Pulmonary surfactant proteins A and D enhance neutrophil uptake of bacteria

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Hartshorn, Kevan L., Erika Crouch, Mitchell R. White, Maria L. Colamussi, Anand Kakkanatt, Benjamin Tauber, Virginia Shepherd, and Kedarnath N. Sastry. Pulmonary surfactant proteins A and D enhance neutrophil uptake of bacteria. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L958–L969, 1998.—The collectins are a class of collagenous lectin proteins present in serum and pulmonary secretions [pulmonary surfactant protein (SP) A and SP-D] that are believed to participate in innate immune responses to various pathogens. With the use of flow cytometric and fluorescent-microscopic assays, SP-A and SP-D were shown to increase calcium-dependent neutrophil uptake of Escherichia coli, Streptococcus pneumoniae, and Staphylococcus aureus. Evidence is provided that the collectins enhance bacterial uptake through a mechanism that involved both bacterial aggregation and direct actions on neutrophils. The degree of multimerization of SP-D preparations was a critical determinant of both aggregating activity and potency in enhancing bacterial uptake. The mechanisms of opsonizing activity of SP-D and SP-A differed in important respects from those of opsonizing antibodies. These results provide the first evidence that surfactant collectins may promote neutrophil-mediated clearance of bacteria in the lung independently of opsonizing antibody.

Escherichia coli; Streptococcus pneumoniae opsonization; collectins; antibodies

EVIDENCE IS RAPIDLY ACCUMULATING that the collectins play an important role in first-line host defense against a variety of pathogens by binding to distinctive microbial carbohydrate determinants (15). Syndromes of increased propensity to childhood bacterial and upper respiratory tract infections have been associated with low levels of the serum collectin mannose-binding lectin (MBL) (24). More recently, low levels of MBL have also been found in patients with immunodeficiency persisting in adulthood (25). The collectins share certain basic structural properties. They contain carbohydrate recognition domains (CRDs), which are globular and composed of trimers with three carbohydrate binding sites per CRD that attach to carbohydrates in a calcium-dependent manner. They tend to occur as large multimeric structures containing multiple CRD heads tethered together by collagen domains that are, in turn, bound together at the amino terminus by disulfide bonds.

Surfactant protein (SP) D and SP-A are collectins present in pulmonary surfactant. SP-D has been shown to bind to gram-negative bacteria through attachment of its CRD to carbohydrate epitopes on lipopolysaccharides (18). In addition, SP-D was shown to cause massive aggregation of these bacteria. Although SP-D has been shown to induce chemotactic responses in neutrophils and macrophages (6), there has thus far been no evidence that it promotes bacterial uptake by phagocytic cells. We have found that SP-D markedly enhances the binding and uptake of influenza virus by neutrophils (10, 11, 13).

Recently, SP-A-deficient mice have been produced by gene targeting and homologous recombination and shown to have increased susceptibility to group B streptococcal infection (19), strongly suggesting a role for SP-A in host defense against pulmonary bacterial infection. SP-A has been shown to enhance uptake of both gram-negative and gram-positive bacteria by rat alveolar macrophages (21, 23, 26). There is a lack of data regarding the effects of surfactant collectins on the neutrophil uptake of bacteria despite the fact that neutrophils are a critical component of host defense against these organisms. In this report, we show that both SP-A and SP-D can enhance neutrophil uptake of a variety of strains of bacteria and initially characterize the mechanisms of these effects.

MATERIALS AND METHODS

Reagents

Formyl-methionyl-leucyl-phenylalanine, cytochalasin B, type II horseradish peroxidase, scopoletin, Ficoll, dextran, sodium citrate, trypan blue, and citric acid were purchased from Sigma (St. Louis, MO). Hypaque was obtained from Winthrop Pharmaceuticals (Des Plaines, IL). Dulbecco’s phosphate-buffered saline was purchased from Flow Laboratories (Costa Mesa, Ca).

Collectin Preparations

Soluble SP-D was purified by affinity chromatography on maltosylagarose, followed by gel-filtration chromatography on 4% agarose (A-15M, Bio-Rad, Hercules, CA) in the presence of 10 mM EDTA. Recombinant human and rat SP-D (rhSP-D and rrSP-D, respectively) were expressed and purified in Chinese hamster ovary K1 cells (American Type Culture Collection CCL-61) as previously described (5, 8). Three rrSP-D preparations including wild-type rrSP-D and two other mutant rrSP-D forms called [Ser12,20]rrSP-D and [Ala72]rrSP-D, which were synthesized with site-directed mutagenesis, were used in these experiments. The methods of preparing rrSP-D wild type, [Ala72]rrSP-D (glycosylation mutant), and [Ser12,20]rrSP-D (cysteine mutant) are outlined in depth elsewhere (2, 3, 5). rrSP-D forms multimeric structures...
and has other biochemical features identical to the native protein. ([Ser\textsuperscript{15,20}]rrSP-D contains serine substitutions at the sites of two NH\textsubscript{2}-terminal cysteine residues involved in the formation of disulfide bonds between collagen domains of SP-D trimers. As a result, this form of SP-D completely lacks the ability to form higher order multimers and exists as a trimer. ([Ala\textsuperscript{72}]rrSP-D is multimerized to a similar extent as wild-type rrSP-D but lacks the single N-linked carbohydrate attachment site present in the collagen domain of rrSP-D (2, 13).)

rhSP-D was prepared and purified as three different fractions by gel-filtration chromatography as previously described (8, 11). These fractions differed in their degree of multimerization, with the highest molecular weight fraction (hereafter termed "rhSP-D multimers") being composed principally of highly multimerized SP-D molecules containing up to 32 CRD heads/molecule. An intermediate-weight fraction (hereafter called "rhSP-D dodecamers") was composed principally of SP-D molecules containing four CRD heads. The final fraction was composed mainly of single-armed SP-D molecules (i.e., mainly trimers).

Native human MBL was purified on mannan-Sepharose and anti-IgG columns (14). MBL was graciously provided by Jens Jenssen (University of Aarhus, Aarhus, Denmark). Native human SP-A was purified from bronchoalveolar lavage fluids of alveolar proteinosis patients as described (10). Prior studies have shown that SP-A isolated by this method is largely composed of multimers with a molecular mass of \~700 kDa (hence probably octadecamers) (14). All of the collectin preparations used in these studies were free of other contaminating proteins as judged by SDS-PAGE analysis.

Biotinylation of SP-D was carried out by incubation of collectins with ImmunoPure NHS-LC-Biotin (Pierce, Rockford, IL) at a ratio of 2:1 of biotin to collectin by weight for 2 h at room temperature in the dark. Excess biotin was then removed by dialysis overnight.

### Bacterial Preparations

Unlabeled and FITC- or rhodamine-labeled Escherichia coli (K12 strain) were obtained from Molecular Probes (Eugene, OR) as was Staphylococcus aureus (Wood strain). These bacterial preparations were heat killed. Streptococcus pneumoniae strains and anti-IgG columns were graciously provided by Dr. Alan J. Parkinson (Jensens, University of Aarhus, Aarhus, Denmark). S. pneumoniae strains were grown in sheep blood agar (Remel, Lenexa, KS) in low oxygen and then in tryptic soy broth with 10% horse serum and killed with Formalin (0.5% Formalin in 0.9% NaCl for 1 h) for experiments described in this paper. FITC labeling of S. pneumoniae particles was carried out as previously described (10). FITC stock was prepared at 1 mg/ml in 1 M sodium carbonate, pH 9.6. Concentrated bacterial stocks were incubated with FITC (10:1 mixture by volume of bacteria in PBS with FITC stock) for 1 h followed by dialysis of the mixture for 18 h against PBS. Before all assays with bacterial particles, the suspensions of these organisms were sonicated to remove aggregates (as assessed by light microscopy). Purified E. coli-specific polyclonal rabbit IgG ("E. coli-opsonizing reagent") was obtained from Molecular Probes. This preparation contains 2 mg/ml of IgG and 0.49 mg/ml of BSA. The concentrations noted in Table 1 reflect only the amount of IgG added.

### Assessment of Bacterial Aggregation

Bacterial aggregation was assessed by several methods. First, we assessed aggregation by measuring changes in light transmission with a highly sensitive SLM/Aminco 8000C (SLM Instruments, Urbana, IL) spectrophotometer through suspensions of bacteria (final concentration 4 mg/ml or 1.8 \times 10^8 bacteria/ml) after the addition of various concentrations of collectins as previously described (12). This method is sensitive enough to detect aggregates of influenza virus or liposomes (12). We also directly evaluated the presence of bacterial aggregates by visual inspection and by fluorescent microscopy with FITC- or rhodamine-labeled bacteria (11). In the fluorescent-microscopic assay, high concentrations of bacteria (72 µg/ml or 3.3 \times 10^8 bacteria/ml) were used to maximize detection of aggregates and because this concentration of bacteria was found to be optimal for subsequent assessment of binding of the bacteria to neutrophils (see Measurement of Bacterial Binding to and Internalization by Neutrophils).

### Assessment of Binding of SP-D to Bacteria

Binding of SP-D to bacteria was also tested with an ELISA assay in which suspensions (~1 µg/ml) of the organisms were allowed to dry onto 96-well plates and fixed on the plates with methanol as described (11). Concentrations of the various bacteria were equalized with the Macarland standard (Remel, Lenexa, KS). To confirm that equal amounts of bacteria in fact bound to the plates, adherent bacterial samples were solubilized with 1% SDS, and the protein concentrations per well were determined. Mean protein concentrations per well were similar for the S. pneumoniae strains and E. coli (range 8–11 µg protein/well). Before the addition of biotinylated SP-D, the plates were blocked with BSA and gelatin. The presence of bound SP-Ds was detected with streptavidin conjugated to horseradish peroxidase and TMB substrate (Bio-Rad). The reaction was stopped with 1 N H\textsubscript{2}SO\textsubscript{4}. Optical density was measured with an ELISA reader. Each individual data point was performed in triplicate. There was minimal background binding of the biotinylated SP-D to wells not containing bacteria.
Neutrophil Preparation

Neutrophils from healthy volunteer donors were isolated to >95% purity with dextran precipitation followed by a Ficoll-Hypaque gradient separation for removal of mononuclear cells and hypotonic lysis to eliminate contaminating erythrocytes as previously described (9). Cell viability was >98% as determined by trypan blue staining, and cells were used within 2 h of isolation. Cells were subsequently washed three times and resuspended in PBS.

Measurement of Bacterial Binding to and Internalization by Neutrophils

Bacterial binding to neutrophils was measured by incubating FITC- or rhodamine-labeled bacteria with neutrophils followed by an evaluation of cell-associated fluorescence with a flow cytometer. First, fluorescent-labeled bacteria were incubated with various concentrations of collectins for 0.5 h at 37°C. The concentration of bacteria incubated with collectins was 3.3 × 10^8 bacteria/ml, which was subjected to further dilution before incubation with neutrophils. Neutrophils and bacteria (5 × 10^5 neutrophils and 1.2 × 10^10 bacteria; ratio of neutrophils to bacteria of 1:24 in 135 µl) were then incubated with these bacterial samples for 1 h at 37°C, washed two times in fresh PBS buffer, and fixed with 2% paraformaldehyde. Cell-associated fluorescence was measured on a Becton Dickinson fluorescence-activated cell sorter scan 2 and analyzed with the Lysis II program. For each neutrophil sample, 2,000 cells were counted, and the results are expressed as mean neutrophil fluorescence of the entire counted population or as the percentage of neutrophils with fluorescence higher than untreated neutrophils (percent positive cells). To discriminate between intra- and extracellular fluorescence, cells were divided into two equal aliquots, one of which was incubated in PBS containing 0.2 mg/ml of trypan blue for 3 min and the other in PBS. This concentration of trypan blue has been shown to quench fluorescence of extracellular FITC and hence is useful for eliminating fluorescence resulting from bacteria attached to the external surface of cells (i.e., bound but not internalized) (28). In some experiments (see Figs. 2B and 5B), neutrophils were preincubated with SP-D or SP-A for 30 min at 4°C followed by washing and the addition of bacteria to neutrophils.

To further confirm internalization of bacteria, we prepared slides of neutrophils by allowing the cells to first adhere to plastic coverslips followed by extensive washing to remove nonadherent cells and then incubation of these coverslips with fluorescent bacterial preparations. These coverslips were then mounted for direct examination with phase-contrast and fluorescent microscopy. Again, trypan blue was used in this microscopic assay to quench extracellular fluorescence.

Statistics

Statistical comparisons were made with Student’s paired t-test.

RESULTS

Interactions of SP-D and SP-A With Gram-Negative Bacteria

SP-D-induced aggregation of E. coli is dependent on the degree of multimerization of SP-D. Brown-Augsburger et al. (3) and Hartshorn and colleagues (8, 11) have previously reported that the ability of various SP-D (or other collectin) preparations to enhance bind-
tion of collectin multimerization to bacterial aggregation, we compared responses obtained with rhSP-D multimers to those obtained with rhSP-D dodecamers. As is apparent in Fig. 1, the rhSP-D multimers caused a greater extent of aggregation compared with the rhSP-D dodecamers, although both were quite effective at causing aggregation. In preliminary dose-response experiments, we found that optimal aggregation of E. coli could be achieved with a concentration of 190 ng/ml of rhSP-D multimers, whereas 380 ng/ml of rhSP-D dodecamers were required to achieve a similar effect (data not shown).

Wild-type rrSP-D caused aggregation of E. coli to a similar extent as rhSP-D dodecamers (Fig. 1B). In contrast, [Ser^{15,20}]rrSP-D caused no aggregation even at concentrations considerably higher than active concentrations of rrSP-D (Fig. 1B; see also Fig. 6C). The [Ser^{15,20}]rrSP-D mutant contains a single trimeric CRD associated with a full collagen domain and NH$_2$ terminus but does not form any higher order multimers (3). Hence the degree of multimerization of SP-D was an important determinant of its aggregating ability. In contrast, N-linked glycosylation of SP-D did not appear to contribute significantly to its aggregating activity because the [Ala$^{72}$]rrSP-D [which lacks the single N-linked oligosaccharide present on SP-D (2, 13)] caused a similar (if not greater) degree of aggregation of E. coli as wild-type rrSP-D (Fig. 1B).

SP-D enhances neutrophil uptake of E. coli. Effect of multimerization and of sequence of incubation of SP-D with bacteria or neutrophils. After incubation of FITC-labeled E. coli with various concentrations of collectins, the opsonized bacterial samples were incubated with freshly isolated human neutrophils for 1 h at 37°C followed by washing and assessment of cell-associated fluorescence with a flow cytometer. Except where otherwise specified, excess SP-D was not washed out before the addition of bacterial samples to neutrophils. As shown in Fig. 2, E. coli bound to neutrophils in the absence of collectins (note mean cell-associated fluorescence in absence of SP-D in Fig. 2A). To assess the extent to which these bacteria were simply attached to the surface of cells as opposed to being internalized, we tested the degree of cellular fluorescence in the presence and absence of 0.2 mg/ml of trypan blue (see Fig. 2A). Note that trypan blue significantly reduced mean neutrophil fluorescence (Fig. 2A) as well as the percentage of neutrophils that fluoresced above control levels (e.g., percentage of fluorescence-positive neutrophils in samples treated with bacteria alone was 75 ± 6 and 65 ± 7% for control and trypan blue-treated cells, respectively; P = 0.002).

As shown in Fig. 2A, preincubation of E. coli with certain concentrations (e.g., 0.44 or 0.88 µg/ml) of rhSP-D multimers caused a significant increase in mean neutrophil-associated fluorescence in the presence or absence of trypan blue. However, use of higher concentrations of this SP-D preparation did not significantly increase neutrophil-associated fluorescence compared with control preparations. As shown in Fig. 2B, rhSP-D dodecamers also promoted neutrophil uptake of E. coli, although the degree of enhancement of uptake...
was less than that for rhSP-D multimers in these experiments. Note that in Figs. 2B and 3–8 the only data shown are neutrophil fluorescence values in the presence of trypan blue. (Neutrophil fluorescence was measured in the presence and absence of trypan blue in many of these experiments, and the effect of trypan blue was consistently similar to that shown in Fig. 2A.)

Another set of experiments directly compared the ability of rhSP-D multimers and polyclonal anti-E. coli antibodies to enhance neutrophil uptake of E. coli. As shown in Table 1, rhSP-D and IgG enhanced bacterial uptake to a similar extent, although optimal concentrations were quite different (i.e., ~44 µg/ml for antibody compared with ~1 µg/ml for rhSP-D multimers). In contrast to the case of rhSP-D multimers (see Fig. 2A), no decline in neutrophil fluorescence occurred when E. coli were pretreated with increasing concentrations of opsonizing antibody. An additional difference was noted in that the antibody preparation enhanced not only the mean neutrophil fluorescence but also the percentage of positive neutrophils as well, whereas rhSP-D multimers enhanced only the mean neutrophil fluorescence (see Table 1). The antibodies, therefore, recruited an additional population of neutrophils to bind E. coli, whereas rhSP-D multimers did not.

The ability of SP-D to increase the uptake of E. coli was calcium dependent because no enhancement of uptake was seen when the assay was carried out in EDTA buffer (Fig. 2B). To further determine the mechanism through which SP-D enhanced neutrophil uptake of E. coli, we separately tested the effect of pretreating neutrophils or bacteria alone with SP-D followed by washing off of unbound SP-D before the neutrophils were incubated with bacteria. As shown in Fig. 2B, preincubation of neutrophils alone with SP-D did enhance subsequent binding of E. coli. As shown in Fig. 2C, preincubation of bacteria with SP-D led to enhanced bacterial uptake to a more limited extent, with significant enhancement only at the highest concentration of rhSP-D tested. In contrast, incubation of E. coli with opsonizing antibodies caused a nearly identical enhancement of bacterial uptake whether unbound antibodies were washed off or not (see Table 1).

To further test the role of multimerization of SP-D in mediating enhanced neutrophil uptake of E. coli, we assessed the effect of [Ser15,20]rrSP-D (cysteine mutant; trimeric) and wild-type rrSP-D in this assay. Figure 3 shows that wild-type rrSP-D caused enhanced neutrophil uptake of E. coli. Note that rrSP-D, like rhSP-D multimers (Fig. 2A), affected uptake in a biphasic manner (i.e., higher concentrations of rrSP-D no longer increased uptake). [Ser15,20]rrSP-D did not cause any significant change in the uptake of E. coli.

rhSP-D dodecamers also enhanced the uptake of bacteria by plastic-adherent neutrophils. As shown in Fig. 4, fluorescent bacteria were seen almost entirely within cells in both control and SP-D-treated samples (indicating effectiveness of trypan blue at quenching extracellular fluorescence). A clear increase in neutrophil-associated fluorescence could be seen in cases where the bacteria had been treated with SP-D (Fig. 4, D and F).

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**Fig. 3.** Wild-type rrSP-D (△) enhances uptake of E. coli by neutrophils but [Ser15,20]rrSP-D (□) does not. E. coli preparations were treated with various concentrations of wild-type rrSP-D or [Ser15,20]rrSP-D, and uptake of these preparations by neutrophils was assessed as in Fig. 2A (in presence of trypan blue). [Ser15,20]rrSP-D is a cysteine mutant version of SP-D that does not form higher order multimers. *Neutrophil fluorescence values significantly greater for samples treated with SP-D compared with values obtained with unopsonized bacteria, P = 0.05.*

**EFFECT OF SEQUENCE OF INCUBATION OF SP-A WITH BACTERIA AND NEUTROPHILS.** As assessed by the flow cytometry method (Fig. 5), SP-A increased neutrophil uptake of E. coli to an extent similar to SP-D. As was the case with SP-D, there was no increase in uptake when the assay was carried out in EDTA-containing buffer. There was a major difference, however, between the concentrations of SP-A and SP-D required to enhance binding (i.e., optimal concentrations of SP-A were more than 10-fold higher than those of SP-D). Unless otherwise specified, SP-A was preincubated with E. coli and excess collectin was not washed off before addition of the bacterial preparation to the neutrophils. When aliquots of these bacterial samples were examined under fluorescence microscopy, SP-A was found to cause aggregation of the bacteria that first became apparent at 2 µg/ml and became marked at 16 µg/ml of SP-A (see Fig. 6). The SP-A concentration dependence was similar for inducing aggregation and enhancing neutrophil uptake of E. coli (see Figs. 5 and 6).

As shown in Fig. 5B, however, SP-A enhanced binding of E. coli to an extent similar to that when the neutrophils were preincubated with lectin (and unbound lectin was washed off) before the addition of bacteria. When E. coli was preincubated with SP-A and excess SP-A was washed off), bacterial uptake was enhanced to a more limited extent and only at the highest concentration of SP-A tested (i.e., neutrophil-associated fluorescence was 166 ± 27% of control value for bacteria preincubated with 68 µg/ml of SP-A; P = 0.02; n = 7 experiments).

**Interactions of SP-D and SP-A With Gram-Positive Bacteria**

Binding to and aggregation of S. pneumoniae. As shown in Table 2, SP-D bound in a calcium-dependent
manner to several clinically important types of S. pneumoniae. This binding was markedly less avid, however, than binding of SP-D to E. coli. The data shown in Table 2 were obtained by fixing bacteria to ELISA plates with methanol (see MATERIALS AND METHODS for a description). We also tested the binding of SP-D to bacteria that were allowed to adhere to the ELISA plates overnight without fixation. Although a similar, or greater, amount of protein bound to the plate by this method, binding of SP-D to S. pneumoniae was somewhat reduced (see Table 2). These results may indicate that fixation with methanol exposed additional SP-D binding sites in these bacteria. As shown in Fig. 7, SP-D induced aggregation of S. pneumoniae as assessed by the fluorescent-microscopic assay, although the concentrations of rhSP-D multimers required to
SP-A increase uptake of *S. pneumoniae* by neutrophils. Table 3 shows the results of experiments in which SP-A, SP-D, and MBL were tested for their ability to enhance neutrophil uptake of several types of *S. pneumoniae*. As judged by mean neutrophil fluorescence, SP-D and SP-A had a consistent enhancing effect on neutrophil uptake of several types of *S. pneumoniae*. The percentage of neutrophils in samples treated with unopsonized *S. pneumoniae* types 4, 19, and 23 were 17 ± 5, 23 ± 7 and 19 ± 5%, respectively, compared with 45 ± 3, 55 ± 4, and 44 ± 6%, respectively, for the bacteria treated with SP-D (P ≤ 0.008 for each). Note that considerably higher concentrations of rhSP-D were required to enhance neutrophil uptake of *S. pneumoniae* than was the case for gram-negative bacteria. By comparison, MBL caused less substantial increases in the uptake of the *S. pneumoniae* types (Table 3). As was the case for *E. coli*, neither SP-D nor SP-A enhanced the uptake of *S. pneumoniae* when the experiments were carried out in EDTA-containing buffer (Table 3). The ability of SP-D to enhance the uptake of *S. pneumoniae* was also confirmed with plastic-adherent neutrophils (Fig. 8). No intracellular fluorescence was seen when the neutrophils were exposed to unopsonized *S. pneumoniae* (Fig. 8B). However, when the cells were exposed to SP-D-treated *S. pneumoniae*, clear intracellular fluorescence was observed (Fig. 8D). Of note, extracellular bacterial aggregates were also apparent in these experiments. Although these aggregates were frequently associated with neutrophils, they were nonfluorescent (see darker areas in Fig. 8D), indicating quenching by trypan blue.

SP-D and SP-A enhance neutrophil uptake of *S. aureus*. SP-D also induced microaggregation of *S. aureus* based on the fluorescent-microscopic assay (Fig. 7, D–F). Similar results were obtained with SP-A (data not shown). Aliquots of these samples were incubated with neutrophils (without washing off of excess collectin). Both SP-D and SP-A enhanced the neutrophil uptake of *S. aureus*. rhSP-D multimers (1.7 µg/ml) increased absolute neutrophil-associated fluorescence from 39 ± 6 (mean fluorescence of neutrophils treated with unopsonized *S. aureus*) to 69 ± 2 (P ≤ 0.05; n = 4 experiments). Lower concentrations of SP-D did not significantly affect neutrophil fluorescence (data not shown). SP-A (64 µg/ml) increased neutrophil-associated fluorescence to 72 ± 15 (P ≤ 0.05; n = 3 experiments).

**DISCUSSION**

Several reports in the literature (7, 20, 23, 26, 27) have shown that SP-A can enhance the uptake of bacteria by alveolar macrophages. Gaynor et al. (7) reported that SP-A enhanced the uptake of *Mycobacterium tuberculosis* by human macrophages through a direct effect on the macrophages themselves. SP-A was internalized after incubation of the macrophages with SP-A (1 h at 37°C), and washing off of unbound SP-A did not alter the enhanced bacterial uptake. Van Iwaarden et al. (27) similarly showed that SP-A directly elicited a chemiluminescent response from alveolar macrophages but not from neutrophils. Tino and Wright (26) found that SP-A enhanced the uptake of *S. pneumoniae* (not typed), *Hemophilus influenzae*, and group A streptococci but that this effect did not correlate with SP-A-induced bacterial aggregation. Pikaar et al. (23) found that SP-A enhanced the uptake of *E. coli* by macrophages, whereas SP-D did not. In contrast, McNeely and Coonrod (20) found that SP-A did not enhance the macrophage uptake of *S. pneumoniae*.
moniae and enhanced the attachment but not the internalization of S. aureus. Although the results vary somewhat, it appears likely that SP-A does enhance phagocytosis of several types of bacteria by alveolar macrophages and that a direct effect of SP-A on macrophages may contribute to this action.

Although neutrophils are a major contributor to host defense against bacteria, there have been no prior reports regarding the ability of surfactant collectins to alter the neutrophil uptake of bacteria. We demonstrate here for the first time that both SP-D and SP-A enhance neutrophil uptake of gram-negative and gram-positive bacteria. Hartshorn and colleagues (11, 13) have previously demonstrated that SP-D enhances the neutrophil uptake of influenza A viruses by neutrophils through a mechanism that depends extensively on multimerization of SP-D and viral aggregation. SP-D was shown to bind in a calcium-dependent manner to carbohydrate epitopes on the virus (13). The “opsonic” activity of SP-D differed in both concentration dependence and mechanism from those of SP-A or anti-viral antibodies.

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The findings presented in this paper indicate that SP-D enhances the uptake of bacteria by neutrophils in a manner similar to that observed with influenza viruses. We provide evidence that the ability of SP-D to aggregate bacteria is a determinant of its ability to enhance neutrophil uptake. First, there was a correlation between the concentrations of SP-D that induced aggregation of E. coli, S. pneumoniae, or S. aureus and those that were active in promoting bacterial uptake by neutrophils. A similar correlation was found for SP-A and E. coli. Second, there was a similar relationship
between the aggregating and opsonizing activity of various preparations of SP-D (i.e., highly multimerized SP-D preparations were more potent at inducing aggregation and enhancing bacterial uptake, whereas the trimeric [Ser\textsuperscript{15,20}]rrSP-D preparation did not aggregate or increase the uptake of bacteria).

However, some of our findings are also consistent with the interpretation that SP-D and SP-A may, in part, enhance bacterial uptake by directly interacting with neutrophils, which have specific SP-A receptors (4). When neutrophils were preincubated with either SP-D or SP-A and then washed before the addition of E. coli, uptake was increased (Figs. 2 and 5). This action was most pronounced in the case of SP-A and suggests similarities between the actions of SP-A on neutrophils with those reported for macrophages. However, it is

Fig. 7. SP-D induces microscopic aggregation of Streptococcus pneumoniae and Staphylococcus aureus. Samples of FITC-labeled type 4 S. pneumoniae were incubated with 0, 2.5, or 10 µg/ml of rhSP-D multimers (A–C, respectively) and examined under fluorescent microscopy. Similar results were obtained with types 19 and 23 S. pneumoniae (data not shown). D–F: rhodamine-labeled S. aureus was treated with 0, 1.75, or 7 µg/ml, respectively, of rhSP-D multimers. Results are representative of at least 3 similar experiments.
unlikely that the effect is identical to that reported by Gaynor et al. (7) with respect to the effects of SP-A on mycobacterial uptake by macrophages. In our experiments, the neutrophils were incubated with SP-A at 4°C rather than at 37°C, making internalization of SP-A unlikely. SP-D has been previously shown to bind to and activate a chemotactic response in neutrophils (6). In the current experiments, SP-A or SP-D could have bound to neutrophils directly and acted as a bridge to increase bacterial binding and/or promoted bacterial binding by activation of the neutrophil. Future experiments will be needed to test out these possibilities.

It is more difficult to account for our finding that preincubation of E. coli with SP-D or SP-A followed by washing off of unbound collectin led to only slight increases in neutrophil uptake. Possible interpretations of this finding include that the washing procedure disrupted bacterial aggregate formation or that unbound collectin contributes importantly to the enhancement of uptake. In any case, experiments in which the collectins were present both before and during bacterial contact with the neutrophils most closely resemble conditions that might occur in vivo, and under such conditions, we obtained highly reproducible enhancement of bacterial uptake.

The mechanism of action of SP-D was clearly distinguishable from that of opsonizing antibodies. With the use of a light-transmission assay, antibodies did not induce aggregation of E. coli (data not shown). A very similar degree of enhancement of E. coli uptake was

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<td>13 ± 3</td>
<td>17 ± 4*</td>
<td></td>
</tr>
<tr>
<td>MBL (44)</td>
<td>16 ± 4</td>
<td>17 ± 5.5</td>
<td>13 ± 1*</td>
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</table>

Values are means ± SE expressed as mean neutrophil fluorescence; n = 2 (SP-A and MBL) and 3 (SP-D) experiments for type 4, 3 experiments for type 19, 3 experiments for type 23, and 2 experiments for type 23 + EDTA; nos. in parentheses, concentration of collectin in µg/ml. MBL, mannose-binding lectin. S. pneumoniae were pretreated with collectins followed by incubation with neutrophils and assessment of mean neutrophil fluorescence (2,000 cells counted). Excess collectin was not washed off bacteria before addition to neutrophils. Experiments were carried out in TBS with calcium except for type 23 + EDTA where TBS with 10 mM EDTA was used. *Significantly greater than neutrophils treated with S. pneumoniae in presence of control buffer, P ≤ 0.05.

Fig. 8. SP-D enhances uptake of S. pneumoniae by adherent neutrophils. These experiments were carried out as described in Fig. 5. FITC-labeled S. pneumoniae (type 23) were preincubated with control buffer (A and B) or 5 µg/ml of rhSP-D multimers (C and D) before incubation with plastic-adherent neutrophils. No fluorescence was seen in neutrophils exposed to unopsonized S. pneumoniae (B). Note presence of extracellular bacterial aggregates (appearing as darker areas on fluorescent micrograph; D) in samples treated with SP-D. In addition, intracellular fluorescence was apparent in SP-D-treated samples. Results are representative of 3 similar experiments.
seen whether or not excess antibodies were washed off after initial incubation with the bacteria. Although SP-D principally increased the mean neutrophil fluorescence without altering the percentage of fluorescence-positive neutrophils, antibodies increased both. Finally, although the enhancing effect of SP-D on E. coli binding was highly concentration dependent (i.e., a fairly narrow range of concentrations enhanced uptake, whereas higher concentrations were not enhancing), antibodies enhanced uptake over a broad range of concentrations. Our current interpretation of these findings is that SP-D enhances neutrophil uptake of bacteria through a mechanism that depends substantially on bacterial aggregation and differs from classical opsonization. It is plausible to speculate that in vivo SP-D might enhance bacterial clearance by phagocytic mechanisms at certain ratios of SP-D to bacteria and by mucociliary mechanisms in other circumstances (e.g., at higher SP-D-to-bacteria ratios).

Enhancement of neutrophil uptake of E. coli or S. pneumoniae by SP-D or SP-A was inhibited by EDTA. EDTA could inhibit the binding of SP-D (or SP-A) to bacteria (e.g., see Table 2) or to neutrophils (6). EDTA could also act on neutrophils directly to inhibit phagocytosis. Further experiments will need to be done to clarify this question. It is, however, of note that binding of SP-A to influenza virus was not inhibited by EDTA (13).

It is difficult to account for differences in our results and those of Pikaar et al. (23), who found that SP-D caused no alteration in the uptake of E. coli by rat alveolar macrophages. Clearly, one possibility is that human neutrophils differ qualitatively in their interactions with SP-D and/or bacteria from human or rat macrophages. From our results, it also appears that variations in concentration or multimerization state of SP-D preparations could also substantially alter the results.

In any case, we have demonstrated that various preparations of SP-D and SP-A are able to enhance the uptake of a variety of bacteria in a reproducible manner. Similar results were obtained with suspended or adherent neutrophils. This is significant because adherent neutrophils behave differently from those in suspension in a variety of systems. It is also notable that SP-A and SP-D were able to enhance neutrophil uptake of several types of S. pneumoniae. These bacteria are major causes of pneumonia and other important infections. Also, S. pneumoniae are not taken up significantly by neutrophils in the absence of opsonins. By comparison with its interactions with E. coli, higher concentrations of SP-D were needed to cause enhanced neutrophil uptake of S. pneumoniae. This may, in part, reflect the fact that SP-D bound to S. pneumoniae with less affinity and aggregated these organisms with less potency. In addition, although SP-D did induce a clearcut increase in uptake of S. pneumoniae by suspended or adherent neutrophils, many bacterial aggregates remained outside the cells (see Fig. 8D). It may be that SP-D is more efficient at enhancing the uptake of pathogens (like E. coli or influenza virus) that neutrophils can ingest to an extent in the unopsonized state.

In our prior studies, complexes of influenza virus formed in the presence of SP-D bound to neutrophils in a neuraminidase-sensitive manner, suggesting that free viral hemagglutinin was still involved in mediating attachment of such complexes to neutrophils (9).

Our results suggest that SP-D and SP-A may play an important role in facilitating neutrophil clearance of bacteria in the airway. The levels of SP-D required to enhance the uptake of E. coli are clearly present in the airway. Honda et al. (16) have found a mean level of SP-D in bronchoalveolar lavage of 0.88 μg/ml. These levels are clearly greatly diluted compared with those present in the airway. In addition, production of surfactant collectins may rise during infection. It is plausible that the levels of SP-D in the airway, therefore, would also be sufficient to induce aggregation and promote uptake of S. pneumoniae. Although we found that higher levels of SP-A were needed to enhance neutrophil bacterial uptake, in vivo levels of SP-A have been found to exceed those of SP-D (e.g., from 1.5 to ~5 μg/ml in bronchoalveolar lavage fluids (22)). Hence, we believe that SP-A and SP-D may play an important role in enhancing neutrophil- and/or mucociliary-mediated clearance of bacteria in the lung.

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