Surfactant proteins A and D enhance the phagocytosis of *Chlamydia* into THP-1 cells

Rebecca E. Oberley,1 Kevin A. Ault,2 Traci L. Neff,2 Kavita R. Khubchandani,1 Erika C. Crouch,3 and Jeanne M. Snyder1

**Departments of 1Anatomy and Cell Biology and 2Obstetrics and Gynecology, University of Iowa College of Medicine, Iowa City, Iowa 52242; and 3Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110**

First published December 2003; accepted in final form 7 April 2004


Chlamydiae are intracellular bacterial pathogens that infect mucosal surfaces, i.e., the epithelium of the lung, genital tract, and conjunctiva of the eye, as well as alveolar macrophages. In the present study, we show that pulmonary surfactant protein A (SP-A) and surfactant protein D (SP-D), lung collectins involved in innate host defense, enhance the phagocytosis of *Chlamydia pneumoniae* and *Chlamydia trachomatis* by THP-1 cells, a human monocyte/macrophage cell line. We also show that BP-A is able to aggregate both *C. trachomatis* and *C. pneumoniae* but that SP-D only aggregates *C. pneumoniae*. In addition, we found that after phagocytosis in the presence of SP-A, the number of viable *C. pneumoniae* pathogens in the THP-1 cells 48 h later was increased ~3.5-fold. These findings suggest that SP-A and SP-D interact with chlamydial pathogens and enhance their phagocytosis into macrophages. In addition, the chlamydial pathogens internalized in the presence of collectins are able to grow and replicate in the THP-1 cells after phagocytosis.

SP-A; SP-D; *Chlamydia trachomatis*; *Chlamydia pneumoniae*

*Chlamydia pneumoniae* and *Chlamydia trachomatis* infect human mucosal surfaces (39). Whereas *C. pneumoniae* preferentially infects the respiratory tract, *C. trachomatis* targets the reproductive tract, the conjunctiva of the eye, and the respiratory tract (39). Infants born to mothers infected with reproductive tract *C. trachomatis* can contract chlamydial infections as a result of bacteria acquired during parturition (6, 7, 19). Infants infected with *C. trachomatis* can develop conjunctivitis, pneumonia, severe respiratory distress, hypoxemia, apnea, and sepsis (19). *C. pneumoniae* is a common cause of pneumonia in adults and children and has also been linked to coronary artery disease (10, 32). Because *C. pneumoniae* survives and replicates inside macrophages, it has been postulated that infected macrophages may be responsible for spreading chlamydial infections from the lung to other sites in the body (10, 32).

Both *C. pneumoniae* and *C. trachomatis* are intracellular pathogens. After the infectious form of the bacteria (elementary bodies) is internalized by the host cell, they occupy vesicles from the exocytic pathway that delay fusion with the plasma membrane by an unknown mechanism (13). Within the intracellular inclusion that contains the endocytosed bacteria, chlamydial elementary bodies transform into much larger reticulate bodies, a form of the bacteria that is able to undergo replication (16). Unlike the phagosome-lysosome compartments present in most cells, mature chlamydial inclusions never acidify (13). Once the bacteria have replicated within the inclusion, they revert back to their infectious elementary body state and are released from the host cell, a process that allows for infection of new cells. This specialized developmental cycle allows the chlamydial bacteria to survive and replicate within cells without entering the phagosome-lysosome compartment. By replicating within cells, *Chlamydiae* evade many immunological defense pathways (13).

Two lung proteins, surfactant protein A (SP-A) and surfactant protein D (SP-D), play a role in innate host defense in the lung (47). SP-A is the major protein component of lung surfactant, whereas SP-D is present in smaller quantities in the lung and in many other mucosal surfaces (47). SP-A and SP-D are collectins, members of a family of proteins that bind carbohydrates in the presence of calcium. Both lung collectins are characterized by four domains, i.e., an amino terminal domain, a type IV collagen-like domain, a neck domain, and a carbohydrate-binding domain (12, 31). SP-A enhances the phagocytosis of many lung pathogens into alveolar macrophages, i.e., *Streptococcus pneumoniae*, *Escherichia coli*, and *Bacillus Calmette-Guerin* (BCG) (35, 43, 46). Not only does SP-A enhance the phagocytosis of bacteria and other pathogens into macrophages, it has also been shown to enhance the killing of phagocytosed bacteria (28). Weikert et al. (46) have shown that SP-A enhances the killing of an intracellular pathogen, BCG, in rat macrophages through a nitric oxide-dependent pathway. In vivo, SP-A knockout mice are more susceptible to infection with group B *S. pneumoniae*, *Pseudomonas aeruginosa*, and respiratory syncytial virus (RSV) compared with wild-type mice, findings that support the concept that SP-A plays an important role in lung host defense (24–26). SP-D has been shown to participate in the uptake and killing of *P. aeruginosa* and *Klebsiella pneumoniae* in vitro (33, 38). It has been demonstrated that SP-D enhances the clearing of influenza A virus, RSV, and *Aspergillus fumigatus* from the lung in vivo (18, 29, 30). SP-D has also been shown to enhance the killing of *K. pneumoniae*, *Mycobacterium tuberculosis*, and yeast (8, 33, 37, 45). The cellular mechanisms involved in SP-A/SP-D-facilitated phagocytosis of pathogens are not understood; however, it is believed that the carbohydrate-binding domain of the collectins plays an important role.
We hypothesized that SP-A and SP-D proteins would enhance the phagocytosis of two lung pathogens, *C. trachomatis* and *C. pneumoniae*, into THP-1 cells, a human macrophage-like cell line, and that these proteins would promote bacterial killing within the macrophages.

**MATERIALS AND METHODS**

**THP-1 and U-937 cells.** THP-1 cells and U-937 cells, human monocyte-derived cell lines (kind gifts from Drs. Buettner and Kusner, University of Iowa), were grown in suspension in T-75 flasks in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic mixture (GIBCO, Grand Island, NY). The cells were passaged at a ratio of 1:2 approximately every 2 days. The cells were stimulated with 1 nM phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO) for 24 h to differentiate the cells into a macrophage-like phenotype (40). The PMA-stimulated THP-1 and U-937 cells were not adherent to plastic but were able to phagocytose bacteria, making them ideal cells to use for measuring bacterial uptake by flow cytometry. However, because the cells were not adherent after 24 h of PMA treatment, they may not have fully differentiated into a macrophage phenotype.

**Purification of native human SP-A and rat recombinant SP-D.** The 600-g pellet of human alveolar protein lavage material (1 ml, a kind gift from Dr. Scott Ferguson, University of Iowa) was delipidated by mixing the material with isopropyl ether (12 ml) and 1-butanol (8 ml) for 30 min. The aqueous phase was collected after centrifugation of the mixture (18,000 g for 30 min) and then precipitated with 100% ethanol (40 ml) at 20°C for 3 h. The precipitated material was centrifuged at 18,000 g for 30 min at 4°C, and the resulting pellet was resuspended in 1 ml of 20 mM KH2PO4. The resuspended material was then eluted over an Affi-gel Blue column (1 ml; Bio-Rad, Hercules, CA). The Affi-gel Blue column binds serum proteins while SP-A protein passes through the column. The column flow-through was collected and then dialyzed against distilled water for 48 h at 4°C. The protein concentration in the sample was determined by a Bradford assay (2). The purity of the purified SP-A protein was characterized by polyacrylamide gel electrophoresis followed by staining with Coomassie blue.

The recombinant rat SP-D protein was stably expressed in CHO-K1 cells as previously described by Crouch et al. (4). The SP-D dodecamers were isolated by sequential maltosyl-agarose and gel filtration chromatography. The recombinant SP-D protein was stored in a buffer containing EDTA and recalcified before use to a final concentration of 2 mM free calcium.

**Chlamydia aggregation.** We labeled *C. trachomatis*, serovar E, or *C. pneumoniae*, TW-183 (kind gift of Dr. Kyle Ramsey, Midwestern University) elementary bodies with fluorescein isothiocyanate (FITC, Sigma) by washing the organisms twice in 1 M sodium carbonate buffer, pH 9.5, and then incubating them with 0.1 mg/ml FITC in sodium carbonate buffer at 37°C for 1 h. The bacteria were then washed twice in 1× phosphate-buffered saline (PBS), followed by centrifugation for 5 min at 5,000 g. The bacteria were resuspended in 1 ml of RPMI 1640 medium (GIBCO) and stored at −80°C. For bacterial aggregation studies, FITC-labeled *Chlamydia trachomatis* or *C. pneumoniae* were incubated in 1 ml of 10% FBS, RPMI 1640 medium alone, or with the medium plus SP-A (25 μg/ml) or SP-D (1 μg/ml) for 1 h at 37°C. In some experiments, the SP-A and SP-D proteins were sonicated twice at a low frequency for 3 s before incubation to break up any protein aggregates. An aliquot of bacteria from each condition (∼20 μl) was placed on a hemacytometer and the fluorescently labeled bacteria were observed using a fluorescence microscope to evaluate bacterial aggregation.

**Phagocytosis assay.** THP-1 cells (1 × 106) or U-937 cells (1 × 106), previously stimulated with PMA for 24 h, were incubated for 1 h at 37°C with FITC-labeled *C. trachomatis* (5 × 107) at a ratio of 50 bacteria to one THP-1 cell [a multiplicity of infection (MOI) equal to 50]. SP-A (0–50 μg/ml) or SP-D (0–5 μg/ml) was added simultaneously with the FITC-labeled bacteria to the THP-1 cells in RPMI medium (which contains glucose). In some experiments, THP-1 cells were preincubated for 30 min with SP-A (25 μg/ml) or SP-D protein (1 μg/ml) and then incubated with *C. trachomatis* (MOI = 50) for 1 h. In further experiments, 1 endotoxin unit (EU) of endotoxin (BioWhittaker, Walkersville, MD) was also added to the incubation. SP-A and SP-D endotoxin contamination was measured with the Limulus Amebocyte Lysate kit (BioWhittaker). In other experiments, mannose (50 and 100 mM) and galactose (50 and 100 mM) were added to the incubation to determine the role of SP-A or SP-D, respectively. After 1 h, the phagocytosis assay was stopped with the addition of ice-cold 1× PBS (500 μl), and the cells were centrifuged for 10 min at 1,000 g. The pelleted cells were resuspended in a trypan blue solution (200 μg/ml in PBS) to quench the fluorescence of bacteria that were attached to the outer surface of the THP-1 cells but not phagocytosed (42). The cells were then fixed with 2% paraformaldehyde, resuspended in 1× PBS, and subjected to analysis in a FACSscan machine (FACS, fluorescence-activated cell sorter; Becton Dickinson Immunometry Systems, San Jose, CA). FACS was used to measure the intracellular fluorescence in the THP-1 and U-937 cells. Light transmission data from cells passing through a laser beam generated by a single air-cooled, argon ion laser (488 nm excitation) were collected by a flow cytometer (FSC detector) and a fluorescence detector. FSC indicates cell size, and SSC indicates the granularity and viability of each cell. Fluorescence data, which indicated internalized bacteria, were collected on a log scale, with green fluorescence measured at 530 nm. Data from 10,000 events (cells) per condition were collected and analyzed with CellQuest software (Becton Dickinson Immunometry Systems). In addition, some cells were observed with a fluorescence microscope.

**Growth and viability of Chlamydia inside THP-1 and U-937 cells.** PMA-stimulated THP-1 cells (1 × 106) were incubated with *C. trachomatis* (0.05 to 2.5 × 107) in the presence or absence of SP-A (25 μg/ml) for 1 h at 37°C. The cells were then centrifuged at 1,000 g for 10 min and resuspended in 10% FBS RPMI medium that contained no antibiotics. These resuspended cells were plated in a six-well culture plate or a chamber slide (four-well slide; Nalgé Nunc, Naperville, IL) and allowed to grow for 48 h. The cells grown on the chamber slide were fixed with 4% paraformaldehyde and stained with hematoxylin. Some cells were also immunostained for the major outer membrane protein (MOMP) of *C. trachomatis*. Infected cells were fixed in ice-cold 50% methanol/50% acetone for 10 min. We then rinsed the slides in 1× PBS and quenched endogenous peroxidase activity by incubating them in 0.3% H2O2 in methanol for 30 min. Slides were rinsed with 1× PBS and incubated in normal rabbit serum (1.5%) for 20 min. The slides were then incubated in *C. trachomatis* MOMP primary antibody (goat anti-MOMP; United States Biological, Swampscott, MA) 1:200 at room temperature for 1 h. The slides were rinsed in 1× PBS and incubated in secondary antibody (1:10,000, anti-goat IgG conjugated to biotin; Cappel, Aurora, OH) for 30 min at room temperature. Slides were then rinsed and incubated in Vectorstain ABC reagent (Vector Labs, Burlingame, CA) for 1 h at room temperature. Slides were then rinsed and incubated in diaminobenzidine (0.7 mg/ml). The slides were then rinsed with 1× PBS followed by H2O2, counterstained with hematoxylin, dehydrated, and mounted with Permount.

We prepared a cell homogenate by removing the cells from the culture dish with a cell scraper and centrifuging and resuspending the cell pellet in PBS. The resuspended cells were then sonicated and centrifuged at 1,000 g, and the supernatants were collected. Equal amounts of homogenate protein were separated by gel electrophoresis on a 10% Tris-HCl polyacrylamide gel. The proteins were then transferred to a nitrocellulose membrane at 100 V for 1 h. The membrane was placed in 7% nonfat dry milk diluted in 0.1% TNT (0.02 M Tris, 0.15 M NaCl, and 0.1% Tween 20) overnight to block nonspecific binding. The blot was then incubated with the *C. trachomatis* MOMP.
primary antibody (United States Biological) at a dilution of 1:200 for 1 h at room temperature, then washed in 0.1% TNT three times at 15 min per wash. The blot was incubated with a secondary antibody (anti-goat conjugated to horseradish peroxidase, 1:1,000; ICN/Cappel, Irvine, CA) for 45 min at room temperature, then washed in 0.1% TNT three times at 15 min per wash, followed by an incubation with enhanced chemiluminescence solution (ECL; Amersham, Buckinghamshire, UK), and exposure to X-ray film.

THP-1 cells and U-937 cells infected with *C. trachomatis* in the presence and absence of SP-A (25 μg/ml) for 1 h were washed, then plated, and allowed to grow for an additional 48 h. After this incubation period, the cells plus media were harvested and centrifuged, the medium supernatant was collected, and the cell pellet was sonicated to release intracellular chlamydial bacteria. The cell homogenates were serially diluted 10^3–10^6, then added to a monolayer of HeLa cells grown in a 24-well plate, along with a positive control (HeLa cells infected with *C. trachomatis* at an MOI of 1.5) (34). HeLa cells are a good host for chlamydial bacteria; thus any viable bacteria obtained from the infected macrophages should readily infect the HeLa cells. The cell supernatants or cell lysates were incubated with the HeLa cells for 3 h then removed and replaced with fresh media that contained cycloheximide (1 mg/l, Sigma). The HeLa cells were then allowed to grow for an additional 48 h. After this interval, the HeLa cells were fixed with 100% methanol for 10 min at room temperature and then stained with a fluorescein-conjugated murine *Chlamydia* culture confirmation monoclonal antibody (Bio-Rad Laboratories, Redmond, WA) at 30 μl/well for 30 min in the dark (34). The stained cells were rinsed in distilled water, and a drop of Fluoromount G mounting medium (Southern Biotechnology, Birmingham, AL) was placed in each well. Using an inverted fluorescence microscope, we examined the cells from each well and assessed the number of fluorescent cells for each condition. A positively stained cell represents a viable infectious organism. Every condition was performed in duplicate.

**Statistics.** All data were derived from at least three experiments. We calculated all fluorescence data as the total number of fluorescent cells or the mean fluorescence per cell and normalized them by making the control condition equal to 100%. The data were analyzed by one-way analysis of variance followed by either a Student-Newman-Keuls test or a paired t-test. Other data were also analyzed by multiple regression analysis.

**RESULTS**

Effects of SP-A and SP-D on the phagocytosis of *C. trachomatis* and *C. pneumoniae*. The effects of SP-A on the phagocytosis of FITC-labeled *C. trachomatis* by PMA-stimulated THP-1 cells were observed via fluorescence microscopy. Photographs of control THP-1 cells incubated with *C. trachomatis* in the absence of SP-A showed that some cells contained small numbers of ingested bacteria (Fig. 1A). The majority of the cells did not contain fluorescent bacteria, and the cells that had phagocytosed bacteria usually contained only one or two *Chlamydia* elementary bodies (Fig. 1A). In the presence of SP-A without the addition of trypan blue, there was an increase in the number of internalized bacteria (arrows), but extracellular bacterial fluorescence was quenched. Magnification bar, 10 μm. Photomicrographs are representative of 5 independent experiments.
numbers of THP-1 cells with phagocytosed bacteria and in the number of bacteria per cell (Fig. 1B). However, extracellular bacteria attached to THP-1 cells also fluoresced (as shown in Fig. 1B). In the presence of SP-A and trypan blue, the fluorescence of extracellular bacteria was quenched, and only bacteria inside the THP-1 cells remained fluorescent (Fig. 1C). In THP-1 cells infected in the presence of SP-D, there were more cells containing chlamydial elementary bodies; however, each infected cell usually contained only one or two bacteria (data not shown). In summary, more THP-1 cells were infected by Chlamydia in the presence of the surfactant proteins.

The phagocytosis of FITC-labeled C. trachomatis and C. pneumoniae by phorbol ester-stimulated THP-1 cells was measured by incubating the cells and bacteria in the presence or absence of SP-A (0–50 μg/ml) or SP-D (0–5 μg/ml) for 1 h at 37°C. These concentrations of SP-A and SP-D proteins are consistent with ranges used in previous studies (14, 21). Intracellular fluorescence was then measured for each condition. Representative FACS profiles are depicted in Fig. 2. There was an overall increase in the proportion of fluorescent cells in cultures of THP-1 cells incubated with fluorescent bacteria in the presence of SP-A and SP-D, for both strains of bacteria, compared with controls (THP-1 cells incubated with chlamydial bacteria in the absence of SP-A or SP-D) (Fig. 2).

In the presence of SP-A, both C. trachomatis and C. pneumoniae were phagocytosed by the THP-1 cells to a significantly greater, dose-dependent degree than in the control condition (without SP-A) (Fig. 3, A and B). In THP-1 cells incubated with C. trachomatis, at the highest SP-A concentration tested (50 μg/ml), there was an ~3.5-fold increase in the relative percentage of fluorescent cells (Fig. 3A) and an ~2.5-fold increase in cells incubated with C. pneumoniae (Fig. 3B). Because some of the SP-A protein used in the present study had detectable levels of endotoxin contamination (~0.1 ng/50 μg of SP-A, or ~1 EU of LPS/50 μg of SP-A), LPS (1 EU) was added to cells and bacteria in the presence and absence of another batch of SP-A that had reduced LPS contamination (0.026 ng/50 μg of SP-A). The addition of LPS (E. coli endotoxin, BioWhittaker) to the incubation mixture had no effect on the ability of SP-A to promote the phagocytosis of Chlamydia (Chlamydia alone: 100 ± 18.8%, Chlamydia plus LPS: 102 ± 6.2%, P = 0.80; Chlamydia plus LPS and SP-A: 310 ± 23%, P < 0.05, n = 3). It has previously been shown that there is no effect of LPS on the phagocytosis of P. aeruginosa in the presence or absence of SP-A (21). SP-A binds mannose via its carbohydrate-binding domain; therefore, to determine whether the carbohydrate-binding domain of SP-A is involved in the SP-A-facilitated phagocytosis, mannosé (50 and 100 mM) was also added to macrophages incubated with C. trachomatis in the presence of SP-A (21). In the presence of SP-A plus mannose (100 mM), the relative amount of intracellular fluorescence due to phagocytosed bacteria was significantly decreased compared with the SP-A-alone condi-

---

Fig. 2. Representative fluorescence-activated cell sorting (FACS) profiles representing fluorescent bacteria phagocytosed by THP-1 cells. A: FACS profiles of THP-1 cells incubated with C. trachomatis (CT). The cells were incubated with C. trachomatis in the absence of surfactant proteins (top), in the presence of SP-D protein (1 μg/ml, middle), or in the presence of SP-A (25 μg/ml, bottom). B: FACS profiles of THP-1 cells incubated with Chlamydia pneumoniae (CPn). The cells were incubated in the absence of surfactant proteins (top), in the presence of SP-D protein (1 μg/ml, middle), or in the presence of SP-A (25 μg/ml, bottom). At left, the graphs depict cell size [forward scatter (FSC), x-axis] vs. the level of FITC fluorescence (y-axis). At right, the graphs depict the level of FITC fluorescence (x-axis) vs. the number of cells (y-axis). The gate (M1) was set such that the fluorescence of the cells to the left of the gate is considered background fluorescence.
tion [SP-A alone, 100 ± 12.3%; SP-A plus 50 mM mannose, 76 ± 3.5%, \( P = 0.06, n = 3 \); SP-A plus 100 mM mannose, 63 ± 2.7% \( P < 0.05, n = 3 \)].

We also investigated the effects of SP-A on the phagocytosis of \( C. trachomatis \) in another human macrophage cell line to determine whether the observed SP-A-enhanced phagocytosis was restricted to the THP-1 cell line. U-937 cells are a human monocyte-derived cell line that can be differentiated into a macrophage-like phenotype with phorbol esters (41). PMA-stimulated U-937 cells were incubated with \( C. trachomatis \) in the presence or absence of SP-A (25 \( \mu \)g/ml). The relative amount of intracellular fluorescence due to phagocyted bacteria in cells incubated in the presence of SP-A was significantly increased compared with the fluorescence in U-937 cells incubated with \( C. trachomatis \) alone (U-937 cells plus bacteria: 100 ± 1.7%, U-937 cells plus bacteria and SP-A: 125.2 ± 2.2%; \( P < 0.05, n = 3 \)). However, the SP-A-mediated increase in phagocytosis observed in the U-937 cells was not as great as that observed in the THP-1 cells.

In the presence of SP-D, there was a significant, dose-dependent increase in the phagocytosis of both \( C. trachomatis \) and \( C. pneumoniae \) by THP-1 cells (Fig. 3, C and D). At the most effective concentration of SP-D (10 \( \mu \)g/ml), there was an \( \sim3.5 \)-fold increase in \( C. trachomatis \) phagocytosis (Fig. 3C). There was an \( \sim2.5 \)-fold increase in \( C. pneumoniae \) phagocytosis in the presence of the most effective concentration of SP-D (2.5 \( \mu \)g/ml, Fig. 3D). A dose-dependent effect was observed with increasing amounts of SP-D; however, at the highest concentration of SP-D tested (2.5 \( \mu \)g/ml for \( C. trachomatis \) and 5.0 \( \mu \)g/ml for \( C. pneumoniae \)), the stimulatory effect declined (Fig. 3, C and D). SP-D is a maltose-binding protein; therefore, to investigate the role of the SP-D carbohydrate-binding domain in enhancing phagocytosis, we added maltose (50 and 100 mM) to macrophages incubated with \( Chlamydia \) in the presence of SP-D (48). Maltose (100 mM) significantly reduced the ability of SP-D to increase the relative intracellular fluorescence due to phagocyted \( C. trachomatis \) [SP-D alone, 100 ± 8.8%; SP-D plus 50 mM maltose, 74.5 ± 6.6%, \( P = 0.19, n = 3 \); SP-D plus 100 mM maltose, 69 ± 6.5%, \( P < 0.05, n = 3 \)].

The data presented in Fig. 3 depict the proportion of THP-1 cells that phagocyted chlamydial elementary bodies in the presence of either SP-D or SP-A. The FACS machine measures the total number of fluorescent cells as well as the mean fluorescence per cell in each experiment. Therefore, we used the FACS data obtained from the experiments presented in Fig. 3 to determine the mean fluorescence per THP-1 cell in the presence of SP-A and SP-D. SP-A at the 10- and 25-\( \mu \)g/ml concentrations caused a significant increase (approximately twofold, \( P < 0.05 \)) in the amount of fluorescence per cell compared with control cells that had taken up the \( C. pneumoniae \) and \( C. trachomatis \) in the absence of SP-A. SP-D, in contrast, had no significant effect on the mean fluorescence per cell compared with cells that took up the bacteria in the absence of SP-D (data not shown).

We also examined the direct effects of SP-A and SP-D protein on the macrophages. THP-1 cells were preincubated with SP-A (25 \( \mu \)g/ml) or SP-D (1 \( \mu \)g/ml) for 30 min, washed, and then incubated with \( C. trachomatis \) (MOI = 50). When the macrophages were preincubated with SP-A or SP-D protein, they ingested fewer bacteria than THP-1 cells co-incubated with SP-A and SP-D.
with the bacteria and SP-A or SP-D protein; however, the effects were not statistically significant (Fig. 4).

**Effects of SP-A and SP-D on Chlamydia aggregation.** SP-A and SP-D have been shown to promote the aggregation of bacteria, which may allow for more efficient uptake of pathogens into phagocytes such as neutrophils and macrophages (14). Therefore, we next evaluated the ability of SP-A and SP-D proteins to aggregate chlamydiae. Using the optimal concentrations of SP-A and SP-D shown to enhance phagocytosis, we incubated FITC-labeled *C. pneumoniae* and *C. trachomatis* in the presence or absence of either SP-A (25 μg/ml) or SP-D (1 μg/ml) for 1 h at 37°C. As shown in Fig. 5, SP-A enhanced the aggregation of both *C. trachomatis* and *C. pneumoniae* (Fig. 5, B and E). SP-D had little effect on the aggregation of *C. trachomatis* compared with controls; however, SP-D aggregated *C. pneumoniae* somewhat (Fig. 5, C and F). SP-D agglutination of *C. pneumoniae* caused very dense compact aggregates, whereas SP-A produced larger and looser bacterial aggregates. Sonication of SP-A or SP-D to disrupt possible protein aggregates before incubation with the bacteria did not alter the extent or pattern of bacterial aggregation.

**Effects of SP-A on chlamydial viability.** Because *Chlamydia* are intracellular pathogens and require a host cell to replicate, we next determined whether the SP-A-enhanced phagocytosis of *Chlamydia* into a human host cell would lead to a change in chlamydial viability within the infected cells. Because *C. trachomatis* infects the respiratory tract and is easier to grow in cultured cells than *C. pneumoniae*, the viability of *C. trachomatis* phagocytosed into THP-1 cells was investigated further. To assess chlamydial viability, we infected the THP-1 cells with *C. trachomatis* in the presence or absence of SP-A, then the cells were washed and allowed to grow for an additional 48 h in antibiotic-free medium. Figure 6, A and B, shows the morphologic changes observed in the infected cells after a 48-h postinfection incubation. Cells grown for 48 h without prior chlamydial infection were healthy, i.e., had a small round shape and stained readily with hematoxylin (Fig. 6A, top).

None of the cells stained positively for MOMP (Fig. 6B, top). In contrast, some of the THP-1 cells that had been infected with *C. trachomatis* showed abnormal morphology consistent with infection (Fig. 6, A and B, middle). The infected cells were larger and contained large inclusions, which displaced the nucleus to one side of the cell (as indicated by the arrow, Fig. 6A, middle). Chlamydia-infected cells also stained positive for MOMP (Fig. 6B, middle). Most of the cells exposed to *Chlamydia* alone were not infected and had a normal phenotype. Chlamydial infection (48 h postinfection) caused a significant increase in cell death compared with PMA-treated THP-1 cells that were not infected (Table 1). In the presence of SP-A, many more cells contained chlamydial inclusions compared with cells incubated with *Chlamydia* in the absence of SP-A (Fig. 6, A and B, bottom). Also, in the presence of SP-A, there was a significant increase in cell death compared with uninfected cells or cells incubated with *Chlamydia* in the absence of SP-A 48 h postinfection (Table 1). SP-A, incubated with the THP-1 cells in the absence of bacteria, had no effect on cell viability (Table 1).

We further assessed the growth and viability of *Chlamydia* within infected THP-1 cells by determining the levels of MOMP within the cells and the ability of *Chlamydia* obtained from homogenates of THP-1 cells to infect a HeLa cell monolayer. Before we examined the effects of infection in the presence of SP-A on the growth and viability of the internalized *Chlamydia* in the THP-1 cells, we characterized and compared both methods of assessing chlamydial infection. THP-1 cells were infected with increasing amounts of *C. trachomatis* (MOI = 0–10) and allowed to grow for an additional 48 h. Cell lysates were prepared and subjected to either MOMP immunoblot analysis or the chlamydial viability assay (Fig. 7, A and B). Densitometric analysis of MOMP immunoblots showed a positive correlation between the MOI used to infect and the amount of MOMP present in the cells 48 h later (Fig. 7A). The viability assay produced very similar results (Fig. 7B). As the MOI used to infect the THP-1 cells increased, the number of viable elementary bodies detected 48 h postinfection increased (Fig. 7B). Multiple regression analysis revealed that there was correlation between the two line graphs shown in Fig. 7, A and B; i.e., the correlation between the number of viable chlamydial bacteria obtained from cells grown for 48 postinfection and the relative amount of MOMP present in the infected cells grown for 48 h postinfection was very high (r = 0.957), indicating that statistically the two curves are the same. Thus both methods of assessing infected cells produced similar results.

Immunoblotting for a *C. trachomatis* antigen, MOMP, was performed on lysates of THP-1 cells infected with *Chlamydia* in the presence or absence of SP-A to estimate the number of bacteria present inside infected cells. In cells infected in the presence of SP-A, there was an increase in immunoreactive MOMP present inside the THP-1 cells (Fig. 8A). Densitometric analysis revealed a statistically significant increase in MOMP levels in the THP-1 cells infected with bacteria in the presence of SP-A (Fig. 8B).

We then determined whether the phagocytosed chlamydial bacteria were viable within the THP-1 cells 48 h postinfection. Cell lysates from THP-1 cells grown for 48 h postchlamydial infection were used to infect a HeLa cell monolayer. THP-1

![Graph](image-url)

**Fig. 4.** THP-1 cells were preincubated with SP-A (25 μg/ml) or with SP-D (1 μg/ml) for 30 min before the addition of *C. trachomatis* [multiplicity of infection (MOI) = 50]. Other macrophages were co-incubated with the respective collectins and bacteria. There was a small decrease in the relative amount of intercellular fluorescence when the cells were preincubated with SP-A or SP-D compared with cells co-incubated with SP-A or SP-D and the bacteria; however, this effect was not significant (ANOVA; SP-A, P = 0.42; SP-D, P = 0.30; n = 3).
cell lysates obtained from cells infected with *C. trachomatis* in the presence of SP-A (25 μg/ml) contained significantly more (>3.5-fold) elementary bodies compared with cells infected with *C. trachomatis* alone (Fig. 9). Cell lysates from U-937 cells grown for 48 h postchlamydial infection were also used to infect HeLa cell monolayers. The lysates obtained from U-937 cells that were infected with *C. trachomatis* in the presence of SP-A contained more elementary bodies than controls. The relative percentages of infectious bacteria in U-937 cells incubated with *Chlamydia* alone were 100% in experiments 1 and 2 and in U-937 cells incubated with *Chlamydia* in the presence of SP-A were 295% in experiment 1 and 343% in experiment 2.

**DISCUSSION**

In the present study, we investigated the effects of two surfactant-associated proteins, SP-A and SP-D, on the phagocytosis of *C. trachomatis* and *C. pneumoniae*. We also examined the effects of SP-A on the subsequent intracellular viability of *C. trachomatis* in macrophages. First, we investigated the effects of SP-A and SP-D on the phagocytosis of chlamydial bacteria. Both proteins have previously been shown to increase the phagocytosis of multiple bacterial species into macrophages, so we hypothesized that both SP-A and SP-D would enhance the phagocytosis of the two chlamydial pathogens into macrophages. SP-A caused an ~3.5-fold increase in the phagocytosis of *C. trachomatis* and an ~2.5-fold increase in *C. pneumoniae* phagocytosis. SP-D enhanced the uptake of *C. trachomatis* ~3.5-fold and also caused an ~2.5-fold increase in the phagocytosis of *C. pneumoniae*. Increased phagocytosis was not observed when the THP-1 cells were preincubated with the collectins before the addition of *Chlamydia*. We hypothesize that the SP-A and SP-D may act as a bridge between the pathogen and phagocytic cell. When the SP-A or SP-D are preincubated with macrophages, the proteins may be unable to clump bacteria as efficiently. In contrast, when the

---

**Fig. 5.** The effects of SP-A on the aggregation of *C. pneumoniae* and *C. trachomatis* in vitro. Bacteria were FITC-labeled and incubated in the presence or absence of SP-A (25 μg/ml) or SP-D (1 μg/ml) for 1 h at 37°C. A: *C. trachomatis* incubated alone displayed little aggregation. B: in the presence of SP-A, large aggregates of bacteria formed. C: in the presence of SP-D, there was minimal aggregation of *C. trachomatis*. D: *C. pneumoniae* incubated alone displayed little aggregation. E: *C. pneumoniae*, in the presence of SP-A, formed large aggregates. F: in the presence of SP-D, *C. pneumoniae* formed some aggregates. Magnification bar, 10 μm. Photomicrographs are representative of 3 independent experiments.

**Fig. 6.** Growth of bacteria phagocytosed into THP-1 cells in the absence or presence of SP-A. A: hematoxylin-stained THP-1 cells 48 h after infection. Top: THP-1 cells that were not infected with bacteria, i.e., cells with a normal morphology. Middle: THP-1 cells infected with *C. trachomatis*. Most of the cells had a normal morphology; however, occasional cells contained a chlamydial inclusion (arrow). Cells with inclusions were much larger, the intracellular chlamydial inclusion did not stain well, and the THP-1 cell cytoplasmic contents were pushed to one side. Bottom: THP-1 cells infected with *C. trachomatis* in the presence of SP-A (25 μg/ml). Many more cells contained chlamydial inclusions (arrows). B: *C. trachomatis* [major outer membrane protein (MOMP)] immunostaining. Top: no MOMP was present in uninfected THP-1 cells. Cultures infected with *C. trachomatis* added alone included a few cells that contained MOMP (arrows, middle). Cultures infected with *C. trachomatis* in the presence of SP-A included many more cells that contained MOMP (arrows, bottom). Magnification bar, 10 μm.
bacteria and macrophages are co-incubated in the presence of the surfactant proteins, SP-A and SP-D may act as bridges and bring the macrophages and clumped bacteria closer for more efficient phagocytosis.

Aggregation of bacteria allows for more efficient clearance of pathogens into phagocytes (14). Bacterial aggregation can be seen under a microscope (9, 14, 22). Also, it has been shown previously that SP-A- or SP-D-mediated aggregation of bacteria involves the collectin carbohydrate-binding domain (5, 11, 33). We hypothesized that the SP-A and SP-D proteins would bind to carbohydrates present on the Chlamydia elementary

Table 1. Macrophage viability in the presence of SP-A and Chlamydia

<table>
<thead>
<tr>
<th>Condition</th>
<th>Minus SP-A</th>
<th>Plus SP-A, 25 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control media</td>
<td>93.8±1.0%</td>
<td>92.8±0.5%</td>
</tr>
<tr>
<td>Chlamydia MOI = 25</td>
<td>74.8±2.5%*</td>
<td>62.0±3.7%*†</td>
</tr>
</tbody>
</table>

Values are means ± SE. SP-A, surfactant protein A; MOI, multiplicity of infection. *Significantly different from the control media minus SP-A condition (P < 0.05); †significantly different from Chlamydia minus SP-A condition (P < 0.05); n = 4 for all conditions.

Fig. 7. MOMP immunoblot and viability assay for assessing the presence of C. trachomatis bacteria in infected THP-1 cells 48 h after infection. A: densitometric measurements from 3 MOMP immunoblot experiments (a representative immunoblot, inset). Increasing the MOI of the bacteria during infection leads to an increase in MOMP in the THP-1 cells. B: C. trachomatis viability assay. Cell lysates obtained from THP-1 cells infected with an increasing MOI of bacteria resulted in an increasing amount of infectious bodies detected in the HeLa cell bioassay. Both methods for assessing chlamydial infection produced similar curves, indicating that both methods can be used to quantify the presence of intracellular C. trachomatis in infected THP-1 cells.

Fig. 8. Growth of bacteria phagocytosed into THP-1 cells in the presence or absence of SP-A. A: immunoblot for MOMP, a protein present on the outer coat of Chlamydia bacteria. The 1st and 3rd lanes contained cell lysates (30 μg) from THP-1 cells infected with C. trachomatis 48 h postinfection. MOMP was present, indicating that there were bacteria in the infected cells. The 2nd and 4th lanes contain cell lysates (30 μg/ml) made from THP-1 cells infected with C. trachomatis in the presence of SP-A (25 μg/ml). There was more MOMP present inside the SP-A-treated infected cells. The last lane contained 150 ng of purified MOMP, as a positive control. This blot is representative of 3 independent experiments. B: densitometric analysis of MOMP immunoblots from 3 separate experiments. The mean of the Chlamydia alone group (CT) was normalized to 100. There was a significant increase in the relative amount of MOMP inside the THP-1 cells infected in the presence of SP-A. *Significant difference from the control condition (P < 0.05). OD, optical density.

Aggregation of bacteria allows for more efficient clearance of pathogens into phagocytes (14). Bacterial aggregation can be seen under a microscope (9, 14, 22). Also, it has been shown previously that SP-A- or SP-D-mediated aggregation of bacteria involves the collectin carbohydrate-binding domain (5, 11, 33). We hypothesized that the SP-A and SP-D proteins would bind to carbohydrates present on the Chlamydia elementary

Fig. 9. Viability of C. trachomatis in THP-1 cells 48 h after infection. The relative number of chlamydial infectious bodies (elementary bodies) present in THP-1 cell lysates was determined. In the presence of SP-A (25 μg/ml), the number of infectious bodies was significantly increased (~3.5-fold) compared with levels in THP-1 cells infected with C. trachomatis alone (CT). *Significant difference from the control condition (P < 0.05). This experiment was repeated 3 times in duplicate each time.
bodies and aggregate the bacteria (15, 27, 44). We found that SP-A aggregates both C. pneumoniae and C. trachomatis. Surprisingly, SP-D did not aggregate C. trachomatis. SP-D did aggregate C. pneumoniae; however, not as much as SP-A did. Both of these collectins have been shown to self-aggregate (47). Therefore, to evaluate whether the bacterial aggregation was the result of bacteria binding to aggregates of SP-A or SP-D, SP-A and SP-D were briefly sonicated, which disrupts protein aggregation. The pattern of bacterial aggregation was identical after incubation with the sonicated proteins.

There were differences between SP-A- and SP-D-enhanced phagocytosis. First, the concentrations of SP-D protein that enhanced phagocytosis were much lower than the corresponding SP-A concentrations. The optimal SP-D concentration was between 1.0 and 2.5 μg/ml, and the optimal concentration of SP-A was 25–50 μg/ml. The different optimal concentrations may reflect the relative concentrations of SP-A and SP-D found in vivo (47). SP-A is the most abundant protein in the lung surfactant, whereas SP-D is the least abundant surfactant protein (47). The actual SP-A concentration found in the lung hypophase is difficult to measure (estimates range from 300 to 1,000 μg/ml); however, total amounts of SP-D obtained from lavage are generally ~10% of the total SP-A (20, 47). Therefore, it is possible that SP-D facilitates the clearance of pathogens at much lower concentrations than SP-A. Second, the SP-D-mediated enhancement of phagocytosis leveled off or decreased at the highest concentrations used, whereas the highest concentration of SP-A still produced an enhancement of phagocytosis. Others have reported a plateau for SP-A-mediated phagocytosis at concentrations of 50 μg/ml, as well as for SP-D at 1 μg/ml (14). Lastly, the proportion of macrophages that contained phagocytosed bacteria was similar in the presence of SP-A and SP-D; however, the flow cytometry patterns of uptake into the macrophages were different. In the presence of SP-A, some macrophages phagocytosed large numbers of bacteria, whereas many cells did not contain any bacteria. In the presence of SP-D, in contrast, the internal fluorescence of many macrophages increased slightly, i.e., most cells contained one or two fluorescent bacteria. These flow cytometry data correlate with the effects of SP-A and SP-D on bacterial aggregation. For example, SP-A aggregates chlamydial bacteria, and when the macrophage phagocytoses the SP-A/bacteria aggregates, they probably engulf a large number of bacteria. SP-D did not clump the chlamydial bacteria as efficiently as SP-A, i.e., SP-D did not aggregate C. trachomatis and was not able to aggregate C. pneumoniae as effectively as SP-A. Therefore, SP-D enhanced the phagocytosis of fewer bacteria per macrophage.

We hypothesize that the difference in aggregation of Chlamydia by SP-A and SP-D is the result of differences in carbohydrate recognition by their respective lectin domains. SP-A has a preference for binding mannose and N-acetylglucosamine (47), sugars that are abundant in the LPSs of Chlamydia (23). SP-D binds most readily to glucose-containing sugars (47); however, SP-D does bind mannose and other monosaccharides with weak affinity. Therefore, it is possible that the carbohydrates present on Chlamydia may be more readily recognized by SP-A than SP-D. The differences in aggregation may also be explained by the number or distribution of binding sites on the bacteria or differences in the composition of the surface glycoconjugates present on C. trachomatis and C. pneumoniae.

Macrophages infected with C. trachomatis in the absence of SP-A and cultured for an additional 48 h contained some chlamydial inclusions; however, in the presence of SP-A there was a large increase in the number of cells with chlamydial inclusions. We used two methods to quantify these observations, i.e., by measuring the amount of bacteria inside the infected THP-1 cells via MOMP immunoblots and bacterial viability assays. Both methods detected increasing levels of Chlamydia when the THP-1 cells were infected with an increasing MOI of Chlamydia. C. trachomatis elementary bodies have large amounts of MOMP present in their outer membrane. Macrophages incubated with C. trachomatis in the absence of SP-A contained some MOMP; however, in the presence of SP-A, the content of MOMP was significantly increased. Thus the MOMP data support the observation that there are more chlamydial inclusions, and thus more chlamydial MOMP, in cells infected in the presence of SP-A.

We next determined whether the bacteria growing inside the macrophages were viable. A cell lysate was prepared from chlamydia-infected macrophages and used to infect a monolayer of HeLa cells. The number of subsequently infected HeLa cells reflects the number of viable infectious bacteria present in the infected THP-1 cells. We found that there was an ~3.5-fold increase in the relative number of infectious bodies present in lysates of THP-1 cells that had been infected in the presence of SP-A compared with controls (which were infected in the absence of SP-A). Interestingly, there was an ~3.5-fold increase in the number of viable bacteria obtained from the cell lysate of THP-1 cells infected with Chlamydia in the presence of SP-A, and there was also a 3.5-fold increase in the phagocytosis of Chlamydia into THP-1 cells in the presence of SP-A. This indicates that the SP-A-enhanced phagocytosis leads to proportionally more viable bacteria in the cells. We also showed that SP-A enhances the number of viable infectious bodies in another human cell line U-937. Thus the SP-A effect on chlamydial viability observed in human macrophage-like cells is not restricted to the THP-1 cell line. It has also been shown that SP-A enhances the phagocytosis of M. tuberculosis (another intracellular pathogen) into macrophages and that these ingested organisms remain viable (1).

Not only were there more viable bacteria in cells infected in the presence of SP-A, there was also a significant increase in macrophage cell death. Chlamydiae are intracellular pathogens that require a live host cell to survive. Therefore, premature host cell death could be detrimental to Chlamydia. It is unclear if the SP-A itself triggered macrophage cell death or if the increased cell death was caused by the large number of phagocytosed Chlamydia. Other investigators have shown that both C. trachomatis and C. pneumoniae can prevent programmed cell death in infected macrophages and epithelial cells (3, 17).

In conclusion, we have demonstrated that both SP-A and SP-D enhance the phagocytosis of C. pneumoniae and C. trachomatis by macrophages. We also show that, in the presence of SP-A, there were more bacterial protein present and more bacterial inclusions present inside the macrophages. Despite the fact that the SP-A-mediated infection caused an increase in macrophage cell death, the number of viable intracellular C. trachomatis bacteria was increased ~3.5-fold. Therefore, we conclude that SP-A enhances the phagocytosis
of the bacteria into macrophages but is unable to facilitate the killing of the pathogen once it has been ingested. From these studies, it appears that SP-A protein may promote the spread of *C. trachomatis* infections by allowing the bacteria to enter, grow, and replicate inside macrophages. Most bacteria phagocytosed into macrophages are killed in the lysosome, but because *Chlamydiae* are intracellular pathogens, the increased phagocytosis leads to more viable organisms. Therefore, SP-A appears to facilitate chlamydial infection of macrophages.

**ACKNOWLEDGMENTS**

The authors thank Paul Reimann and Dennis Dunnwald for help preparing the photomicrographs for this manuscript. We also thank Dr. Kyle Ramsey (Midwestern University, Downers Grove, IL) for providing us with the *C. pneumoniae* used in this study.

**REFERENCES**


