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Contributions of the N- and C-Terminal Domains of Surfactant Protein D to the Binding, Aggregation, and Phagocytic Uptake of Bacteria

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Collectins play important roles in host defense against infectious microorganisms. We now demonstrate that the serum collectins mannose-binding lectin (MBL) and conglutinin have less ability to bind to, aggregate, and enhance neutrophil uptake of several strains of gram-negative and gram-positive bacteria than pulmonary surfactant protein D (SP-D). Collectins are composed of four major structural domains (i.e., N-terminal, collagen, and neck and carbohydrate recognition domains). To determine which domains of SP-D are responsible for its greater bacterial binding or aggregating activity, activities of chimeric collectins containing the N-terminal and collagen domains of SP-D coupled to the neck recognition domains and carbohydrate recognition domains (CRD) of MBL or conglutinin (SP-D/Cong$_{neck+CRD}$ and SP-D/MBL$_{neck+CRD}$) were tested. The SP-D/Cong$_{neck+CRD}$ and SP-D/MBL$_{neck+CRD}$ chimeras bound to and aggregated the bacteria more strongly than did wild-type MBL or conglutinin. SP-D/MBL$_{neck+CRD}$ also enhanced neutrophil uptake of bacteria more so than MBL. Hence, the SP-D N-terminal and/or collagen domains contribute to the enhanced bacterial binding and aggregating activities of SP-D. In prior studies, SP-D/Cong$_{neck+CRD}$ and SP-D/MBL$_{neck+CRD}$ had increased ability to bind to influenza virus compared not only with that of conglutinin or MBL but with that of wild-type SP-D as well. In contrast, the chimeras had either reduced or unchanged ability to bind to or aggregate bacteria compared to that of wild-type SP-D. Hence, although replacement of the neck recognition domains and CRDs of SP-D with those of MBL and conglutinin conferred increased viral binding activity, it did not favorably affect bacterial binding activity, suggesting that requirements for optimal collectin binding to influenza virus and bacteria differ.

Collectins are present in mammalian serum, lung secretions, and on various mucosal surfaces, where they play important roles in innate immunity against bacterial, viral, and fungal pathogens. Humans with heritable deficiencies of the serum collectin mannose-binding lectin (MBL) are at increased risk for viral, bacterial, and fungal infections (20, 34). The lung collectin surfactant protein D (SP-D) is expressed in the respiratory tract and some other mucosal surfaces (26). SP-D binds to a broad range of pathogens and has antimicrobial effects in vitro. Deletion of the SP-D gene in mice leads to pulmonary lipidosis, abnormal alveolar macrophage activation, deficient phagocytosis of bacteria by alveolar macrophages, and increased pulmonary inflammation after bacterial infection (21, 23).

There is in vitro and in vivo evidence that the collectins play important roles in innate host defense against influenza viruses (4, 29). SP-D−/− mice exhibit deficient clearance of influenza virus and markedly increased pulmonary inflammation after influenza virus infection (24). We have demonstrated that the collectins differ significantly in their interactions with influenza virus. SP-D causes much greater viral aggregation and neutralization of viral infectivity than MBL (11). The bovine serum collectin conglutinin causes greater neutralization of infectivity of influenza viruses than either MBL or SP-D (10, 11). Collectins are composed of four domains, including an N-terminal domain involved in disulfide bond formation, a collagen domain, an alpha-helical neck domain involved in trimerization, and a C-terminal carbohydrate recognition domain (CRD) involved in calcium-dependent binding to carbohydrates. We have constructed chimeras containing the N-terminal and collagen domains of SP-D linked to the neck recognition domains and CRDs of conglutinin or MBL (SP-D/Cong$_{neck+CRD}$ and SP-D/MBL$_{neck+CRD}$, respectively). A diagram of these constructs is shown in Fig. 1. These chimeras have greater viral neutralizing and aggregating activity than wild-type SP-D, MBL, or conglutinin (10, 12, 36). The presence of the MBL or conglutinin CRD increased viral binding activity compared to that of SP-D, while the N-terminal and collagen domains of SP-D increased viral aggregation and neutrophil uptake of the virus compared to that of wild-type MBL or conglutinin.

Given the above findings we hypothesized that the chimeric collectins SP-D/Cong$_{neck+CRD}$ and SP-D/MBL$_{neck+CRD}$ would have similarly enhanced antibacterial properties compared to those of the wild-type collectins. We have previously shown that wild-type SP-D can bind to bacterial lipopolysaccharides (LPS) with resulting bacterial aggregation and enhanced neutrophil uptake of several strains of gram-negative and gram-positive bacteria (9, 22). In these studies we used the collectin chimeras to further characterize the specific contributions of specific domains of SP-D to its interactions with bacteria.

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TABLE 1. Description of recombinant collectins used in this study

Collectin	Description	Animal of origin	Mulitmerization state	Monosaccharide preferred	Reference
RrSP-D	Wild type	Rat	Dodecamer	Glucose, inositol	3
RbConglutinin	Wild type	Cow	Dodecamer	GlcNAc	10
SP-D/Cong$_{neck+CRD}$	Chimera	Rat and cow	Dodecamer	GlcNAc	10
RhSP-D	Wild type	Human	Dodecamer	Glucose, inositol	8
RhMBL	Wild type	Human	Octadecamer	Mannose	33
SP-D/MBL$_{neck+CRD}$	Chimera	Human	Dodecamer	Mannose	36
SP-D/MBL$_{neck+CRD}$	Chimera	Human	Trimer	Mannose	36
RrSP-D$_{ala72}$	Mutant	Rat	Dodecamer	Glucose, inositol	2
RrSP-D$_{CDM}$	Mutant	Rat	Trimer	Glucose, inositol	28

MATERIALS AND METHODS

Reagents. Formyl-methionyl-leucyl-phenylalanine, cytochalasin B, horseradish peroxidase-Type II, scopoletin, Ficoll, dextran, trypan blue, and the mannose-, glucose-, or galactose-bovine serum albumin (BSA) conjugates were purchased from Sigma Co. (St. Louis, Mo.), and Hypaque was obtained from Winthrop Pharmaceuticals (Des Plaines, Ill.). Dulbecco's phosphate-buffered saline (PBS) was purchased from Flow Laboratories (Costa Mesa, Calif.).

Collectin preparations. Table 1 summarizes the various recombinant collectin preparations used in this paper. All preparations were expressed in CHO-K1 cells as previously described (3, 8). Wild-type recombinant collectins included recombinant rat and human SP-Ds (RrSP-D and RhSP-D), recombinant bovine conglutinin (RbConglutinin), and recombinant human MBL (RhMBL). The detailed characterization of these preparations was reported in the references listed in Table 1. RbConglutinin, RrSP-D, and RhSP-D consisted of the purified dodecamer fraction, which was isolated by gel filtration chromatography under nondenaturing conditions. Natural bovine serum conglutinin and human serum MBL were also used in some experiments (as noted in Results), purified as described previously (6), and graciously provided by Jens Jensenius (University of Aarhus, Aarhus, Denmark). Unlike the other collectins used in these studies, natural and recombinant human MBL are composed principally of octadecamers (16, 33).

Two mutated recombinant forms of RrSP-D were also used in these experiments: RrSP-D$_{CDM}$ and RrSP-D$_{ala72}$. RrSP-D$_{CDM}$ is a trimeric form of SP-D that lacks the entirety of the collagen domain (28). RrSP-D$_{CDM}$ was the gracious gift of Dennis Voelker (National Jewish Medical and Research Center, Denver, Colo.). RrSP-D$_{ala72}$ was generated by substituting alanine for serine at position 72, thereby disrupting the consensus for N-linked glycosylation at Asn 70. RrSP-D$_{ala72}$ dodecamers were prepared as previously described (2).

Two chimeric collectins were also employed (see Fig. 1). The first consisted of the N-terminal and collagen domains of RrSP-D joined to the neck domain and CRD of conglutinin (termed SP-D/Cong$_{neck+CRD}$). The SP-D/Cong$_{neck+CRD}$ chimera was constructed by thermal cycling with overlap extension and was expressed in CHO-K1 cells (10). The secreted protein was isolated by GlcNAc affinity chromatography, and the SP-D/Cong$_{neck+CRD}$ dodecamers were purified by gel filtration chromatography under nondenaturing conditions. The second chimera consists of the N-terminal and collagen domains of recombinant human SP-D (RhSP-D) joined to the neck domain and CRD of human MBL (SP-D/MBL$_{neck+CRD}$) (36). The secreted protein was isolated from the medium by saccharide affinity chromatography on mannose-agarose. The dodecameric and trimeric forms of the SP-D/MBL$_{neck+CRD}$ chimera were then purified by gel filtration chromatography.

Biotinylation of collectins (or antibodies) was performed by incubation of collectins with Immunopure NHS-LC-Biotin (Pierce, Rockford, Ill.), 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. Biotinylation did not alter the ability of the collectins to induce bacterial aggregation or to inhibit hemagglutination activity of influenza virus (data not shown).

Bacterial preparations. Bacterial strains were selected to facilitate comparisons with the published literature (see Table 2). Rough strains of *Escherichia coli* have been extensively used in previous studies of SP-D interactions with gram-negative bacteria and LPS (9, 22). Likewise, *Salmonella enterica* serovar Typhimurium has been used in studies of MBL-bacterial interactions. Both gram-negative organisms offer the advantage of a number of well-characterized mutants that vary in the structure of their LPS molecules. Unlabeled and fluorescein isothiocyanate- or Rhodamine-labeled *E. coli* (K-12 strain) cells were obtained from Molecular Probes (Eugene, Oreg.), as were Zymosan particles. *Streptococcus pneumoniae* strains were the gracious gift of Alan J. Parkinson (Centers for Disease Control Arctic Investigation Laboratory, Anchorage, Alaska). The TV119, TV160, and SL1102 strains of *S. enterica* serovar Typhimurium were kind gifts of H. Nikaido (University of California, Berkeley) and were grown in our laboratory. These are rough strains with known LPS oligosaccharide structure (17). The TV119 strain is also known as the Ra strain and has an outer-core oligosaccharide terminating in GlcNAc. The TV160 and SL1102 mutants contain capsular polysaccharide terminating in galactose or inner-core polysaccharides (i.e., 2-keto-3-deoxyoctonic acids), respectively (17). Table 2 summarizes the gram-negative bacterial strains used in this paper.

Live *E. coli* or *S. enterica* serovar Typhimurium cells for experiments were prepared by growing bacteria overnight on Luria-Bertani agar, selecting several colonies the morning of experiments, and resuspending these in Luria-Bertani medium. Bacteria were incubated until an optical density at 600 nm (OD_{600}) of ~0.6 was reached (usually ~2 h at 37°C). Bacteria were then counted with a Hausser counting chamber and were resuspended in PBS. *S. pneumoniae* strains were grown in sheep blood agar (Remel, Lenexa, Kans.) in low oxygen and then in tryptic soy broth with 10% horse serum. Live *S. pneumoniae* cells were grown and counted on the day of experiments as described above.

FITC labeling of formalin-fixed bacteria was carried out as previously described (14). The FITC stock was prepared at 1 mg/ml in 1 M sodium carbonate, pH 9.6. Concentrated bacterial or yeast 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 in PBS. FITC labeling of live bacteria was

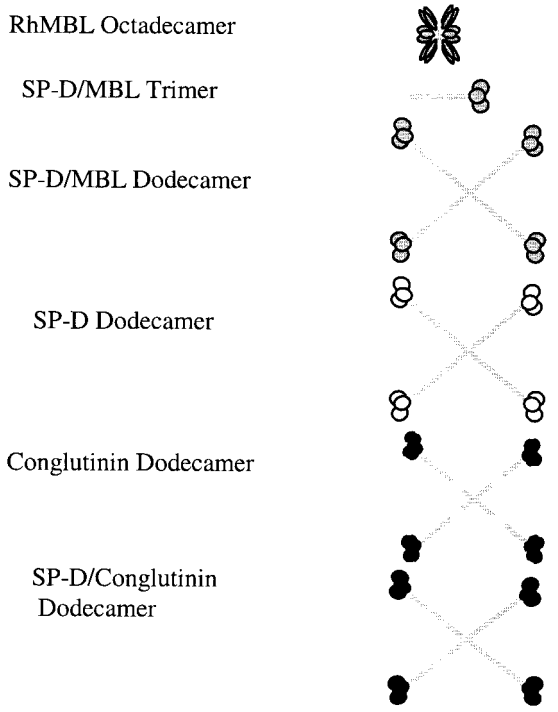

FIG. 1. Diagram of wild-type and chimeric collectins.

TABLE 2. Description of gram-negative bacterial strains used in this study

Bacteria	Strain (alternate designation)	Outer-core oligosaccharide	Binding and aggregating results from prior literature	References
E. coli	K-12	GlcNAc, glucose	MBL and SP-D bind and aggregate	9, 18, 22
S. enterica serovar Typhimurium	TV119 (Ra)	GlcNAc	MBL binds	5, 17
S. enterica serovar Typhimurium	TV160 (Rb2)	Galactose	MBL has minimal binding	5
S. enterica serovar Typhimurium	SL1102 (Re)	None	MBL does not bind	5, 17

performed in the same manner except that the bacteria were not dialyzed but were washed in PBS by repeated centrifugation to allow their immediate use in the neutrophil uptake assays. Prior to all assays using bacteria or yeast particles, the suspensions of these organisms were gently sonicated to disrupt aggregates, as monitored by light microscopy.

Assessment of binding of collectins to bacteria. Binding of collectins to bacteria or influenza virus was tested by enzyme-linked immunosorbent assay (ELISA) as described previously (9). Suspensions of formalin-fixed bacteria (~1 μg [dry weight] of bacteria/ml) were allowed to dry onto 96-well plates, followed by washing and incubation with biotinylated collectins. The presence of bound collectins was detected by using streptavidin conjugated to horseradish peroxidase and 3,3′,5,5′-tetramethylbenzidine substrate (Kierkegaard and Perry, Gaithersburg, Md.). The reaction was stopped by using 1 N H_2SO_4. Optical density was measured with an ELISA reader. A modification of the ELISA was also employed to exclude artifacts resulting from formalin treatment or drying of bacteria. Freshly isolated, live bacteria (~3×10^6 bacteria/ml) were allowed to settle on ELISA plates overnight at 4°C in liquid buffer, followed by centrifugation of plates at 2,500 rpm in a Sorvall RT7 centrifuge prior to assay to maximize bacterial adhesion. Each experimental condition was tested in duplicate or triplicate wells and was averaged for analysis. The background binding of collectins to wells coated with BSA alone was subtracted from the experimental values. The background binding levels were in the range of 0.03 to 0.2 at OD_{450}. More than three separate ELISAs were performed for each variable tested.

Assessment of bacterial aggregation. Bacterial aggregation was examined by several methods. First we assessed aggregation by measuring changes in light transmission through suspensions of bacteria after addition of various concentrations of collectins as previously described (9) by using an SLM/Aminco 8000C (SLM Instruments, Urbana, Ill.) spectrofluorometer. Viable bacteria were grown to an OD_{600} of ~0.6 on the day of assay and then were counted and resuspended in PBS at a final concentration of 3×10^6 bacteria/ml. Another method for assessing bacterial aggregation involved fluorescence microscopy by using FITC- or Rhodamine-labeled bacteria (9). For this assay, higher concentrations of bacteria (3.3×10^8 bacteria/ml) were used in order to maximize the detection of aggregates and to optimize the binding of the bacteria to neutrophils (see below).

Bacterial colony formation was measured after incubation of live bacteria for aggregation experiments. This was done by taking samples of bacteria that had been incubated with collectins for 30 min at 37°C during the aggregation assay, diluting these bacterial samples in fresh, sterile PBS at 1:1,000 dilution, and plating the samples on LB agar. Plating was performed by taking 50 μl of samples and dispersing on the agar by using sterile glass beads. Colonies were counted after incubation for 24 h at 37°C. Results were expressed as percentages of colonies formed in collectin-treated samples compared to those of bacteria treated in an identical manner with control buffer (e.g., incubated in aggregation assay and plated in identical manner).

Neutrophil preparation. Neutrophils from healthy volunteer donors were isolated to >95% purity as previously described by using dextran precipitation followed by a Ficoll-Hypaque gradient separation for removal of mononuclear cells and hypotonic lysis to eliminate contaminating erythrocytes (13). 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 uptake by neutrophils. Bacterial uptake by neutrophils was measured by incubating FITC- or Rhodamine-labeled bacteria with neutrophils, followed by evaluation of cell-associated fluorescence by using a flow cytometer as described previously (9). Bacteria were preincubated with collectins in the manner described above for evaluation of bacterial aggregates under fluorescent microscopy (i.e., 72 μg of bacteria/ml and various concentrations of collectins for 30 min at 37°C). For experiments using live bacteria, bacteria were grown up on the day of assay as described above, incubated with FITC as described for 1 h followed by washing, and then directly incubated with neutrophils. Neutrophils were then incubated with these bacterial samples (5×10^5 neutrophils and 1.2×10^7 bacteria at a ratio of 1 neutrophil to 24 bacteria in 135 μl) for 1 h at 37°C and washed twice in fresh PBS buffer and fixed with 2% paraformaldehyde. The mean cell-associated fluorescence of 2,000 cells was measured by using flow cytometry. These assays were performed after incubation with 0.2 mg of trypan blue/ml for 3 min to eliminate extracellular fluorescence (9).

Statistics. Statistical comparisons were made using Student's paired *t* test.

RESULTS

Wild-type SP-D causes greater aggregation of *S. enterica* serovar Typhimurium TV119 or *E. coli* K-12 than wild-type MBL or conglutinin. Initially FITC- or rhodamine-labeled bacteria were incubated with SP-D, human serum MBL, or bovine serum conglutinin, and aggregate formation was assessed by fluorescence microscopy (Fig. 2). Recombinant human and rat SP-D dodecamers caused marked aggregation of formalin-fixed *E. coli* K-12 and *S. enterica* serovar Typhimurium TV119. By contrast, a mutant trimeric form of SP-D ($RrSP\text{-}D_{CDM}$) lacked aggregating activity in this assay. Human serum MBL or RhMBL caused formation of small aggregates of *E. coli* K-12 as described by Kawasaki and coworkers (18) (data not shown). Similar small aggregates of *S. enterica* serovar Typhimurium TV119 were formed by MBL (Fig. 2). Unexpectedly, bovine serum conglutinin caused very little aggregation of *E. coli* K-12 or *S. enterica* serovar Typhimurium TV119 (Fig. 2).

Quantitative comparison of bacterial aggregating activity of MBL and SP-D was obtained by use of light transmission assay (Fig. 3). RhMBL, which is predominantly composed of octadecamers, caused significantly less aggregation of formalin-fixed *E. coli* K-12 or live *S. enterica* serovar Typhimurium TV119 than RhSP-D dodecamers (Fig. 3).

The chimeric collectin, $SP\text{-}D/MBL_{neck+CRD}$, causes significantly greater aggregation of *E. coli* K-12 and *S. enterica* serovar Typhimurium TV119 than MBL. $SP\text{-}D/MBL_{neck+CRD}$ contains the N terminus and collagen domain of human SP-D and the neck domain and CRD of human MBL. As shown in Fig. 3, dodecamers of $SP\text{-}D/MBL_{neck+CRD}$ caused significantly greater aggregation of formalin-treated *E. coli* than RhMBL. Similar results were obtained with live *E. coli* (data not shown) and with live *S. enterica* serovar Typhimurium TV119 (Fig. 3B). The degree of aggregation of *E. coli* by $SP\text{-}D/MBL_{neck+CRD}$ dodecamers was comparable to that induced by RhSP-D dodecamers. After completion of aggregation assays with live bacteria, the ability of collectin- or buffer-treated bacteria to form colonies was also tested. This was done by making dilutions of the bacterial samples and culturing them overnight on LB agar. As shown in Table 3, $SP\text{-}D/MBL_{neck+CRD}$ dodecamers caused significantly greater inhibition of colony growth than RhMBL. This provides further confirmation of the altered ability of $SP\text{-}D/MBL_{neck+CRD}$ dodecamers to interact

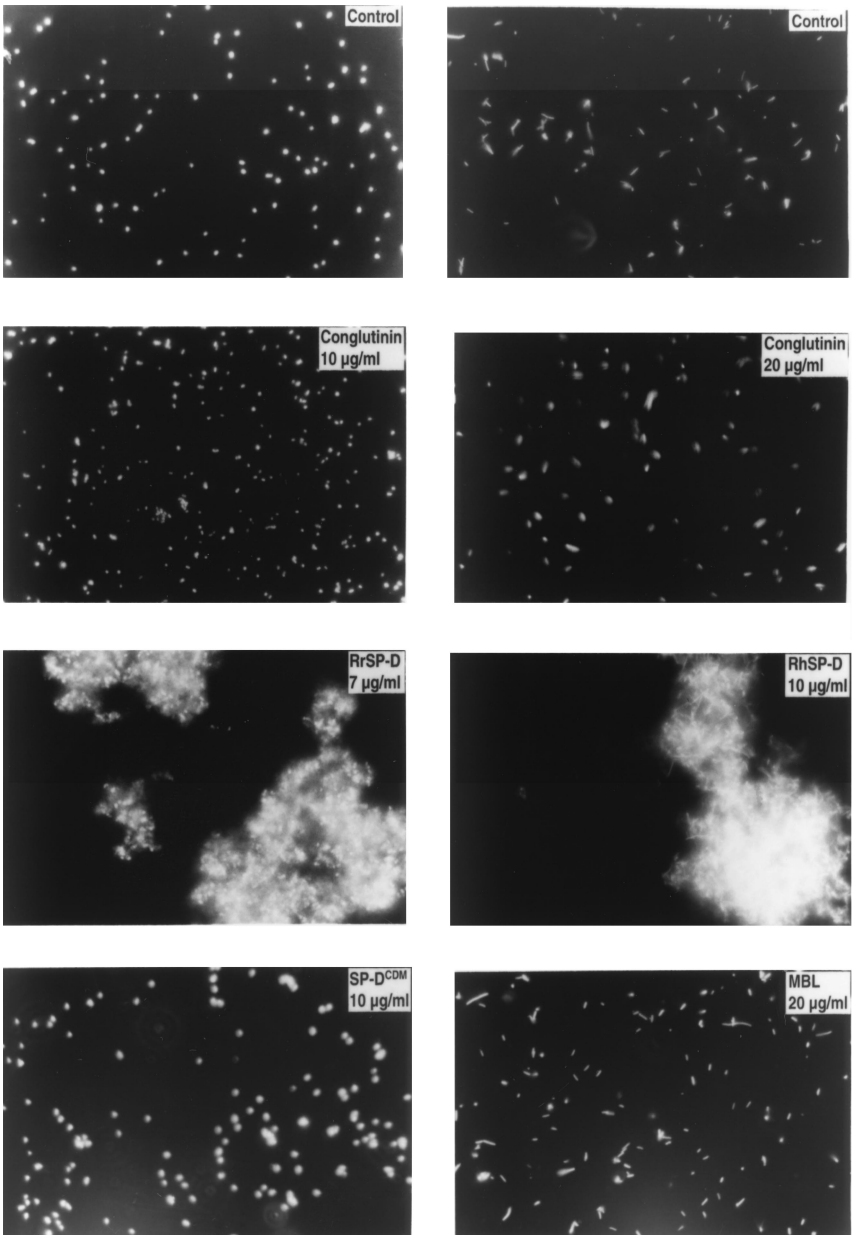

FIG. 2. Comparison of the ability of bovine conglutinin, RrSP-D, RhSP-D, human serum MBL, or RrSP-D_{CDM}(SP-D^{CDM}) to cause aggregation of *E. coli* K-12 or *S. enterica* serovar Typhimurium TV119 as assessed by fluorescent microscopy. Samples of rhodamine-labeled *E. coli* (left panels) or FITC-labeled *S. enterica* serovar Typhimurium TV119 (right panels) were incubated for 30 min at 37°C with either control buffer or various collectins as indicated, followed by examination of samples under fluorescent microscopy. Pictures are representative of three or more experiments. No (or minimal) aggregation was evident in samples treated with conglutinin or RrSP-D_{CDM} (a collagen domain deletion form of RrSP-D). MBL caused formation of small aggregates of *S. enterica* serovar Typhimurium. RrSP-D or RhSP-D (not shown) induced massive aggregation of *E. coli*, and RhSP-D induced massive aggregation of *S. enterica* serovar Typhimurium. Control samples were treated with buffer alone without added collectins.

with bacteria compared to that of RhMBL. The trimeric fraction of SP-D/$MBL_{neck+CRD}$ caused significantly less aggregation of bacteria than SP-D/$MBL_{neck+CRD}$ dodecamers even when a significantly higher concentration of SP-D/$MBL_{neck+CRD}$ was added (Fig. 3 and data not shown).

SP-D/$MBL_{neck+CRD}$ binds *E. coli* K-12 and *S. enterica* serovar Typhimurium TV119 more strongly than MBL. As shown in Fig. 4A, the dodecameric fraction SP-D/$MBL_{neck+CRD}$ bound to formalin-fixed *E. coli* significantly more strongly than RhMBL. In contrast, the trimeric fraction of SP-D/$MBL_{neck+CRD}$ did not bind to bacteria to a significantly greater extent than RhMBL. Of note, at the lower concentrations tested, binding of SP-D/$MBL_{neck+CRD}$ dodecamers to *E. coli* was significantly less than binding of dodecamers of RhSP-D in these experiments (Fig. 4B).

RhMBL also bound to live *S. enterica* serovar Typhimurium

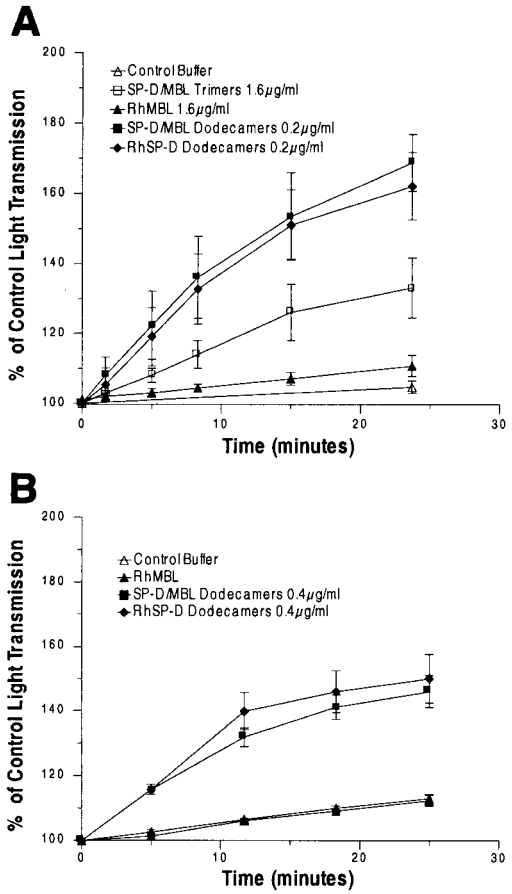

FIG. 3. RhSP-D or SP-D/$MBL_{neck+CRD}$ causes greater aggregation of *E. coli* K-12 (A) or *S. enterica* serovar Typhimurium TV119 (B) than RhMBL. Bacterial aggregation was assessed by using light transmission assay (see Materials and Methods). (A) The indicated concentration of RhMBL, RhSP-D (dodecameric fraction), or SP-D/$MBL_{neck+CRD}$ (dodecameric or trimeric fraction) was added at time zero. Dodecamers of RhSP-D and SP-D/$MBL_{neck+CRD}$ had similar potency in causing aggregation of *E. coli*. In contrast, even a substantially higher concentration of RhMBL or of the trimeric fraction of SP-D/$MBL_{neck+CRD}$ caused significantly ($P \leq 0.05$) less aggregation than either RhSP-D or SP-D/$MBL_{neck+CRD}$ dodecamers. (B) RhMBL also caused significantly less aggregation of live *S. enterica* serovar Typhimurium TV119 than either RhSP-D or SP-D/$MBL_{neck+CRD}$ dodecamers. Results are means ± SEM of four or more experiments.

TV119 significantly less than RhSP-D or SP-D/$MBL_{neck+CRD}$ dodecamers (Fig. 4B). For this assay, freshly grown bacteria were allowed to adhere to the ELISA plates without fixation (i.e., by incubation overnight at 4°C followed by centrifugation of plates at 2,500 rpm in a Sorval RT7 centrifuge to maximize bacterial adhesion). Binding of either RhSP-D or SP-D/$MBL_{neck+CRD}$ dodecamers was essentially abolished by EDTA and was significantly reduced by addition of mannose, glucose, or GlucNAc (Fig. 4C). Of interest, binding of SP-D/$MBL_{neck+CRD}$ dodecamers was more strongly inhibited than that of RhSP-D by these monosaccharides. Mannose or glucose inhibited binding of RhSP-D more strongly than GlucNAc.

To further assess the specificity of these interactions we examined the ability of these collectins to bind to or aggregate strains of *S. enterica* serovar Typhimurium that differ in the structure of their LPS, TV160 and TV119. The TV160 strain differs from TV119 in lacking terminal glucose and GlcNAc residues on its outer-core oligosaccharide. The SL1102 strain lacks the outer-core oligosaccharide. Neither RhSP-D nor SP-D/$MBL_{neck+CRD}$ showed significant binding to the TV160 or SL1102 strains of *S. enterica* serovar Typhimurium (Table 4). These results are consistent with the possibility that these collectins preferentially bind to glucose or GlcNAc residues in the outer-core oligosaccharide of *S. enterica* serovar Typhimurium TV119.

SP-D/$MBL_{neck+CRD}$ dodecamers increase in neutrophil uptake of *E. coli* K-12 to a greater extent than wild-type MBL. As shown in Table 5, SP-D/$MBL_{neck+CRD}$ dodecamers also caused significantly greater increase in uptake of FITC-labeled, formalin-treated *E. coli* K-12 than RhMBL. The trimeric fraction of SP-D/$MBL_{neck+CRD}$ was less potent at increasing the uptake of *E. coli* than SP-D/$MBL_{neck+CRD}$ dodecamers. This difference was statistically significant at the concentration of 1.4 μg/ml of the collectins. SP-D/$MBL_{neck+CRD}$ dodecamers also caused significantly greater neutrophil uptake of freshly isolated, live *E. coli* than RhMBL or SP-D/$MBL_{neck+CRD}$ trimers. SP-D/$MBL_{neck+CRD}$ dodecamers (4 μg/ml) increased uptake of live *E. coli* to 319% ± 67% of control, compared to 106% ± 5.6% and 154% ± 36% of control for RhMBL and SP-D/$MBL_{neck+CRD}$ trimers, respectively ($n = 4$; $P < 0.04$ comparing SP-D/$MBL_{neck+CRD}$ dodecamers to either RhMBL or SP-D/$MBL_{neck+CRD}$ trimers).

Conglutinin causes less aggregation of *E. coli* K-12 and *S. enterica* serovar Typhimurium TV119 than SP-D. As noted above, bovine serum conglutinin did not cause aggregation of formalin-fixed *E. coli* K-12 or *S. enterica* serovar Typhimurium TV119 in fluorescent microscopic assays (Fig. 2). This result was unexpected, since conglutinin strongly aggregates influenza virus (15). Similar results were obtained by using light

TABLE 3. Effects of collectins on bacterial colony formation[a]

Strain	% Control colony formation after treatment with collectin		
	RhSP-D dodecamers	SP-D/$MBL_{neck+CRD}$ dodecamers	RhMBL
S. enterica serovar Typhimurium TV119	74 ± 15 (<0.07)	48 ± 12 (<0.006)	127 ± 9
E. coli K-12	50 ± 24 (<0.048)	29 ± 14 (<0.003)	94 ± 11

[a] Colony formation was assessed after incubation of bacteria with the collectins in the aggregation assays (see Fig. 3). Postaggregation bacterial samples were diluted in PBS at a 1:1,000 dilution and were plated on LB agar. Colonies were counted after 24 h. The results are means ± SEM of three to six experiments. The mean number of colonies in control samples was 294 colonies/agar plate. The numbers in parentheses are *P* values, shown for comparison of colonies formed in the presence of collectin to colonies formed after similar treatment with control buffer alone. Results obtained with SP-D/$MBL_{neck+CRD}$ dodecamers were also significantly lower than those obtained with RhMBL-treated bacteria ($P < 0.05$). Differences in activity between SP-D and SP-D/$MBL_{neck+CRD}$ dodecamers were not statistically significant.

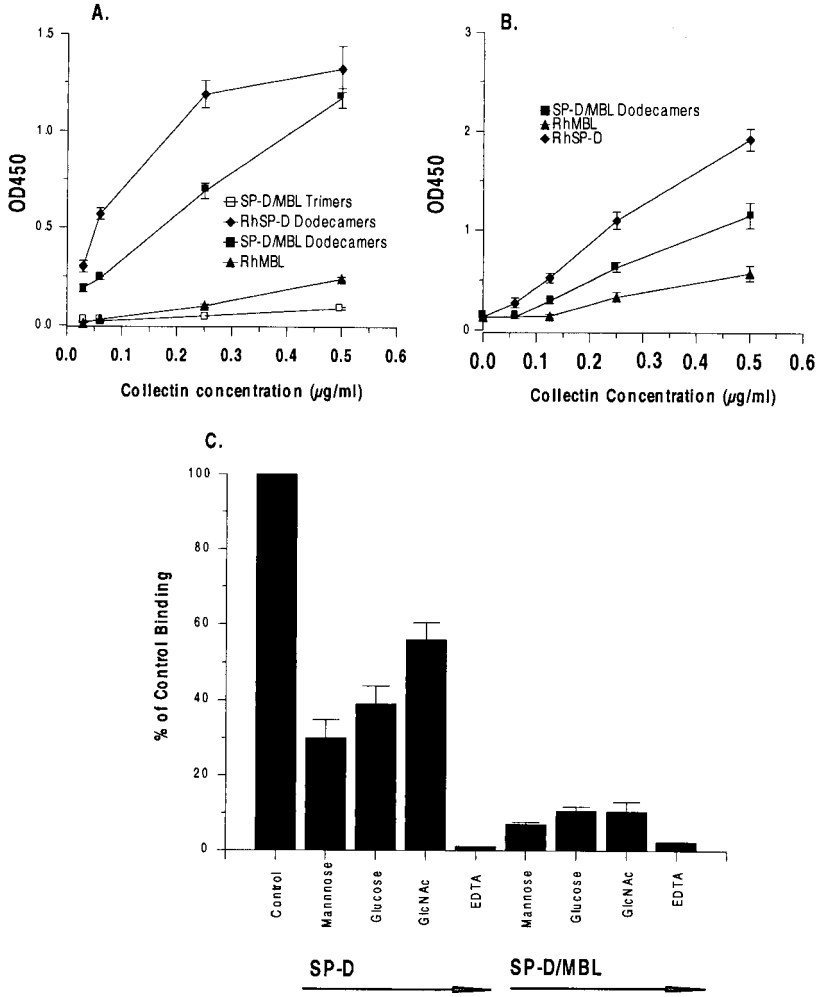

FIG. 4. RhSP-D or SP-D/MBL$_{neck+CRD}$ binds to *E. coli* K-12 and *S. enterica* serovar Typhimurium TV119 more strongly than RhMBL. Binding to formalin-fixed *E. coli* was measured by ELISA. (A) SP-D/MBL$_{neck+CRD}$ dodecamers and RhSP-D bound to *E. coli* significantly more strongly than either RhMBL or SP-D/MBL$_{neck+CRD}$ trimers. RhSP-D dodecamers also bound to *E. coli* to a significantly greater extent than SP-D/MBL$_{neck+CRD}$ dodecamers. (B) RhMBL bound to *S. enterica* serovar Typhimurium significantly less strongly than either RhSP-D or SP-D/MBL$_{neck+CRD}$ dodecamers. RhSP-D dodecamers bound to *S. enterica* serovar Typhimurium TV119 significantly more strongly than SP-D/MBL$_{neck+CRD}$ dodecamers. (C) The effect of adding the indicated monosaccharides (160 mM) or EDTA (10 mM) to assay buffer was tested. All monosaccharides and EDTA significantly inhibited binding of either RhSP-D or SP-D/MBL$_{neck+CRD}$ dodecamers to *S. enterica* serovar Typhimurium TV119. However, binding of SP-D/MBL$_{neck+CRD}$ dodecamers was inhibited to a significantly greater extent than binding of RhSP-D dodecamers by the monosaccharides. GlucNAc inhibited binding of RhSP-D dodecamers significantly less than mannose or glucose. Results are means ± SEM of three or more experiments where significant differences are noted ($P < 0.05$).

TABLE 4. RhSP-D and SP-D/MBL$_{neck+CRD}$ do not bind to *S. enterica* serovar Typhimurium TV160 or SL1102

Strain	Binding of SP-D to bacteria[a]	Binding of SP-D/MBL$_{neck+CRD}$ to bacteria[b]
TV119	2.1 ± 0.13	1.35 ± 0.14
TV160	0.17 ± 0.05	0.17 ± 0.06
SL1102	0.18 ± 0.06	0.19 ± 0.05

[a] Results shown are means ± SEM of OD_{450} from three separate experiments involving binding of (0.5 μg/ml) RhSP-D dodecamers or SP-D/MBL$_{neck+CRD}$ dodecamers to ELISA plates coated with the indicated strains of bacteria. Binding of SP-D to plates coated with BSA alone was 0.21. Background binding was not subtracted from the results shown in the table.

[b] Binding of SP-D/MBL$_{neck+CRD}$ to ELISA plates coated with BSA alone was 0.207.

TABLE 5. Effect of RhMBL or SP-D/MBL$_{neck+CRD}$ on neutrophil uptake of *E. coli*

Collectin	% Neutrophil fluorescence at collectin concentration (μg/ml)[a]:				
	0	0.7	1.4	2.8	5.7
RhMBL	100	ND	113 ± 21	102 ± 9	104 ± 8
SP-D/MBL$_{neck+CRD}$ dodecamers	100	207 ± 51	217 ± 38*	236 ± 65*	199 ± 68
SP-D/MBL$_{neck+CRD}$ trimers	100	116 ± 8	164 ± 25**	170 ± 33*	164 ± 40

[a] Results shown are means ± SEM of six experiments and are expressed as percent of control neutrophil fluorescence. An asterisk indicates concentrations of SP-D/MBL$_{neck+CRD}$ dodecamers or trimers that caused significant increases ($P < 0.05$) in neutrophil uptake of *E. coli.* Two asterisks indicate that a 1.4-μg/ml concentration of SP-D/MBL$_{neck+CRD}$ trimers caused significantly less increase in uptake than the same concentration of SP-D/MBL$_{neck+CRD}$ dodecamers.

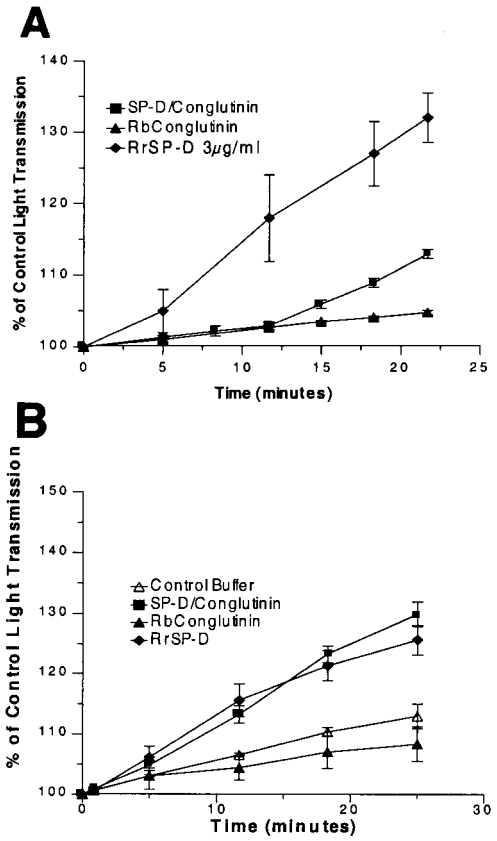

FIG. 5. Comparison of the ability of RrSP-D, RbConglutinin, and SP-D/$Cong_{neck+CRD}$ to aggregate *E. coli* K-12 or *S. enterica* serovar Typhimurium TV119. Collectins (final concentration, 3 μg/ml) were added at time zero followed by monitoring of light transmission through stirred suspensions of formalin-fixed *E. coli* K-12 (A) or live *S. enterica* serovar Typhimurium TV119 (B). Results shown are means ± SEM of three or more experiments. In the case of *E. coli* all of the collectins caused significant increases in light transmission (indicating aggregation of bacteria) compared to that of the control buffer (not shown). However, SP-D/$Cong_{neck+CRD}$ caused significantly greater aggregation than RbConglutinin, and RrSP-D caused significantly greater aggregation than either RbConglutinin or SP-D/$Cong_{neck+CRD}$ ($P \leq 0.05$ in each case). In the case of *S. enterica* serovar Typhimurium, SP-D/$Cong_{neck+CRD}$ and RrSP-D caused a similar degree of aggregation that was significantly greater than that caused by RbConglutinin or control buffer ($P < 0.05$). RbConglutinin did not cause greater aggregation of *S. enterica* serovar Typhimurium than control buffer.

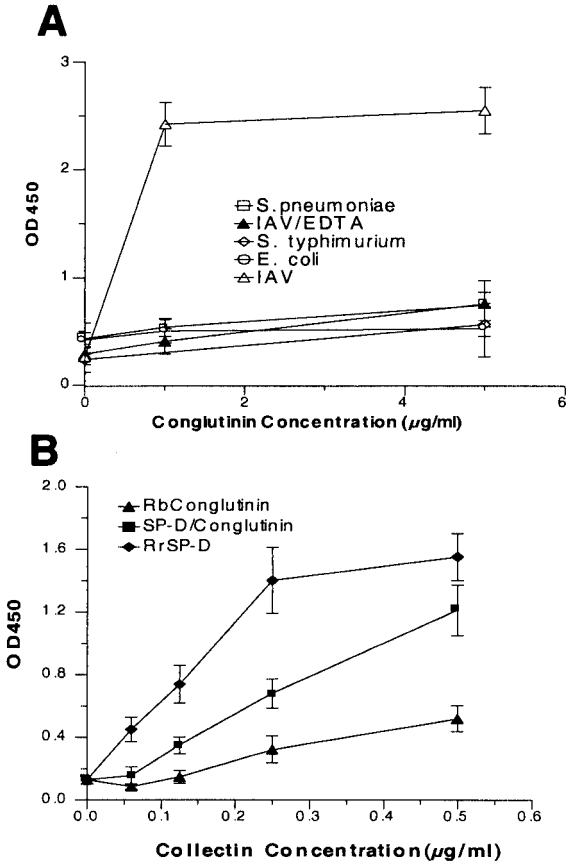

FIG. 6. Binding of conglutinin, RrSP-D, or SP-D/$Cong_{neck+CRD}$ to bacteria or Influenza A virus (IAV) as assessed by ELISA. (A) IAV or formalin-fixed bacteria (*S. pneumoniae* Type 23, *E. coli* K-12, or *S. enterica* serovar Typhimurium TV119, as indicated) were coated onto ELISA plates and were incubated with biotinylated bovine serum conglutinin. Bound conglutinin was detected by using avidin-horseradish peroxidase and peroxidase substrates. Conglutinin bound significantly to IAV ($P < 0.05$) in calcium-containing buffer but not in buffer containing 10 mM EDTA (IAV/EDTA). (B) Live *S. enterica* serovar Typhimurium TV119 was coated onto ELISA plates by sedimentation and binding of biotinylated RrSP-D and RbConglutinin, and SP-D/$Cong_{neck+CRD}$ was assessed by ELISA. All of the collectins showed significant binding to live *S. enterica* serovar Typhimurium; however, RrSP-D bound significantly more than RbConglutinin or SP-D/$Cong_{neck+CRD}$, and SP-D/$Cong_{neck+CRD}$ bound significantly more than RbConglutinin. Results are means ± SEM of three or more separate ELISAs.

transmission assays for aggregation of the bacteria and RbConglutinin (Fig. 5). Note that freshly isolated, live *S. enterica* serovar Typhimurium TV119 also was not aggregated by RbConglutinin (Fig. 5).

SP-D/$Cong_{neck+CRD}$ causes greater aggregation of *E. coli* K-12 and *S. enterica* serovar Typhimurium TV119 than RbConglutinin. SP-D/$Cong_{neck+CRD}$ aggregated *E. coli* K-12 and *S. enterica* serovar Typhimurium TV119 to a significantly greater extent than RbConglutinin (Fig. 5). SP-D/$Cong_{neck+CRD}$ aggregated *S. enterica* serovar Typhimurium to an extent similar to that of RrSP-D; however, it aggregated *E. coli* significantly less than RrSP-D. The reactions showed specificity given that neither RrSP-D nor SP-D/$Cong_{neck+CRD}$ caused aggregation of the TV160 or SL1102 strains of *S. enterica* serovar Typhimurium. After 25 min, light transmission through suspensions of the TV160 or SL1102 strains was not significantly greater for samples treated with the collectins than for samples maintained in control buffer ($n = 2$ for each; data not shown).

Conglutinin binds less efficiently to several strains of bacteria than does SP-D. Since conglutinin has high affinity for GlcNAc and is structurally similar to SP-D, it was unexpected that conglutinin would not aggregate either *E. coli* K-12 or *S. enterica* serovar Typhimurium TV119. We therefore compared the ability of conglutinin and SP-D to bind to these bacteria. We found minimal binding of bovine serum conglutinin to several strains of formalin-fixed bacteria, despite strong bind-

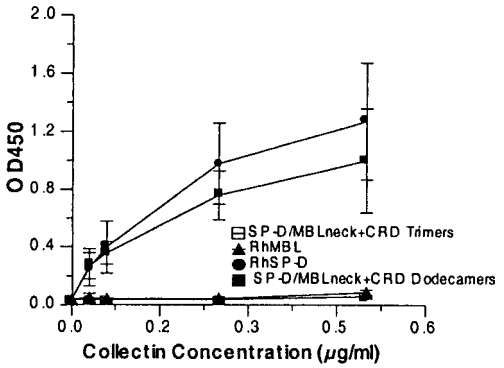

FIG. 7. RhSP-D, RhMBL, or SP-D/$MBL_{neck+CRD}$ bind to *S. pneumoniae* significantly more strongly than RhMBL. *S. pneumoniae* Type 23 (formalin-fixed) cells were coated onto ELISA plates. RhSP-D dodecamers and SP-D/$MBL_{neck+CRD}$ dodecamers bound to the bacteria significantly more avidly than either RhMBL or SP-D/$MBL_{neck+CRD}$ trimers.

ing to influenza virus (Fig. 6). Although RbConglutinin showed detectable binding to freshly isolated, live *S. enterica* serovar Typhimurium TV119 (Fig. 6B) at physiological concentrations with this method, binding was still significantly lower than observed for RrSP-D.

SP-D/$Cong_{neck+CRD}$ binds *S. enterica* serovar Typhimurium TV119 more extensively than RbConglutinin. SP-D/$Cong_{neck+CRD}$ bound to *S. enterica* serovar Typhimurium TV119 to a greater extent than RbConglutinin but to a lesser extent than RrSP-D (Fig. 6B). Thus, exchange of the N-terminal and collagen domains of RrSP-D for those of conglutinin resulted in significantly increased binding to and aggregation of *E. coli* K-12 or *S. enterica* serovar Typhimurium TV119. These results cannot be attributed to differences in the extent of multimerization of RbConglutinin or SP-D/$Cong_{neck+CRD}$ because both preparations consist of dodecamers (10).

One potentially important difference between the collagen domains of conglutinin and SP-D is the presence of an N-linked oligosaccharide at asparagine 70 of SP-D. Conglutinin lacks N-linked oligosaccharide attachments. To exclude the possibility that the difference in ability to bind to or aggregate bacteria resulted from interactions of bacteria with the N-linked sugar on SP-D, we compared the abilities of RrSP-D_{ala72} and wild-type RrSP-D to aggregate *E. coli*. The RrSP-D_{ala72} mutant lacks a consensus sequence (NGS) for N-linked glycosylation (2). RrSP-D_{ala72} had a significantly greater bacterial aggregating activity than wild-type SP-D (i.e., mean percentages ± standard errors of the means [SEM] for light transmission 20 min after addition of collectins was 170% ± 7% for RrSP-D_{ala72} versus 134% ± 4% for wild-type RrSP-D; $n = 6$; $P < 0.01$).

SP-D, or chimeras containing the N-terminal and collagen domains of SP-D, bind to *S. pneumoniae* significantly more than RhMBL or RbConglutinin. *S. pneumoniae* strains are common causes of gram-positive infection and are the most common cause of community-acquired bacterial pneumonia. As shown in Fig. 7, RhSP-D or SP-D/$MBL_{neck+CRD}$ dodecamers bound to a formalin-fixed preparation of *S. pneumoniae* Type 23 (a prevalent strain) more extensively than RhMBL. The enhanced binding activity of SP-D/$MBL_{neck+CRD}$ was dependent on formation of higher-order multimers, since SP-D/$MBL_{neck+CRD}$ trimers did not have greater binding activity than RhMBL. The results were confirmed by using live *S. pneumoniae* Type 23 (Fig. 8). Although binding of SP-D/$MBL_{neck+CRD}$ dodecamers to live *S. pneumoniae* Type 23 was also significantly greater than that of RhMBL, it was slightly, but significantly, less than that for binding of RhSP-D dodecamers. SP-D/$Cong_{neck+CRD}$ also bound to *S. pneumoniae* to a significantly greater extent than RbConglutinin (Fig. 8). RbConglutinin also bound significantly less than RrSP-D to several other capsular types of *S. pneumoniae* (i.e., types 4, 6A, 14, and 19) by using this ELISA method (data not shown).

DISCUSSION

In these studies, SP-D was found to have significantly greater ability than MBL or conglutinin to aggregate and/or bind to several bacterial strains. Initial experiments were carried out with formalin-fixed bacterial strains. The use of formalin-fixed strains allowed standardization and also excluded possible effects of bacterial proteases on collectin activities (e.g., proteolytic degradation of conglutinin [19]). However, important observations were confirmed by using freshly isolated, live bacteria to exclude artifacts resulting from fixation.

We examined interactions of these collectins with gram-negative bacteria that have known LPS carbohydrate structure and interactions with serum collectins and/or SP-D (see Table 2). These strains are useful because they allow comparisons with published literature and assessment of the contribution of LPS-associated carbohydrates. Both the *E. coli* K-12 strain and *S. enterica* serovar Typhimurium TV119 strain have been shown to be susceptible to complement-dependent lysis by MBL and are among the bacterial strains to which MBL binds most strongly (5, 17, 18). It is notable, therefore, that SP-D aggregated and bound to these strains more strongly than MBL.

It is likely that the monosaccharide-binding affinities of the collectins play an important role in their attachment to these gram-negative bacteria (e.g., note the failure of SP-D or MBL to bind to the TV160 or SL1002 strains of *S. enterica* serovar Typhimurium); however, other factors may be important as well (see below). Because MBL has higher affinity for GlcNAc than SP-D, our initial hypothesis was that the reduced ability of MBL to aggregate or bind to *E. coli* K-12 and *S. enterica* serovar Typhimurium TV119 resulted from differences in the N-terminal and collagen domains of the two molecules. Both of these bacterial strains contain LPS-associated sugars (e.g., GlcNAc) that should allow strong attachment of the MBL CRD. SP-D/$MBL_{neck+CRD}$ dodecamers did bind to and aggregate the bacteria to a greater extent than comparable concentrations of MBL. These results were dependent on formation of dodecamers, since the trimeric fraction of SP-D/$MBL_{neck+CRD}$ did not have increased bacterial binding or aggregating activity compared to that of MBL. SP-D/$MBL_{neck+CRD}$ dodecamers also suppressed colony formation of live bacteria to a greater extent than RhMBL. Although further studies are needed to determine the mechanism of this effect (i.e., aggregation versus other effects on bacterial viability), it provides further evidence of enhanced interaction of the chimera compared to that of MBL.

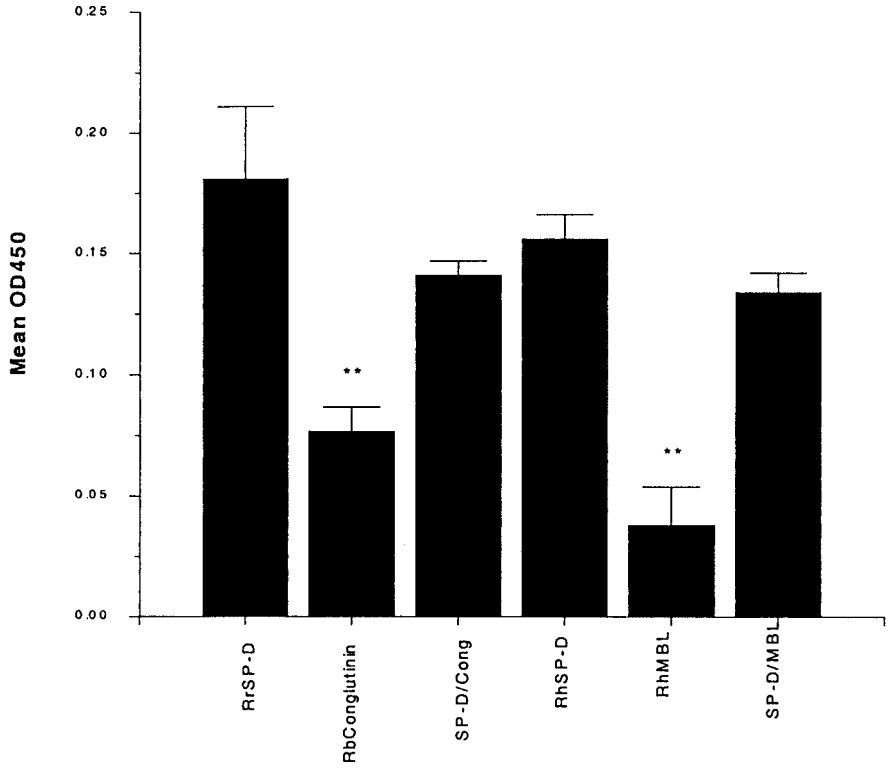

FIG. 8. Comparison of ability of SP-D, conglutinin, MBL, and collectin chimeras to bind to live *S. pneumoniae* type 23. Results are means ± SEM of three or four ELISA experiments. Background binding of the collectins to wells with blocking buffer alone was subtracted from the results shown. Wild-type RbConglutinin and RhMBL bound to the bacteria to a significantly lower extent than the other collectins tested (**, $P < 0.05$). RrSP-D and RhSP-D bound to the bacteria significantly more strongly than the SP-D/$\text{Cong}_{\text{neck+CRD}}$ or SP-D/$\text{MBL}_{\text{neck+CRD}}$ chimera, respectively.

On the basis of prior studies with influenza virus (35, 36), we anticipated that SP-D/$\text{MBL}_{\text{neck+CRD}}$ dodecamers would have enhanced bacterial aggregating and binding activity compared to that of wild-type SP-D as well. However, SP-D/$\text{MBL}_{\text{neck+CRD}}$ dodecamers had approximately the same bacterial aggregating activity as SP-D and bound the bacteria less extensively than SP-D. It is unclear why SP-D/$\text{MBL}_{\text{neck+CRD}}$ dodecamers bound bacteria less strongly than RhSP-D dodecamers while having similar aggregating activity, although it is possible that different interactions can occur in suspension during the aggregation assay compared to that for the ELISA, which was in solid phase.

Recombinant or native conglutinin and SP-D/$\text{Cong}_{\text{neck+CRD}}$ had markedly reduced ability to bind to *S. enterica* serovar Typhimurium TV119 and other bacteria compared to that of SP-D. Despite strong homologies between conglutinin and SP-D (25), the CRDs of these proteins appear to have significant differences in biological activity. The CRD of conglutinin binds to iC3b and mediates conglutination (37). Also, the CRD of conglutinin has significantly greater ability to bind to and neutralize influenza viruses than does of SP-D (10, 12). In vivo, replacement of wild-type SP-D in SP-D-gene-deleted (SP-D−/−) mice with the SP-D/$\text{Cong}_{\text{neck+CRD}}$ chimera through transgenic expression in the lung does not restore all normal activities of SP-D (37). These SP-D−/−, SP-D/$\text{Cong}_{\text{neck+CRD}}$ overexpressing mice have restored ability to process pulmonary phospholipids and to inhibit pulmonary influenza infection but develop macrophage abnormalities and emphysema, like SP-D−/− mice. Results of the present paper provide further evidence of functional differences between the CRD of conglutinin and SP-D. Of note, *S. enterica* serovar Typhimurium TV119 contains LPS with an O-polysaccharide that terminates in a monosaccharide (GlcNAc), for which MBL and conglutinin have higher affinity than SP-D. Hence, the reduced ability of conglutinin, SP-D/$\text{MBL}_{\text{neck+CRD}}$, or SP-D/$\text{Cong}_{\text{neck+CRD}}$ to bind to these bacteria compared to binding of SP-D is striking and suggests that the identity of the terminal LPS monosaccharide does not alone determine collectin binding.

A variety of factors could account for differences in the abilities of the collectins to bind to specific bacterial strains apart from the terminal sugar present on the bacterial coat proteins. First, it has been demonstrated that collectins can bind to nonterminal sugar residues in a polysaccharide (1). Furthermore, the orientation of a polysaccharide relative to the protein backbone can markedly affect binding. Conglutinin can bind to oligosaccharides on RNase only when the protein is reduced (30). The ability of conglutinin to bind to a high mannose oligosaccharide on iC3b, whereas MBL cannot, also appears to depend on the relationship of the oligosaccharide to the underlying protein backbone (31). The density or topographical arrangement of LPS on the surface of some bacteria may strongly affect collectin binding, as suggested by Devyatyarova-Johnson et al. (5) with respect to binding of MBL to

TABLE 6. Comparison of interactions of collectins with IAV and *S. enterica* serovar Typhimurium TV119[a]

Collectin	Binding to IAV	Aggregation of IAV	Binding to S. enterica serovar Typhimurium	Aggregation of *S. enterica* serovar Typhimurium
RhSP-D	3+	3+	4+	4+
RhMBL	1+	1+	1+	1+
SP-D/MBL dodecamers	4+	4+	2+	4+
SP-D/MBL trimers	1+	1+	1+	1+
RrSP-D	3+	2+	3+	3+
RbConglutinin	3+	2+	1+	+/−
SP-D/conglutinin	4+	4+	2+	3+

[a] Results with IAV are from references 10 and 36. Results with *S. enterica* serovar Typhimurium are from this paper.

Salmonella and *Neisseria* strains. The ability of a collectin molecule to bind to many sugar moieties simultaneously on the surface of a pathogen can greatly affect binding affinity. Finally, it is also possible that the CRD of SP-D contains ancillary binding sites (apart from the principle calcium and carbohydrate binding pocket) that contribute to its enhanced ability to bind to certain bacteria, as suggested by comparisons of the crystal structures of the SP-D and MBL CRDs (7). The fact that binding of SP-D to *S. enterica* serovar Typhimurium TV119 was less readily inhibited by monosaccharides than binding of SP-D/$MBL_{neck+CRD}$ dodecamers (Fig. 4C) supports the hypothesis that ancillary binding interactions may contribute to binding of SP-D to bacteria.

In any case, our studies indicate that significant differences exist in the ability of neck domains and/or CRDs of SP-D, MBL, and conglutinin to mediate binding to some strains of bacteria. Prior studies demonstrated differences in the ability of SP-D/$MBL_{neck+CRD}$ and SP-D/$Cong_{neck+CRD}$ to interact with influenza virus compared to that of wild-type SP-D, as summarized in Table 6 (10, 35, 36). It is notable, however, that these prior studies showed clearly increased ability of the chimeras to bind to, aggregate, or neutralize influenza viruses compared to that of SP-D (or compared to that of wild-type MBL and conglutinin). Hence, the requirements for optimal binding of a collectin to influenza virus or bacteria appear to differ. Further studies with chimeric SP-D molecules containing smaller portions of the CRDs of serum collectins should clarify whether or not specific segments of the CRD are responsible for differences in bacterial or viral binding activity of SP-D and serum collectins.

Our studies also demonstrate that the N terminus and collagen domains of SP-D have significant functional differences from the comparable domains of MBL or conglutinin. Dodecamers of the SP-D/$MBL_{neck+CRD}$ or SP-D/$Cong_{neck+CRD}$ chimera bound to and aggregated all of the bacterial strains tested to a greater extent than MBL or conglutinin, respectively. As noted, SP-D/$MBL_{neck+CRD}$ caused significantly greater increases in bacterial uptake by neutrophils than RhMBL. Similar results were obtained from comparing the ability of SP-D/$Cong_{neck+CRD}$ and conglutinin to increase neutrophil uptake of *E. coli* (data not shown). We hypothesize that the greater bacterial or viral aggregating activity of SP-D/$MBL_{neck+CRD}$ dodecamers compared with that of MBL indicates that the much larger collagen domain of SP-D allows greater opportunities for cross-linking of microbial particles than the more compact domain of MBL.

The greater ability of SP-D/$Cong_{neck+CRD}$ to aggregate influenza virus (10) or bacteria compared with that of conglutinin is less readily explained given greater similarity between the N-terminal and collagen domains of SP-D and conglutinin. Our results obtained with the $RrSP\text{-}D_{ala72}$ mutant version of SP-D indicate that the N-linked sugar on SP-D is not responsible for the enhanced interactions of SP-D/$Cong_{neck+CRD}$ with influenza virus (10, 11) or bacteria (this paper). In fact, removal of the N-linked sugar from SP-D increased its aggregating activity. This finding requires further study but suggests that the presence of the N-linked sugar in the collagen domain of SP-D may limit mobility of the arms of multimeric SP-D molecules. Other differences in the N-terminal domains of SP-D and conglutinin include the presence of interchain disulfide cross-links, the interruption in the collagen sequence, and the shorter length of the collagen domain of conglutinin (32). Further analysis of the structural features of the N terminus and collagen domains of SP-D, MBL, and conglutinin that are responsible for enhanced microbial aggregating and opsonizing activities of SP-D, or chimeras containing the N terminus and collagen domains of SP-D, are warranted.

In summary, we demonstrate that the serum collectins and SP-D differ significantly in their interactions with bacteria. Conglutinin and MBL show significantly less bacterial binding and aggregating activity than SP-D. These differences are, in part, secondary to differences in binding properties of the SP-D CRD compared to those of the CRDs of MBL or conglutinin but also reflect differences in the N-terminal and collagen domains. Although most of the experiments described in this paper involved laboratory-derived gram-negative bacterial strains, similar differences in bacterial binding activity were found with clinically relevant strains of *S. pneumoniae*. The enhanced binding activity of SP-D/$MBL_{neck+CRD}$ dodecamers compared to that of RhMBL may also allow this molecule to interfere with complement activation by MBL. Further studies will address this question, since such complement activation could be deleterious in certain settings.

ACKNOWLEDGMENTS

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