Surfactant protein A enhances the phagocytosis of C1q-coated particles by alveolar macrophages

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Received 14 September 2001; accepted in final form 10 July 2002

Surfactant protein A enhances the phagocytosis of C1q-coated particles by alveolar macrophages. Am J Physiol Lung Cell Mol Physiol 283: L1011–L1022, 2002. First published July 22, 2002; 10.1152/ajplung.00366.2001.—Surfactant protein-A (SP-A) plays multiple roles in pulmonary host defense, including stimulating bacterial phagocytosis by innate immune cells. Previously, SP-A was shown to interact with complement protein C1q. Our goal was to further characterize this interaction and elucidate its functional consequences. Radiolabeled SP-A bound solid-phase C1q but not other complement proteins tested. The lectin activity of SP-A was not required for binding to C1q. Because C1q is involved in bacterial clearance but alone does not efficiently enhance the phagocytosis of most bacteria, we hypothesize that SP-A enhances phagocytosis of C1q-coated antigens. SP-A enhanced by sixfold the percentage of rat alveolar macrophages in suspension that phagocytosed C1q-coated fluorescent beads. Furthermore, uptake of C1q-coated beads was enhanced when either beads or alveolar macrophages were preincubated with SP-A. In contrast, SP-A had no significant effect on the uptake of C1q-coated beads by alveolar macrophages adhered to plastic slides. We conclude that SP-A may serve a protective role in the lung by interacting with C1q to enhance the clearance of foreign particles.

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16, 28), although via different mechanisms. Another lung collectin, SP-D, functions as an opsonin by either binding to microorganisms and mediating their interaction with receptors on phagocytic cells or by binding to and aggregating microorganisms and presenting them to phagocytes in a multivalent fashion (20, 32). In contrast, C1q is thought to function primarily as an activation ligand by binding to C1q receptors on monocytes and directly stimulating the cells to phagocytose microorganisms opsonized with other proteins, such as IgG or C4b (36). SP-A functions as both an opsonin (39) and as an activation ligand (36). In addition, SP-A, MBL, and C1q are all reported to interact with the same C1q receptors (12, 26, 29, 37), further demonstrating their functional similarities.

Tenner and colleagues (36) showed that both C1q and SP-A stimulated monocytes to phagocytose sheep erythrocytes coated with either IgG or C4b, and Nepomuceno et al. (29) showed that enhancement of phagocytosis by C1q and SP-A is through the C1q receptor that mediates phagocytosis (C1qRρ). C1q is present at a low concentration in normal human lavage fluid where it may aid in the recognition and clearance of microorganisms from the lung (42). However, C1qRρ is not present on AM (Dr. Zissis Chrones, University of Texas Health Center at Tyler, personal communication); therefore, C1q may have a minimal direct effect on the clearance of opsonized particles in the lung, where AM are the primary immune cells. Because SP-A enhances the phagocytosis of bacteria to which it binds (39) and because SP-A binds directly to C1q (30), we investigated the hypothesis that SP-A specifically enhances the phagocytosis of C1q-coated particles.

MATERIALS AND METHODS

Purified proteins. Purified complement proteins, C1q, proenzyme C1r, activated Cls, C4, C3, C3b, and factor B, were purchased from Advanced Research Technologies (San Diego, CA). Human serum albumin (HSA) and BSA were obtained from Sigma (St. Louis, MO). SP-A was purified from the alveolar lavage fluid of alveolar proteinosis patients. SP-A preparations used in these studies were treated with polymyxin agarose to remove contaminating endotoxin (27) and contained <0.2 µg endotoxin/µg protein. Recombinant rat SP-D was purified as described previously (7).

Buffers. Isotonic veronal buffers were prepared as described previously (18). Normal-ionic-strength (145 mM NaCl) veronal-buffered saline contained 1 mM MgCl₂, 0.15 mM CaCl₂, and gelatin (GVBS⁺). Low-ionic-strength (65 mM NaCl) veronal-buffered saline contained dextrose and one 1 mM MgCl₂ and 0.15 mM CaCl₂ (DGVB₅⁻) or 10 mM EDTA. Phagocytosis buffer was prepared by adding 1 mM CaCl₂ and 0.1% BSA to Dulbecco’s PBS, pH 7.4 (DPBS; Gibco, Rockyville, MD). Fluorescence-activated cell sorting (FACS) buffer was phagocytosis buffer that also contained 1% formaldehyde solution to fix cells.

Sensitization of sheep erythrocytes with IgG. Sheep erythrocytes were diluted to 5 × 10⁹/ml in GVBS⁺, and 15 ml of sheep erythrocytes were warmed to 37°C for 15 min. Rabbit anti-sheep erythrocyte IgG was diluted 1:1,600 in 15 ml GVBS⁺ and also warmed to 37°C for 15 min. The entire volume of the antibody dilution was added dropwise to the sheep erythrocytes with constant stirring. The cells were sensitized with antibody for 15 min at 37°C with frequent mixing. Antibody-sensitized sheep erythrocytes were pelleted at 1,900 g and washed one time in GVBS⁺ before being resuspended in GVBS⁺ and adjusted to 5 × 10⁹/ml.

Iodination of SP-A. SP-A was labeled with 125I using iodobeads (Pierce, Rockford, IL). Two prewashed iodobeads were incubated with 2 µl 125I (0.2 nCi) in 50 µl phosphated-buffered water, pH 7.0, for 5 min at room temperature. SP-A was then added to a final concentration of 1 mg/ml, and the reaction proceeded for 30 min at room temperature. Unconjugated iodine was removed using a Micro Bio-spin 6 chromatography column (Bio-Rad, Hercules, CA). Three microliters of the sample were counted in a gamma counter. The concentration of iodinated SP-A was determined by the bicinchoninic acid protein assay (BCA; Pierce) and used to calculate the specific activity of the labeled protein.

SP-A binding studies. To test the interaction of SP-A with various proteins, 2 µg purified protein were coated on the wells of a 96-well MaxiSorp BreakApart microtiter plate (Nunc, Rochester, NY) in 0.1 M sodium bicarbonate buffer, pH 9.7, for 1 h at +2°C. All microtiter plate incubations were done using a total volume of 200 µl/well for all experiments. The wells were washed three times in DPBS containing 0.05% Tween 20 (DPBS-T). Iodinated SP-A was added to the wells at a final concentration of ~1 µg/ml in GVBS⁺, DGVB₅⁻, or EDTA-DGVBS and incubated for 30 min at room temperature. Wells were washed three times in the same buffer. Finally, wells were broken apart and counted in a gamma counter to determine the amount of SP-A associated with the wells.

Experiments were also performed to show that unlabeled SP-A could compete with labeled SP-A for binding to C1q. Wells were incubated with 2 µg C1q or HSA in 0.1 M sodium bicarbonate buffer, pH 9.6, overnight at 4°C. Next, wells were washed three times with DPBS-T and incubated for 30 min with 3% BSA in DPBS. Dilutions of iodinated SP-A, unlabeled SP-A, and HSA were made in DGVBS⁺. Iodinated SP-A was added to unlabeled protein just before addition to wells, for a final concentration of 0.2 µg/ml iodinated SP-A. BSA-blocked wells were washed three times with DPBS-T, and iodinated SP-A/unlabeled protein solutions were added to wells. Wells were incubated for 30 min at room temperature, washed three times with DPBS-T, and counted in a gamma counter.

SP-A binding to sheep erythrocytes was also investigated. Sheep erythrocytes or IgG erythrocytes were resuspended to 5 × 10⁹/ml in DGVBS⁺ buffer. Either sheep erythrocytes or IgG erythrocytes (50 µl) was centrifuged at 1,900 g and resuspended in 50 µl DGVBS⁺ containing no protein, C1q, or SP-A at 15 µg/ml. The cells were preincubated with protein for 15 min at 37°C. After this time, an equal volume of iodinated SP-A (~1 µg/ml in DGVBS⁺) was added. Cells were incubated an additional 30 min and then washed twice in DGVBS⁺. The cell pellets were transferred to new tubes and counted in the gamma counter to determine how much iodinated SP-A was cell associated.

Protein adsorption of fluorescent beads. Fluorescent latex beads were used to investigate the effect of a specific interaction between SP-A and C1q on particle phagocytosis by AM. Fluorescein isothiocyanate (FITC)-labeled plain microspheres measuring 1 µm in diameter (Fluoresbrite microspheres; Polysciences, Warrington, PA) were adsorbed with BSA or C1q as described below. A 2.5% suspension of plain beads (500 µl) was washed three times with 1 ml of 0.1 M borate buffer, pH 8.5 (pH 9.5 for SP-A beads). Beads were resuspended in 1.3 ml of 0.1 M borate buffer and incubated with BSA, C1q, or SP-A at a final concentration of 230 µg/ml.
The beads were incubated with the protein overnight at room temperature with gentle, end-over-end mixing. The next day, the beads were pelleted, and the supernatant was removed and saved for protein determination by BCA. Possible non-specific binding sites on the beads were blocked by incubating them in 0.1 M borate buffer, pH 8.5 (pH 9.5 for SP-A beads), containing 10 mg/ml BSA for 30 min at room temperature. This blocking step was repeated three times. Beads were resuspended in storage buffer (0.1 M PBS containing 5% glycerol, 1% BSA, and 0.1% sodium azide, pH 7.4) and kept at 4°C. Adsorption efficiency was calculated by subtracting the amount of protein recovered in the supernatant from the initial protein added. The amount of protein bound to the beads was estimated. On average, BSA, C1q, and SP-A beads were coated with ~31,000, 15,000, and 8,000 molecules/ bead, respectively.

The concentration of the adsorbed bead preparations was determined so that equal numbers of the different bead preparations could be added to the cells. Briefly, the original stock of plain beads (4.55 × 10^8 beads/ml) was diluted serially. Each solution (200 μl) was added to a fluorescence microtiter plate reader at an excitation of 485 nm and emission of 530 nm to create a standard curve. Dilutions of the protein-adsorbed beads were read, and bead concentration for each sample was determined using the standard curve.

Isolation of rat AM. Pathogen-free male rats (250 g) were obtained from Taconic Farms (Germantown, NY) and housed in a pathogen-free facility until used. AM were isolated from rats. Rats were anesthetized with an overdose of pentobarbital sodium (~400 mg/kg body wt). The chest cavity was opened, and the trachea was cannulated. The lungs were removed and lavaged sequentially with 15- to 20-ml volumes of prewarmed lavage buffer (PBS containing 0.2 mM EGTA) that had been prewarmed to 37°C. Each set of lungs was lavaged with a total of 150 ml lavage buffer, and the lavages were pooled and centrifuged at 228 g to recover the cell pellet. The pellet was washed one time in 10 ml phagocytosis buffer and gently resuspended in the same buffer for use in phagocytosis assays.

Phagocytosis assays by FACS. AM were isolated from rat lungs as described above. Cells were washed and resuspended to a final concentration of 2 × 10^6/ml in phagocytosis buffer. AM (250,000 cells) were incubated with either BSA-coated beads or C1q-coated beads at a ratio of 10 beads/AM (10 μl beads at 2.5 × 10^8 beads/ml in storage buffer) with increasing concentrations of SP-A at 37°C in a total volume of 250 μl phagocytosis buffer. All incubations of cells with beads were for 30 min, unless otherwise stated. After the incubation, cells were washed twice in phagocytosis buffer and resuspended in FACS buffer that contained 1% formaldehyde solution. Cells were stored in the dark until analyzed for cell-associated fluorescent beads by FACS.

AM pretreated with SP-A. We tested the hypothesis that SP-A directly stimulates AM to phagocytose beads. AM were preincubated in the presence or absence of 25 μg/ml SP-A for 1 h at 37°C, pelleted at 228 g, and washed two times with 1 ml phagocytosis buffer to remove unbound SP-A. These cells were then incubated with BSA- or C1q-coated beads at a ratio of 10 beads/AM for 1 h at 37°C and analyzed by FACS as before.

Phagocytosis assay by confocal microscopy. Confocal microscopy was used to determine the extent to which beads were internalized by AM, since FACS analysis could not distinguish between intracellular and extracellular beads. AM were incubated with BSA- or C1q-coated beads in the presence or absence of 25 μg/ml SP-A. After the incubation, cells were washed, resuspended in phagocytosis buffer, and attached to glass slides by spinning at 400 revolutions/min (14 g) for 4 min in a cytospin. AM were fixed with 1% paraformaldehyde for 10 min and counterstained with 1% Evan’s blue for 5 min. Cells were analyzed using a Zeiss LSM410 laser-scanning microscope with a krypton/argon laser, dual-channel FITC/rhodamine settings, and ×63 lens. Slides were blinded, and 20 images of randomly selected cells were stored for each slide. Each image represented a 0.5-μm section of the cells. For each slide, >100 AM were counted to determine both the percentage of AM with at least one internalized bead and the number of internalized beads per cell.

Phagocytosis by adherent and nonadherent cells. To compare bead phagocytosis by cells in suspension with that by cells adhered to slides, we tested the following three experimental conditions: 1) AM in suspension in phagocytosis buffer (same method as above phagocytosis experiments), 2) AM adhered to slides for 3 h in RPMI (control for 3-h adherence). All three conditions were done on the same day using the same initial cell population. For the adherence conditions, cells were diluted to 6.8 × 10^5 cells/ml in RPMI and added to Permanox-coated chamber slides (2 well; Lab-Tek) at 1 × 10^6 cells/well. Slides were incubated for 3 h at 37°C with 5% CO2 and washed two times with phagocytosis buffer, and 1.5 ml phagocytosis buffer containing BSA or C1q beads (10 beads/AM) with or without 25 μg/ml SP-A was added to each slide. Beads were spun on slides for 3 min at 50 g, and slides were incubated at 37°C with 5% CO2 for 1 h. Alternatively, freshly isolated AM were diluted to 2 × 10^6 cells/ml in RPMI and incubated in suspension for 3 h at 37°C with agitation before being resuspended in phagocytosis buffer. Suspension AM were then incubated for 1 h at 37°C with BSA or C1q beads in the presence or absence of 25 μg/ml SP-A. Meanwhile, a third group of cells was diluted to 2 × 10^6 in phagocytosis buffer and directly incubated with BSA or C1q beads in the presence or absence of 25 μg/ml SP-A at 37°C for 1 h as for previous experiments. For cells in suspension, final reactions had a volume of 250 μl containing 250,000 AM and 10 beads/AM.

After incubation with beads, cells in suspension were washed and prepared for analysis by FACS or confocal microscopy as in the above experiments. Likewise, cells adhered to slides were washed two times with DPBS and prepared as other slides for confocal microscopy.
Statistical analysis. All data are expressed as means ± SE for three or more experiments. Comparisons between two groups were performed using a two-tailed Student t-test with unequal variances. All samples were unpaired. For multiple comparisons between three or more groups, a Tukey test was used. Differences were considered statistically significant at P ≤ 0.05.

RESULTS

SP-A binds specifically to C1q. SP-A has been reported previously to bind to C1q (30), but little is known about the properties of this interaction. We first studied the specificity of the interaction using a microtiter plate binding assay. Radiolabeled SP-A bound significantly to wells coated with C1q but not to wells coated with control proteins, such as BSA or HSA, or with various other complement proteins, including C1r, C1s, C4, C3, C3b, or factor B, indicating that the interaction is specific (Fig. 1A). SP-A bound to C1q-coated wells to a much greater extent than to wells coated with structurally similar molecules, SP-A and SP-D (Fig. 1B). Fifteen percent of the total added SP-A bound to C1q-coated wells. Also, excess unlabeled SP-A inhibited binding of labeled SP-A to plate-bound C1q in a concentration-dependent manner. HSA, used as a control, did not inhibit binding (Fig. 1C).

The domain of SP-A involved in binding to C1q was also investigated. SP-A binding to C1q was partially calcium dependent, since binding in 10 mM EDTA buffer was only 50% of the binding in calcium-containing buffer (Fig. 2). However, SP-A binding to C1q was not inhibited by the addition of 500 mM mannose, suggesting a lack of involvement of the lectin activity of SP-A (Fig. 2). Furthermore, the binding of SP-A to C1q was eightfold greater in low-ionic-strength DGVBS++ buffer than in normal-ionic-strength GVBS++ buffer (Fig. 3).

SP-A binding studies were also performed using antibody-coated sheep erythrocytes as a model immune complex to determine if SP-A also binds to C1q that is associated with immune complexes. Erythrocytes were coated with or without antibody and incubated with or without purified human C1q. SP-A bound to a much greater extent to sheep erythrocytes sensitized with both antibody and C1q than to uncoated erythrocytes or erythrocytes sensitized with antibody alone (Fig. 4), showing that SP-A binding to sheep erythrocytes was C1q dependent. Twenty percent of the total added SP-A bound to the C1q-coated erythrocytes.

SP-A enhances the phagocytosis of C1q-coated particles. Because SP-A binds to C1q, we hypothesized that SP-A may aid in the phagocytosis of particles opsonized with C1q. A FACS-based phagocytosis assay was used to determine the contribution of SP-A and/or C1q to the phagocytosis of fluorescent polystyrene latex beads coated with C1q or BSA, as a control protein, by AM.

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Fig. 1. Surfactant protein (SP)-A binds specifically to C1q. A: BSA, human serum albumin (HSA), C1q, C1r, C1s, C4, C3, C3b, or factor B was immobilized on the wells of a microtiter plate and incubated with radiolabeled SP-A in low-ionic-strength DGVBS++ buffer. After the incubation, wells were washed and counted in a gamma counter to determine how much SP-A bound to each protein. B: the specificity of the interaction between SP-A and C1q was tested further by incubating radiolabeled SP-A with wells coated with proteins structurally similar to C1q in DGVBS++ buffer. *P < 0.05 compared with all other wells; †P < 0.05 compared with HSA, Tukey test; n = 3 experiments. C: to test whether unlabeled SP-A competes with labeled SP-A for binding to C1q, C1q was immobilized on the wells of a microtiter plate and incubated with a mixture of 0.2 μg/ml radiolabeled SP-A and increasing concentrations of unlabeled SP-A or HSA. 2P < 0.02 compared with no unlabeled protein, Tukey test; $P ≤ 0.01 compared with the same concentration of unlabeled HSA, Student’s t-test; n = 3; cpm, counts/min.
The addition of SP-A enhanced the association of C1q-coated particles with AM in a concentration-dependent manner (Fig. 5). SP-A increased the percentage of AM with associated C1q beads two- to threefold over that of BSA beads at all of the SP-A concentrations tested (Fig. 5A). In addition, SP-A enhanced the average number of C1q beads associated with each AM, which can be seen when data are expressed as relative fluorescence units per AM (Fig. 5B). In the presence of 25 μg/ml SP-A, AM have approximately sixfold more C1q beads associated with them compared with BSA beads. SP-A significantly enhanced the percentage of AM with associated C1q beads at a concentration of SP-A as low as 1 μg/ml, the lowest concentration tested. SP-A enhanced the association of BSA-coated beads with AM at concentrations of 10 μg/ml and greater (not at 1 or 5 μg/ml), although not to the same extent as C1q-coated beads.

In addition, uptake of beads coated with SP-A was evaluated. These beads showed increased association with AM compared with BSA- or C1q-coated beads (Table 1), consistent with the hypothesis that the binding of SP-A to the beads is responsible for the enhancement of phagocytosis. However, association of SP-A-coated beads with AM was much less than association of C1q-coated beads when AM were incubated with beads and soluble SP-A at the same time.

Confocal microscopy was used to confirm that the beads were internalized, since the FACS-based phagocytosis assay cannot distinguish between internalized beads and beads bound to the outside of the AM. These experiments showed that SP-A specifically enhanced the internalization of C1q-coated beads by AM (Fig. 6 and Table 1). SP-A modestly enhanced the uptake of BSA-coated beads. There was no consistent difference in the uptake of C1q- and BSA-coated particles in the absence of SP-A. The percentage of AM with internalized C1q-coated particles was threefold greater than that of BSA-coated particles in the presence of 25 μg/ml SP-A for one experiment and was enhanced sevenfold in a second experiment (Table 1). The number of beads internalized by 100 AM (phagocytic index) in the presence of SP-A was also 4- to 12-fold greater.
In the above experiments, we examined phagocytosis when AM were incubated with beads and soluble SP-A at the same time. Here, we further investigate the mechanism for the enhancing effect on phagocytosis of C1q-coated particles by SP-A. BSA- or C1q-coated beads were preincubated with SP-A and washed to remove soluble SP-A before addition to AM. The effect of this treatment on phagocytosis by AM was determined using the FACS-based phagocytosis assay. SP-A significantly enhanced the phagocytosis of C1q-coated beads, which can be seen when data are based on average fluorescence (Fig. 7A). SP-A also enhanced the percentage of AM with associated BSA-coated and C1q-coated beads (Fig. 7B). Although these data are consistent with an opsonic role of SP-A, confocal microscopy of AM with SP-A-pretreated beads showed cells that had internalized both individual and aggregated beads (data not shown). Aggregation of both BSA-coated and C1q-coated beads, resulting from the SP-A pretreatment procedure, may contribute to their phagocytosis. This may explain the unexpectedly high response for the BSA-coated beads pretreated with SP-A.

SP-A may function as an activation ligand in the clearance of C1q-coated beads. We tested the hypothesis that SP-A directly stimulates AM by an activation ligand mechanism. AM were preincubated with soluble SP-A and washed before the addition of C1q- or BSA-coated beads. The FACS-based phagocytosis assay was performed with SP-A-treated or mock-treated macrophages. Significant enhancement of phagocytosis of C1q-coated but not BSA-coated beads was seen in AM preincubated with SP-A when data are based on average fluorescence of each macrophage (Fig. 8A) or percent positive AM (Fig. 8B).

These results are consistent with SP-A acting directly on the cells to change their ability to phagocytose C1q-coated beads. However, it is also possible that some SP-A bound to cells during pretreatment remains on the cell surface. This cell surface-bound SP-A could then bind to and enhance the uptake of C1q beads subsequently added through an opsonic mechanism. SP-A does not affect C1q bead uptake by adherent AM in the same way as suspended AM. We wanted to test further the hypothesis that SP-A acts directly on AM to enhance the uptake of C1q beads. Previously, others have demonstrated that SP-A acts as an activation ligand to enhance phagocytosis by AM and monocytes (9, 36). In their experiments, cells were allowed to adhere to slides that had been coated with SP-A before the addition of phagocytic targets. In this way, one can demonstrate that plate-bound SP-A is interacting with the phagocytic cell, but not the phagocytic targets, which enter the opposite side of the adhered cell.

However, our findings demonstrate that AM adhered to a slide do not respond to C1q beads and soluble SP-A in the same way as cells in suspension, and these findings preclude our ability to test SP-A as an activation ligand in this experimental setting. Figure 9 shows the difference in bead uptake between adherent and nonadherent AM. For each experiment, a single preparation of cells was divided into three groups for phagocytosis reactions. One group of cells was allowed to adhere to slides for 3 h at 37°C before beads and SP-A were added. Two groups of cells in suspension were used for comparison with the adherent cells. First, beads and SP-A were added to cells immediately after cell preparation, as was done for previous phagocytosis experiments. Second, cells were incubated 3 h (37°C, in the same buffer used for cells to adhere) before beads and SP-A were added. For both groups of cells in suspension, SP-A significantly enhanced the

![Fig. 5. SP-A enhances association of C1q-coated particles with alveolar macrophages (AM) in suspension. C1q-coated or BSA-coated fluorescent beads were incubated with AM and increasing concentrations of SP-A. The effect of SP-A on association of beads with cells was determined using fluorescence-activated cell sorter (FACS) analysis. The percentage of cells with associated fluorescence (A) and the mean fluorescence of all cells counted (B) was determined. RFU, relative fluorescence units. *P ≤ 0.05 compared with no SP-A, Tukey test; †P ≤ 0.05 compared with BSA-coated beads at the same SP-A concentration, Student’s t-test; n = 3.]
uptake of C1q-coated beads, as shown by confocal microscopy (Fig. 9) and FACS analysis (data not shown). However, there was no significant change in the uptake of C1q-coated beads by adherent AM in the presence of SP-A (Fig. 9). Interestingly, SP-A did significantly enhance the uptake of BSA-coated beads by adherent AM. A similar trend appeared for the cells in suspension, but the difference was not statistically significant.

### DISCUSSION

The goal of this study was to determine the effect of the previously reported interaction of SP-A with C1q on phagocytosis of particles by AM. Although C1q alone is not an efficient opsonin, C1q in combination with antigen-specific antibody is more effective (4). C1q greatly enhances the phagocytosis of particles opsonized with other proteins such as C4b and IgG through

![Image](http://ajplung.physiology.org/)

Fig. 6. SP-A enhances internalization of C1q-coated particles. AM were incubated with BSA-coated beads alone (A), with BSA-coated particles and 25 μg/ml SP-A (B), with C1q-coated beads alone (C), or with C1q-coated beads and 25 μg/ml SP-A (D) and fixed to slides. Images of each slide of cells were taken using dual-channel confocal microscopy and examined for internalized beads.
the C1q receptor that stimulates phagocytosis, C1qR$_p$ (29, 36). However, C1qR$_p$ is absent on AM (Dr. Zissis Chronos, personal communication), the primary immune cell in the noninflamed lung. Therefore, opsonization of microorganisms by C1q alone may have minimal effects on their clearance from the airspaces where AM are the resident immune cells. SP-A was previously shown to bind directly to C1q, but the functional significance of the interaction was not investigated (30). Because SP-A has been shown to enhance the phagocytosis of particles to which it binds (39) and because SP-A binds to C1q, we hypothesized that SP-A enhances the clearance of particles opsonized with C1q from the alveolar airspaces.

The current study investigated the properties of the interaction between SP-A and C1q. We first confirmed the previously reported interaction between SP-A and C1q by showing that SP-A binds to C1q immobilized on microtiter plate wells (Fig. 1). The interaction was very specific, since SP-A bound significantly less to other...
structurally related proteins (Fig. 1B). The interaction between SP-A and C1q was also sensitive to ionic strength. Although the specificity of the binding was similar in both low- and normal-ionic-strength (145 mM NaCl) GVBS++ buffer, the level of binding was reduced significantly overall in normal-ionic-strength buffer compared with low-ionic-strength buffer (Fig. 3). This finding differs from that reported by Oosting and Wright (30) who showed that the binding of SP-A to C1q was less than the binding of SP-A to solid-phase SP-A and SP-D in normal-ionic-strength PBS. These differences may be because of the different buffers used or the fact that milk was used as a blocking reagent in the previous study. It is possible that proteins in milk bind to C1q and preclude the binding of SP-A to C1q.

Experiments were also performed to define the mechanism of the interaction between SP-A and C1q. The binding of SP-A to C1q is partially calcium dependent (Fig. 2), suggesting either that part of the binding is mediated by the calcium-dependent carbohydrate recognition domain of SP-A or that calcium induces conformational changes in SP-A that favor binding. However, the inability of 500 mM mannose to inhibit SP-A binding to C1q suggests that the lectin activity of SP-A is not involved in binding to C1q. Therefore, calcium-induced changes in the conformation of SP-A is a more likely explanation of the calcium-dependent binding.

The ability of SP-A to bind to C1q on the commonly used complement target, sheep erythrocytes, was also tested to ensure that the SP-A-C1q interaction was not merely an artifact of the microtiter plate-binding assay. Sheep erythrocytes sensitized with antibody and/or C1q were used in SP-A-binding experiments that again showed that SP-A interacted specifically with C1q bound to the surface of the erythrocytes (Fig. 4). This finding demonstrated that SP-A binds to C1q associated with immune complexes that activate the classical pathway.

The functional significance of the interaction between SP-A and C1q was investigated by examining the effects of SP-A and C1q on the phagocytosis of fluorescent polystyrene latex beads by AM. FACS analysis showed that SP-A enhances the association of C1q-coated beads with AM in a concentration-dependent manner while having little effect on the uptake of BSA-coated beads. Confocal microscopy confirmed that beads were internalized and demonstrated that SP-A enhanced both the percentage of AM taking up C1q-coated beads and the average number of internalized C1q-coated beads. Furthermore, pretreatment of either beads or macrophages with SP-A enhanced the uptake of C1q-coated beads.

Aggregation of beads pretreated with SP-A complicated our efforts to determine the extent to which SP-A functions as an opsonin, since SP-A aggregated and enhanced the uptake of both C1q-coated beads and BSA-coated beads. Confocal microscopy of beads internalized by AM confirmed that both BSA- and C1q-coated beads pretreated with SP-A were significantly aggregated. Fluorescent microscopy of beads showed...
that most of the aggregation occurred when the beads were pelleted to separate unbound SP-A from that associated with the beads (data not shown). Therefore, aggregation of SP-A-pretreated beads may explain the finding that opsonization of BSA- and C1q-coated beads with SP-A enhances phagocytosis of both types of beads.

Our data suggest that SP-A may act as an activation ligand to enhance the uptake of C1q-coated beads. There was enhanced association of C1q-coated beads, but not BSA-coated beads, with AM that had been pretreated with SP-A. This finding suggests a direct effect on AM by SP-A. SP-A has been shown to upregulate mannose receptor function on monocyte-derived macrophages (9) and could be acting on AM in this experiment to upregulate expression of a C1q receptor. Tenner and coworkers (36) showed that SP-A functions as an activation ligand by stimulating complement receptor 1 (CR1)-mediated phagocytosis by monocytes and monocyte-derived macrophages. CR1 binds C1q (19), C3b, and C4b, and, unlike C1qR, CR1 is expressed by AM (10, 14). In the current study, SP-A-enhanced phagocytosis of C1q-coated particles could be due in part to upregulation of CR1 (or another C1q receptor) on AM.

Others have shown that SP-A augments the phagocytosis of serum-opsonized bacteria by functioning as an activation ligand. Van Iwaarden and colleagues (40) first reported that AM preincubated with pulmonary surfactant showed enhanced phagocytosis of serum-opsonized Staphylococcus aureus, and this could be reproduced by preincubating AM with SP-A alone. Although the opsonic components of serum responsible for enhancing phagocytosis were not identified, it is possible that C1q could have contributed to SP-A-mediated phagocytosis in those experiments.

Although pretreatment of AM with SP-A enhanced the phagocytosis of C1q-coated beads, the effects of SP-A were much more dramatic when C1q-coated beads were incubated with AM in the presence of soluble SP-A. It is not clear why this coincubation results in the greatest enhancement of phagocytosis. It may be that the effect we observe when pretreating AM with SP-A is because of a small amount of SP-A remaining on the cell surface that can then act as an opsonin for C1q-coated beads. Another possible explanation for these data is that the signal from SP-A to cells to phagocytose C1q-coated beads is more effective when soluble SP-A is present during bead phagocytosis than when cells are pretreated with SP-A. Furthermore, because AM actively internalize and degrade SP-A (45), the SP-A concentration in AM pretreatment experiments may be reduced further in the time before AM are incubated with beads. Thus pretreating AM with SP-A may not accurately represent the condition where soluble SP-A is constantly present during the phagocytosis experiment. The large effect of SP-A on C1q bead uptake that we see when soluble SP-A is present during phagocytosis could be because of a cooperative effect of both opsonic and activation ligand mechanisms.

An interesting outcome of our efforts to determine the mechanism by which SP-A enhanced uptake of C1q-coated beads was that AM adhered to a plastic substrate had a reduced response to SP-A compared with suspended AM (Fig. 9). This difference seems to be caused by the act of adherence, because when cells from the same preparation were incubated for a comparable time at the same temperature, and in the same buffer, but were not adhered to a slide, they still responded to SP-A. In every condition tested, these control cells were not as phagocytic as either the non-adhered cells in suspension or the adhered cells, but in every experiment we saw that SP-A enhanced C1q bead uptake by these control cells, as shown by both confocal microscopy and FACS analysis.

Others have seen changes in cell function or protein phosphorylation induced by adherence of AM. For example, Quintero and Wright (31) saw that AM adhered to plastic for 1.5 h internalized 10 times as much lipid as AM in suspension. Bates et al. (2) compared AM that had been adhered to plastic for 1 or 24 h. They observed changes in the rate of SP-A degradation but not in lipid uptake between these two groups of cells. In addition, Hirano and Kanno (17) found that phosphorylation of Syk and paxillin resulted when AM adhered to a plastic substrate. These studies and our results present interesting questions about how the act of adherence may change the phagocytic and/or signaling properties of AM and also lead us to wonder whether the properties of adherent AM, nonadherent AM, or both are relevant to how these cells behave in the lung.

The results of the phagocytosis assays show functional significance for the interaction of C1q and SP-A at normal ionic strength. However, the binding of SP-A to C1q at normal ionic strength is reduced compared with binding at low ionic strength (Fig. 3). Other studies have shown that C1q binding to peripheral blood leukocytes varies inversely with ionic strength (34). It has been proposed that the low affinity of C1q for immune cells in normal-ionic-strength buffer in these studies is because the C1q was presented in monomeric form; when C1q is clustered on a surface, as it would be on a complement activator, the affinity may be enhanced greatly. This hypothesis is consistent with the ability of C1q-coated erythrocytes to cause rosetting (indicating binding) with peripheral blood leukocytes at normal ionic strength (8) and the finding that aggregated, but not monomeric, C1q stimulates the oxidative response in polymorphonuclear leukocytes (35). Moreover, clustering of lectin domains is an important mechanism by which collectins gain high affinity for their ligands (43, 6).

SP-A greatly enhances the phagocytosis of C1q-coated particles by AM at normal ionic strength, despite the relatively lower affinity binding of SP-A to C1q at normal ionic strength compared with binding at low ionic strength. The binding of SP-A to clustered C1q may explain these results. Because the number of C1q molecules adsorbed to the polystyrene beads approaches the protein capacity of the beads (based on the bead and protein surface areas), the C1q-coated
beads likely present clustered C1q molecules. Therefore, the beads may present C1q clustered on their surfaces in a fashion similar to C1q displayed on the surface of a complement activator.

The collectins have limited, but varying, carbohydrate specificities. For example, SP-A binds with high affinity to fucose and mannose (13); in contrast, SP-D binds more avidly to maltose than either fucose or mannose (6). Multiple collectins with different carbohydrate specificities are thought to exist to promote the clearance of diverse bacteria in the absence of specific antibody. By also binding to particles opsonized with C1q, SP-A may recognize an additional repertoire of microorganisms that it would not recognize in the absence of C1q.

In summary, we have found that SP-A significantly enhances the uptake of C1q-coated particles by AM in suspension but not by AM adhered to a plastic substrate. Our data support the hypothesis that SP-A either acts as an opsonin or induces aggregation to enhance C1q bead uptake; our data also suggest that SP-A may act as an activation ligand to produce the same effect. These results imply that in vivo SP-A could function as a necessary second signal in stimulating the phagocytosis of particles opsonized with other proteins, including C1q.

We thank Hollie Garner and Eric Walsh for purification of alveolar proteinosis patient SP-A and recombinant rat SP-D, respectively. We also thank Haixiang Jiang for help with binding studies. We are grateful to J. Michael Cook and Lynn Martinek at the Duke University Flow Cytometry Facility for assistance in FACS samples. Also, Dr. Rick Fehon and Heather Solari provided excellent instruction and technical support during our use of the Confocal Facility. We express our appreciation to Timothy A. Bolger for critical review of this manuscript.

This work was supported jointly by National Heart, Lung, and Blood Institute Grants RO1HL-51134 (J. R. Wright) and RO1HL-63937 (M. M. Frank).

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