slide agglutination assay (data not shown), indicating that the antigens were adsorbed on their surfaces. The LPS-coated beads were agglutinated by SP-D, whereas LTA-coated beads were not (Fig. 3). As with whole bacteria, the agglutination of the LPS-coated beads was calcium dependent (data not shown) and inhibited by maltose. In addition, direct binding of SP-D to LPS ladders was demonstrated by lectin blotting of purified LPS isolated from various strains of Klebsiella (Fig. 4).

Coating with SP-D enhances the association of Klebsiella with AM. For these and the following experiments, K50-3OF were incubated with SP-D and then washed three times with endotoxin-free PBS prior to their addition to the AM. Associated organisms were then enumerated by light microscopy as described in Materials and Methods. When uncoated unencapsulated bacteria were incubated with AM, only small numbers of organisms were associated with the macrophages following incubation for 30 min at 37°C (4 ± 2 bacteria/AM [mean of triplicates ± standard deviation]), and most macrophages (94 ± 3%) showed no associated bacteria. However, the number of bound organisms was significantly increased when bacteria were precoated with 3.3 μg of SP-D per ml (54 ± 16 bacteria/AM; P < 0.001), and many of the macrophages (55 ± 12%) showed bacteria and microaggregates of bacteria within cytoplasmic vacuoles and associated with the cell membrane by light microscopy. Bacterial association with the macrophages was specific and lectin mediated, because the numbers of assoc-

### Table 1. Agglutination of K. pneumoniae by SP-D

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean capsule formation ± SD (μg of GluA/10¹¹ bacteria)</th>
<th>Slide agglutination witha:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>SP-D</td>
<td>SP-D + Ca²⁺</td>
</tr>
<tr>
<td>K50, parent</td>
<td>15 ± 2</td>
<td>–</td>
</tr>
<tr>
<td>K50-3OF</td>
<td>&lt;1</td>
<td>–</td>
</tr>
<tr>
<td>K21a parent</td>
<td>9.1 ± 1</td>
<td>–</td>
</tr>
<tr>
<td>K21a/8/30</td>
<td>&lt;1</td>
<td>–</td>
</tr>
</tbody>
</table>

*a* GluA, glucuronic acid

b Agglutination was subjectively graded according to the following scale: -, absence of microscopic agglutination; +++, small numbers of microscopic aggregates with the majority of the bacteria unagglutinated; and ++++, essentially complete agglutination with a predominance of large aggregates and few unagglutinated bacteria.

![FIG. 1. SP-D-induced agglutination of unencapsulated K. pneumoniae.](http://iai.asm.org/)

The time course of the decrease in absorbance for mixtures of Klebsiella strain K50-3OF and RrSP-D (10 μg/ml) in the presence of calcium (open squares), absence of calcium (solid squares), or competing maltose in the presence of calcium (circles) was measured as described in Materials and Methods. Each point is the average of three experiments; each determination was performed in triplicate. The standard deviation is indicated by error bars.
associated bacteria were significantly decreased (to 16 ± 8 bacteria/AM; \(P < 0.001\)) when the bacteria were incubated with SP-D in the presence of maltose.

In other experiments, the parental, encapsulated K50 organisms were preincubated with 3.3 μg of SP-D per ml before addition to the macrophages. The number of SP-D-treated bacteria associated with the macrophages was not significantly different from that of untreated encapsulated Klebsiella (36 ± 4 and 41 ± 5 bacteria/AM, respectively). The results suggest that SP-D does not affect the magnitude of binding of encapsulated Klebsiella to AM via the mannose receptor (3).

Coating with SP-D enhances the internalization of Klebsiella by AM. In order to more accurately assess bacterial binding

FIG. 2. Inhibition of SP-D-induced agglutination of K. pneumoniae and E. coli with LPS. The percent inhibition of LPS from Klebsiella strain K50-3OF (squares), E. coli Y1088 (diamonds) and Klebsiella strain K21a (circles) was calculated from the change of the transmission of SP-D induced-aggregates of K50-3OF and of Y1088 strains in the presence of indicated concentrations of LPS (see Fig. 1). Each point is the mean of three experiments; each determination was performed in triplicate. The standard deviation did not exceed 20% of the mean for any of the data points.

FIG. 3. SP-D-induced agglutination of latex beads coated with LPS. Latex beads coated with LPS from Klebsiella strain K50-3OF and with LTA from S. pyogenes were exposed to the indicated concentration of SP-D in HBS supplemented with 10 mM CaCl₂, with or without 50 mM maltose. After 45 min of rotation end-over-end, the mixture was allowed to stand in spectrophotometer holder and the optical density at 660 nm was recorded after 15 min. Each point is the mean of three experiments; each determination was performed in triplicate. The standard deviation is indicated by error bars. Values of both LTA and LPS coated latex with maltose, however, were significantly different from those of LPS coated latex at corresponding SP-D concentrations of 0.8 to 3.3 μg/ml (\(P < 0.01\) at the highest concentration).

FIG. 4. Binding of SP-D to LPS extracted from Klebsiella strain K50-3OF. The Klebsiella strain K50-3OF LPS used for the agglutination studies was examined by silver staining (Ag) as described in Materials and Methods. Consistent with our previous studies, the LPS showed a predominance of rough forms with a faint ladder of larger species. Direct binding of SP-D to the LPS was visualized by lectin blotting (Blot) as described. The strongest band corresponds to the rough forms identified on the silver stain; however, there was also detectable labeling of the larger species. The spot approximately one-third of the distance from the top of the blot is an artifact.
TABLE 2. Phagocytosis of unencapsulated Klebsiella strain K50-3OF as determined by ELISA

<table>
<thead>
<tr>
<th>Pretreatment of Klebsiella</th>
<th>No. of bound bacteria (mean ± SD) after:</th>
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<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>Untreated</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>SP-D</td>
<td>0.43 ± 0.15</td>
</tr>
</tbody>
</table>

* Monolayers of rat AM were exposed to Klebsiella strain K50-3OF at room temperature for 30 min. After washing off the unbound bacteria (0 min), the plates were further incubated at the indicated temperature and time, and the bacteria were fixed to stop the ingestion process, and the number of extracellular, bound bacteria was quantified by ELISA using antibodies to Klebsiella as described in Materials and Methods. Even after 90 min at 4°C there was no significant decrease in ELISA signal, consistent with an energy dependent process. The signal obtained after 30 min at 37°C (*) was significantly less than that for the 0 min time point (P < 0.001). Binding in the absence of SP-D was near the limits of detection for this assay.

![FIG. 5. Tetrazolium dye reduction assay of bacterial killing. Monolayers of rat AM were exposed to SP-D-coated (filled bars) or uncoated (open bars) Klebsiella strain K50-3OF for 30 min. The macrophages were washed free of nonbound bacteria and incubated in medium. At the indicated time and temperature 0.1% Tween was added to selectively lyse the phagocytic cells. Viable bacteria were allowed to grow for 3 h at 37°C, at which time the tetrazolium dye was added, and the absorbency was recorded after 10 min. Each point is the mean of three experiments; each determination was performed in triplicate. The standard deviation is indicated by error bars.](http://iai.asm.org/)

Coating with SP-D enhances the killing of Klebsiella by AM. Bacterial viability was assessed using standard growth assays in parallel with the light microscopic binding assays described above. As shown in Table 2, nearly two-thirds of the bound bacteria were killed within 90 min at 37°C. There was only a small decrease in growth following a parallel 90-min incubation at 4°C, consistent with internalization of approximately two-thirds of the bound bacteria. Following a 90-min incubation at 4°C there was a negligible decrease in absorbance, indicating that the bacteria were still extracellular, and that the decrease in absorbance at 37°C is not a consequence of bacterial aggregation. Approximately 90% of the bacteria can be detected following lysis of the macrophages with distilled water (2).

Coating with SP-D enhances NO production by AM. For these experiments the NR-8383 rat AM cell line was used. Because these cells exhibit many of the characteristics of normal AM, including NO production in response to various stimuli, they provide a more convenient and phenotypically homogenous population of cells for the NO stimulation assays (18, 23). SP-D coated K50-3OF gave significantly higher stimulation of NO production by the rat alveolar macrophage cell line than uncoated bacteria (Fig. 6). There was no significant increase in the release of NO when bacteria were incubated with SP-D in the absence of calcium or presence of maltose or when the macrophages were incubated with SP-D chromatography buffer.

Interestingly, there was minimal increase in NO production following the addition of sonicates of SP-D coated Klebsiella (Materials and Methods). In control experiments we confirmed that sonication does not decrease the bacterial agglutinating activity of SP-D. Furthermore, the addition of intact
SP-D and LPS greatly exceed those estimated to exist in association with 10^5 SP-D-coated bacteria (bar 2), SP-D-coated K50-3OF Klebsiella in buffer depleted of calcium with EDTA (10 mM) (bar 3), or SP-D-coated bacteria in the presence of maltose (bar 4). Control cultures included macrophages incubated with sonicated bacteria in the absence of SP-D (bar 5), sonicates of organisms coated with 7 μg of SP-D per ml (bar 6), sonicates of organisms coated with 14 μg of SP-D per ml (bar 7), or medium alone. The amount of nitrite released in quadruplicate cultures of macrophages stimulated by the indicated agents was determined. The data represent the means of three experiments. Because the absolute amount of nitrite produced varied between experiments, the results are presented as the percent of the nitrite released by macrophages triggered with 0.1 μg of LPS per ml, which stimulated the production of a mean ± standard deviation of 33 ± 12 μM per well. Comparisons of the means were made using the Student t test, and a P value of <0.05 was considered significant. Differences between the values of cultures depleted of calcium (bar 3) or samples containing SP-D-coated bacteria in the presence of maltose (bar 4) and the samples containing SP-D-coated bacteria (bar 2) were significant. There was no statistical difference (P > 0.1) between the means of the control cultures (bars 5, 6, and 7) and those of cultures containing bacteria alone (bar 1). However, the differences between the values of each of the control mixtures or of the mixtures containing uncoated bacteria and those of SP-D-coated bacteria were highly significant (P < 0.01).

SP-D to bacterial sonicates did not cause an increase in NO stimulation. For example, no enhancement was observed when 100 pg of SP-D was added to sonicates, which contained approximately 0.17 pg of LPS. For reasons discussed in the following paragraph, this amount of SP-D is estimated to be in excess of the amount associated with 10^5 SP-D-coated bacteria.

Coating of LPS-latex beads with SP-D enhances NO production by AM. Given that constituents solubilized by sonication from SP-D-coated bacteria are not active in stimulating NO production, the possibility that stimulation primarily results from the presentation of LPS-decorated particles to macrophages by SP-D was considered. Significantly, precoating of LPS-latex beads with SP-D gave NO levels >10-fold higher than those obtained for LPS-latex beads or latex beads alone (Table 4). In preliminary experiments we observed that incubation of latex beads with the same amount of SP-D does not enhance the stimulation observed with beads alone, suggesting that binding to SP-D may alter the presentation or distribution of the bound protein (data not shown).

This stimulation cannot be attributed to the presence of soluble SP-D or LPS. There was no effect of 10 ng of SP-D or LPS per ml, alone or in combination (Table 4). The amounts of SP-D and LPS greatly exceed those estimated to exist in association with the beads, <54 and <1 pg/well, respectively. The latter concentration estimates were based on the surface area of the 1.1-μm-diameter bead, the number of beads per well, a packing density of adsorbed LPS comparable to the gram-negative bacterial cell wall (60%, undoubtedly a vast overestimate), a maximum binding of one molecule of SP-D per molecule of LPS, and the molecular mass of SP-D dodecamers (approximately 540 kDa) as compared to K50-3OF LPS (>10 kDa).

In other experiments, 100-fold-higher concentrations of purified SP-D (1,000 ng/ml) failed to stimulate macrophage NO production. Previous studies by others have also shown that preparations of soluble SP-D or SP-A with comparably low levels of endotoxin contamination do not stimulate macrophage NO production (65). Although the addition of purified LPS at concentrations slightly greater than 10 ng/ml elicited detectable stimulation, approximately 10-fold higher concentrations were required to achieve levels of NO comparable to those obtained with SP-D coated LPS-latex beads. Furthermore, the addition of 10 μg of polymixin B per ml showed no effect on the stimulation of SP-D coated LPS-latex beads but completely blocked stimulation by 10 μg of LPS per ml (data not shown).

**DISCUSSION**

We have demonstrated that SP-D can agglutinate unencapsulated Klebsiella strain K50-3OF phase variants but not the parental encapsulated strains. When the unencapsulated K50-3OF phase variant was coated with SP-D and washed free of unbound SP-D it showed enhanced binding and temperature-dependent internalization and killing by rat alveolar macrophages. By contrast, binding to the encapsulated parental strain did not augment the already-efficient phagocytosis and killing of these organisms by alveolar macrophages.

Furthermore, SP-D-coated bacteria increased NO production by NR-8383 AM. The observed stimulation of NO production cannot be attributed to LPS associated with the cell wall, because NO production was not significantly increased when macrophages were treated with bacterial sonicates that contained the majority of the bacterial LPS. This is not surprising given that the total LPS content of the 10^5 bacteria used for these studies is approximately 0.3 pg, which is far less than the minimal amount of LPS required to stimulate macro-

**TABLE 4. NO Production by N-8383 macrophages in response to LPS-coated latex beads**

<table>
<thead>
<tr>
<th>Stimulant(s)</th>
<th>Nitrite production (μM/well)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS, 10 ng/ml</td>
<td>&lt;1</td>
</tr>
<tr>
<td>SP-D, 10 ng/ml</td>
<td>&lt;1</td>
</tr>
<tr>
<td>LPS + SP-D (10 ng/ml each)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Latex beads alone</td>
<td>4.50 ± 1.02</td>
</tr>
<tr>
<td>Latex beads + 10 ng/ml SP-D</td>
<td>&lt;1</td>
</tr>
<tr>
<td>LPS-latex beads</td>
<td>4.03 ± 1.67</td>
</tr>
<tr>
<td>LPS-latex beads–SP-D</td>
<td>5.19 ± 1.19</td>
</tr>
</tbody>
</table>

* Assays were performed as described for experiments using intact Klebsiella. Data are for three experiments and are expressed as the mean ± the standard deviation.

* This amount of purified LPS and SP-D is estimated to be greater than the maximum amount of LPS and SP-D present on the SP-D coated LPS-latex beads (see text).

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**FIG. 6. NO production by NR-8383 rat AM.** Macrophages were stimulated with: 10^6 CFU/ml bacteria (bar 1), SP-D-coated bacteria (bar 2), SP-D-coated K50-3OF Klebsiella in buffer depleted of calcium with EDTA (10 mM) (bar 3), or SP-D-coated bacteria in the presence of maltose (bar 4). Control cultures included macrophages incubated with sonicated bacteria in the absence of SP-D (bar 5), sonicates of organisms coated with 7 μg of SP-D per ml (bar 6), sonicates of organisms coated with 14 μg of SP-D per ml (bar 7), or medium alone. The amount of nitrite released in quadruplicate cultures of macrophages stimulated by the indicated agents was determined. The data represent the means of three experiments. Because the absolute amount of nitrite produced varied between experiments, the results are presented as the percent of the nitrite released by macrophages triggered with 0.1 μg of LPS per ml, which stimulated the production of a mean ± standard deviation of 33 ± 12 μM per well. Comparisons of the means were made using the Student t test, and a P value of <0.05 was considered significant. Differences between the values of cultures depleted of calcium (bar 3) or samples containing SP-D-coated bacteria in the presence of maltose (bar 4) and the samples containing SP-D-coated bacteria (bar 2) were significant. There was no statistical difference (P > 0.1) between the means of the control cultures (bars 5, 6, and 7) and those of cultures containing bacteria alone (bar 1). However, the differences between the values of each of the control mixtures or of the mixtures containing uncoated bacteria and those of SP-D-coated bacteria were highly significant (P < 0.01).
phages (7, 39). Numerous controls further excluded a contribution of contaminating soluble endotoxin. As shown by Wright and coworkers (65) and confirmed here, low-endotoxin, soluble SP-D is not an effective stimulator of macrophage NO production.

The SP-D-induced agglutination of the unencapsulated phase variant K50-3OF and its association with macrophages was sensitive to maltose, insensitive to lactose, and calcium dependent, strongly suggesting that the SP-D carbohydrate recognition domain mediates the interactions. LPS purified from *E. coli* or *K. pneumoniae* inhibited the SP-D-induced agglutination of either *E. coli* or unencapsulated *K. pneumoniae*. Furthermore, SP-D agglutinated latex beads coated with purified *Klebsiella* LPS but not beads coated with another cell wall glycolipid, streptococcal LTA. Binding of SP-D to the K50-3OF LPS was confirmed in blotting assays of LPS ladders resolved by SDS-PAGE. Because agglutination was not inhibited by purified capsular polysaccharides from *K. pneumoniae*, we infer that SP-D does not efficiently bind to the capsular glycopolysaccharides. By contrast, both macrophage mannose receptor and SP-A do not interact with these unencapsulated variants (unpublished data). The two C-type lectins do, however, interact with di-mannose residues of the *Klebsiella* capsular polysaccharide (3, 22).

Together, the available data demonstrate that SP-D interacts with a common structure of enterobacterial LPS that is likely exposed on the surfaces of unencapsulated organisms (28, 35). Our data provide strong evidence that this interaction can be sterically inhibited by the presence of a capsule. In this regard, capsular expression has been shown to be associated with decreased SP-D-induced agglutination of *Cryptococcus neoformans* (56). However, the underlying structures that react with SP-D in this eukaryotic organism have not been defined.

The length of the O-polysaccharide chain of LPS could also influence the accessibility of the inner core region for SP-D binding. This possibility is consistent with the observation that K21a LPS, which shows a significantly larger and more complex O-polysaccharide on silver-stained gels, is a less-effective inhibitor of SP-D-induced agglutination of *E. coli* and *K. pneumoniae* than LPS from the K50-3OF strain (Fig. 3). It is also supported by the observation that changing the growth conditions to favor the synthesis of LPS molecules with large O-polysaccharide side chains dramatically reduces the agglutinability of the unencapsulated K50-3OF by SP-D (12). Variations in the length of the O-polysaccharide chain and capsule expression (and possibly other cell surface structures) probably account for the strain variability in SP-D-mediated agglutination of enteric bacteria noted in previous studies. We suggest that the precise structure and density of LPS moieties determine strain-specific differences in the interactions with SP-D, and this can vary with the growth conditions.

Various inflammatory cytokines can directly or indirectly mediate antibacterial effects by increasing proteolytic activity, augmenting the respiratory burst response, upregulating the expression of specific adhesion molecules in the lung (25), and increasing the expression of inducible nitric oxide synthetase (34). The effect of SP-D on macrophage nitric oxide production in response to *Klebsiella* is of particular interest given that NO is required for effective protection against *K. pneumoniae* (61). Our data strongly suggest that the effects of SP-D on macrophage NO production depend on its interaction with LPS in association with a particulate (or microbial) surface. Sonicates of SP-D bacterial complexes or SP-D-treated bacterial sonicates, which contained the majority of the total LPS, did not elicit NO production. In addition, coating of LPS-latex beads with purified SP-D through interactions with its CRD domains markedly increased NO production, and this effect was not inhibited with polymyxin B. Furthermore, purified SP-D and/or K50-3OF LPS at concentrations much greater than those present on the surface of a bead showed no detectable effect on NO production. Thus, these data suggest that “charging” of SP-D molecules with a particulate ligand (or microorganism) may be necessary for proinflammatory effects on macrophage function. Such a mechanism could limit the activation of leukocytes by SP-D in the absence of a significant challenge by microorganisms or other particulate ligands in vivo. Future studies will examine the mechanisms by which ligand binding modifies the biological activity of SP-D. Although SP-D-coated latex beads did not stimulate NO production, studies using beads coated with other SP-D ligands are needed.

Bacterial aggregation (or the aggregation of LPS-latex beads) was always observed under the conditions of our assays. However, we have no direct evidence to indicate that particle aggregation per se is required for the SP-D-mediated enhancement of uptake and killing of K50-3OF, or effects on NO production. Light microscopic analysis suggests that both aggregated and unaggregated organisms are bound and internalized by the macrophages. On the other hand, smaller aggregates may be more efficiently internalized than large aggregates. In the presence of SP-D we often observed very large aggregates associated with the cell surface. Inefficient internalization of very large bacterial aggregates might in part account for the failure of SP-D to enhance the internalization of *E. coli* J5 (50); the J5 strain is a deep rough mutant and therefore expected to be extremely sensitive to agglutination. On the other hand, Restrepo et al. have shown that aggregation is not required for the internalization and killing of a strain of mucoid *P. aeruginosa* (54). Planned studies, using functionally univalent, nonaggregating SP-D mutants could help resolve this issue.

Although SP-D did not agglutinate encapsulated K50 or K21a parental strains, there was detectable binding of SP-D to these organisms. For example, when bacteria were incubated with SP-D, extensively washed, lysed in SDS-PAGE buffer, and subjected to SDS-PAGE, SP-D was detected by immunoblotting using antibodies to SP-D (data not shown). Significantly, the signal was partially blocked by incubating in the presence of maltose. In this regard, it was previously reported that fluorescein isothiocyanate-labeled SP-D bound to various smooth strains of *E. coli* and an encapsulated clinical strain of *K. pneumoniae* that were not detectably agglutinated in the spectrophotometric assay (28). Given the inability of purified capsular polysaccharides to inhibit agglutination, it seems likely that the lectin-dependent binding to encapsulated *Klebsiella* is mediated by interactions of SP-D with LPS, or possibly other surface glycoconjugates, rather than with capsular polysaccharides. Antibodies specific for LPS have been shown to penetrate the capsule and interact with subcapsular antigenic determinants (40). In any case, the quantity and/or quality of
binding to encapsulated organisms appears insufficient to cause agglutination. In addition, binding does not further augment the host defense activities of macrophages, which express mannose receptor and efficiently bind to and internalize encapsulated parental strains.


Klebsiella pneumoniae capsule-mediated resistance to opsonophagocytosis in human lung epithelial cells (14). We speculate that SP-D-mediated aggregation and enhanced phagocytic clearance contribute to the first line of defense against encapsulated phase variants. It is possible that in some compromised patients these mechanisms will be insufficient to rapidly eradicate the organism, thereby favoring the selection of encapsulated organisms.

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REFERENCES


lated Klebsiella pneumoniae to assemble functional type 1 fimbriae on their surface. FEMS Microbiol. 179:123–130.